

**CRANFIELD UNIVERSITY**

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**PATHOGEN RESPONSES IN FOODS: UNDERESTIMATED  
ECOPHYSIOLOGICAL FACTORS**

**SCHOOL OF CRANFIELD HEALTH**

**PhD THESIS**



CRANFIELD UNIVERSITY  
SCHOOL OF CRANFIELD HEALTH

PhD THESIS

Academic Year 2008-2009

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“Pathogen Responses in Foods:  
Underestimated Ecophysiological Factors”

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January 2009

This thesis is submitted in partial fulfilment of the requirements  
for the degree of Doctor of Philosophy

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## ABSTRACT

Accurate prediction of the fate of microbial foodborne pathogens in foods is of great concern for anyone involved in the food chain. Factors that may influence microbial responses in foods and food environments, such as food structure and composition, microbial interactions and mode of growth were identified and assessed in the present study.

The fate of *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* O157:H7 was monitored both in and on teewurst, a raw spreadable sausage, at different storage temperatures. Regardless of the storage temperature and inoculation type, pathogen numbers decreased during storage. The increase of endogenous lactic acid bacteria and the concomitant reduction of pH mostly accounted for this reduction. The inactivation of all three pathogens inoculated into batter or onto slices varied considerably among trials possibly due to variations in the initial batch-to-batch levels of lactic acid microflora and the associated microbial interactions.

The effect of structure, composition and microbial interactions on the growth kinetics of *L. monocytogenes* was evaluated in different growth substrates, including broth, agar, sterile meat blocks, naturally contaminated meat blocks and minced meat. The growth responses of *L. monocytogenes* were significantly different in the different growth media and food products tested. These differences were more pronounced at low temperatures. The validation of a model based on data from broth against the observed growth of the pathogen in the rest of the tested media showed that broth models may result in significant prediction errors.

The potential for mono- or multi-strain cultures of *Escherichia coli* O157:H7 to attach and form biofilm in combinations of food-contact surfaces, growth substrates and storage temperatures was examined. The susceptibility of biofilms to sanitizers was also evaluated. Attachment and biofilm formation was strain dependent. The presence of food residues (liquid or solid) facilitated the attachment/transfer of *E. coli* O157:H7 on food-contact surfaces. At moderately cold temperatures culture broth was more

conducive to subsequent growth. At chill temperatures the presence of natural microflora in liquid residues enhanced further attachment of the pathogen. Biofilms were less susceptible to sanitation treatments as compared to planktonic cells. Biofilm cells surviving sanitation were able to survive and present slight increases at refrigeration and abuse temperatures, respectively, in cross-contaminated ground meat.

Acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2) signalling molecules in the cell-free supernatants of *Pseudomonas aeruginosa*, *Yersinia enterocolitica*-like, *Serratia proteamaculans* and a mixture of two *Yersinia enterocolitica* strains were found to affect the growth kinetics of two *Salmonella* Enteritidis and *S. Typhimurium* strains, respectively. *P. aeruginosa* synthesized quorum sensing signals that accelerated the metabolic activity of *Salmonella* strains. All other quorated bacteria tested had a negative effect on both initiation of growth and metabolic activity. The effect seems to be strain and QS signal dependent.

## ACKNOWLEDGEMENTS

First of all, I am deeply indebted to both my supervisors in Cranfield University, Dr. David Aldred and Professor Naresh Magan for their time, valuable guidance and support throughout this study.

I wish to express my gratitude to Professor George-John Nychas in Laboratory of Food Microbiology and Biotechnology, Agricultural University of Athens, Greece for giving me the opportunity to have this rewarding and enjoyable “journey” through the world of food microbiology. I would also like to thank him for his trust, support, scientific guidance, patience, understanding and enthusiasm all these years.

I would also like to thank Professor John Sofos, in Center for Meat Safety and Quality, Department of Animal Sciences, Colorado State University, USA, for his warm welcome and valuable advice on biofilm experiments. To my colleagues, Gina, Catie, Camelia, Mawill, Alex, Shivani, Chen and Jeremy for their help and great collaboration.

My greatest thanks to Dr. Luchansky and all the great scientists I have met and worked with in Agricultural Research Service, Easter Regional Research Service, United States Department of Agriculture, USA. To Edu, Joe Lee, Stefanie, Denise, Lupe and Fernando for making my staying pleasant.

To Dr. Konstantinos Koutsoumanis, in Laboratory of Food Microbiology and Hygiene, Faculty of Agriculture, Aristotle University of Thessaloniki, Greece, Dr. Efstathios Panagou, Laboratory of Food Microbiology and Biotechnology, Agricultural University of Athens, Greece and Dr. Panagiotis Skandamis, Laboratory of Food Hygiene, Agricultural University of Athens, Greece for sharing their knowledge in the difficult field of Predictive Microbiology.

Additionally, my thanks goes also to all my colleagues in the Laboratory of Food Microbiology and Biotechnology that have been close to me all these years and managed to put up with me. To Litsa, Aspasia, Virginia, Dafni, Tasos, Antonia, Nikos

and Stathis for their great collaboration, help, valuable conversations and all the great moments we had both inside and outside the lab. Most of them became best friends.

I also wish to express my thankfulness to my family for their encouragement, love and support all these years of my studies. They were always there for me. Finally, I would also like to thank Makis for his love and patience, and for always giving me the best motivation to keep going.

Dimitra

January 2009

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**CHAPTER 1**  
**GENERAL INTRODUCTION**





# Chapter 1

## GENERAL INTRODUCTION

### 1.1 MICROBIAL FOOD SAFETY

Microorganisms are ubiquitous in nature as they can be easily dispersed and tolerate extreme environmental conditions. Consequently, they can contaminate and grow in almost all food products. The microbiological safety and stability of foods depends mostly on limiting the initial microbial contamination, preventing or limiting the growth rate of microorganisms and destroying microbial populations through the application of combined preservative factors (Leistner, 2000).

During the last years, despite the progress made for their control, a number of new (not previously known; e.g. *Escherichia coli* O157:H7), emerging (known but not associated with foods; e.g. *Listeria monocytogenes*) and reemerging (evolved or associated with different foods or appearing in new geographic locations; e.g. *Salmonella*) foodborne pathogens are and will continue to be of great concern (Buchanan, 1997; Schlundt, 2002; Sofos, 2008). Changes in the agricultural practices and technology, mass food production, decreasing trade barriers, changes in the eating habits of consumers and microbial adaptation have disseminated and increased the incidence and severity of foodborne outbreaks and diseases worldwide (Todd, 1997).

In response to these challenges, the introduction of preventive strategies [e.g. the application of hazard analysis critical control point (HACCP)], quantitative microbial risk assessment and predictive microbiology disciplines seem to lead to fundamental changes in the approach of food safety and prevention of foodborne disease. For the successful performance of such strategies a detailed knowledge of the effects of food processing, storage and distribution conditions on the responses of microbial foodborne pathogens is required.

## 1.2 FACTORS AFFECTING MICROBIAL GROWTH AND SURVIVAL

The dynamics of microbial populations in foods (growth, survival, or death) are primarily determined by their exposure to combinations of intrinsic food properties and extrinsic storage conditions.

### 1.2.1 Intrinsic factors

Intrinsic factors of a food include nutrients, water activity, pH, redox potential (oxidation reduction potential) and naturally occurring or added antimicrobials. In foods these factors affect microbial growth either favourably or adversely (Mossel and Ingram, 1955; Mossel et al., 1995; Adams and Moss, 2000).

#### *Nutrients*

The chemical composition of the food is decisive on microbial growth as it determines the quantities of the vital nutrients available for the growth of microorganisms. These nutrients include water, a source of energy (carbohydrates), nitrogen (proteins), vitamins and minerals. Water is not considered a nutrient but is essential for the synthesis of cell mass and energy. All foods contain these five major nutrient groups, either naturally or added, with the amount of each nutrient varying considerably with the type of food. Microorganisms differ greatly in nutrient requirements and in their ability to utilise large and complex carbohydrates (e.g. starch, cellulose and glycogen), large proteins (e.g. casein) and lipids.

#### *pH and acidity*

Increasing the acidity of foods, either through fermentation or addition of weak acids, has been one of the most ancient methods of food preservation. The pH is a function of the hydrogen ion ( $[H^+]$ ) concentration in a food:

$$pH \approx -\log_{10} [H^+]$$

It is well known that groups of microorganisms have pH optimum, minimum and maximum for growth to occur. When the pH in a food is reduced to below the minimum

limit for growth of a microbial species, the cells not only stop growing but also lose viability, the rate of which depends on the extent of pH reduction (Mossel and Ingram, 1955). This is more apparent with weak lipophilic acids that do not completely dissociate. Undissociated acid molecules, unlike protons and other charged molecules may pass through the cell membrane and acidify the cytoplasm. The cell, trying to maintain its high (ca. 7.5) internal pH by neutralising or expelling the protons in the environment, slows growth as it diverts energy from growth-related functions. Depending on the external pH and concentration of acid the cell may eventually die (Adams and Moss, 2000).

#### *Water activity*

Microbial growth is remarkably associated with the presence of water in its liquid phase. Water requirements of microorganisms are generally described in terms of water activity ( $a_w$ ) of the food or environment. In other words, the water activity of a food describes the degree to which water is “bound” in the food, its availability to participate in chemical and biochemical reactions, and its availability to facilitate microbial growth. Most foods have water activity values that are close to the optimum growth levels of most microorganisms (0.97-0.99). However, the exact range of water activities allowing growth of bacteria is influenced by other physicochemical and nutritional factors in the environment.

#### *Redox potential*

Redox potential (Eh) or oxidation-reduction is the measure of the ease by which a substrate loses or gains electrons. Growth of microorganisms and their ability to generate energy by specific metabolic reactions depends on Eh of foods. The Eh range at which different microorganisms can grow are as follows: aerobes, +500 to +300mV; facultative anaerobes, +300 to +100mV; and anaerobes, +100 to -250mV or lower. The measured Eh in foods varies greatly according to the ingredients, packaging, pH and microbial growth. The presence and absence of oxygen and the Eh of a food determines the growth ability of a microorganism and the specific metabolic pathways used during growth to generate energy and metabolic end-products.

### *Naturally occurring or added antimicrobials*

Some product tissues may contain naturally occurring compounds that inhibit the growth of microorganisms, the concentration of which often increases with physical damage. In plants breakage of the tissue releases various antimicrobial constituents such as essential oils, phytoalexins and lectins. Animal products also present a range of antimicrobial constituents. Examples include lactoferrin and lactoperoxidase system in milk, lysozyme in eggs and milk, ovotransferrin in eggs. Antimicrobial substances differ in their spectrum of activity and their effect may be insignificant either due to processing factors (e.g. heating and cold storage) or their low concentration. Microbial activity in foods may also result in the production of antimicrobial substances, such as bacteriocins, acids and other inhibitory compounds. In addition to naturally occurring antimicrobials, a variety of chemical preservatives or additives may enhance food quality and safety (e.g. citric acid, ascorbic acid, sodium chloride and sodium ascorbate).

### **1.2.2 Extrinsic factors**

Extrinsic factors important in microbial growth in a food include the environmental conditions in which the products are stored. These include temperature, relative humidity and gaseous environment (Mossel and Ingram, 1955; Mossel et al., 1995; Adams and Moss, 2000). The latter two influence  $a_w$  and Eh, and have been presented in section 1.2.1. As such, the influence of storage temperature on microbial growth will only be discussed.

#### *Temperature*

Microbial growth in foods is accomplished through enzymatic reactions. A rule of thumb in chemistry suggests that simple reaction rates double with every 10°C increase in temperature. Similarly, by decreasing the temperature by 10°C, the enzymatic reactions are reduced to half (Mossel and Ingram, 1955). This assumption for microbial growth is only valid over a limited range of organism-dependant temperatures. Above the optimal growth temperature, growth rate decreases precipitously. On the contrary, below the optimum growth temperature, growth rate decreases but more gradually.

According to how temperature influences growth, microorganisms are divided into four groups: thermophiles, mesophiles, psychrophiles and psychrotrophs. At low storage temperatures, cells do not only grow slower than in optimum temperatures, but they also express different genes and are physiologically different. Moreover, expression of virulence genes in several pathogens is governed by temperature.

## **1.3 PREDICTIVE MICROBIOLOGY**

### **1.3.1 Introduction**

Predictive or quantitative microbiology focuses on the development of mathematical models to accurately predict the growth of microorganisms in foods (McMeekin et al., 1993). The fundamental premise of predictive microbiology is that responses of populations of microorganisms to environmental factors are reproducible and that, by characterizing environments in terms of those factors that most affect microbial growth and survival, it is possible from past observations to predict responses of those microorganisms, in other, similar environments (Ross et al., 2000). Knowledge of microbial responses and models can be stored in databases and used to interpret the effect of processing, distribution and storage conditions on microbial growth. This approach provides precision in estimating the safety and shelf life of foods. In addition, the combination of data on the temperature history of a product and mathematical models may lead to “intelligent” product management systems for the optimization of food quality and safety at the time of consumption (Koutsoumanis et al., 2005).

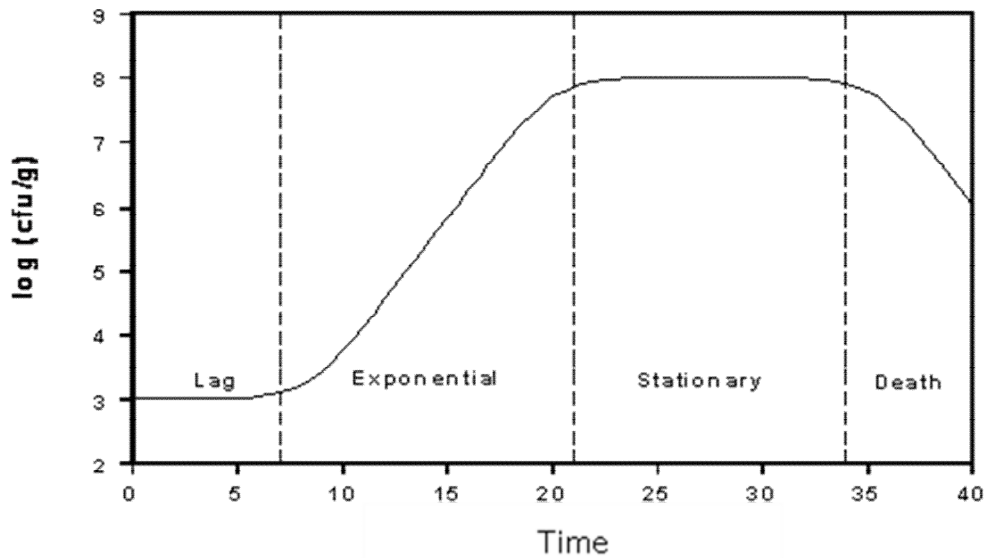
### **1.3.2 Principles of microbial growth**

In an environment where nutrients are not limiting to microbial growth, bacterial populations will proliferate and increase in number. By plotting the logarithm of microbial density against time, a characteristic curve will result as shown in Figure 1.1. The growth curve exhibits four different phases: the lag phase, exponential phase, stationary phase and death phase.

During lag phase bacterial cells are involved in a series of biochemical activities in order to allow themselves to adapt to the new environment and initiate growth. In this

phase, cells increase in size but not in number. After the end of these activities cells multiply exponentially at a constant rate. During the exponential phase cell components are synthesized so that they are present in constant proportions to cover the requirements for production of new cells which are considered to be identical with regards to their physiological state. As the population continues to increase the accumulation of metabolic end-products of cells leads to the reduction of the growth rate and cells are introduced into stationary phase. The duration of this phase varies depending on the microorganism and growth medium. Eventually, as further toxic metabolic end-products accumulate, the cells may be led to death. Boyd (1988) recognizes two additional periods of growth; an acceleration phase between lag and exponential phase and a deceleration phase between exponential and stationary phase.

Food microbiologists are particularly interested in growth kinetics (lag phase and exponential growth rate) of microorganisms. It has been shown that in most cases the spoilage of foods is caused by microbial action, thus, the ability to predict the microbial growth and particularly the duration of lag phase is very important for the prediction of the shelf-life of foods. In the case of pathogenic bacteria this knowledge contributes to their efficient control.



**Figure 1.1 Typical bacterial growth curve.**

### 1.3.3 Classification of predictive models

In the area of predictive microbiology different model classification schemes have been proposed in the literature.

#### *Kinetic and probability models*

Kinetic models attempt to explain the time needed for a specific growth response in terms of environmental variables such as temperature, pH, water activity, gaseous atmosphere, redox potential, biological structure, relative humidity, nutrient content and antimicrobial properties (Buchanan, 1993). In the fields of kinetic models two different approaches have been reported. With the first approach, the growth rate is calculated and the models are used for prediction based on the exponential phase of microorganisms. With the second approach, sigmoid functions are fitted to the experimental data and the models are used for the description of the effect of various environmental factors. Evaluation of this fitted sigmoid curve may allow researchers to make predictions for the studied microorganisms in a particular food system. In both

approaches, models are generated by using data of microbial growth under various combinations of environmental factors in order to provide information on lag time, generation time, growth rate and maximum population density (Baranyi and Roberts, 1994, 1995). However, kinetic models can be difficult to develop as they require the accumulation of a large amount of data (Baranyi and Roberts, 1995).

Probability-based models are routinely used to predict the chance of a pathogenic organism growing or forming toxin under specific environmental conditions (McDonald and Sun, 1999). The foundation of probability models is the relationship between the microbial growth and the physicochemical properties of the environment. Probability models are appropriate in cases where toxin is produced while they provide little information on the growth responses of the organism. However, a disadvantage of these models is that probability increases with time. As such, probability models are in fact a combination of both probability and kinetics and that can make them confusing.

#### *Empirical and mechanistic models*

Empirical (or black box) models, such as the Gompertz function, describe simply experimental conditions in a form of acceptable mathematical equations (Gibson et al., 1988). Polynomial equations are the most common empirical models. These models are easy to use and their application requires no knowledge of biochemical or other activities (Whiting, 1995). However, polynomial equations do not have parameters with biological meaning. Therefore, empirical models do not contribute any knowledge to the intrinsic mechanism by which the data are generated. Understanding the mechanisms that regulate cell metabolism may lead to the development of mechanistic (or white box) models. Such models could express the activity of cells with greater accuracy and be used as tools for the generation of prediction models from hypotheses (Bazin and Prosser, 1992). However, purely mechanistic models that incorporate all intrinsic and extrinsic factors that affect microbial growth have not yet been developed (Ross et al., 2000). Notwithstanding, it is generally accepted by most researchers that mechanistic models are superior to empirical models (Van Impe et al., 1992; Zwietering et al., 1993).



### *Primary, secondary and tertiary models*

The classification system proposed by Whiting and Buchanan (1993) groups most models into primary, secondary and tertiary based models.

Primary models describe the responses of bacterial populations as a function of time, under particular environmental and cultural conditions. These models are used for the calculation of kinetic parameters of growth such as maximum growth rate, generation time, lag phase duration and maximum population density (Whiting and Buchanan, 1993; Whiting, 1995;).

Secondary models are used for the quantification of the effects of various environmental factors such as temperature, pH, water activity, redox potential, on each and every kinetic parameter of microbial growth. For example, the growth rate of an organism can be calculated for the corresponding temperature by using a primary model. Then the growth rates corresponding to different temperatures are collated in a secondary model so that the effect of temperature is expressed quantitatively with a mathematic equation allowing the user to determine the growth rate in any given temperature (Whiting and Buchanan, 1993).

Tertiary models bring predictive microbiology to its final form. These models basically combine primary and secondary models in a user-friendly computer software package. End users of such systems are not required to have knowledge of microbiological techniques or of the primary and secondary models used. Tertiary models change predictive food microbiology into an accessible and powerful tool for the food industry. There are several microbial modelling software packages currently available. The most important of these are: Pathogen Modelling Program, (PMP) developed by the United States Department of Agriculture, Food Safety Research Unit (<http://ars.usda.gov/Services/docs.htm?docid=6786>); the Food Micromodel (FMM), developed by the Institute of Food Research (IFR); Combined Database for Predictive Microbiology (ComBase), a combination of FMM and PMP software supplemented with data collated in research establishments and from publications (<http://www.combase.cc>); Sym'previous, developed by the Institut National de

Recherche Agronomique; Seafood Spoilage Predictor (SSSP), developed by the Danish Institute for Fisheries Research (<http://www.dfu.min.dk/micro/sssp/Home/Home.aspx>).

### **1.3.4 Applications of predictive food microbiology**

The ability of predictive models to predict microbial responses in foods makes them an invaluable research tool. Many applications of predictive food microbiology have also been proposed with relevance to the food industry (McDonald and Sun, 1999), the most important of which, are presented in this section.

#### *Hazard analysis critical control point (HACCP)*

The HACCP concept was introduced to food microbiology as a proactive, preventive system of quality and safety control as a means to replace the time consuming and potentially less effective inspection processes and end product testing (Hartmann, 1997). As such, it requires the ability to quantitatively deal with all variables influencing food safety. However, it is not possible to determine quantitatively all aspects of microbial growth kinetics in a complex production process. On the other hand, predictive food microbiology is a quantitative method which describes the effects of these variables on microbial growth, survival or inactivation. Having that in mind, predictive microbiology can contribute to HACCP with preliminary hazard analysis, identification and establishment of critical control points, and corrective actions to be taken (McDonald and Sun, 1999). A combination of predictive food microbiology and HACCP can offer the food industry a systematic structured approach of solving problems, with quantitative calculations when necessary.

#### *Microbial Risk assessment*

Microbial risk assessment is a new and emerging discipline in the area of food safety. Its application provides a scientific description of foodborne risks related to the occurrence of pathogenic microorganisms in the whole food chain. The risk assessment process consists of four stages that include hazard identification, hazard characterization, exposure assessment and risk characterization. One of the greatest difficulties in microbial risk assessment is in determining the levels of pathogenic

microorganisms in food at the moment of consumption, i.e. exposure assessment. At this point predictive microbiology can be used to estimate changes in bacterial numbers even under dynamically changing conditions (e.g. fluctuating temperatures) (Walls and Scott, 1997b; Koutsoumanis, 2000).

#### *Microbial shelf-life studies*

It is generally acknowledged that in most cases food spoilage (i.e. changes in the sensory characteristics of the food that make it unacceptable to consumers) is the result of microbial activity (Nychas 2007, 2008). Even if the microbial flora of more products is made up of a variety of microorganisms, it has been proven that under certain conditions only a fraction of the total microflora is responsible for spoilage, termed as specific spoilage organisms (SSO). Therefore the duration of the shelf-life of a certain product could be calculated as the time that is required for the SSO to multiply to a level that causes observed spoilage (Koutsoumanis and Nychas, 2000). The use of predictive microbiology for prediction of growth of SSO to the level that causes spoilage can lead to the prediction of the product shelf-life. The prediction of shelf-life, in contrast to prediction of food safety, requires extended research since spoilage is a complicated process and factors such as the type of SSO and the level of spoilage varies depending on the type of product and storage conditions.

#### *Product research and development*

Changes in the composition or processing of a product may have important effects on the behavior of spoilage microorganisms or potential growth of pathogenic microorganisms. Predictive microbiology in this case can be used for the rapid determination of the consequences of such changes. It could also considerably contribute to fast decision making on whether to introduce modifications in preservation technologies. The combined use of predictive microbiology with HACCP in the initial stages of product development will allow for the determination of risks related to raw materials, processing, distribution or consumption (McMeekin et al., 1993).

### **1.3.5 Constraints of predictive microbiology in practical situations**

The successful application of predictive models under real life conditions has been the subject of numerous commentaries and debates. Several researchers have postulated that predictive models may provide accurate or usually conservative (i.e. fail-safe) predictions for the responses of pathogenic microorganisms in foods (Hudson and Mott, 1993; Walls and Scott, 1996, 1997a). On the contrary, others reported that predictions are not always valid for actual foods (Dalgaard and Jorgensen, 1998; Geysen et al., 2006; Xanthiakos et al, 2006).

The uncertainty in the performance of predictive models derives either from the unknown initial status of a food (e.g. initial numbers, types and physiological status of microorganisms) or from the microbial responses in a given or changing environment. Our inability to implicitly consider the effect of all these factors (variables) in foods while generating a model, termed as “completeness error” (Ross et al., 2000), may explain why predictive models remain a research tool rather than an effective industrial application (McDonald and Sun, 1999). Some of these “underestimated” factors will be discussed in the following section.

### **1.4 “UNDERESTIMATED” FACTORS AFFECTING MICROBIAL GROWTH**

In the majority of foods, the *in situ* environment will mean association of microbial cells with a solid substrate either through entrapment or attachment, or a combination thereof. As a result, the cells are immobilised and localised in high densities and may grow as microcolonies or biofilms (Katsaras and Leistner, 1991; Delaquis et al., 1992). At different sites within the food there may be variation in the levels of oxygen, pH, water activity, nutrients and, in certain foods, preservatives. This results in a series of interconnected micro-environments, some of which may be preferential for microbial growth (Wimpenny et al., 1995).

With the possible exception of highly processed products, foods harbour a variety of microorganisms which include different species of bacteria and strains within these species. The growth responses and activity of any one species or strain, whether it is an

unwanted spoilage or pathogenic microorganism, or a desirable biocontrol organism, will, in most cases, be determined by the presence of other species (Boddy and Wimpenny, 1992) and the *in situ* cell-to-cell ecological interactions which often occur in the solid phase of foods. Detailed knowledge on how these “underestimated” factors interact with spoilage and pathogenic microorganisms may allow for their efficient control.

#### **1.4.1 Food structure**

Food structure can be defined as the spatial organization of identifiable elements in a food and their interactions at levels below 100µm (Aguilera and Stanley, 1999; Aguilera et al., 2000). These elements include water and oil droplets, fat crystals, cell walls, proteins, gas bubbles and starch granules. Food microstructure may be naturally imparted or produced by processing (e.g. heating, drying and fermentation). In the latter case multicomponent structured matrices derive from the reassembling of food components such as lipids, proteins and carbohydrates into colloidal dispersions, emulsions or gel phases by heating, cooling and application of shear forces. Knowledge of the microstructural features of a food not only will lead to proper control of its properties (mechanical, textural and chemical; Aguilera et al., 2000) but also of its microbiological stability and safety (Dodd and Waites, 1992).

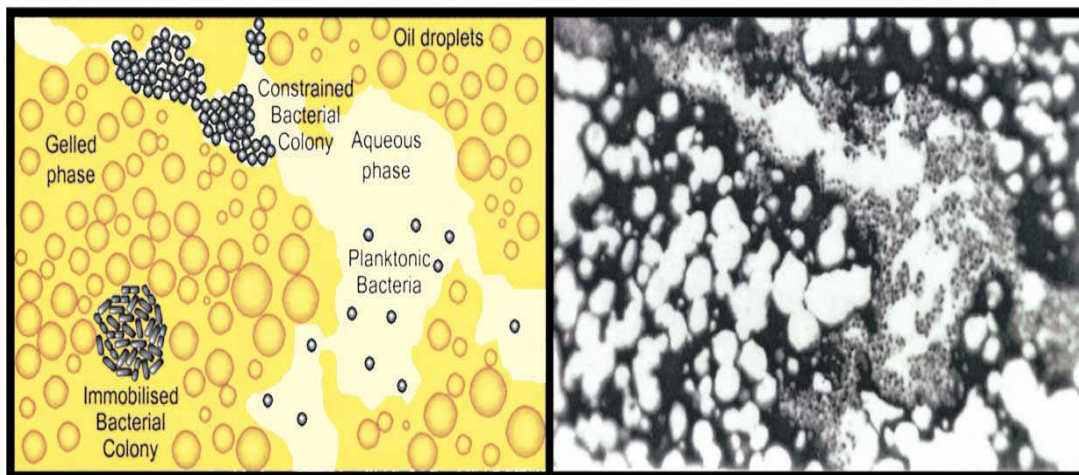
##### **1.4.1.1 Site and form of growth**

Microbial growth in foods takes place in the aqueous phase and is affected by the microstructural features of this phase. The aqueous phase may be uniform throughout food or particular food regions may have different structures (Wilson et al., 2002). Examples of this microarchitecture in foods include liquids (e.g. soups and juices), gels (e.g. pâté and cheeses made with low-fat milk), oil-in-water emulsions (e.g. milk, mayonnaise and dairy cream), water-in-oil emulsions (e.g. butter and margarine), gelled emulsions (e.g. sausages and whole-milk cheeses) and surface (e.g. meat and vegetables tissues).

According to the food microarchitecture, microorganisms may grow as individual free-floating (planktonic) cells and if accompanied by motility they have accessibility to

preferred regions of the food, or immobilized and constrained to grow as microcolonies (Figure 1.2) (Katsaras and Leistner, 1991; Delaquis et al., 1992; Brocklehurst et al., 1995; Wimpenny, et al., 1995; Wilson et al., 2002). The mode of growth (immobilized versus planktonic growth) affects significantly the growth dynamics of microorganisms especially when approaching environmental conditions stressful for growth (Robins and Wilson, 1994; Brocklehurst et al., 1997; Wilson et al., 2002; Meldrum, et al., 2003; Koutsoumanis et al., 2004).

Microbial cells, apart from immobilizing on food surfaces (Mattila and Frost, 1988; Katsaras and Leistner, 1991), may attach to food-contact surfaces, form microcolonies and ultimately establish a microbial community, known as biofilm (Carpentier and Cerf, 1993; Costerton et al., 1995). Biofilm cells exhibit an altered phenotype with respect to growth rate and gene transcription (e.g. acquisition of resistant phenotypes) compared to their planktonic counterparts. This is of primary importance for the food industry, as pathogens may attach to food-contact surfaces and equipment and lead to cross-contamination of foods (Kumar and Anand, 1998). Special features of the biofilm mode of growth will be discussed in detail in section 1.4.3.



**Figure 1.2 Schematic diagram (left) and light micrograph (right) showing different environments (microstructures) for microbial growth in a sample of cheese. (Adapted from Wilson et al., 2002)**

#### **1.4.1.2 Food structure induced mechanisms that affect microbial growth**

According to Robins and Wilson (1994), several mechanisms may account for the restriction of microbial growth in structured foods, with the most important being nutrient availability and supply, accumulation of metabolic-end products in the site of growth and physical constraints on motility and expansion of microcolonies.

##### *Nutrient availability and supply*

The supply of nutrients to the colony occurs by means of diffusion from the bulk medium, and depending on the nutrient requirements of bacterial cells, may limit growth (Robins and Wilson, 1994). Stecchini et al. (1998) investigated the influence of agar concentration on the growth responses of *Bacillus cereus* and on diffusivity of glucose and a low-molecular-weight protein. Increasing agar concentration did not influence the diffusivity of sucrose or the small protein ( $6.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$  and  $6.2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ , respectively) but did influence the colony growth, with smaller colonies and of lower viable cells per colony observed with increasing agar concentration. They concluded that the microbial growth in the agar concentration range tested was influenced by the reduction in pore size and the increase in the bundle thickness of the gel, but not by limited diffusion of nutrients. However, that seems to be the case only for low-molecular-weight molecules (e.g. glucose and small proteins) as the diffusion of high-molecular-weight molecules (e.g. dextran) may be restricted even at very low agar concentrations (0.5%) (Lebrun and Junter, 1994). At this point it should be noted that limitations in nutrient diffusion are more enhanced on the surface than in the matrix of foods (Brochlehurst et al., 1997).

##### *Physical constraints on motility and expansion of microcolonies*

Food microstructure may impose a physical restraint on the increase of colony size if the expansion rate of the growing colony is limited by its ability to displace the structural components of the food (Robins and Wilson, 1994). Growth may occur from any position of the colony and, as a consequence, colony structure is subjected to rearrangement in order to create space for the new cells. The ability of the colony to rearrange by displacing the microstructural components of the food depends on the

mechanical properties of the food matrix and microbial cell interactions. The range of pore sizes and bundle thickness of a gel influences the colony size, morphology and maximum cells concentration. Robins and Wilson (1994) reported that structural constraints due to immobilization of *Yersinia enterocolitica* cells in gelatin gel resulted in a reduced exponential growth rate and population yields compared to liquid medium. Physical constraints imposed by the food structure on the morphology and dimension of the colonies seem to be more enhanced in case of motile bacterial. For motile strains, the microcolony morphology may change from large and diffuse through compact and round, up to a more compact and elliptic with increasing gel concentration. In case of non-motile strains no decrease in colony size can be observed with increasing agar concentration, although a change from a compact lobed, to a compact lenticular structure may occur (Mitchell and Wimpenny, 1997; Wimpenny et al., 1995; Antwi et al., 2006).

#### *Local accumulation of microbial metabolites*

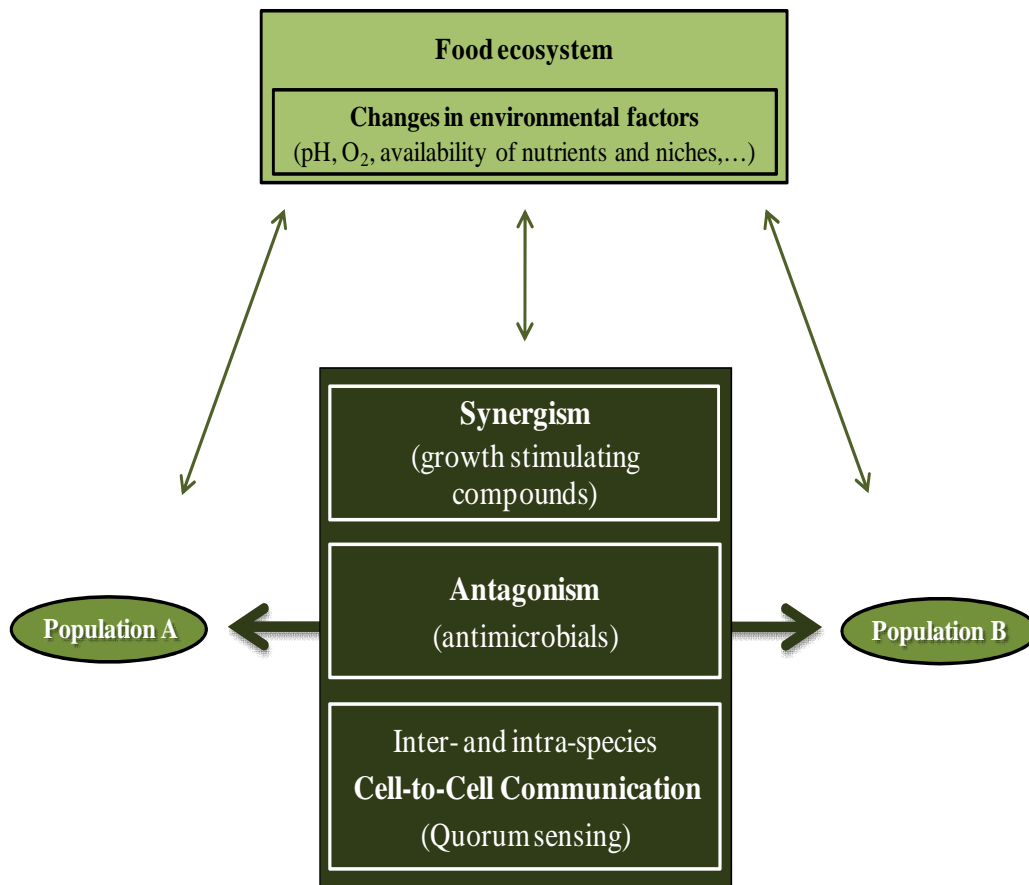
The immobilization of microorganisms on a food surface or matrix may lead to alteration of the local microenvironment within or around the growing colony due to accumulation of microbial metabolites, especially if diffusion of the toxic products is limited. It has been shown that the size of a bacterial colony increases exponentially first and then linearly, and this can be attributed to transport limitations both of nutrients and waste products (Wimpenny et al., 1995). Localization of metabolic-end products directs the formation of gradients, for example, of redox potential, pH and nutrients and as a result some regions of the colony, usually those lying in the centre, may present a reduced metabolic activity and thus slower or no growth (Wimpenny et al., 1995; Walker et al., 1997; Skandamis et al., 2000; Meldrum et al., 2003). Because of the local accumulation of metabolic end-products interactions between colonies may occur and slow down the growth (Malakar et al., 2000). In an isolated colony production of acidic metabolites creates gradients of pH in the colony. Interactions in the colony itself (intra-colony interaction) occur and cells in the outer edge of the colony grow the fastest as conditions there are more favorable. When the colony density is high, the size of the growing colonies is small, due to constraints in expansion, and as a result the acid produced by one colony may reach and affect the neighboring colony in shorter time



compared to an isolated colony. Consequently, the surrounding medium becomes saturated with organic acid and interactions between the colonies (inter-colony interactions) approximate to those encountered in homogeneous environments (Walker et al., 1997; Malakar et al., 2000).

#### **1.4.2 Microbial interactions**

A microbial ecosystem represents a delicately balanced association of microorganisms each interacting with and influencing the other members of the association (Figure 1.3). Several types of microbial interactions have been described in complex food ecosystems (Boddy and Wimpenny, 1992) which can be broadly classified on the basis of their effects as being detrimental or beneficial. Typical examples of microbial interactions in foods are antagonism (i.e. one population benefits at the expense of the other, and the latter is restricted) and synergism (i.e. one or both populations benefit in the presence of other). Recently, it became evident that bacteria possess sophisticated systems of cell-to-cell communication (generically termed as quorum sensing) that enable them to interact with the same or different bacterial species via specific diffusible signal molecules (Williams et al., 2001).



**Figure 1.3 Schematic representation of interactions occurring between microbial populations in food ecosystems (Modified from Leroy and De Vuyst, 2007).**

#### **1.4.2.1 Synergism and antagonism**

In any food ecosystem, the initial microbial contamination and environmental conditions during processing, transportation and storage influence the establishment of a particular microbial association and determine the rate of attainment of a climax population of so called “ephemeral (specific) spoilage microorganisms” (Nychas et al., 2007, 2008). The growth and metabolic activity of the ephemeral microbial association leads to the manifestation of changes in foods that are characterised as spoilage. The ephemeral spoilage microorganisms are usually expected to be several logarithmic cycles higher than pathogens adventitiously introduced into foods and as a consequence

are assumed to have a competitive advantage for nutrient uptake. This may lead to the alteration of the intrinsic properties (e.g. pH, water activity, atmosphere, nutrient catabolism and production of inhibitory metabolites) of foods in ways that the survival and growth of pathogens may be stimulated (Farrag and Marth, 1989; Marshall et al., 1992; Tsigarida et al., 2000) or suppressed (Drosinos and Board, 1994; Cheng et al., 1995; Buchanan and Bagi, 1997; Fett, 2006; McKellar, 2007).

Synergistic microbial interactions may frequently occur in food environments. Examples include the degradation of complex proteins and carbohydrates by some spoilage organisms to produce simple substrates for the growth of pathogenic organisms, utilisation of organic acids by yeasts and moulds to favour the growth of bacteria, production of micronutrients by some species that will assist the growth of other species and alteration of the microclimate of the food system (Fleet, 1999). *L. monocytogenes* growth, for instance, has been found to be enhanced through the degradation of proteins by *Pseudomonas* spp. that leads to the production of simple nutrients (protein hydrolysates) (Farrag and Marth, 1989; Marshall et al., 1992). Moreover, removal of oxygen by a Gram-negative microflora allowed the growth of the anaerobic *Clostridium botulinum* (Huss et al., 1980).

Antagonism between spoilage microorganisms and foodborne pathogens is probably the most well studied type of microbial interactions as it provides a potentially simple approach to limiting the growth of pathogens in food ecosystems. Several microorganisms important in food spoilage have been shown to inhibit pathogenic bacteria. Dominant *Pseudomonas*, for example, in aerobically stored foods may interfere with pathogens by lengthening their lag phase, slowing growth or suppressing the maximum population yields (Buchanan and Bagi, 1999). Possible mechanisms that account for the antimicrobial effect of pseudomonads are thought to be competition for nutrients and space, production of secondary metabolites known as siderophores or production of unidentified substances (Cheng et al., 1995; Fett, 2006; McKellar, 2007). In fermented foods or foodstuffs stored under anoxic conditions, lactic acid bacteria may grow quickly and outcompete the growth of the undesired microorganisms such as *L. monocytogenes*, *E. coli* and *Salmonella* spp. (Drosinos and Board, 1994; Adams and

Nicolaides, 1997; Buchanan and Bagi, 1997). The inhibitory action of lactic acid bacteria against pathogens has been suggested to be the result of the decrease of the local pH by the production of organic acids, inhibitory action of undissociated organic acid molecules, competition for nutrients and production of specific antibacterial substances such as bacteriocins (Adams and Nicolaides, 1997).

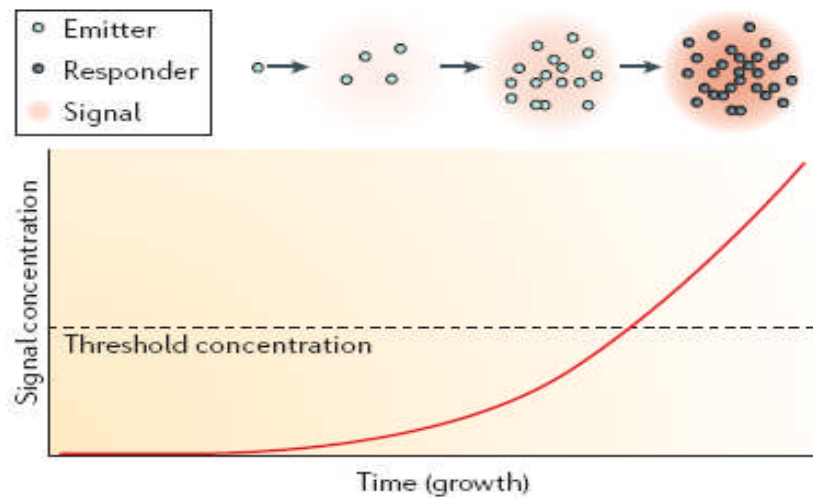
Despite the plethora of studies concerning microbial interactions between spoilage and pathogenic bacteria the mechanisms that govern the competitive advantage of the diverse spoilage bacteria are poorly understood. Possible reasons for this may include inadequate quantitative knowledge on the diversity, taxonomic identity and spatial distribution of bacterial species in foods, relationship between growth and activity of individual (specific) microorganisms and product safety/quality, and biochemical and physiological data on the food colonisation process (Fleet, 1999).

#### **1.4.2.2 Cell-to-cell communication (Quorum sensing)**

Bacteria have most often been considered as populations of cells that act individually, but it is now increasingly apparent that there is much interaction and communication among neighboring cells (Keller and Surette, 2006).

In most natural ecosystems including foods, microorganisms have to commingle with each other, compete for nutrients and survive in growth-restrictive environments. In response to these challenges bacteria coordinate a communal behavior as a function of population density through the synthesis of low-molecular-weight diffusible signalling molecules. When a critical level of these molecules in the extracellular environment is reached, microorganisms sense that a sufficient level of “quorum” of bacteria is present in the specific niche and consequently respond through a coordinated expression of certain genes that allows them to mount a unified response favorable to population survival (Figure 1.4) (Smith et al., 2004). Such a response may include regulation of virulence, competence for DNA uptake, biofilm development and sporulation (Balaban and Koyfman, 2001).

Several types of bacterial signalling molecules have been now described. Generalizing, these can be divided into three broad categories: (i) N-acyl homoserine lactones (AHLs) or autoinducer-1 (AI-1), produced and utilised by Gram-negative bacteria mainly for intraspecies communication (Whitehead et al., 2001), (ii) autoinducing peptides (AIPs), produced and used by Gram-positive bacteria for intraspecies communication (Sturme et al., 2002; Dunny and Leonard, 1997) and (iii) furanosyl borate diester or autoinducer-2 (AI-2), which is produced by both Gram-positive and Gram-negative bacteria and thought to serve as a universal language for inter- and intra-species communication (Federle and Bassler, 2003).



**Figure 1.4 Generic scheme for quorum sensing. In its simplest form, cell-to-cell signaling results from the production of signalling molecules by emitter cells and their accumulation in the surrounding environment. At some threshold concentration, the signaling molecules bind to receptors on or in the bacterial cell, leading to changes in gene expression in the responding cell. For intraspecies quorum sensing, the emitter and the responder are usually the same cells, as illustrated here (Adapted from Keller and Surette, 2006).**

### *Intraspecies cell-to-cell communication*

A great number of Gram-negative bacteria synthesize multiple AHLs. AHLs are characterized by a homoserine lactone ring which is N-acylated with a fatty acyl group at the C1 position. The N-acyl chain may vary in length, saturation level and oxidation state. Typically, the acyl chains range from 4 to 18 carbons, may contain double bonds, and often contain an oxo- or hydroxyl- substituent at the C3 position (Whitehead et al., 2001). AHLs are synthesized with the reaction of S-adenosyl-methionine (SAM); with an acyl-acyl carrier protein, which is typically carried out by an enzyme of the LuxI family of the AHL synthases, and sensed by the response transcriptional regulators of the LuxR family. The LuxR/AHL complex is responsible for up- or down- regulation of multiple target genes (Swift et al., 2001). Bacterial species may synthesise more than one type of AHL while the same type of AHL may be produced by representatives of different bacterial genera (Pearson et al. 1994, 1995; Eberl et al., 1996; Swift et al., 1997). Short-chain AHLs are generally diffusible throughout the bacterial membrane while long-chain AHLs seem to be actively transported in and out of the cells via efflux and influx systems (Whitehead, et al., 2001). Several factors may influence the concentration and type (i.e. length and substitution or not of the C3 of the acyl chain) of AHLs, including temperature, pH, NaCl, growth media, and bacterial growth phase (Gonzalez et al., 2001; Medina-Martinez et al., 2006, 2007 ).

In Gram-positive bacteria, cell-to-cell communication is accomplished via peptides or modified peptides (auto-inducing peptides - AIPs). AIPs are characterized by a small size (i.e. ranging from 5 to 26 amino acid residues), high stability, specificity and diversity, and can be linear or cyclic (Dunny and Leonard, 1997) These peptides are ribosomally synthesized as precursor peptides, subsequently processed to form the active mature peptide autoinducer signal molecule and then secreted via an ATP-Binding Cassette (ABC) transporter. Depending on whether the sensor is on the cell surface or cytoplasm, the peptides can exert their function either intercellularly or extracellularly (Dunny and Leonard, 1997; Sturme et al., 2002).

### *Interspecies cell-to-cell communication*

Bacteria not only communicate with members of the same species, but may also “eavesdrop” the “conversation” of other species and modulate their behaviour in response to signal molecules they do not synthesize. The only presently known family of signal molecules shared by more than 70 species of both Gram-negative and Gram-positive bacteria is autoinducer-2 (AI-2) (Federle and Bassler, 2003). AI-2 signal molecules are considered to be a universal language because they not only allow bacteria to respond to endogenously produced AI-2, but also to AI-2 produced by other bacterial species in the vicinity. The biosynthetic pathway for AI-2 has been described (Schauder et al., 2001). AI-2 is synthesized in three enzymatic steps from SAM. Following methyl transfer from SAM, S-adenosyl-homocysteine (SAH) is formed. Subsequently, Rfs enzymes remove adenine from SAH to form S-ribosyl-homocysteine (SRH). Finally the LuxS protein cleaves SRH to produce homocysteine (HC) and AI-2 precursor, 2,4-dihydroxy-2-methyldihydro-3-furanone (DHMF). The latter cyclises spontaneously and gives rise to a number of related furanone derivatives. The exact structure of AI-2 furanone has not yet been determined (Schauder et al., 2001). AI-2 production may be influenced by temperature and growth medium (Cloak et al., 2002; Brandl et al., 2005).

### *Quorum sensing and food safety*

Quorum sensing is one of the mechanisms used by bacteria to sense changes in their environment and coordinate gene expression in favour of survival of the population. Enhanced access to nutrients or environmental niches, mounting defensive responses against eukaryotic hosts and competing organisms (i.e. secretion of virulence factors), optimization of the ability of the cell to differentiate into morphological forms (i.e. biofilm formation, sporulation) and adaptation/survival in hostile, growth restrictive environments are some bacterial behaviours dictated by the use of signal-response systems (Balaban and Koyfman, 2001; Swift et al, 2001; Smith et al. 2004).

The role of cell-cell communication in food ecological niches has only recently received attention from food microbiologists and a growing body of evidence has been collected suggesting that bacterial food spoilage may be regulated by quorum sensing systems

(Smith et al. 2004; Ammor et al. 2008). Knowledge, however, concerning the role of cell-to-cell signalling on the growth, survival, and virulence expression of pathogens in food ecosystems and environments is scarce. Few studies have, thus far, investigated the influence of food system conditions on autoinducer signals production by foodborne pathogens (Cloak et al., 2002; Lu et al., 2004; Brandl, et al., 2005; Medina-Martinez et al., 2006, 2007) and the influence of these quorum sensing signals on the survival/growth of pathogenic bacteria in foods (Brandl et al., 2005; Soni et al., 2008). Soni et al. (2008), for example, reported that the survival and virulence expression of a LuxS mutant strain of *E. coli* O157:H7 was enhanced in the presence of AI-2. Similarly, production of AI-2 by *S. enterica* serovar Typhimurium contributed significantly to its fitness in chicken compared to the LuxS<sup>-</sup> mutant strain (Brandl et al., 2005).

It is challenging to understand which factors in foods may influence cell-to-cell signalling and how pathogens respond in the presence of signals produced by other quorated bacteria (Smith et al., 2004). This could potentially lead to identification of species-specific molecules and/or development of interventions that could be employed to control or inhibit the quorum sensing-regulated behaviours of pathogens, ultimately impacting food safety.

### **1.4.3 Bacterial mode of growth (Biofilms)**

During the last few decades one of greatest advances in the research field of microbiology was the recognition that bacteria prefer a community-based, surface-bound, sedentary lifestyle instead of living alone as free-floating organisms. The first predication of this theory derived from direct microscopic observations and quantitative recovery techniques in natural aquatic ecosystems, in which, more than 99% of bacteria were shown to grow as biofilms on a wide range of surfaces (Costerton et al., 1978).

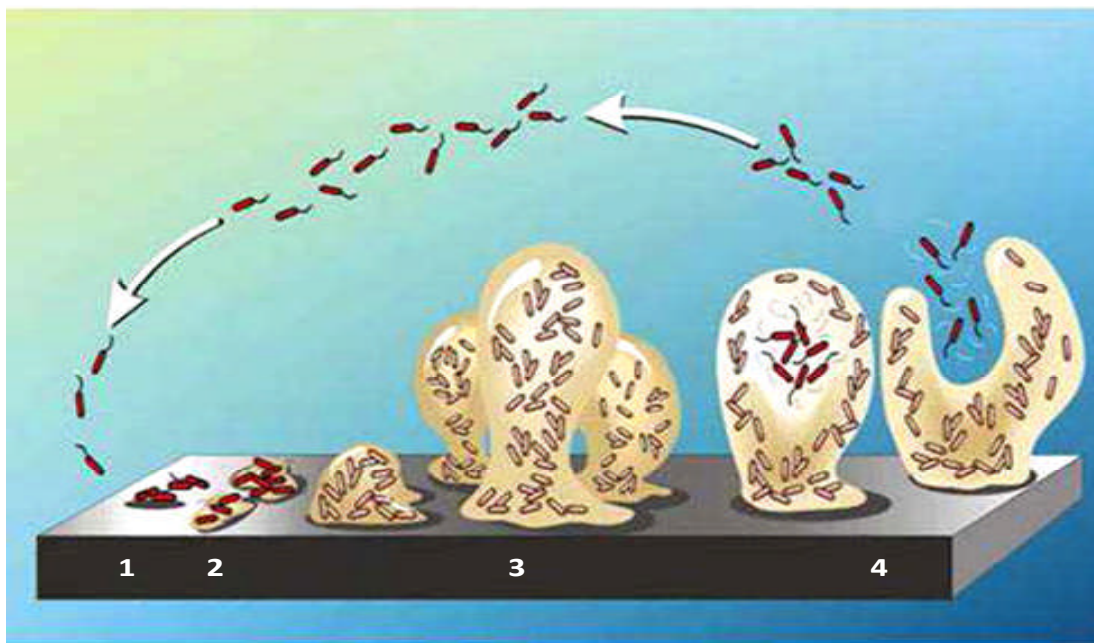
According to the most recent and integrated definition biofilm is «a microbiologically derived microbial community characterised by cells irreversibly attached to a substratum, or interface or to each other, embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002).



### 1.4.3.1 Biofilm life cycle

Biofilm formation is a dynamic process that involves a series of distinct developmental steps including the initial attachment to a surface, the formation of microcolonies, the maturation of microcolonies into an EPS-encased mature biofilm and finally the detachment and dispersal of cells (Figure 1.5).

Initial surface colonization (reversible attachment) depends on the transportation of microbial cells in close proximity with a surface. Bacterial transport can be passive or active. Passive transport is accomplished through physical mechanisms such as Brownian movement, gravitational forces and hydrodynamic forces of the bulk fluid. Active transfer is mediated by the bacterial surface organelles (e.g. flagella, pili and other surface adhesins) and chemotaxis.



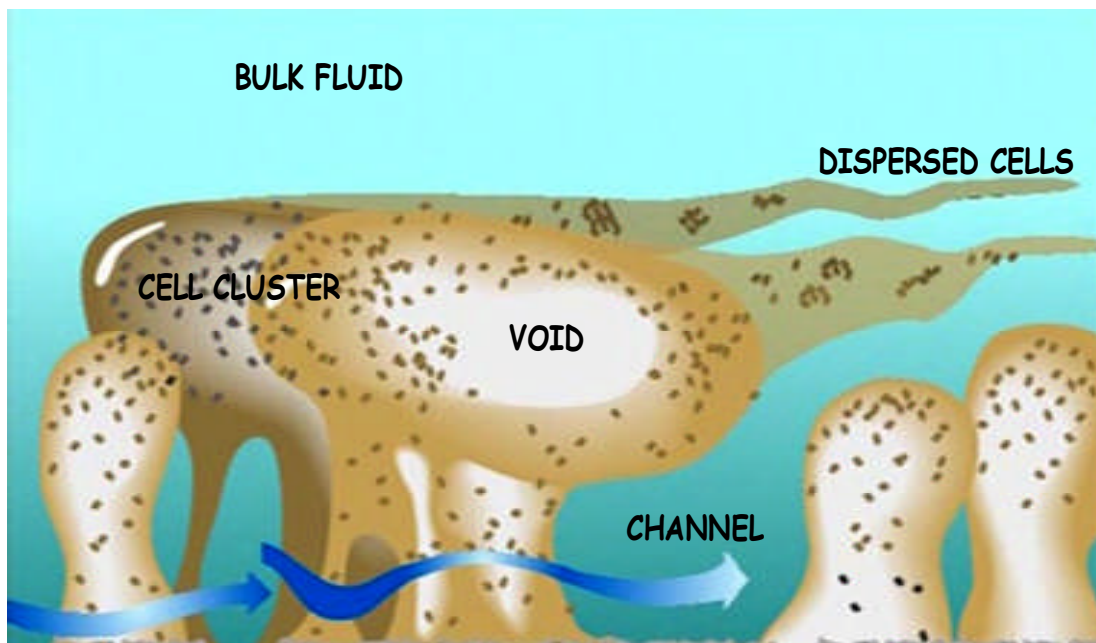
**Figure 1.5 Conceptual illustration of biofilm life cycle. Step 1: attachment of bacterial cells onto surface (reversible attachment), Step 2: growth of colonies and production of extracellular polymeric substances (irreversible attachment), Step 3: growth and maturation of biofilm architecture, and Step 4: detachment (dispersal of single cells or clusters) (Adapted from Centre for Biofilm Engineering, Montana State University, USA, <http://www.erc.montana.edu>).**

Once a bacterium cell reaches critical proximity to a surface (<1 nm), then the final adhesion depends on the net sum of attractive and repulsive forces between the microbial cell surface and the substratum. These forces include van der Waals and electrostatic forces, and hydrophobic interactions (Chmielewski and Frank, 2003).

Once attachment to a surface has been achieved, microorganisms must maintain contact with the surface and grow, in order to form a mature biofilm. The change from reversible to irreversible attachment is mainly accomplished through the production of EPS. Microbial EPS are biosynthetic polymers that can be highly diverse in chemical composition and may include polysaccharides, proteins, nucleic acids, and phospholipids (Wingender et al., 1999). Apart from the contribution of EPS in the reversible attachment process, various proteinaceous organelles (e.g. pili and flagella) have been shown to direct this phase (Stoodley et al., 2002).

The next step in biofilm formation, maturation, constitutes in the production of a complex and heterogeneous (in both space and time) microarchitecture (Costerton et al., 1994). The use of modern microscopy methods, and primarily of confocal scanning laser microscopy, led to the creation of a conceptual image of bacterial architecture as exists in natural environments. Although each microbial community presents a unique architecture, however, some structural attributes can be considered common. Recent studies have shown that the biofilm has a porous structure with capillary water channels within which the water and nutrients are distributed (Costerton et al., 1995). Living, fully hydrated biofilms contain microcolonies of bacterial cells that are in matrix enclosed “mushrooms” or “towers” and separated from other microcolonies by interstitial voids (water channels) (Figure 1.6). The architecture of biofilms is strongly affected by the hydrodynamic forces in the surrounding environment and the composition of microbial community (Costerton et al., 1995). Biofilm maturation depends on nutrient availability in the immediate environment, perfusion of those nutrients to cells within the biofilm and removal of waste. Moreover, pH, temperature, diffusion of oxygen, carbon source, and osmolarity are other important factors that control biofilm maturation (Carpentier and Cerf, 1993; Costerton et al., 1995).

The biofilm developmental life cycle comes full circle with cell dispersal. Dispersal is a general term that describes the release of bacterial cells (individual or in clusters) either by shedding daughter cells from actively growing cells, shearing of biofilm aggregates because of hydrodynamic forces or detachment as a result of cell-to-cell communication or low nutrient levels which allow bacteria to seek new nutrient-rich environments through unknown mechanisms (Donlan, 2002; Stoodley et al., 2002). The mode of dispersal affects the phenotypic characteristics of bacteria. Detached or sheared cells from the biofilm are likely to retain certain biofilm characteristics (e.g. antimicrobial resistance), whereas, cells that have been shed as a result of growth most likely revert quickly to the planktonic mode of growth (Donlan, 2002).



**Figure 1.6 Conceptual illustration of the heterogeneity of biofilm structure showing bacterial clusters, water channels and detached cells. Biofilm structure may vary within bacterial species and environmental conditions (Adapted from Centre for Biofilm Engineering, Montana State University, USA, <http://www.erc.montana.edu>).**

#### **1.4.3.2 Ecological advantages of biofilm formation**

In most natural ecosystems bacteria live in association with surfaces known as biofilms, which is the prevailing microbial lifestyle. Possible reasons for bacteria to adapt to this mode of growth instead of living as individual cells include protection from the environment, nutrient availability and metabolic cooperativity, transfer of genes, and cell-to-cell communication (Jefferson, 2004).

Bacterial cells residing within a biofilm experience a certain degree of shelter and homeostasis. Thus, living in a biofilm provides protection against environmental challenges, such as nutrient deprivation and starvation (Costerton et al., 1995), exposure to UV radiation, pH shifts and osmotic shock (Flemming, 1993), and exposure to antimicrobials (Lewis, 2001). The increased biofilm invulnerability is believed to be the result of various factors, acting alone or in combination. These include decreased microbial growth rate, gene expression that leads to a resistant phenotype (persister cells) and restricted penetration through the production of EPS (Lewis, 2001). EPS may prevent penetration and diffusion of antimicrobial agents from the surrounding milieu into the biofilm (Gilbert et al., 1997) while at the same time it has the ability to bind nutrients creating a nutrient-rich microenvironment within a nutrient-poor microenvironment (Costerton et al., 1994).

The highly permeable water channels interspersed throughout the biofilm provide an effective means of exchanging nutrients and metabolites with the bulk aqueous phase, thereby enhancing nutrient availability and removal of potentially toxic metabolic-end products (Costerton et al., 1995). In most natural ecosystems, biofilms consist of diverse populations of microorganisms. A range of metabolic interactions have been observed among microorganisms in biofilms, including mutualistic and commensal relationships, through the exchange of substrates and the removal or distribution of metabolic products. Moreover, metabolic interactions within biofilms may be facilitated by the spatial arrangement of interacting cells (Davey and O' Toole, 2000).

Horizontal gene transfer is crucial for evolution and genetic diversity of microbial communities and can be increased in environments where bacteria are attached to

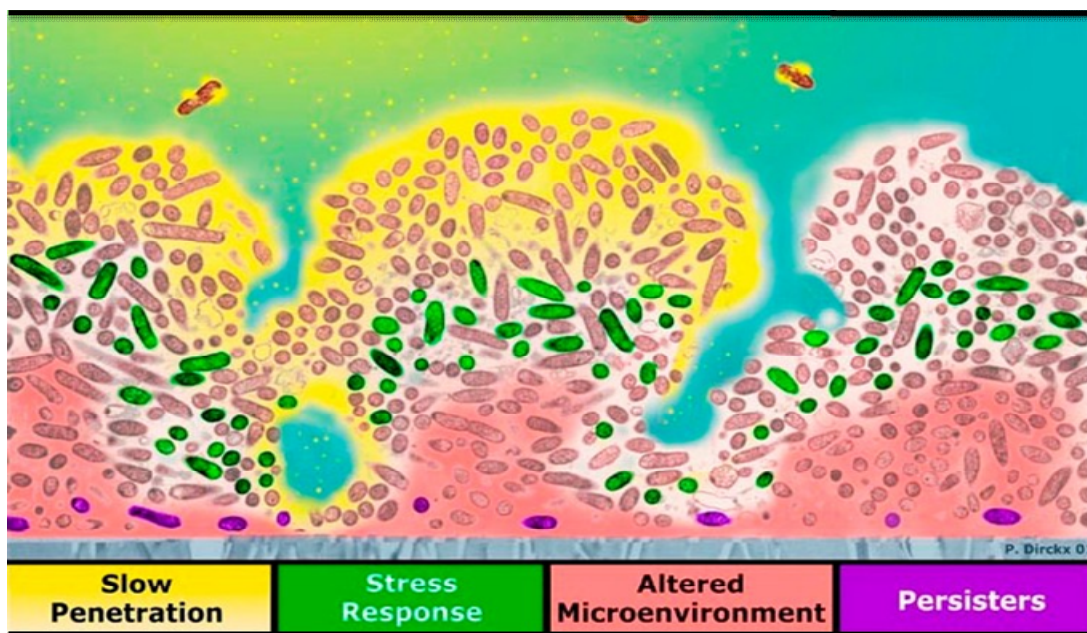
surfaces or interfaces. With bacterial conjugation being the most effective mechanism for dissemination of the genetic information, gene transfer enhances the physiologic and metabolic performance of complex biofilm communities (Hausner and Wuertz, 1999; Davey and O'Toole, 2000).

Up to now (2009), various studies have shown that initial attachment, maturation and breakdown of a biofilm can be regulated through a controlled expression of different genes which depends on the levels of the population and is controlled through the production of cell-to-cell signalling molecules (quorum sensing) (Eberl et al., 1996; McLean, et al., 1997; Davies, et al., 1998). The first example of “attached” (surface-associated) behavior depending on cell-to-cell communication was bacterial translocation (Elbert, et al., 1996). Cells of *Serratia liquefaciens* exposed to the surface of a semi-solid surface substratum resulted in highly motile cells which were then moved in groups colonizing the whole surface. The high degree of synchronization among cells within the “swarm” colony, while single cells exhibited uncoordinated movement, led to the assumption that this characteristic is a social phenomenon (Elbert et al., 1996). Davies et al. (1998) also showed that two cell-to-cell signalling systems in *P. aeruginosa*, lasR-lasI and rhlR-rhII that direct the synthesis of the diffusible extracellular signals, N-(3-oxododecanoyl)-L-homoserine lactone and N-butyryl-L-homoserine lactone, respectively, were involved in biofilm formation. Mutants of the pathogen, unable to produce both signals, formed a thin and uniform biofilm which lacked the typical biofilm architecture of the wild strains.

#### **1.4.3.3 Resistance of biofilms to antimicrobial treatments**

Biofilm resistance to various biocides, disinfectants and antibiotics is more enhanced compared to their planktonic counterparts (Stewart and Costerton, 2001; Donlan and Costerton, 2002). Indeed, biofilm cells can be up to 1000 times more resistant to antimicrobial agents (Costerton et al., 1999). Resistance mechanisms developed from biofilm cells exhibit a broad spectrum defense that is effective against many types of antimicrobials (Parsek and Singh, 2003). However, when cells detach from biofilms they acquire a non-resistant phenotype. This probably indicates that protection due to the biofilm mode of growth is not always the result of mutations or of acquisition of

resistance genes. Biofilm resistance is affected by the depth of the biofilm, the cellular density, the concentration and exposure time of the antimicrobial, the bacterial strain as well as the interactions between species in case of mixed-species biofilms (Lerliche et al., 2003; Burmolle et al., 2006). Possible mechanisms that may account for the increased resistance of biofilms to antimicrobial compounds are illustrated in Figure 1.7. Briefly, these mechanisms include the slow penetration and/or neutralization of the antimicrobial agent within the biofilm, the acquisition of resistant phenotypes, the altered microenvironment within the biofilm that contributes to slow microbial growth and the presence of persister cells (Lewis, 2001; Mah and O' Toole, 2001; Stewart and Costerson, 2001; Nikolaev and Plakunov, 2007).



**Figure 1.7 Proposed mechanisms of increased biofilm tolerance to antimicrobials (Adapted from Centre for Biofilm Engineering, Montana State University, USA, <http://www.erc.montana.edu>).**

#### **1.4.3.4 Biofilms in food-processing environments**

In food-processing environments the presence of inorganic and organic material absorbed onto food-contact surfaces facilitates bacterial attachment and subsequent biofilm formation (Hood and Zottola, 1997). The fact that parts of the biofilm may dislodge from the surface is of great concern to the food industry due to the risk for contamination of food products. The risk becomes even more serious as pathogenic microorganisms in biofilms may express an increased resistance to disinfectants (Carpentier and Cerf, 1993; Costerton et al., 1999; Lewis, 2001). Improperly cleaned and sanitized food-contact surfaces and equipment are considered to be the major sources of contamination. The transmission of pathogens may also result from aerosols produced during the cleaning of these surfaces. Other common sources involved in biofilm accumulation are the floors, waste water pipes, bends in pipes, etc (Kumar and Anand, 1998). However, biofilms are not always detrimental as cases in which attachment of pathogenic bacteria was inhibited through the formation on surfaces of biofilms constituting of technological bacteria, such as *Lactococcus lactis* and *Staphylococcus sciuri*, have been reported (Leriche et al., 1999; Leriche and Carpentier, 2000; Zhao et al., 2004). Biofilm formation by technological bacteria could potentially be used as an alternative and novel intervention strategy against settlement, growth and/or survival of pathogenic bacteria on food-contact surfaces and equipment of dairy or sausage-making plants in combination with appropriate hygienic operations.

## 1.5 AIM OF THE RESEARCH

This research provides insights into the role of food structure, composition, microbial interactions and mode of growth on the responses of foodborne pathogenic microorganisms.

### **The main questions addressed in this research were:**

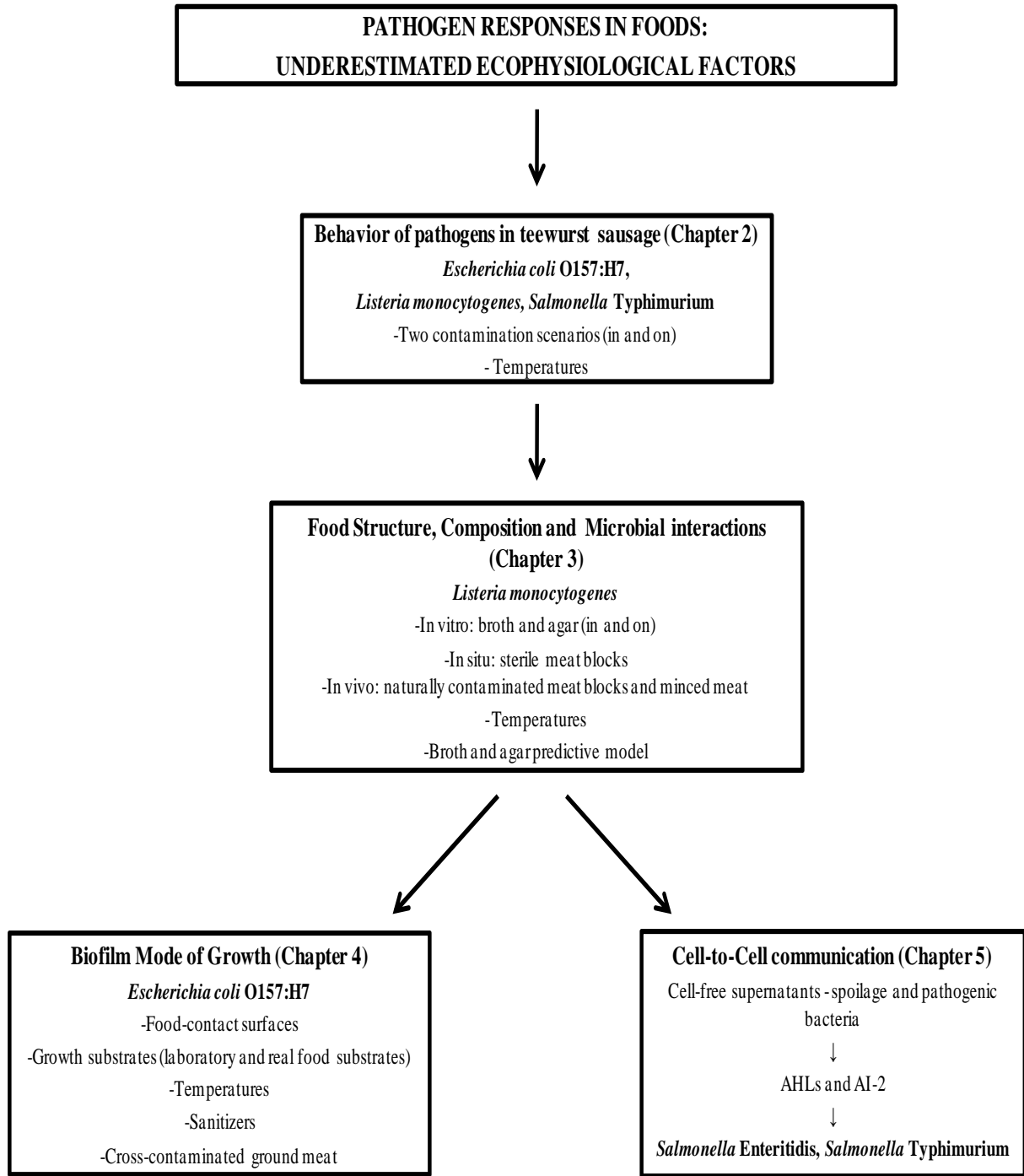
- Fate of foodborne pathogens in teewurst, a raw spreadable sausage, that represents a complex food ecosystem;
- Influence of food microstructure, composition and microbial interactions on the growth kinetics of *L. monocytogenes*;
- Potential of quorum sensing signalling compounds to affect the physiological aspects of *Salmonella* growth;
- Formation, destruction and removal of biofilms of *E. coli* O157:H7 on food-contact surfaces under conditions encountered in food environments.

### **The following studies have been carried out to achieve these objectives:**

- The responses of three pathogenic bacteria namely *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* Typhimurium were investigated in teewurst spreadable sausage under two different inoculation scenarios and at four different storage temperatures (1.5, 4, 10, 21°C).
- *L. monocytogenes* was inoculated into or onto laboratory media (laboratory broth and agar medium) and real food (sterile or naturally contaminated meat blocks and naturally contaminated minced meat). Growth was monitored at 3.5, 5, 10, 15 and 20°C. Data collected from growth in laboratory media were used to generate models describing the effect of temperature on the growth kinetics of the pathogen. The performance of each model was evaluated against the observed growth in the rest of the substrates.



- Cell-free supernatants of *Serratia proteamaculans*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* strains were prepared and tested for the presence of quorum sensing signalling molecules (AHLs and AI-2) using different bacterial biosensors and thin-layer chromatography. The effect of these signalling molecules on the growth kinetics of two *Salmonella* serotypes was evaluated by using conductance measurements.
- Nine different strains of *Escherichia coli* O157:H7 were assessed for their ability to attach and form biofilms on stainless steel under two inoculation scenarios. A multi-strain composite of the pathogen was further utilized to examine the attachment and subsequent biofilm formation on stainless steel and high-density polyethylene surfaces under the effect of laboratory and real food (liquid and solid) substrates and two storage temperatures. The efficacy of two sanitizers on biofilms formed on acetal surfaces and the potential of survivor cells to survive and/or grow in cross-contaminated meat was also assessed.



**Figure 1.8 Schematic representation of the contents of this thesis.**

## **CHAPTER 2**

# **BEHAVIOR OF *Escherichia coli* O157:H7, *Listeria monocytogenes*, AND *Salmonella* Typhimurium IN TEEWURST, A RAW SPREADABLE SAUSAGE**



## Chapter 2

### **Behavior of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium in teewurst, a raw spreadable sausage**

*Part of the work described in this chapter has been accepted for publication in the International Journal of Food Microbiology (The experimental work has been performed by the author of this thesis)*

#### **ABSTRACT**

The fate of *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli* O157:H7 were separately monitored both in and on teewurst, a traditional raw and spreadable sausage of Germanic origin. Multi-strain cocktails of each pathogen (ca. 5.0 log CFU/g) were used to separately inoculate teewurst that was stored at 1.5, 4, 10, and 21°C. When inoculated into commercially-prepared teewurst batter, in general, the higher the storage temperature, the greater the reduction in pathogen levels. Depending on the storage temperature, pathogen levels in the batter decreased by 2.3 to 3.4, ca. 3.8, and 2.2 to 3.6 log CFU/g for *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*, respectively, during storage for 30 days. When inoculated onto both the top and bottom faces of sliced commercially-prepared product, the results for all four temperatures showed a decrease of 0.9 to 1.4, 1.4 to 1.8, and 2.2 to 3.0 log CFU/g for *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*, respectively, over the course of 21 days. With the possible exceptions of salt and carbohydrate levels, chemical analyses of teewurst purchased from five commercial manufacturers revealed only subtle differences in proximate composition for this product type. The obtained data established that teewurst does not provide a favourable environment for the growth/survival of *E. coli* O157:H7, *S. Typhimurium*, or *L. monocytogenes* inoculated either into or onto the product.

## 2.1 INTRODUCTION

Teewurst (Figure 2.1) is a traditional sausage of Germanic origin, typically made from pork and beef, which is characterized by a soft spreadable texture. It is usually produced by small producers and sold under refrigeration as a raw spreadable meat (USDA/FSIS, 1993, 2005). At present, there is a general lack of criteria for both the manufacture and the compositional descriptions for fresh and raw spreadable sausages, including teewurst (Islam and Jockel, 2005).

Teewurst is grouped with other meat products such as mettwurst that display a relatively low acid content (e.g., pH 5.3-5.5) and high moisture content (e.g.,  $a_w \geq 0.95$ ) (Brown, 2000). Processing and preparation of this product does not typically include any heat treatment or antimicrobial interventions other than the salts, spices, nitrites, and perhaps phenolics contributed by liquid smoke, that are added directly to the batter (Brown, 2000). The teewurst manufactured in the USA is the same as the teewurst produced in Germany, unless it is cooked, as is practiced by some manufacturers. As USA regulations stipulate, true product names and a “safe handling statement” must be accurately affixed to the label to provide consumers with the ability to choose between “teewurst uncooked, cured meat spread” or “cooked teewurst”, with the former being “raw”.

The association of teewurst with foodborne illnesses in recent years is well documented (Ammon et al., 1999, Werber et al., 2006). In Germany, consumption of raw spreadable sausages, including teewurst, was identified as a risk factor for sporadic illnesses associated with Shiga toxin producing *E. coli* (STEC) in persons aged 10 years or older (Werber et al., 2006). Similarly, a large outbreak (28 cases, 3 deaths) of haemolytic uremic syndrome (HUS) caused by a sorbitol-fermenting strain of *E. coli* O157:H- was associated with consumption of teewurst, a raw pork product, and mortadella, a cooked pork product (Ammon et al., 1999). Although teewurst is intended to be cooked by the consumer, its production includes ingredients such as nitrites that cause the raw sausage to appear as a ready-to-eat (RTE) product; therefore, teewurst is notoriously eaten without proper cooking, either by preference or by misperception. In a survey conducted

in Germany in 2001 related to knowledge and handling of raw meat, and in particular teewurst, ca. 50% of the 510 participants reported eating teewurst and, somewhat surprisingly, only ca. 36% of them recognised it as a raw meat product (Bremer et al., 2005).

Thus, this study was conducted to evaluate the behaviour of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* inoculated either into the batter or onto the surface of sliced teewurst, both of which were subsequently stored under aerobic conditions at refrigeration and abuse temperatures. Proximate composition analyses of commercial teewurst produced by five relatively small processors were also conducted to address the potential variety and range of chemical traits, since a standard of identity does not currently exist for this product.



**Figure 2.1 Teewurst, a raw spreadable sausage.**

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Bacterial strains**

Multi-strain cocktails of *L. monocytogenes* (MFS2, MFS102, MFS104, MFS105, and MFS110), *E. coli* O157:H7 (EC505B, C7927, and SLH21788), and *S. Typhimurium* (H3278, G7601, H3402, H2662, H3380, and G8430) were used in this study to account for potential variation (Porto-Fett et al., 2008). All strains were individually maintained at -80°C in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) plus 10% glycerol (Sigma Chemical Co., St. Luis, Mo.). A portion of ≤100 µl of a frozen suspension of each isolate was streak plated onto BHI agar and incubated overnight at 37°C. A single colony of each isolate was then individually transferred into 50 ml of BHI broth and incubated at 37°C overnight with shaking at 100 rpm. A 100 µl portion was thereof added to 50 ml of fresh BHI broth and cells were incubated for additionally 18 h with shaking. The multi-strain composite for each pathogen was prepared by combining equal volumes of the individual cell suspensions to yield ca. 9.0 log CFU/ml for each pathogen.

### **2.2.2 Formulation and manufacture of teewurst sausage**

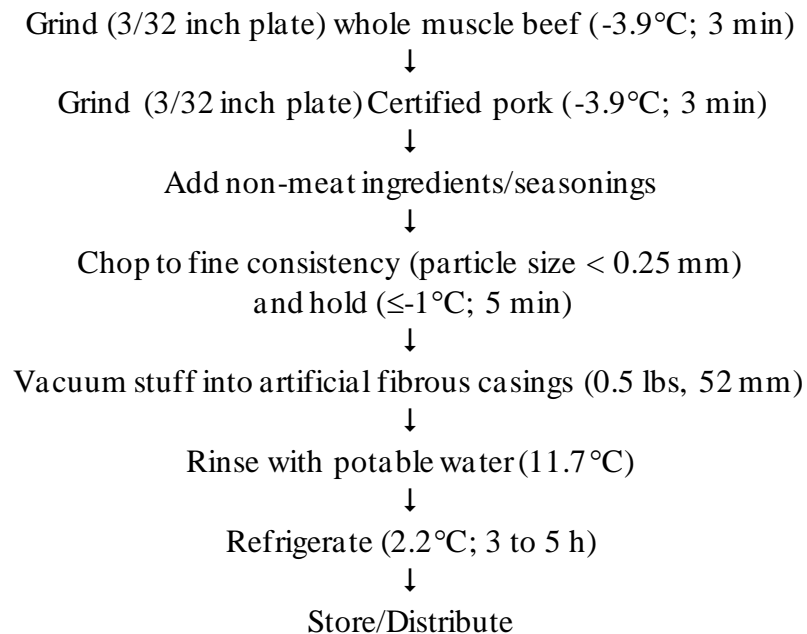
The formulation of teewurst batter, as purchased from a local manufacturer (Ernst A. Illg Meats, Inc.; Chalfont, PA), consisted of certified pork trimmings (27.22 Kg; fat-lean ratio 70%-30%), full muscle beef (18.14 Kg; fat-lean ratio 70%-30%) and 1.74 Kg of the following non-meat ingredients: seasoning spices (First Spice Mixing Co., Long Island City, NY), sodium nitrite curing salt, liquid smoke flavoring, paprika, cardamom and sugar. The manufacturing process for this brand of teewurst is shown in Figure 2.2.

### **2.2.3 Inoculation of teewurst batter**

To simulate contamination at the processing plant, three batches (one batch per trial) of freshly-processed teewurst batter were separately inoculated with ca. 5.2 log CFU/g of each multi-strain pathogen cocktail. After inoculation, the batter was mixed at ambient temperature (22 ± 1°C) using a commercial countertop mixer (Univex SRM12; Salem, NH) for ca. 2 min to ensure for relatively even distribution of the inoculum. The batter



was stuffed using a commercial (manual) stuffer (D-73779; Dick, Deizisau, Germany) into 4.5 cm diameter artificial “fibrous” casings (F Plus; Walsroder GMBH, Germany) in portions of ca. 100 g. The resulting chubs were stored at 1.5, 4, 10, or 21°C for up to 30 days. In each of the three trials two chubs were sampled at each sampling interval (N = 3 trials; n = 2 replicates/chubs per sampling interval per trial). It should be noted that the terms “batter” and “chub” herein refer to teewurst inoculated prior to stuffing.



**Figure 2.2 Flow diagram describing the teewurst manufacturing process used in this study.**

#### **2.2.4 Inoculation of the surface of teewurst slices**

To simulate post-process contamination in the home or in a food service establishment, three batches (one batch per trial) of freshly-processed teewurst were obtained from a collaborating manufacturer as above. Teewurst was transferred aseptically from the original packages onto sterile styrofoam trays (1012S; Genpak, Glens Falls, NY) and sliced (ca. 20 g each slice, ca. 5 cm diameter) with the aid of an ethanol-sterilized knife. Individual slices were placed onto styrofoam trays (Genpak) and separately inoculated on the top surface of each slice with 50 µl of each multi-strain pathogen cocktail. Cells were then distributed with the aid of a sterile plastic cell spreader (Midsci; St. Luis, MO). The trays containing the inoculated teewurst were placed into a biological safety cabinet and held for ca. 15 min at ambient temperature ( $22 \pm 1^\circ\text{C}$ ) to allow for the inocula to better attach to the meat slices. Next, the slices were inverted and the process was repeated on the opposite side. The final concentration of each pathogen was ca. 4.5 log CFU/g. Inoculated slices were then placed into sterile polyethylene bags. The bags were stored at 1.5, 4, 10, or 21°C for up to 21 days. In each of the three trials two bags/slices were sampled at each sampling interval (N = 3 trials; n = 2 replicates/slices per sampling interval per trial).

#### **2.2.5 Microbiological analyses**

Initial and final populations of total plate count (TPC) and total lactic acid bacteria (LAB) were enumerated on slices and in chubs as follows. A total of three slices (ca. 20 g each) or three chubs (ca. 5 g each), from each of the three trials/batches tested, were separately transferred into plastic filter stomacher bags (Spiral Biotech, Norwood, MA) containing 15 or 45 ml of 0.1% sterile peptone water (Difco, Becton, Dickinson and Co., Sparks, MD), respectively, and stomached for ca. 2 min (Stomacher 400; Seward, Cincinnati, OH). The TPC were enumerated by spread-plating 100 µl of the resulting slurry, with or without prior dilution in sterile peptone water, onto Brain Heart Infusion agar plates (BHI; Difco) and aerobic incubation at 30°C for 72 h. For enumeration of LAB, appropriate dilutions of the slurry were spread-plated (100 µl) onto deMan Rogosa Sharpe agar (MRS; Difco) and incubated anaerobically at 37°C for 72 h (10.1% carbon dioxide, 4.38 % hydrogen and the balance in nitrogen; Bactron IV Anaerobic/Environmental Chamber; Sheldon Manufacturing Inc., Cornelius, OR). For

enumeration of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* on teewurst slices or teewurst chubs the inoculated samples were treated as above and appropriate dilutions of the resulting slurry were surface-plated (100  $\mu$ l) onto Modified Oxford agar for enumeration of *L. monocytogenes* (MOX; Difco), MacConkey sorbitol agar for enumeration of *E. coli* O157:H7 (SMAC; Difco), and xylose lysine tergitol-4 agar for enumeration of *S. Typhimurium* (XLT4; Difco). Typical colonies of each pathogen were counted after aerobic incubation of plates at 37°C for 48 h (MOX) or 24 h (SMAC and XLT4). When pathogen numbers in batter decreased to  $\leq 1$  log CFU/g by direct plating, the presence or absence of the pathogens were determined by enrichment.

### **2.2.6 Physicochemical analyses**

At both the beginning and at the end of storage, control (non-inoculated) teewurst samples [N = 3 trials; n = 3 slices (ca. 20 g each) or n = 3 chubs (ca. 5 g each) per trial] were analyzed for pH and  $a_w$  by using a model 6000P pH/temperature electrode and a model 5500 pH meter (Daigger, Vernon Hills, IL) and a water activity meter (Decagon Aqualab Model series 3; Decagon Devices, Pullman, WA), respectively, according to the manufacturer's instructions. For the market basket survey, two chubs from each brand were analyzed to determine the proximate composition of the teewurst purchased from five commercial processors as determined by a commercial laboratory using methods approved and described by the Association of Official Analytical Chemists (McNeal, 1990).

### **2.2.7 Statistical analyses**

Microbial counts were transformed to logarithms before means and standard deviations were computed, and counts were reported in terms of log CFU/g. When bacterial counts in teewurst batter decreased to below the threshold of detection ( $\leq 1$  log CFU/g), a value of 1 was used for positive samples after enrichment for determination of the arithmetic mean. Differences in the proximate composition of teewurst sausages manufactured by different processors were evaluated using SPSS 12.0 for windows (SPSS, 1997). Least squares means separation was performed using the Tukey procedure at a significance level of  $p < 0.05$ .

### 2.2.8 Modeling inactivation kinetics

Counts of *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* inoculated either in teewurst batter or on slices and stored at 4°C (typical storage temperature for fresh and raw meat products) were modeled separately as a function of time for each of the three trials. The model of Geeraerd et al. (2000) originally developed for modelling microbial inactivation during a mild heat treatment was used. The explicit solution of the original dynamic model for static conditions (when the model parameters do not depend on time) which describes the cell concentration ( $N(t)$ ) reads as follows:

$$N(t) = (N(0) - N_{res}) \cdot e^{-k_{max}t} \cdot \left( \frac{e^{k_{max}S_1}}{1 + (e^{k_{max}S_1} - 1) \cdot e^{-k_{max}t}} \right) + N_{res}$$

where  $N(0)$  (log CFU/g) is the initial microbial population,  $N_{res}$  (log CFU/g) is the residual proportion of the population,  $k_{max}$  (1/days) is the specific inactivation rate and  $S_1$  (days) is the shoulder of the inactivation curve. For curve fitting the GInaFiT (Geeraerd and Van Impe Inactivation Model fitting Tool; Geeraerd et al., 2005), a freeware Add-in for Microsoft Excel<sup>®</sup> was used.

## 2.3 RESULTS

### 2.3.1 Microbiological analyses of teewurst

Direct plating of samples of control (non-inoculated) teewurst slices or control batter/chubs taken from each of the three trials/batches tested revealed the absence ( $\leq 0.2$  and  $\leq 1$  log CFU/g for teewurst slices and batter/chubs, respectively) of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*. These samples also tested negative for each of the same three pathogens following enrichment. The average initial TPC and LAB levels were  $7.2 \pm 0.7$  (ranging from 6.4 to 7.9) log CFU/g and  $5.7 \pm 0.9$  (ranging from 4.3 to 6.5) log CFU/g, respectively, for teewurst batter, whereas for teewurst slices the average initial TPC and LAB levels were  $6.5 \pm 0.7$  (ranging from 5.3 to 7.1) log CFU/g and  $5.5 \pm 0.9$  (ranging from 4.3 to 6.3) log CFU/g, respectively (Table 2.1). Average initial values of pH were  $5.87 \pm 0.25$  and  $6.18 \pm 0.19$  for teewurst batter and teewurst slices, respectively, whilst thereafter the pH decreased to about pH 4.39 and 4.78,

respectively, at the end of storage for both batter and slices. Average initial  $a_w$  values were about 0.960 ( $SD \leq 0.005$ ) for both slices and batter, and  $a_w$  changed relatively little over the storage period. For both slices and batter, numbers of TPC and LAB were similar at the end of the respective storage period (Table 2.1).

**Table 2.1 Evaluation of native flora, pH, and  $a_w$  of non-inoculated teewurst before and after storage**

Product Type	Storage time (days)	Temperature (°C)	TPC	LAB	pH	$a_w$
Teewurst	0		$7.2 \pm 0.7^*$	$5.7 \pm 0.9$	$6.18 \pm 0.19$	$0.957 \pm 0.004$
	30	1.5	$6.5 \pm 1.0$	$7.0 \pm 1.5$	$4.85 \pm 0.19$	$0.955 \pm 0.002$
		4	$6.3 \pm 0.7$	$6.6 \pm 1.2$	$4.58 \pm 0.15$	$0.953 \pm 0.002$
		10	$6.6 \pm 0.6$	$6.8 \pm 0.5$	$4.45 \pm 0.15$	$0.953 \pm 0.003$
		21	$6.7 \pm 0.3$	$6.6 \pm 0.5$	$4.39 \pm 0.10$	$0.945 \pm 0.003$
Teewurst slices	0		$6.5 \pm 0.7$	$5.5 \pm 0.9$	$5.87 \pm 0.25$	$0.960 \pm 0.005$
	21	1.5	$7.0 \pm 1.1$	$6.4 \pm 1.2$	$4.66 \pm 0.48$	$0.958 \pm 0.004$
		4	$7.6 \pm 0.7$	$7.2 \pm 0.7$	$5.13 \pm 0.81$	$0.958 \pm 0.003$
		10	$8.3 \pm 0.3$	$8.0 \pm 0.2$	$5.51 \pm 0.40$	$0.956 \pm 0.003$
		21	$8.1 \pm 0.1$	$8.0 \pm 0.1$	$4.78 \pm 0.76$	$0.952 \pm 0.002$

TPC; total plate count.

LAB; Lactic acid bacteria.

\* Values are mean log CFU/g  $\pm$  standard deviation; n=9 slices or chubs per sampling interval.

### **2.3.2 Viability of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* inoculated into teewurst batter or onto the surface of teewurst slices**

Regardless of the storage temperature, numbers of all three pathogens inoculated into the batter decreased after 30 days of storage (Table 2.2). With the exception of storage at 21°C which generated the greatest overall lethality, the observed reductions were not appreciably different for the other temperatures tested. More specifically, when chubs inoculated with *E. coli* O157:H7 prior to stuffing were subsequently stored at 1.5, 4, and 10°C pathogen numbers decreased by 2.3, 3.2, and 3.0 log CFU/g, respectively, after 30 days of storage. When chubs inoculated with *L. monocytogenes* prior to stuffing were stored at 1.5, 4, and 10°C pathogen numbers decreased by 2.2, 2.6, and 2.6 log CFU/g, respectively, after 30 days of storage. *E. coli* O157:H7 and *L. monocytogenes* levels decreased to below the level of detection by both direct plating ( $\leq 1$  log CFU/g) and enrichment after 25 and 18 days of storage at 21°C, respectively. *S. Typhimurium* levels decreased below detectable levels by direct plating within 15, 18, and 11 days at 1.5, 4, and 10°C, respectively. The absence of *S. Typhimurium* was confirmed by the inability to recover cells of this pathogen even by enrichment after 30 days at 1.5 and 4°C, after 21 days at 10°C, and after 11 days at 21°C. In general, *S. Typhimurium* was inactivated at a greater rate and to a greater extent (absent by enrichment within 11 days at 21°C) than *E. coli* O157:H7 or *L. monocytogenes* when inoculated into batter (Table 2.2).

Regarding survival on teewurst slices, pathogen numbers remained relatively unchanged after four days of storage for all temperatures tested (Table 2.3). Storage at 1.5, 4, 10, and 21°C for up to 21 days resulted in reductions of *E. coli* O157:H7 and *S. Typhimurium* from ca. 4.8 log CFU/g to 3.7, 3.7, 3.9, and 3.4 log CFU/g and from ca. 4.3 log CFU/g to 2.5, 2.8, 2.9, and 2.7 log CFU/g, respectively. When slices were inoculated with *L. monocytogenes* and stored at 1.5, 4, 10, and 21°C for up to 21 days, pathogen numbers decreased from ca. 4.5 log CFU/g to 1.8, 2.3, 1.8, and 1.5 log CFU/g, respectively. In general, *L. monocytogenes* was inactivated at a greater rate and to a greater extent than *S. Typhimurium* and *E. coli* O157:H7 at all temperatures tested. Moreover, the decrease in levels of *E. coli* O157:H7, *S. Typhimurium*, or *L. monocytogenes* when inoculated onto slices of teewurst was not appreciably ( $p > 0.05$ )

affected by the storage temperature (Table 2.3), that being so, similar reductions in pathogen levels were observed at all temperatures tested for a given pathogen.

The observed lethality of the three pathogens inoculated into batter or onto slices varied considerably among trials for all temperatures tested (Tables 2.2. and 2.3). In order to quantify this variation, microbial counts generated from storage of chubs and slices at 4°C were fitted using the model of Geeraerd et al. (2000). This temperature was selected as it is the typical storage temperature for fresh and raw meat products. Inactivation parameters for *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* are shown in Table 2.4. Differences in the residual population, specific inactivation rate, and shoulder duration were observed among the trials for each of the pathogens tested. These data suggest that factor/s intrinsic to teewurst and/or its production may affect differently the fate of the pathogens in the product during storage.

**Table 2.2 Counts (mean log CFU/g  $\pm$  standard deviation; n = 6 chubs for each sampling interval) of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* inoculated into teewurst batter**

Microorganism/ Temperature (°C)	Storage time (days)								
	0	3	8	11	15	18	21	25	30
<i>E. coli</i> O157:H7									
1.5	5.3 $\pm$ 0.0 <sup>aA</sup>	4.9 $\pm$ 0.3 <sup>abAB</sup>	4.9 $\pm$ 0.1 <sup>abA</sup>	4.8 $\pm$ 0.1 <sup>abA</sup>	4.3 $\pm$ 0.6 <sup>bcA</sup>	3.6 $\pm$ 0.6 <sup>cdA</sup>	3.5 $\pm$ 0.4 <sup>dA</sup>	3.4 $\pm$ 0.5 <sup>dA</sup>	3.0 $\pm$ 0.6 <sup>dA</sup>
4	5.3 $\pm$ 0.0 <sup>aA</sup>	5.1 $\pm$ 0.1 <sup>aAB</sup>	5.0 $\pm$ 0.1 <sup>aA</sup>	4.7 $\pm$ 0.3 <sup>aA</sup>	4.0 $\pm$ 0.3 <sup>bA</sup>	3.7 $\pm$ 0.5 <sup>bcA</sup>	3.6 $\pm$ 0.3 <sup>bcA</sup>	3.3 $\pm$ 0.4 <sup>cAB</sup>	2.1 $\pm$ 0.7 <sup>dA</sup>
10	5.3 $\pm$ 0.0 <sup>aA</sup>	5.2 $\pm$ 0.1 <sup>aA</sup>	4.6 $\pm$ 0.4 <sup>aA</sup>	4.5 $\pm$ 0.7 <sup>aA</sup>	2.9 $\pm$ 0.7 <sup>bB</sup>	2.7 $\pm$ 0.4 <sup>bAB</sup>	2.3 $\pm$ 1.1 <sup>bAB</sup>	2.5 $\pm$ 0.8 <sup>bB</sup>	2.3 $\pm$ 1.1 <sup>bA</sup>
21	5.3 $\pm$ 0.0 <sup>aA</sup>	4.8 $\pm$ 0.3 <sup>abB</sup>	3.5 $\pm$ 0.4 <sup>bcB</sup>	2.4 $\pm$ 1.1 <sup>cdB</sup>	1.7 $\pm$ 0.6 <sup>dC</sup>	2.3 $\pm$ 1.4 <sup>cdB</sup>	1.9 $\pm$ 0.9 <sup>cdB</sup>	ND <sup>1</sup>	ND
<i>S. Typhimurium</i>									
1.5	4.9 $\pm$ 0.1 <sup>aA</sup>	4.2 $\pm$ 0.5 <sup>aA</sup>	3.2 $\pm$ 1.6 <sup>abA</sup>	2.0 $\pm$ 1.5 <sup>bcA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	ND
4	4.9 $\pm$ 0.1 <sup>aA</sup>	4.3 $\pm$ 0.3 <sup>aA</sup>	3.1 $\pm$ 0.7 <sup>bA</sup>	2.5 $\pm$ 1.2 <sup>bA</sup>	1.2 $\pm$ 0.3 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	ND
10	4.9 $\pm$ 0.1 <sup>aA</sup>	3.8 $\pm$ 1.0 <sup>bAB</sup>	1.6 $\pm$ 0.5 <sup>cB</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	ND	ND	ND
21	4.9 $\pm$ 0.1 <sup>aA</sup>	2.6 $\pm$ 1.6 <sup>bB</sup>	1.1 $\pm$ 0.2 <sup>cB</sup>	ND	ND	ND	ND	ND	ND
<i>L. monocytogenes</i>									
1. <sup>o</sup>	5.4 $\pm$ 0.2 <sup>aA</sup>	5.3 $\pm$ 0.1 <sup>aA</sup>	4.5 $\pm$ 0.9 <sup>abA</sup>	4.2 $\pm$ 0.9 <sup>abcA</sup>	4.1 $\pm$ 0.8 <sup>bcA</sup>	3.5 $\pm$ 1.0 <sup>bcA</sup>	3.8 $\pm$ 0.3 <sup>bcA</sup>	3.6 $\pm$ 0.6 <sup>bcA</sup>	3.2 $\pm$ 0.5 <sup>cA</sup>
4	5.4 $\pm$ 0.2 <sup>aA</sup>	5.2 $\pm$ 0.1 <sup>abA</sup>	4.4 $\pm$ 0.7 <sup>bcA</sup>	4.6 $\pm$ 0.1 <sup>bA</sup>	3.7 $\pm$ 0.2 <sup>cdAB</sup>	3.5 $\pm$ 0.3 <sup>dA</sup>	3.1 $\pm$ 0.8 <sup>dA</sup>	2.9 $\pm$ 0.2 <sup>dA</sup>	2.8 $\pm$ 0.6 <sup>dA</sup>
10	5.4 $\pm$ 0.2 <sup>aA</sup>	5.3 $\pm$ 0.1 <sup>aA</sup>	3.9 $\pm$ 0.4 <sup>bAB</sup>	3.5 $\pm$ 0.6 <sup>bcAB</sup>	3.1 $\pm$ 0.3 <sup>cdB</sup>	3.4 $\pm$ 0.3 <sup>bcdA</sup>	3.1 $\pm$ 0.4 <sup>cdA</sup>	3.2 $\pm$ 0.4 <sup>bcdA</sup>	2.8 $\pm$ 0.6 <sup>dA</sup>
21	5.4 $\pm$ 0.2 <sup>aA</sup>	3.9 $\pm$ 0.9 <sup>bB</sup>	2.6 $\pm$ 1.0 <sup>cB</sup>	2.2 $\pm$ 1.4 <sup>cB</sup>	1.8 $\pm$ 0.6 <sup>cC</sup>	ND	ND	ND	ND

Means with different lowercase letters within a row are significantly different ( $p < 0.05$ ). Means with different uppercase letters within a column for each organism are significantly different ( $p < 0.05$ ).

<sup>1</sup>ND; not detected by either direct plating or by enrichment.



**Table 2.3 Counts (mean log CFU/g  $\pm$  standard deviation; n=6 slices for each sampling interval) of *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* inoculated onto teewurst slices**

Organism/ Temperature (°C)	Storage time (days)									
	0	1	2	4	6	8	11	13	18	21
<i>E. coli</i> O157:H7										
1.5	4.8 $\pm$ 0.2 <sup>aA</sup>	4.4 $\pm$ 0.2 <sup>abA</sup>	4.5 $\pm$ 0.2 <sup>abA</sup>	4.5 $\pm$ 0.1 <sup>abA</sup>	4.4 $\pm$ 0.1 <sup>abA</sup>	4.1 $\pm$ 0.3 <sup>abcA</sup>	4.4 $\pm$ 0.1 <sup>abcA</sup>	3.9 $\pm$ 0.7 <sup>bcAB</sup>	3.7 $\pm$ 0.4 <sup>cA</sup>	3.7 $\pm$ 0.5 <sup>cA</sup>
4	4.8 $\pm$ 0.2 <sup>aA</sup>	4.4 $\pm$ 0.3 <sup>abA</sup>	4.5 $\pm$ 0.1 <sup>abA</sup>	4.5 $\pm$ 0.1 <sup>abA</sup>	4.1 $\pm$ 0.2 <sup>bcdA</sup>	4.1 $\pm$ 0.2 <sup>bcdA</sup>	4.2 $\pm$ 0.3 <sup>bcA</sup>	4.1 $\pm$ 0.5 <sup>bcdA</sup>	3.7 $\pm$ 0.1 <sup>cdA</sup>	3.7 $\pm$ 0.3 <sup>dA</sup>
10	4.8 $\pm$ 0.2 <sup>aA</sup>	4.5 $\pm$ 0.3 <sup>abA</sup>	4.5 $\pm$ 0.2 <sup>abA</sup>	4.4 $\pm$ 0.2 <sup>abA</sup>	4.1 $\pm$ 0.1 <sup>abA</sup>	4.0 $\pm$ 0.3 <sup>bA</sup>	4.2 $\pm$ 0.3 <sup>abA</sup>	3.8 $\pm$ 0.0 <sup>bAB</sup>	4.5 $\pm$ 0.8 <sup>abA</sup>	3.9 $\pm$ 0.2 <sup>bA</sup>
21	4.8 $\pm$ 0.2 <sup>aA</sup>	4.6 $\pm$ 0.1 <sup>abA</sup>	4.6 $\pm$ 0.3 <sup>abA</sup>	4.2 $\pm$ 0.1 <sup>abcB</sup>	4.1 $\pm$ 0.2 <sup>abcA</sup>	3.8 $\pm$ 0.5 <sup>bcdA</sup>	3.7 $\pm$ 0.4 <sup>bcdA</sup>	3.0 $\pm$ 0.2 <sup>dB</sup>	3.4 $\pm$ 1.1 <sup>cdA</sup>	3.4 $\pm$ 0.5 <sup>cdA</sup>
<i>S. Typhimurium</i>										
1.5	4.3 $\pm$ 0.2 <sup>aA</sup>	4.1 $\pm$ 0.2 <sup>abAB</sup>	3.7 $\pm$ 0.2 <sup>abcA</sup>	3.7 $\pm$ 0.2 <sup>abcA</sup>	3.4 $\pm$ 0.4 <sup>bcA</sup>	3.2 $\pm$ 0.2 <sup>cA</sup>	3.1 $\pm$ 0.4 <sup>cdA</sup>	3.1 $\pm$ 0.2 <sup>cA</sup>	2.5 $\pm$ 0.4 <sup>dA</sup>	2.5 $\pm$ 0.5 <sup>dA</sup>
4	4.3 $\pm$ 0.2 <sup>aA</sup>	4.1 $\pm$ 0.2 <sup>abAB</sup>	4.0 $\pm$ 0.2 <sup>abA</sup>	3.6 $\pm$ 0.2 <sup>abcA</sup>	3.5 $\pm$ 0.2 <sup>bcdA</sup>	3.2 $\pm$ 0.1 <sup>cdeA</sup>	2.7 $\pm$ 0.3 <sup>eA</sup>	2.5 $\pm$ 0.2 <sup>eAB</sup>	2.9 $\pm$ 0.5 <sup>deA</sup>	2.8 $\pm$ 0.8 <sup>deA</sup>
10	4.3 $\pm$ 0.2 <sup>aA</sup>	3.9 $\pm$ 0.1 <sup>abcB</sup>	4.0 $\pm$ 0.1 <sup>abA</sup>	3.5 $\pm$ 0.3 <sup>abcA</sup>	2.9 $\pm$ 0.1 <sup>cB</sup>	2.9 $\pm$ 0.5 <sup>cA</sup>	3.1 $\pm$ 0.4 <sup>bcA</sup>	3.2 $\pm$ 0.1 <sup>bcA</sup>	3.1 $\pm$ 0.3 <sup>bcA</sup>	2.9 $\pm$ 0.6 <sup>cA</sup>
21	4.3 $\pm$ 0.2 <sup>aA</sup>	4.2 $\pm$ 0.2 <sup>aA</sup>	4.0 $\pm$ 0.3 <sup>abA</sup>	3.5 $\pm$ 0.1 <sup>abA</sup>	3.5 $\pm$ 0.5 <sup>abA</sup>	3.2 $\pm$ 0.5 <sup>abcA</sup>	2.6 $\pm$ 1.0 <sup>bcA</sup>	2.0 $\pm$ 0.6 <sup>cB</sup>	3.0 $\pm$ 1.4 <sup>abcA</sup>	2.7 $\pm$ 0.8 <sup>bcA</sup>
<i>L. monocytogenes</i>										
1.5	4.5 $\pm$ 0.1 <sup>aA</sup>	4.3 $\pm$ 0.5 <sup>aA</sup>	4.6 $\pm$ 0.2 <sup>aA</sup>	4.3 $\pm$ 0.3 <sup>aA</sup>	4.3 $\pm$ 0.2 <sup>aA</sup>	4.0 $\pm$ 0.8 <sup>abA</sup>	3.1 $\pm$ 1.3 <sup>abcA</sup>	2.5 $\pm$ 1.6 <sup>bcA</sup>	1.9 $\pm$ 1.1 <sup>cA</sup>	1.8 $\pm$ 0.8 <sup>cA</sup>
4	4.5 $\pm$ 0.1 <sup>aA</sup>	4.3 $\pm$ 0.5 <sup>abA</sup>	4.6 $\pm$ 0.1 <sup>aA</sup>	3.8 $\pm$ 0.8 <sup>abcA</sup>	3.9 $\pm$ 0.9 <sup>abcAB</sup>	3.5 $\pm$ 1.4 <sup>abcA</sup>	2.7 $\pm$ 1.1 <sup>bcA</sup>	2.6 $\pm$ 1.3 <sup>bcA</sup>	2.2 $\pm$ 1.2 <sup>cA</sup>	2.3 $\pm$ 0.9 <sup>cA</sup>
10	4.5 $\pm$ 0.1 <sup>aA</sup>	4.4 $\pm$ 0.5 <sup>aA</sup>	4.6 $\pm$ 0.5 <sup>aA</sup>	3.4 $\pm$ 1.2 <sup>abA</sup>	3.1 $\pm$ 1.2 <sup>abAB</sup>	3.0 $\pm$ 1.3 <sup>abA</sup>	3.7 $\pm$ 0.9 <sup>abA</sup>	2.2 $\pm$ 1.3 <sup>abA</sup>	2.4 $\pm$ 1.9 <sup>abA</sup>	1.8 $\pm$ 1.7 <sup>bA</sup>
21	4.5 $\pm$ 0.1 <sup>abA</sup>	4.3 $\pm$ 0.6 <sup>abcA</sup>	4.8 $\pm$ 0.5 <sup>aA</sup>	4.0 $\pm$ 0.3 <sup>abcA</sup>	2.3 $\pm$ 1.4 <sup>cdB</sup>	2.8 $\pm$ 1.0 <sup>bcdA</sup>	3.0 $\pm$ 1.7 <sup>abcdA</sup>	2.4 $\pm$ 0.1 <sup>cdA</sup>	1.8 $\pm$ 1.0 <sup>dA</sup>	1.5 $\pm$ 1.1 <sup>dA</sup>

Means with different lowercase letters within a row are significantly different ( $p < 0.05$ ). Means with different uppercase letters within a column for each organism are significantly different ( $p < 0.05$ ).

### **2.3.3 Proximate composition analyses**

With possible exceptions of the carbohydrate levels that were not statistically ( $p \geq 0.05$ ) different among the five brands and the salt level for brand A that was significantly ( $p \leq 0.05$ ) lower compared to the other four brands, chemical analyses revealed subtle differences ( $p \leq 0.05$ ) for a given chemical trait among the five commercial brands tested (Table 2.5). Proximate composition analyses also revealed that teewurst in general has relatively low nitrite and salt levels and a relatively high moisture and high fat content, characteristics that typically do not provide a sufficient barrier to microbial persistence in such products.

**2.3.4 Market basket survey.** As a final component of this study, a market basket survey of commercially available teewurst was conducted. With reference to USDA/FSIS directive 7235.1 (USDA, 1994) for raw or partially cooked meat and poultry products, the labels from four of the five brands tested herein declared teewurst as an uncooked product and/or provided safe handling instructions, that being “Keep refrigerated” and/or “Cook thoroughly” (Table 2.6). A lack of uniformity in the listed ingredients and additives used by these five processors was also observed and subsequently confirmed by proximate composition analyses (Tables 2.5 and 2.6).

**Table 2.4 Inactivation kinetic parameters of *E. coli*, *S. Typhimurium*, and *L. monocytogenes* during storage for 21 days (slices) and 30 days (chubs) at 4°C**

Microorganism	Sample	Trial	Initial population (log CFU/g)	Residual population (log CFU/g)	$S_1^a$ (days)	$K_{max}^b$ (1/days)	$R^{2c}$
<i>E. coli</i> O157:H7	Teewurst batter	1	5.19	- <sup>d</sup>	9.44	0.38	0.95
		2	5.31	- <sup>d</sup>	1.79	0.25	0.95
		3	5.25	- <sup>d</sup>	9.97	0.27	0.92
	Teewurst slices	1	4.84	3.60	- <sup>e</sup>	0.37	0.91
		2	4.72	3.63	- <sup>e</sup>	0.24	0.84
		3	4.49	- <sup>d</sup>	- <sup>e</sup>	0.07	0.74
<i>S. Typhimurium</i>	Teewurst batter	1	4.99	- <sup>d</sup>	0.93	0.56	0.98
		2	4.85	- <sup>d</sup>	1.78	0.93	0.99
		3	4.74	- <sup>d</sup>	2.60	0.68	0.99
	Teewurst slices	1	4.44	2.30	- <sup>e</sup>	0.39	0.93
		2	4.21	2.32	- <sup>e</sup>	0.34	0.94
		3	4.13	3.20	- <sup>e</sup>	0.35	0.87
<i>L. monocytogenes</i>	Teewurst batter	1	5.42	3.46	7.25	0.49	0.90
		2	5.45	2.59	4.33	0.36	0.99
		3	5.12	2.40	9.41	0.71	0.94
	Teewurst slices	1	4.72	- <sup>d</sup>	9.07	0.55	0.96
		2	4.56	1.33	1.64	1.32	0.91
		3	4.54	2.87	4.65	0.30	0.89

<sup>a</sup>Shoulder length of inactivation curve.

<sup>b</sup>Specific inactivation rate.

<sup>c</sup>Coefficient of determination.

<sup>d</sup>Residual population was not observed.

<sup>e</sup>Shoulder was not observed.

**Table 2.5 Proximate composition analyses of five brands of commercial teewurst<sup>1</sup>**

	Teewurst A <sup>2</sup>	Teewurst B	Teewurst C	Teewurst D	Teewurst E
Phenolics(g/100g)	0.07 ± 0.00 <sup>ab3</sup>	0.07 ± 0.01 <sup>ab</sup>	0.09 ± 0.00 <sup>bc</sup>	0.05 ± 0.00 <sup>a</sup>	0.11 ± 0.01 <sup>c</sup>
Salt (g/100g)	1.26 ± 0.16 <sup>a</sup>	2.10 ± 0.22 <sup>b</sup>	2.12 ± 0.10 <sup>b</sup>	2.16 ± 0.00 <sup>b</sup>	2.34 ± 0.00 <sup>b</sup>
Nitrite (µg/g)	<1.00 <sup>a</sup>	4.03 ± 0.07 <sup>b</sup>	1.19 ± 0.26 <sup>a</sup>	1.61 ± 0.24 <sup>a</sup>	<0.10 <sup>c</sup>
Moisture (g/100g)	44.35 ± 0.21 <sup>ab</sup>	40.65 ± 0.07 <sup>a</sup>	52.60 ± 0.99 <sup>c</sup>	51.90 ± 0.14 <sup>c</sup>	50.10 ± 3.11 <sup>bc</sup>
Protein (g/100g)	12.35 ± 0.64 <sup>a</sup>	12.95 ± 0.21 <sup>ab</sup>	15.60 ± 0.71 <sup>bc</sup>	15.00 ± 0.28 <sup>abc</sup>	16.20 ± 1.13 <sup>c</sup>
Fat (g/100g)	39.00 ± 0.42 <sup>a</sup>	41.60 ± 1.84 <sup>a</sup>	26.15 ± 0.21 <sup>b</sup>	25.85 ± 0.21 <sup>b</sup>	28.80 ± 0.57 <sup>b</sup>
Acidity <sup>4</sup> (%)	0.35 ± 0.06 <sup>a</sup>	0.40 ± 0.13 <sup>a</sup>	0.67 ± 0.11 <sup>ab</sup>	0.94 ± 0.04 <sup>b</sup>	0.65 ± 0.06 <sup>ab</sup>
CHO <sup>5</sup> (g/100g)	1.77 ± 0.11 <sup>a</sup>	1.27 ± 1.65 <sup>a</sup>	2.75 ± 0.15 <sup>a</sup>	4.17 ± 0.75 <sup>a</sup>	1.70 ± 2.40 <sup>a</sup>
Ash (g/100g)	2.54 ± 0.11 <sup>a</sup>	3.57 ± 0.00 <sup>b</sup>	2.91 ± 0.22 <sup>ac</sup>	3.08 ± 0.11 <sup>bc</sup>	3.38 ± 0.10 <sup>bc</sup>
pH	6.11 ± 0.01 <sup>a</sup>	NT <sup>6</sup>	NT	5.69 ± 0.04 <sup>b</sup>	6.09 ± 0.03 <sup>a</sup>
a <sub>w</sub>	0.956 ± 0.004 <sup>a</sup>	NT	NT	0.973 ± 0.001 <sup>b</sup>	0.967 ± 0.001 <sup>b</sup>

<sup>1</sup> Proximate analyses were performed on two samples from each processor (mean values ± standard deviation).

<sup>2</sup> Teewurst A (Ernst A. Illg Meats Inc.) product was utilized in all challenge experiments conducted in this study.

<sup>3</sup> Means with different letter (a, b, c) within a row are significantly different (p < 0.05).

<sup>4</sup> Acidity titratable as acetic acid.

<sup>5</sup> CHO; carbohydrates.

<sup>6</sup> NT; not tested.

**Table 2.6 Labeling information from five brands of commercial teewurst<sup>a</sup>**

Ingredients/Other information	Teewurst A <sup>b</sup>	Teewurst B	Teewurst C	Teewurst D	Teewurst E
Pork	x	x	x	x	x
Beef	x	x	x		
Salt	x	x	x	x	x
Carbohydrates	x		x		x
Spices	x	x		x	x
Paprika	x	x	x	x	x
Oleoresin of paprika				x	
Rum		x		x	
Smoke flavor/natural smoke	x			x	x
Flavoring			x		x
Sodium nitrite	x	x	x	x	x
Sodium erythorbate			x	x	x
Sodium acetate			x		
“Uncooked product”	x	x	x		x
“Cook thoroughly”	x				x
“Keep refrigerated”	x		x	x	x

<sup>a</sup>According to the labeling information declared from processor.

<sup>b</sup>Teewurst A (Ernst A. Illg Meats Inc.) product was utilized in all challenge experiments conducted in this study.

## 2.4 DISCUSSION

Teewurst is a very popular traditional/ethnic sausage, typically consumed raw, that remains in demand, albeit in the face of generally declining sales (Ernst K. Illg, personal communication, 2008). In the USA, it is produced by a limited number of small plants that are located primarily in the northeast and upper midwest regions of the country.

From a public health perspective spreadable sausages such as teewurst are considered to be higher-risk products, presumably because consumers are not aware of the safe-handling requirements for teewurst as a product that may contain raw meat (Bremer et al., 2005) and/or due to their preference to consume it “as is”. In recent years, consumption of teewurst has caused human illnesses due to its contamination with *E. coli* O157:H7 and *L. monocytogenes* and, therefore, such products are potentially a vehicle for harborage and/or transmission of foodborne pathogens (Timm et al., 1999; Brown, 2000; Goulet et al., 2002; FAO, 2004; Pichner et al., 2004).

If opened/sampled, teewurst has a refrigerated shelf life of between 1 to 5 days (Campbell-Platt, 1995; Brown, 2000; Ockerman and Basu, 2007), whereas if left unopened the shelf life could extend for up to 7 to 21 days at 4°C (Ernst K. Illg, personal communication, 2008). In the present study, however, visible mould-like spoilage was evident on teewurst slices within 21 days of refrigerated storage (1.5 and 4°C) or within 5 days of storage at abuse temperatures (10 and 21°C). Regardless, teewurst did not support the growth of the pathogens which were observed to decrease during extended storage; however, in the event of post-process contamination with relatively high levels of these pathogens, as seen for other meat products, teewurst could possibly expose some consumers to a health risk especially within its shelf life (Yang et al., 2006; Gounadaki et al., 2007; Matargas et al., 2008).

Levels of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* decreased in teewurst chubs during storage for 30 days (Table 2.2). Greater reductions in pathogen numbers were observed at 10 and 21°C as compared to 1.5 and 4°C. However, at the

end of storage, with the exception *S. Typhimurium* for which the most significant lethality was observed, surviving numbers of each pathogen were of similar levels. Moreover, in agreement with related studies conducted on salami and soudjouk (Nightingale et al., 2006; Porto-Fett et al., 2008), *S. Typhimurium* inoculated into teewurst batter/chubs was less viable than *L. monocytogenes* and *E. coli* O157:H7.

Pathogen lethality may be mainly attributed to the presence of native LAB as the relatively low nitrite and salt levels and a relatively high moisture and high fat content do not typically provide a sufficient barrier to microbial persistence (Table 2.5). According to Rödel and Scheuer (2006) the inhibition of *E. coli* in fermented raw sausages was enhanced due to the acidification of the product by LAB and reduction of  $a_w$  while the presence of sodium nitrite had only a weak effect. The proliferation and metabolic activity of LAB are known to inhibit undesirable bacteria, mainly through the production of lactic acid and the subsequent pH reduction of foods, but also by the production of CO<sub>2</sub>, hydrogen peroxide, ethanol, diacetyl, and/or bacteriocins (Hugas, 1998).

The reduction of pH and proliferation of LAB due to carbohydrates added in teewurst, were indicative of spontaneous fermentation occurring during storage of the product (i.e. chubs and slices) (Table 2.1). The uncontrolled fermentation in addition to the batch-to-batch levels and diversity of LAB naturally present in raw meat and associated microbial interactions (i.e. chemical changes in product), could possibly explain the observed variability in lethality expressed by differences in the inactivation parameters for each pathogen among trials and between chubs and slices (Table 2.4; Kaya and Gökalp, 2004; Comi et al., 2005; Skandamis and Nychas, 2007).

The findings of the present study suggest that inclusion of a defined lactic starter culture(s) and perhaps a limited/controlled fermentation during manufacture would improve the reproducibility from batch-to-batch and enhance both the quality and safety of the finished product (Lucke, 2000; Calicioglou et al., 2001). As previously reported, fermentation of a German-style uncooked sausage (24°C/24 h) followed by smoking (22°C/20 h) resulted in a 2- to 3-log reduction of *L. monocytogenes* (Farber et al., 1993).

In fact, fermentation of spreadable raw sausages in Germany constitutes a critical element of the manufacturing process so as to insure that the final product is characterised by an appropriate flavour, colour, texture, and acidification level ( $\leq$  pH 5.6; D-lactic acid  $\geq$  0.2 g/100 g; Islam and Jockel, 2005). In the case of teewurst, however, the addition of a starter culture and the ensuing production of organic acid(s) and other compounds could possibly have a detrimental effect on product taste, that being too sour, and on product texture, that being too firm and, as such, less spreadable (Ernst K. Illg, personal communication, 2008).

Regardless, the need for a more precise standard of identity was evident from the differences among brands in the various physicochemical traits measured, as well as from differences in the information included on product labels (Table 2.5 and 2.6). In the absence of any readily accessible and/or published information, the data in Tables 2.5 and 2.6 may serve as a starting point for assisting in the development of a list of ingredients and range of attendant concentrations for defining a standard of identity for teewurst.

Limited scientific literature on the fate of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* either “on” or “in” teewurst exists. This study provides valuable information to small and very small plants producing teewurst and to regulatory authorities overseeing its production for assessing product safety from these foodborne pathogens. The need to establish both a standard of identity and guidelines for its manufacture are critical given that teewurst is a raw rather than ready-to-eat product, as well as given that despite labeling instructions to the contrary, this product is frequently ingested in the raw state without cooking. The data herein also suggest that the fate of pathogens in food ecosystems may be significantly affected by the presence and initial numbers of endogenous lactic acid bacteria and highlight the complexity of microbial interactions.



**CHAPTER 3**  
**EFFECT OF FOOD STRUCTURE, COMPOSITION AND**  
**MICROBIAL INTERACTIONS ON THE GROWTH KINETICS**  
**OF *Listeria monocytogenes***



## Chapter 3

### **Effect of food structure, composition and microbial interactions on the growth kinetics of *Listeria monocytogenes***

#### **ABSTRACT**

In the present study, the effect of food structure, composition and microbial interactions on the growth kinetics of *Listeria monocytogenes* Scott A was evaluated using different growth substrates including broth, agar (spread and pour plate technique), sterile meat blocks, naturally contaminated meat blocks and minced meat. The laboratory medium used was tryptone soy broth (with or without agar addition) with pH adjusted approximately to that of meat (5.7). Inoculated samples were incubated at 3.5, 5, 10, 15 and 20°C. Data generated were fitted to the primary model of Baranyi and Roberts (Baranyi, J., and T. A. Roberts. 1994. *Int. J. Food Microbiol.* 23:277-294) and growth kinetics parameters of *L. monocytogenes* were calculated. The results showed that the growth kinetics of *L. monocytogenes* were significantly different at the different growth media and food products tested. These differences were more pronounced at low temperature conditions. The validation of a model based on data from broth against the observed growth of the pathogen in the rest of the tested media showed that broth models may result in significant prediction errors especially under stressful environmental conditions (i.e. low temperature) in which factors such as food structure and microbial interactions may become very significant in microbial growth.

### 3.1 INTRODUCTION

Microbial contamination of foods by spoilage and pathogenic bacteria is unavoidable despite the progress made for their control. Food spoilage and primarily food safety has been at the forefront of societal concerns due to high number of microbial foodborne diseases and outbreaks, and associated product recalls (Sofos, 2002). In response to these challenges, there is a growing interest worldwide in the research field of predictive microbiology as a tool for improving food safety management in terms of HACCP implementation and quantitative microbial risk assessment (Walls and Scott, 1997b; Nauta, 2002; McMeekin et al., 2006). Predictive or quantitative microbiology involves the development of mathematical models to quantitatively assess and predict the evolution of microorganisms under environmental conditions encountered in foods (McMeekin et al., 1993).

Over the last two decades, models have been published describing the responses of new, evolving or emerging pathogenic microorganisms (e.g. *Escherichia coli* O157: H7, *Salmonella* and *Listeria monocytogenes*) to a wide range of chemical, environmental and physical factors (Buchanan and Philips., 1990; Farber et al., 1996; George et al., 1996; Fernandez et al, 1997; McClure et al., 1997; Koutsoumanis et al., 1998; Parente et al., 1998; Oscar, 1999; Cheroute-Vialette and Lebert, 2000; Devlieghere et al., 2001; Coleman et al., 2003). However, it is generally acknowledged that most existing models remain a research tool rather than an effective industrial application due to the growing debate on whether these models can reliably predict the growth of the modelled organisms in real food environments which are complex, being spatially and temporarily heterogeneous (Ross and McMeekin, 1994; McDonald and Sun, 1999; Pin et al, 1999). This was evident since recent validation studies have shown that the predictions from these models are not necessarily valid for actual foods (Dalgaard and Jorgensen, 1998; Lebert et al., 1998; Giffel and Zwietering, 1999; Geysen et al., 2006; Xanthiakos et al, 2006).

The majority of predictive models have been traditionally generated using data obtained from experiments conducted in simple model systems, that is, well-defined and

controlled liquid laboratory media. Therefore, while current predictive models for foodborne pathogenic or spoilage organisms take into account a precise description of the effect of environmental conditions (i.e. temperature, pH, water activity and preservatives) on microbial growth, they often neglect or underestimate factors such as the food matrix microstructure and composition (Robins and Wilson, 1994; Wilson et al., 2002), and the influence of the background flora and associated microbial interactions (Gram and Melchiorson, 1996; Pin *et al*, 1999)

Indeed, while temperature and the chemical composition of foods appears to be the most important factors that influence their safety and spoilage (Koutsoumanis et al., 2005), the food microstructure has also been suggested to significantly affect the survival and/or growth of microorganisms (Brochlehurst et al., 1997; Koutsoumanis et al., 2004). In contrast to liquid broths where bacterial cells live as free floating organisms having accessibility to the nutrients within the bulk of the liquid, in the majority of foods which are highly structured (e.g. meat), bacterial cells are immobilized and constrained to form microcolonies (Wilson et al., 2002). The physical structure of foods affects bacterial growth by imposing physical constraints (by limiting the expansion rate of the growing microcolony and the motility of microorganisms), by limiting the diffusion of essential nutrients, oxygen and water, or by preventing the diffusion of metabolic products away from the site of growth (Robins and Wilson, 1994; Stecchini et al., 1998; Wilson et al., 2002; Dykes, 2003; Koutsoumanis et al., 2004).

Another important determinant of microbial growth is the influence of spoilage and other natural food microflora on pathogenic microorganisms. Depending on the food type, processing conditions and food distribution chain, a fraction of the microbial association, the so called ephemeral (specific) spoilage organisms, will establish and may potentially influence the growth responses of other spoilage or pathogenic bacteria present in the same ecosystem via the production of metabolites, competition for available growth substrates and/or production of signal molecules that enable cell-to-cell communication (Nychas et al., 2007, 2008; Ammor et al., 2008). Although many researchers claim that interactions between the natural microflora and pathogens occur only at high population densities of the specific spoilage microflora (i.e. at the stage of

spoilage) and thus their incorporation in predictive models is most of the times unnecessary, others support the idea that the dynamics of mixed microbial populations can be highly complex within the food microenvironment and thus microbial interactions should be considered while generating a predictive model (Jay, 1997; Lebert et al., 2000; Lucke, 2000; Malakar et al., 2003; Powell et al., 2004).

The objectives of the present study were i) to evaluate the effect of food structure and microbial interactions on the growth kinetics of *L. monocytogenes*, a ubiquitous microorganism able to survive and multiply even under cold storage temperatures, ii) to identify and isolate the sources of prediction error related with a model for the effect of temperature on the growth of *L. monocytogenes*, iii) to evaluate the extent of prediction error from each source and iv) to analyze the prediction error in relation to the environmental conditions.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Organism and growth conditions**

*L. monocytogenes* Scott A (kindly donated by Dr Eddy Smid, ATO-DLO, Bornsesteeg 59, AA-Wageningen, The Netherlands) was used throughout this study. The organism was maintained frozen (-80°C) in vials of treated beads in a cryoprotective fluid (Protect, Technical Service Consultants Ltd., Lancashire, England) until use. *L. monocytogenes* was propagated by adding one bead to 10 ml of tryptone soy broth (TSB; pH 7.3 ± 0.2, LAB M, International Diagnostics Group Plc, Lancashire, England) and incubating aerobically without agitation at 30°C for 24 h. Aliquots (50 µl) of the activated culture were transferred to 10 ml of TSB and incubated at 30°C for 24 h, at which time late exponential phase was attained (working culture).

### **3.2.2 Kinetic experiments**

The growth kinetic parameters of *L. monocytogenes* were evaluated using laboratory growth media (broth and agar), naturally contaminated minced meat and naturally contaminated meat blocks, and sterile meat blocks. Experiments were performed at least

once with an overall number of replicate samples varying between two and six. Preparation of samples was performed as follows:

#### *Experiments in laboratory growth media*

TSB medium supplemented with 0.5% yeast extract was used in all experiments in broth. The composition of TSB was tryptone (casein digest USP, 17 g/l), soy peptone (3 g/l), sodium chloride (5 g/l), dipotassium hydrogen phosphate (2.5 g/l) and dextrose (2.5 g/l). Appropriate dilutions of the working culture were made and aliquots (0.5 ml) were inoculated into 250-ml conical flasks containing 50 ml TSBYE to yield an initial population of ca.  $10^3$  CFU/ml. TSB medium supplemented with 0.5% yeast extract and solidified with the addition of agar ( $15\text{g l}^{-1}$ ; LAB M) (TSB+agar), was used in all experiments in agar. Inoculation of solid TSB was conducted using two different techniques; spread plate technique and pour plate technique. Appropriate dilutions of the working culture were made and aliquots (0.1 ml) were inoculated onto the surface of sterile Petri dishes containing 20 ml of solid TSB+agar to obtain a target inoculum of ca.  $10^3$  CFU/cm<sup>2</sup> (agar-spread). Aliquots of 0.5 ml of the appropriate dilution were also added in 500 ml of molten (47°C) TSB+agar to obtain ca.  $10^3$  CFU/g and the agar-culture solutions were immediately poured (20 ml) into Petri dishes (agar-pour). The pH of all laboratory media was adjusted approximately to the pH of raw meat (5.7) with 1N HCl before autoclaving. Inoculated samples were stored under controlled isothermal conditions (3.5, 5, 10, 15 and 20°C).

#### *Experiments in naturally contaminated meat*

Fresh (<24 h after slaughter) pork muscle purchased from a butcher-shop and two batches of minced pork provided by a Greek meat product manufacturer (batch 1) or purchased from a central market (batch 2), were used in this study. The pork muscle was cut under aseptic conditions into blocks of ca. 46 cm<sup>2</sup> (3x5x1 cm) prior to inoculation. Appropriate dilutions of the working culture were made and a target inoculum of ca.  $10^3$  CFU/g or cm<sup>2</sup> was obtained for ground meat and meat blocks, respectively. After inoculation, two 50 g portions of minced meat and two meat blocks were individually placed on each end of a meat retail foam tray (12x15 cm) and over-wrapped with air-permeable polyethylene film. Inoculated samples were stored under

controlled isothermal conditions (3.5, 5, 10, 15 and 20°C). The temperature was monitored using electronic temperature monitoring devices (Cox tracer, Cox Technologies, Belmont, NC, USA). The presence of *L. monocytogenes* was tested in minced meat and meat blocks through enrichment technique prior to inoculations and the pathogen was not detected.

#### *Experiments in sterile meat*

Fresh pork muscle, purchased from a butcher-shop was used for preparation of sterile meat blocks. In order to obtain meat samples without the endogenous microflora (sterile) the meat surface was sprayed with 100% alcohol and burned with a gas burner in order to reduce the initial microbial load (Tsigarida et al., 2000). The burned surface tissue was removed aseptically and the sterile tissue below was excised and cut into pieces (ca. 70 g, 1 cm thickness). Appropriate dilutions were made to obtain a target inoculum of ca.  $10^3$  CFU/g. Samples of sterile meat blocks were packaged in sterile Petri dishes and stored under controlled isothermal conditions (3.5, 5, 10, 15 and 20°C).

#### **3.2.3 Microbiological analysis**

Broth (1 ml) from conical flasks was transferred aseptically to a test tube containing 9ml of Ringer solution (pH  $6.8 \pm 0.2$ , LAB M). Agar samples (agar-spread and agar-pour) from Petri dishes were transferred to a stomacher bag (Seward, London, United Kingdom) containing 100 ml of Ringer solution and the mixture was homogenised for 60 s with the aid of a stomacher (Lab Blender 400, Seward Medical, London, UK). Ground meat portions (25 g) were transferred to stomacher bags, 225 ml of Ringer's solution were added and the contents were homogenised for 60 s with a stomacher. Meat blocks were transferred to a stomacher bag, 100 ml of Ringer solution were added, and the mixture was homogenised for 60 s with a stomacher. Portions (1 g) of sterile meat blocks were transferred to 9 ml Ringer solution and vortexed for 60 s to allow for bacterial cells detachment from the tissue surface.

Samples (0.1 ml), from *in vitro* and *in situ* experiments, of the appropriate 10-fold serial dilutions were spread on the surface of tryptone soy agar (TSA, LAB M) for enumeration of *L. monocytogenes* and incubated at 30°C for 48 h. Samples (0.1 ml),



from *in vivo* experiments, of the appropriate 10-fold serial dilution were spread on the surface of the appropriate media in Petri dishes for enumeration of (i) total aerobic viable count on plate count agar (PCA; Merck 1.05463) incubated at 25°C for 72 h, (ii) pseudomonads on cefrimide fucidin cephaloridine agar (CM559 [Oxoid, Basingstoke, United Kingdom] supplemented with selective supplement SR 103E) and incubated at 25°C for 48 h (32), (iii) *Brochothrix thermosphacta* on streptomycin-thallos acetate-cycloheximide (actidione) agar (STAA; this medium was made from basic ingredients in the laboratory) incubated at 25°C for 72 h (12), (iv) *L. monocytogenes* on Palcam Listeria Selective Agar supplemented with Palcam Listeria Selective Supplement (Merck), (v) *Enterobacteriaceae*, and (vi) lactic acid bacteria (LAB). For *Enterobacteriaceae* and LAB, 1.0 ml was inoculated into 10 ml of molten (45°C) violet red bile glucose agar (VRBGA; Merck, Darmstadt, Germany) and Man Rogosa Sharpe agar (MRS; Merck), respectively. After setting, a 10 ml overlay of molten medium was added. VRBGA and MRS plates were incubated at 37 and 25°C for 24 and 96 h, respectively.

All plates were examined visually for typical colony types and morphological characteristics that were associated with each growth medium. In addition, the selectivity of each medium was checked routinely by Gram staining and microscopic examination of smears prepared from randomly selected colonies obtained from all media.

### **3.2.4 Growth monitoring**

*L. monocytogenes* growth data (log CFU/ml, g or cm<sup>2</sup>) generated from every treatment were fitted as a function of time with the primary model of Baranyi and Roberts (1994) and the maximum specific growth rate ( $\mu_{\max}$ , h<sup>-1</sup>) of the pathogen was calculated. For curve fitting the in-house program DMFit, kindly provided by J. Baranyi (Institute of Food Research, Norwich, United Kingdom), was used.

### 3.2.5 Development and validation of broth and agar-spread model

The maximum specific growth rate ( $\mu_{\max}$ ) of *L. monocytogenes* grown in broth and agar-spread, respectively, was modelled as a function of temperature using the square root-type model (Ratkowsky et al., 1982):

$$\sqrt{\mu} = b(T - T_{\min})$$

where  $b$  is a constant,  $T$  is the storage temperature ( $^{\circ}\text{C}$ ), and  $T_{\min}$  is the theoretical minimum temperature for growth of the organism.

Both models were validated against growth of the pathogen in the laboratory growth media (i.e. agar-pour, agar-spread and/or broth) or food products (i.e. sterile meat blocks and naturally contaminated meat blocks and minced meat) by calculating the bias and accuracy factors (Ross, 1996) and/or the percent relative prediction error (Delignette-Muller et al., 1995):

$$\text{Bias factor } (Bf) = 10^{(\sum \log(\mu_{\max \text{ predicted}} / \mu_{\max \text{ observed}}) / n)}$$

$$\text{Accuracy factor } (Af) = 10^{(\sum |\log(\mu_{\max \text{ predicted}} / \mu_{\max \text{ observed}})| / n)}$$

$$\% \text{ relative error } (RE) = (\mu_{\text{observed}} - \mu_{\text{predicted}} / \mu_{\text{observed}}) \cdot 100$$

The bias factor indicates a systematic over- or under- prediction of growth and the accuracy factor is a measure of the average difference between observed and predicted values.

### 3.2.6 Modelling the growth of *L. monocytogenes* in naturally contaminated meat blocks and minced meat-batch 2 under dynamic pH conditions

The concentration (log CFU/cm<sup>2</sup> or g) of *L. monocytogenes* in naturally contaminated meat blocks stored at 5 and 20 $^{\circ}\text{C}$  was estimated under static (initial pH of naturally contaminated meat) and dynamic pH (pH of naturally contaminated meat measured

during storage at each temperature). The maximum specific growth rate ( $\mu_{\max}$ ) was calculated from the model of Buchanan and Philips (2000):

$$\begin{aligned} \ln(GT) = & 227.7984 - 0.2465T - 380.8103a_w - 8.4117pH - 0.0287Ta_w \\ & + 0.00829TpH + 3.0406a_w pH + 0.00274T^2 + 1747631a_w^2 + 0.3882pH^2 \end{aligned}$$

where GT is the generation time ( $GT = \log(2)/\mu_{\max}$ ) in hours.  $a_w$  was set at the value of 0.99. Subsequently, the growth of the pathogen was calculated using the explicit solution of the primary model of Baranyi and Roberts (1994):

$$y(t) = y_o + \mu_{\max} A(t) - \frac{1}{m} \ln \left( 1 + \frac{e^{m\mu_{\max}A(t)} - 1}{e^{m(y_{\max} - y_o)}} \right)$$

and

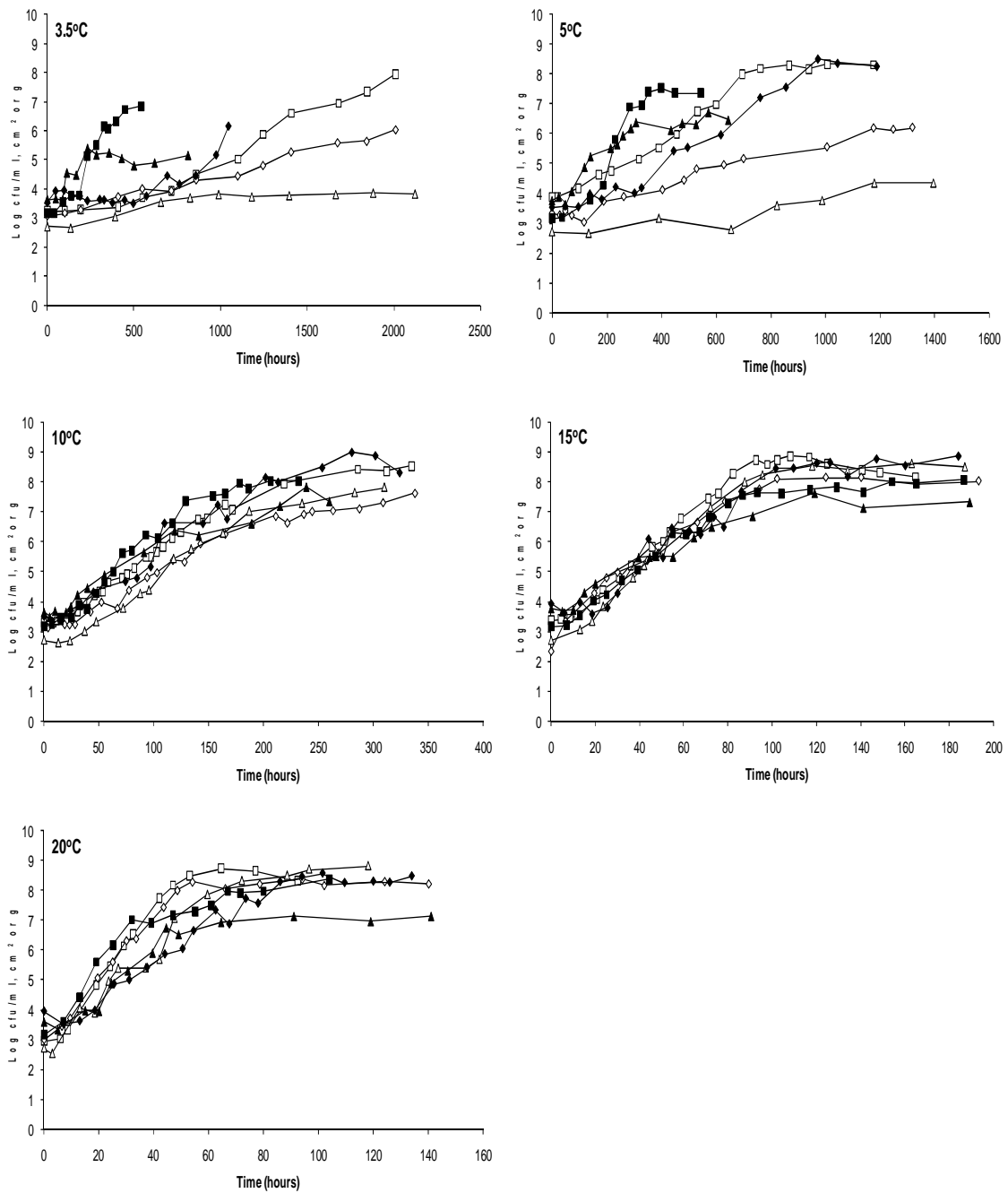
$$A(t) = t + v^{-1} \ln \left( \frac{e^{-vt} + q_o}{1 + q_o} \right)$$

where  $y(t)$  [ $\ln(\text{CFU}/\text{cm}^2)$ ] is the natural logarithm of cell density at time  $t$ ,  $y_o$  and  $y_{\max}$  the natural logarithm of initial and maximum cell density, respectively,  $\mu_{\max}$  (1/h) is the maximum specific growth rate of cell population,  $A(t)$  is a delayed time variable (lag phase, h),  $m$  is a curvature parameter,  $v$  is the rate for the transition from the lag to the exponential growth phase and  $q_o$  is a measure of the initial physiological state of the cells.

### 3.3 RESULTS

#### 3.3.1 Growth of *L. monocytogenes* in laboratory media and in real food environment

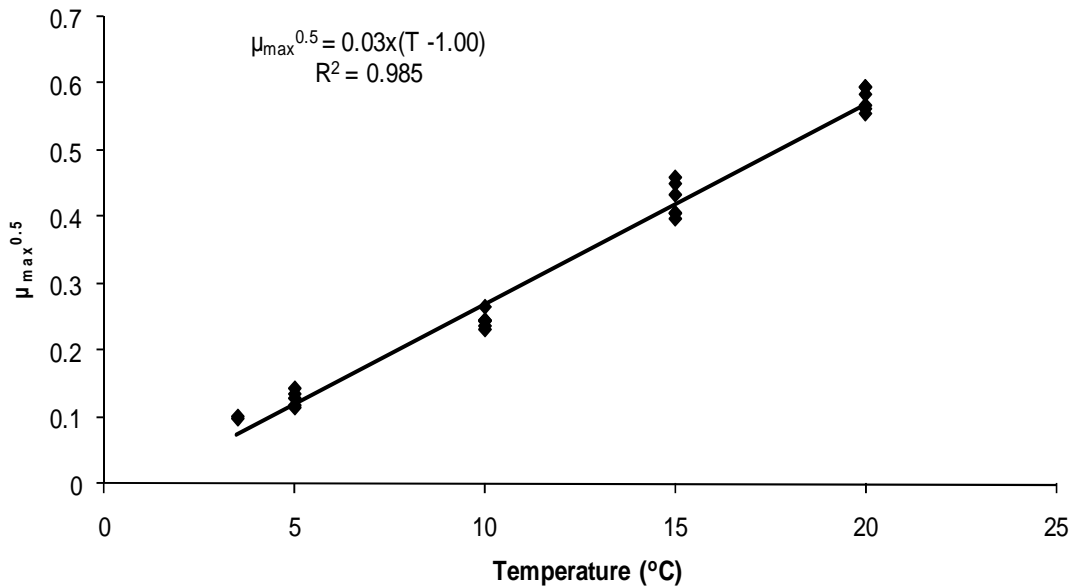
*L. monocytogenes* was inoculated in broth, on agar (spread plated and pour plated), on naturally contaminated meat blocks, in naturally contaminated minced meat-batch 1 and in sterile meat blocks to evaluate the effect of temperature and growth substrate on its growth. The population dynamics of *L. monocytogenes* on the laboratory and real food substrates stored at different isothermal temperatures (3.5, 5, 10, 15 and 20°C) are presented in Figure 3.1. *L. monocytogenes* grew under all conditions studied and, as expected, growth was enhanced with increasing temperature. In high storage temperatures (e.g. 15 and 20°C) the growth of the pathogen was similar in the different growth media tested with that in broth being slightly faster than in rest of the media. As the storage temperature decreased, however, differences on the behavior of the pathogen were observed. While *L. monocytogenes* grew faster in broth than on agar (agar-spread and agar-pour) at all temperatures below 10°C (i.e. 3.5 and 5°C), in the case of real food substrates the pathogen grew faster in naturally contaminated minced meat-batch 1 and meat blocks as compared to laboratory media.



**Figure 3.1 Growth of *L. monocytogenes* in laboratory media (broth (□), agar-spread (◇), agar-pour (Δ)) and in real food environment (sterile meat blocks (◆), naturally contaminated meat blocks (▲), naturally contaminated minced meat-batch 1 (■)) during storage at 3.5, 5, 10, 15 and 20°C.**

### 3.3.2 Identifying and isolating the sources of prediction error

Typically, predictive models are generated from experiments conducted in broth. Therefore, the maximum specific growth rate ( $\mu_{\max}$ ) of *L. monocytogenes* in broth as derived from the primary model of Baranyi and Roberts (1994) was modeled as a function of temperature using the square root-type model ( $b=0.0030$ ,  $SE=0.0008$ ;  $T_{\min}=1.0024$ ,  $SE=0.3239$ ) (Figure 3.2). The model fitted the data well according to the coefficient of determination ( $R^2 = 0.985$ ) and the root mean squared error (RMSE = 0.016) (Figure 3.2).



**Figure 3.2 Development of a square-root type model describing the effect of temperature on the maximum specific growth rate ( $\mu_{\max}$ ) of *L. monocytogenes* based on data from broth.**

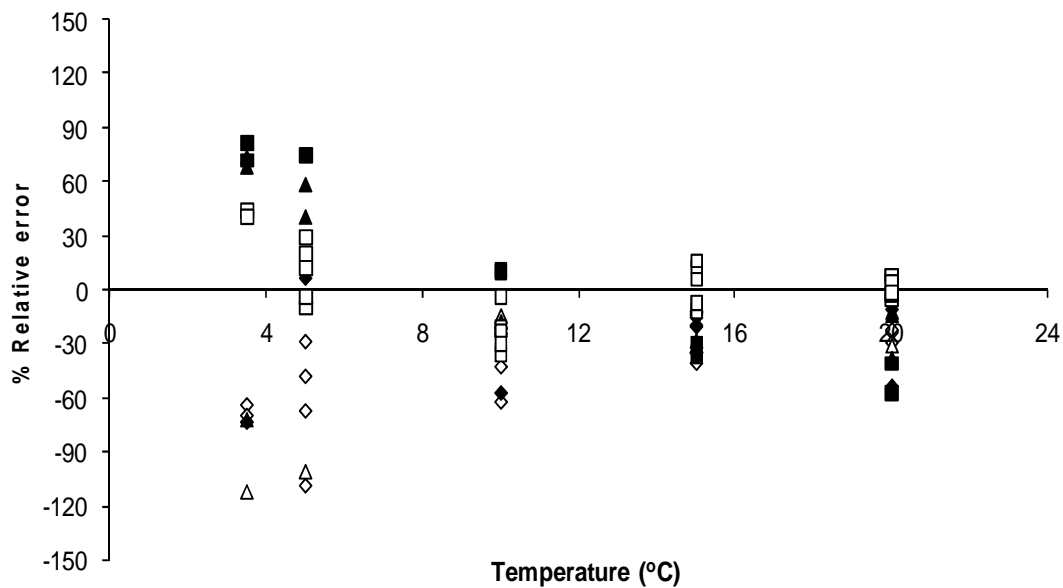
Subsequently, the performance of the derived broth model was validated against the observed growth of the pathogen in the rest of the substrates by calculating the bias and accuracy factors (Table 3.1). The broth model was found, on average, to overpredict the growth of *L. monocytogenes* in agar-spread, agar-pour and sterile meat blocks by exhibiting Bf values of 1.22 to 1.42 and with predictions differing from observations by 28 to 42 %. In contrast, when observed data on naturally contaminated meat blocks and minced-batch 1 were used, the broth model was found to significantly underpredict the growth of *L. monocytogenes* (Table 3.1).

**Table 3.1 Validation of the square root-type model generated from data in broth against the tested growth substrates**

Growth substrate	Bias* factor	Accuracy factor	Prediction error source
Broth	0.97	1.18	Primary error
Agar-spread	1.42	1.42	Structure
Agar-pour	1.37	1.37	Structure
Sterile Meat Blocks	1.22	1.28	Structure-Composition
Nat. Cont. Meat Blocks	0.77	1.69	Structure-Composition-Microbial interaction
Nat. Cont. Minced Meat-batch 1	0.63	2.10	Structure-Composition-Microbial interaction

Bias and accuracy factors were calculated using the approach of Ross (1996).

The prediction error was further analysed as a function of temperature by calculating the “per cent relative error” which expresses the error of predicted from broth model  $\mu_{\max}$  values of *L. monocytogenes* and the observed values in agar (agar-spread and agar-pour) and real food substrates (sterile meat blocks and naturally contaminated meat blocks and minced meat-batch 1) (Figure 3.3). The results showed that at high storage temperatures the model predictions were in general satisfactory. As the temperature decreased however, predictions overestimated growth on agar medium (agar-spread and agar-pour) and underestimated the growth of the pathogen in real food substrates. The above results indicated that factors other than the storage temperature influence the growth of the organism.

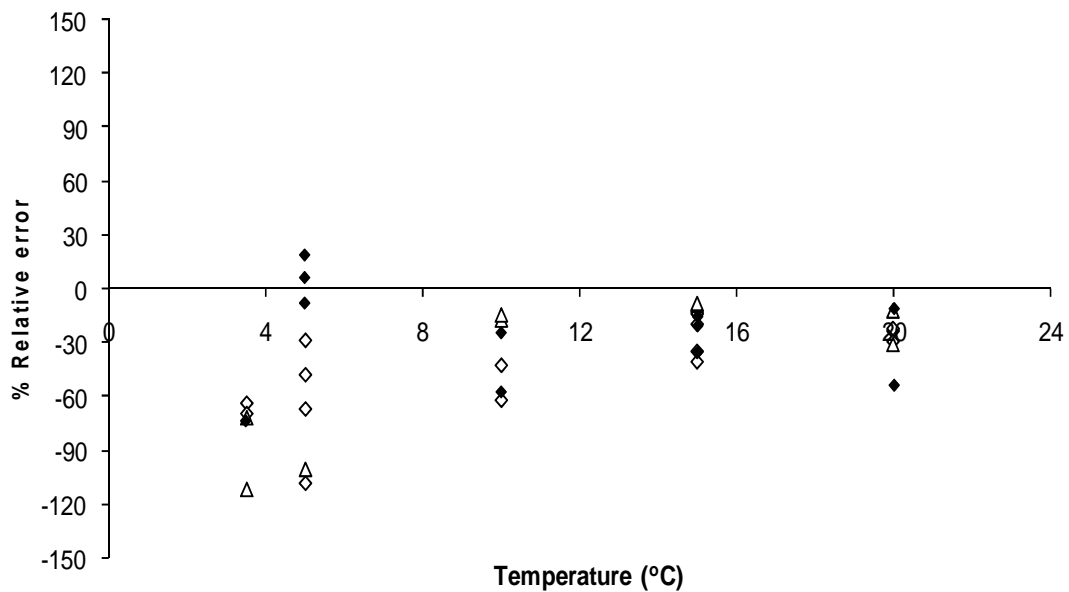


**Figure 3.3** Relative error between the predicted from broth  $\mu_{\max}$  values of *L. monocytogenes* and the observed values on laboratory media (broth (□), agar-spread (◇), agar-pour (△)) and real food environment (sterile meat blocks (◆), naturally contaminated meat blocks (▲), naturally contaminated minced meat-batch 1 (■)) at 3.5, 5, 10, 15 and 20 °C.



### Effect of structure

By comparing the predicted growth of *L. monocytogenes* in broth versus the observed growth on and in agar, and sterile meat blocks it was possible to designate substrate structure as the source of prediction error since the only difference among these growth media was the structure (in case of broth and agar) or the structure and composition (in case of broth and sterile meat blocks). To be specific, the predicted  $\mu_{\max}$  of *L. monocytogenes* from the broth model was higher than the observed  $\mu_{\max}$  in the structured substrates (Figure 3.4). This overprediction was more pronounced at low storage temperatures (i.e. 3.5 and 5°C).

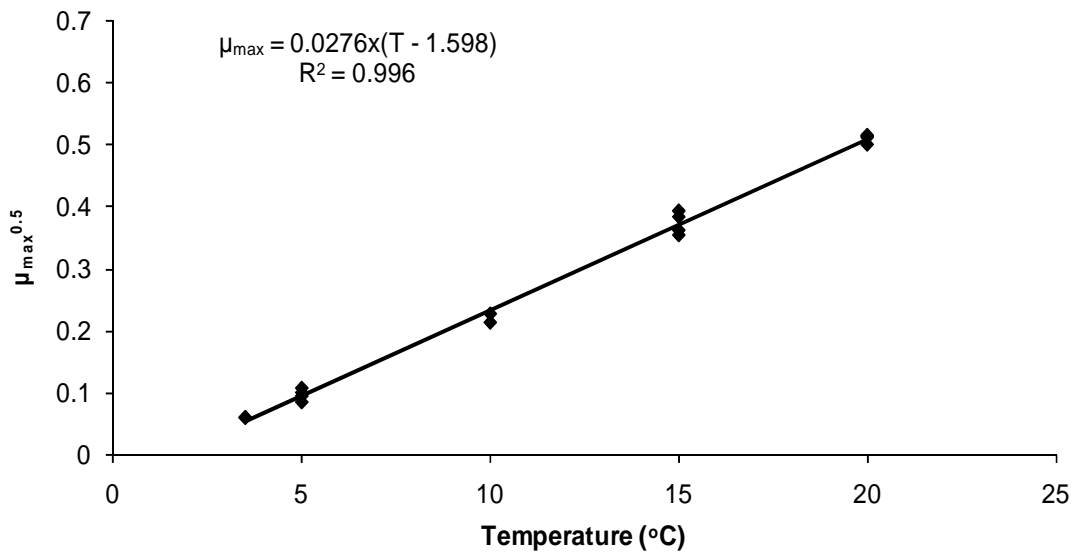


**Figure 3.4** Effect of structure on the growth of *L. monocytogenes* as expressed by the relative error between the predicted from broth and the observed  $\mu_{\max}$  values on agar-spread (◇), agar-pour (△) and sterile meat blocks (◆).

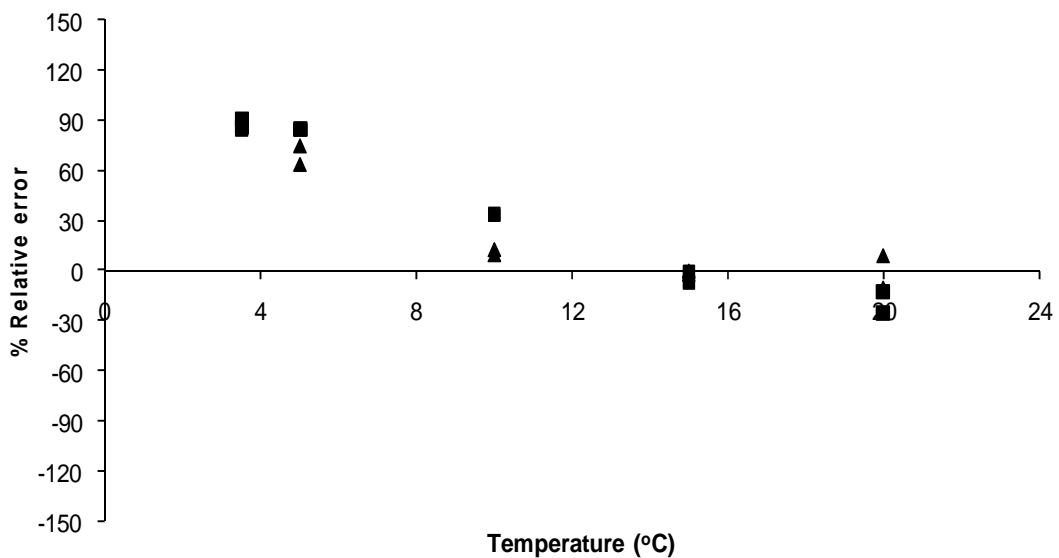
### *Effect of microbial interactions*

A square root-type model describing the effect of temperature on  $\mu_{\max}$  of *L. monocytogenes* was developed based on data from agar-spread ( $b=0.0276$ ,  $SE=0.0005$ ;  $T_{\min}=1.5965$ ,  $SE=0.2015$ ;  $R^2 = 0.996$ ;  $RMSE = 0.008$ ) (Figure 3.5). The performance of the model was validated against observed  $\mu_{\max}$  values on other structured foods such as naturally contaminated meat blocks and minced meat-batch 1 (Figure 3.6). At high growth temperatures the agar-spread model could adequately estimate the observed behaviour of the pathogen in the real food environment. However, at low storage temperatures the model underestimated the growth of the pathogen with predictions being within the 64 to 91 % RE zone. From the above results it was evident that even when the growth substrate microstructure was taken into account while generating a predictive model (i.e. agar-spread model), the accurate prediction of *L. monocytogenes* in real food environment was unattainable. Instead, the presence of the background flora and associated microbial interactions could explain the observed discrepancy.

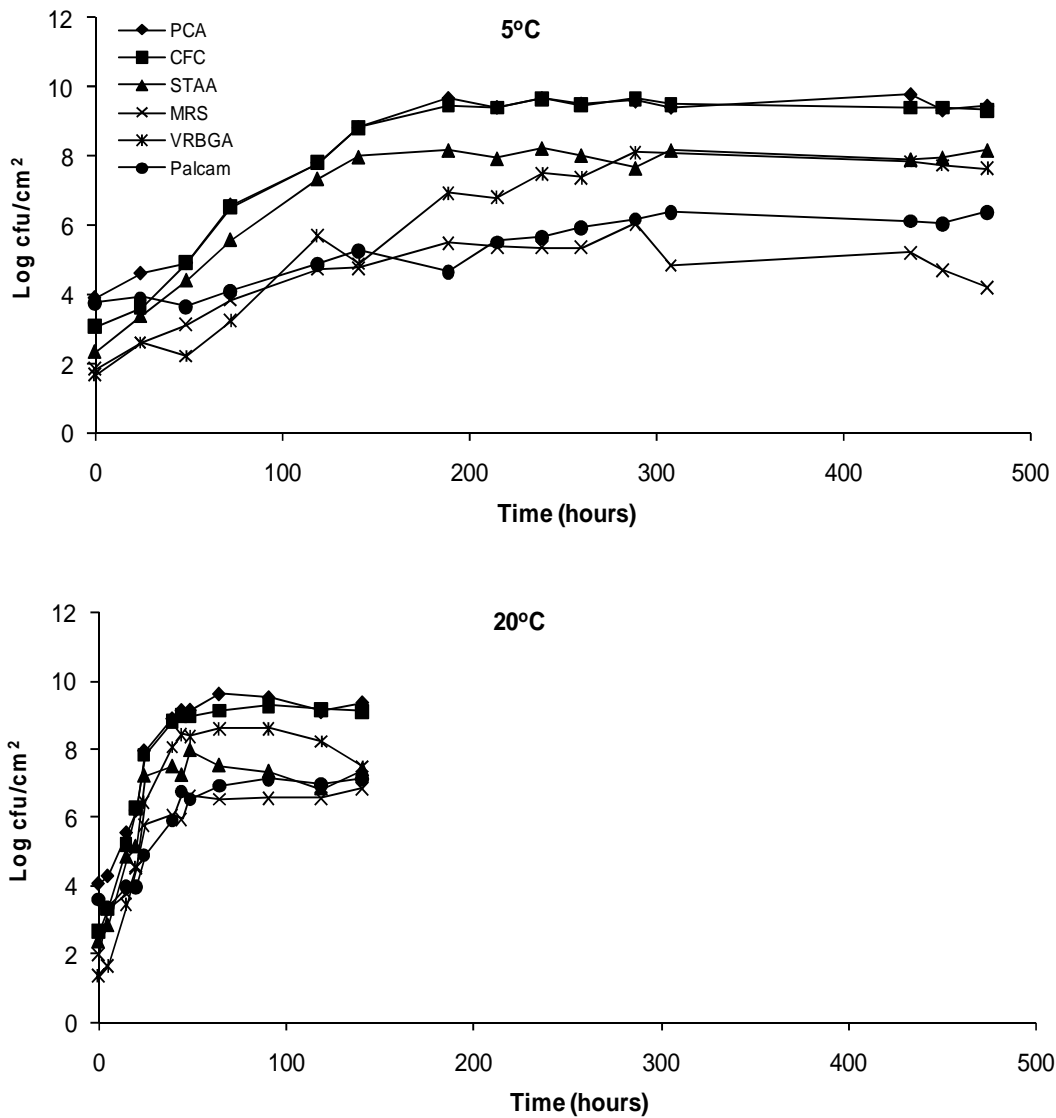
Pseudomonads were the dominant spoilage microorganisms at all temperatures studied followed by *B. thermosphacta* and/or *Enterobacteriaceae* while LAB remained at lower levels (Figures 3.7 and 3.8; also figures A.1 and A.2 in the Appendix). The growth of *L. monocytogenes*, pseudomonads and the associated pH increase in naturally contaminated meat blocks stored at 5 and 20°C is presented in Figure 3.9. At 5°C, *L. monocytogenes* growth until stationary phase (308 h) was followed by an increase in the pH by 2.8 units as a result of the fast growth of psychrotrophic pseudomonads. On the contrary, at 20°C the pH increased only by 0.5 units (44.5 h) probably due to the growth of other mesophilic members of the meat microflora (e.g. LAB) to higher population levels compared to 5°C. This influenced the pH increase through the production of metabolic end-products (e.g. lactic acid). The above results suggest that the effect of microbial interactions in a real food environment is different at different environmental conditions.



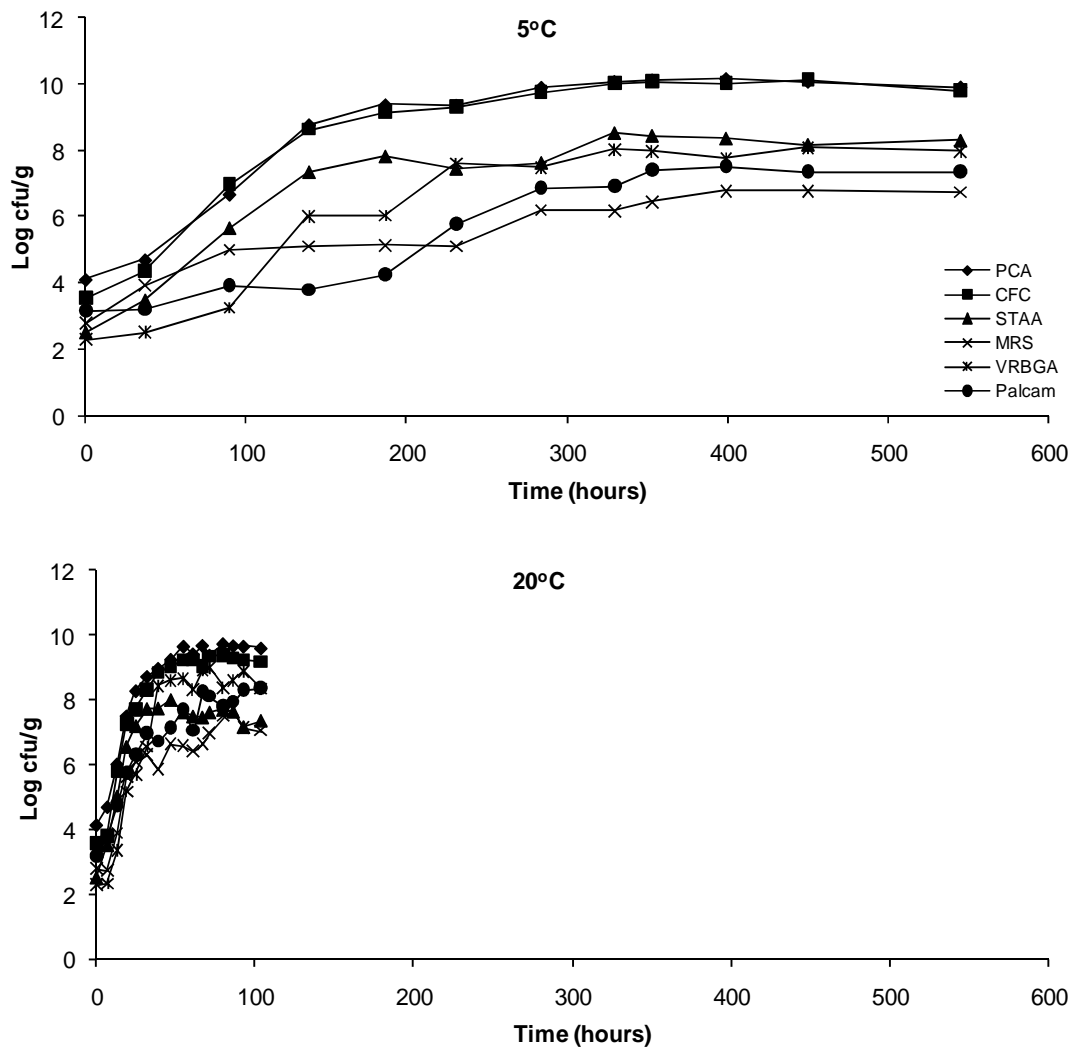
**Figure 3.5** Development of a square-root type model describing the effect of temperature on the maximum specific growth rate ( $\mu_{\max}$ ) of *L. monocytogenes* based on data from agar-spread.



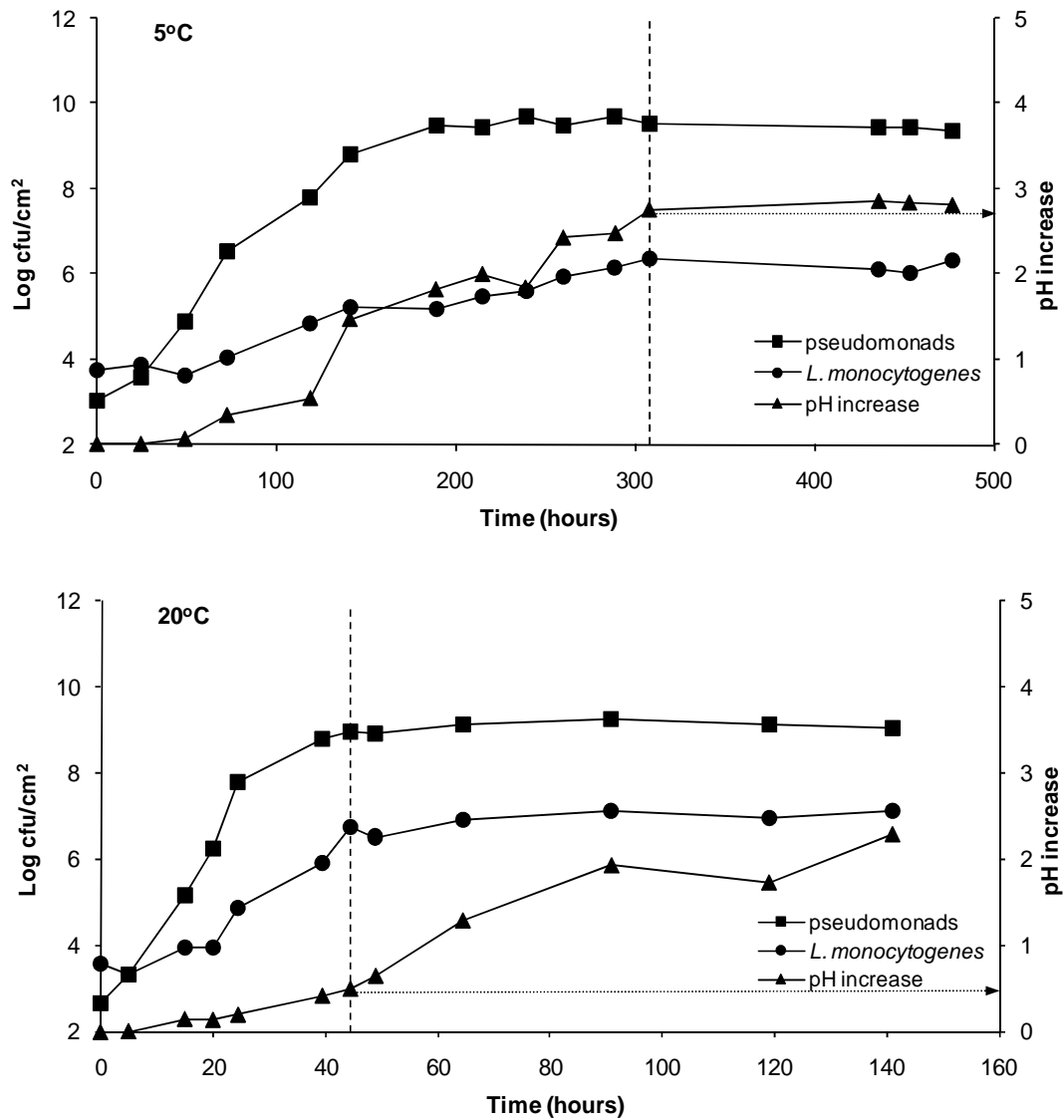
**Figure 3.6** Effect of microbial interactions on the growth of *L. monocytogenes* as expressed by the relative error between the predicted from agar-spread and the observed  $\mu_{\max}$  values on naturally contaminated meat blocks (▲) and naturally contaminated minced meat-batch 1 (■).



**Figure 3.7** Representative growth curves of the spoilage microflora and *L. monocytogenes* on naturally contaminated meat blocks stored aerobically at 5 and 20°C. Media: PCA, plate count agar (total aerobic populations); CFC, cetrinide fucidin cephaloridine (pseudomonads); STAA, streptomysin-thallos acetate (actidionine) agar (*B. thermosphacta*); MRS, Man Rogosa Sharp (lactic acid bacteria); VRBGA, violet red bile glucose agar (*Enterobacteriaceae*); Palcam, Palcam *Listeria* Selective Agar (*L. monocytogenes*).



**Figure 3.8** Representative growth curves of the spoilage microflora and *L. monocytogenes* on naturally contaminated minced meat-batch 1 stored aerobically at 5 and 20°C. Media: PCA, plate count agar (total aerobic populations); CFC, cetrimide fucidin cephaloridine (pseudomonads); STAA, streptomycin-thallos acetate (actidionine) agar (*B. thermosphacta*); MRS, Man Rogosa Sharp (lactic acid bacteria); VRBGA, violet red bile glucose agar (*Enterobacteriaceae*); Palcam, Palcam Listeria Selective Agar (*L. monocytogenes*).



**Figure 3.9** Growth of *L. monocytogenes*, pseudomonads and pH increase in naturally contaminated meat blocks stored at 5 and 20°C. Growth of *L. monocytogenes* until stationary phase was concomitant with an increase of pH by 2.8 at 5°C, whereas, at 20°C the pH increased by 0.5 units.

Further, the equation of Buchanan and Philips (2000) was used to model the effect of pH on the growth of *L. monocytogenes*. In figure 3.10, the predictions of the model under static (initial pH of naturally contaminated meat blocks) and dynamic pH conditions in naturally contaminated meat blocks were graphically compared to the observed growth at 5 and 20°C. Under static pH conditions, the model could satisfactorily describe the growth of the pathogen at 20°C, but not at 5°C. In the latter case the predicted values from the model were systematically lower than the experimentally observed values, indicating underprediction of growth. However, when dynamic pH conditions were considered while generating the model, the observed and the predicted growth of *L. monocytogenes* showed good agreement at both storage temperatures (Figure 3.10).

The above observations were also validated in naturally contaminated minced meat-batch 2. The growth responses of the endogenous meat microflora and *L. monocytogenes* are presented in Figure 3.11 (also figure A.3 in the appendix). As expected, pseudomonads dominated over the rest members of the microbial association. However, and in contrast to meat blocks or minced meat-batch 1, LAB were found to reach population levels similar to that of *Brochothrix thermosphacta* and/or *Enterobacteriaceae*. A possible explanation would be their high initial (day-0) population (4.60 log CFU/g), which was comparable to that of the total aerobic populations (4.88 log CFU/g) (Figure 3.11; also figure A.3 in the appendix). The growth responses of the pathogen in minced meat-batch 2 under static and dynamic pH conditions were then evaluated at 5 and 20°C (Figure 3.12). Under all conditions studied the model gave significant overpredictions of the growth of *L. monocytogenes* as compared to the observed growth.

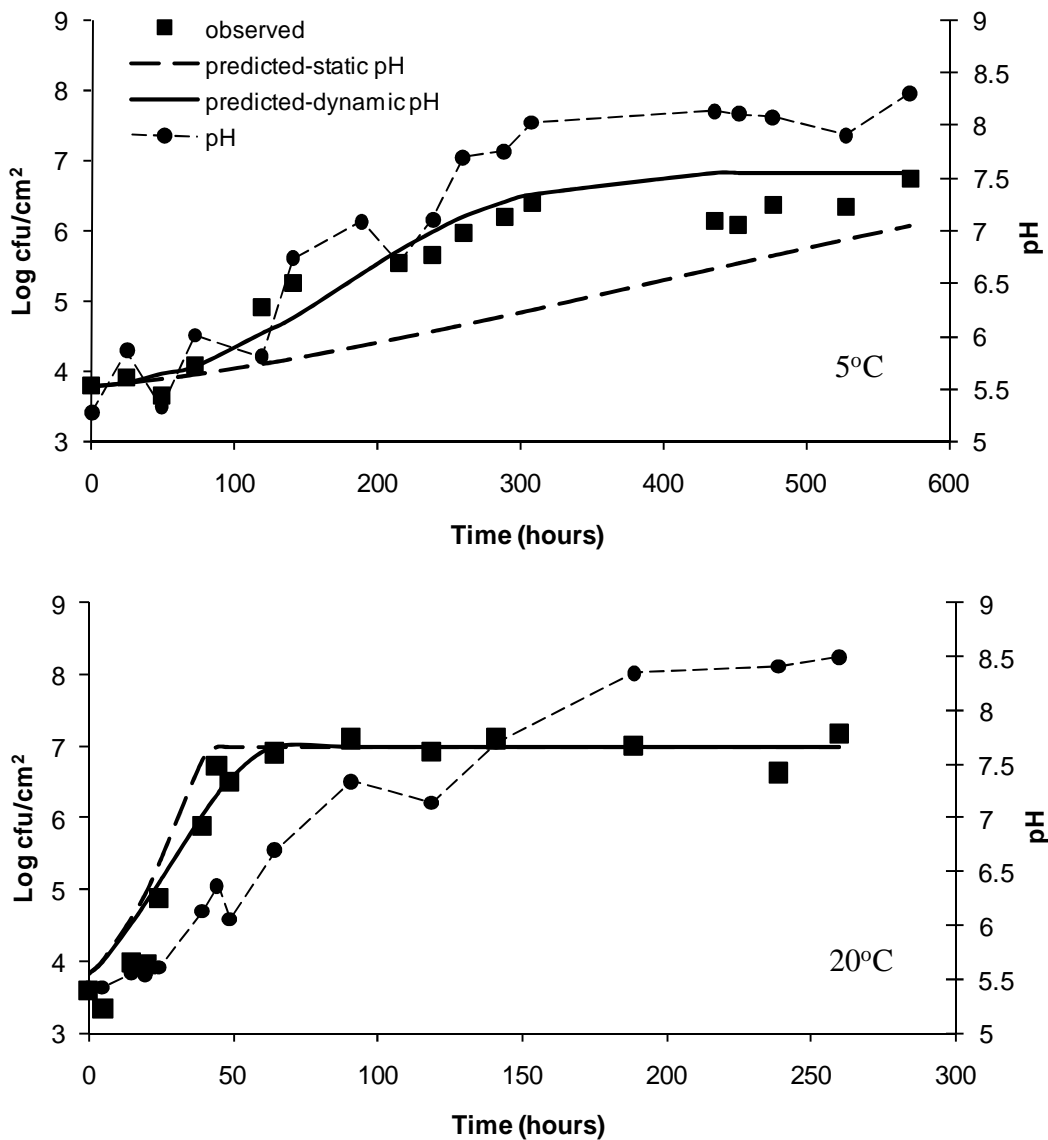
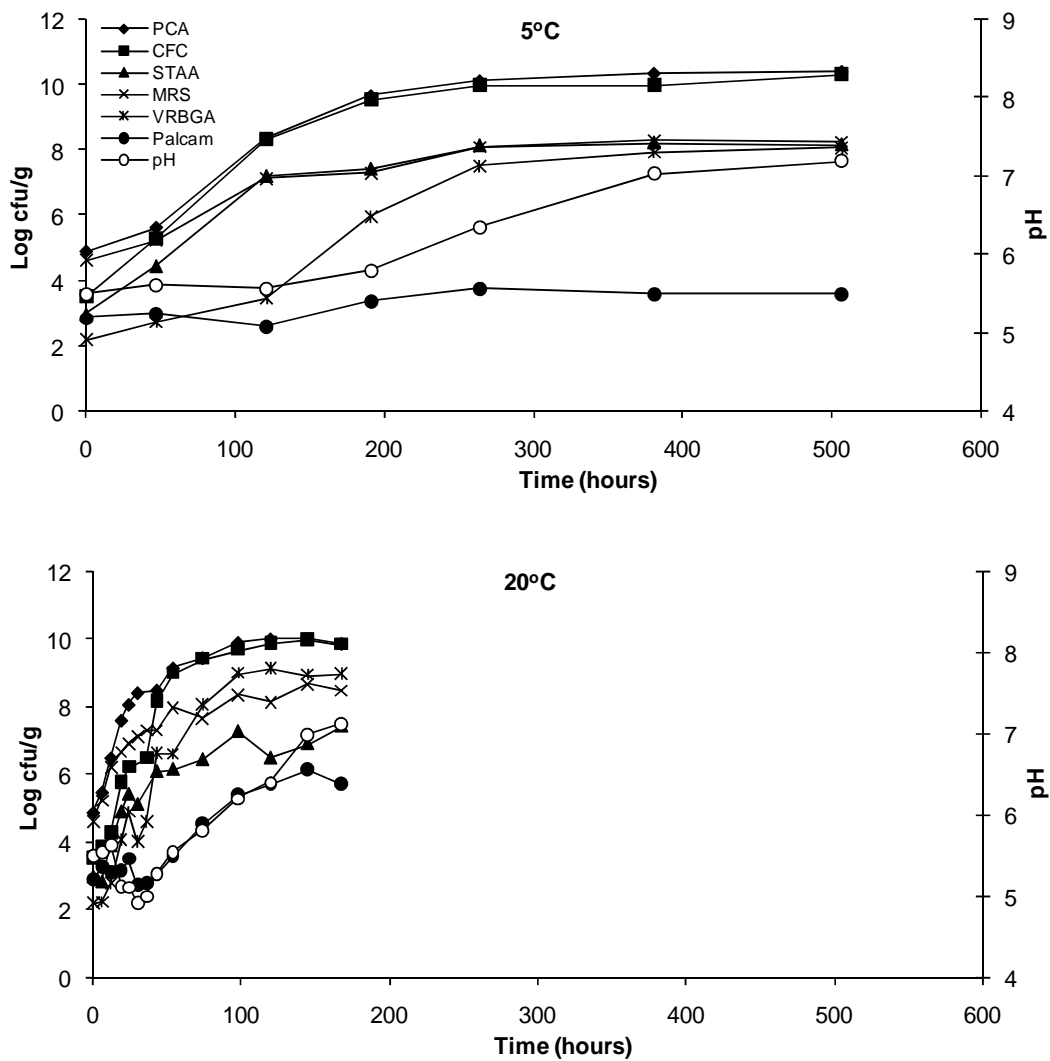


Figure 3.10 Observed and predicted growth of *L. monocytogenes* under static (initial pH value of naturally contaminated meat blocks) and dynamic pH in naturally contaminated meat blocks stored at 5 and 20°C.





**Figure 3.11** Representative growth curves of the spoilage microflora and *L. monocytogenes* and pH changes on naturally contaminated minced meat-batch 2 stored aerobically at 5 and 20°C. Media: PCA, plate count agar (total aerobic populations); CFC, cetrimide fusidin cephaloridine (pseudomonads); STAA, streptomycin-thallos acetate (actidionine) agar (*B. thermosphacta*); MRS, Man Rogosa Sharp (lactic acid bacteria); VRBGA, violet red bile glucose agar (*Enterobacteriaceae*); Palcam, Palcam Listeria Selective Agar (*L. monocytogenes*).

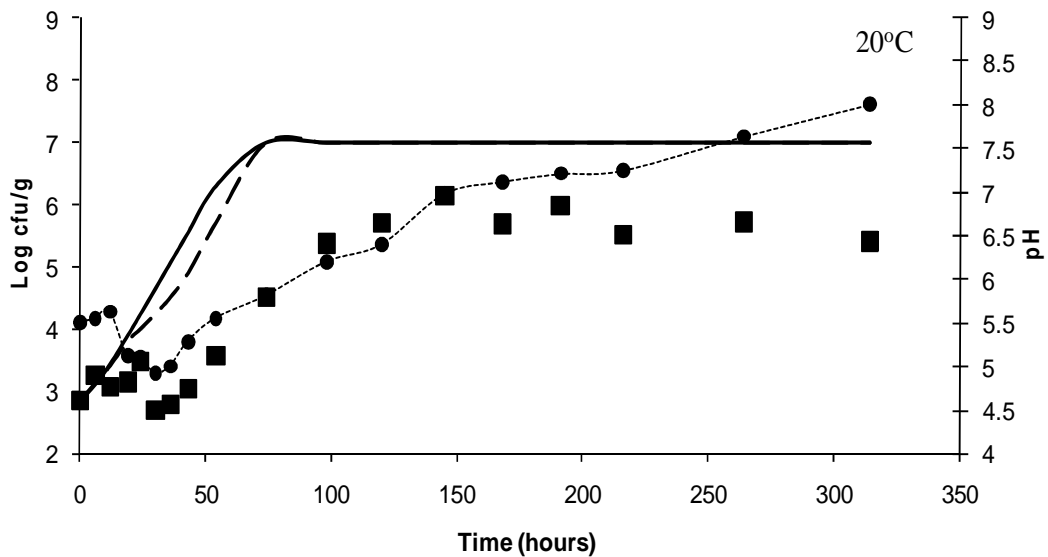
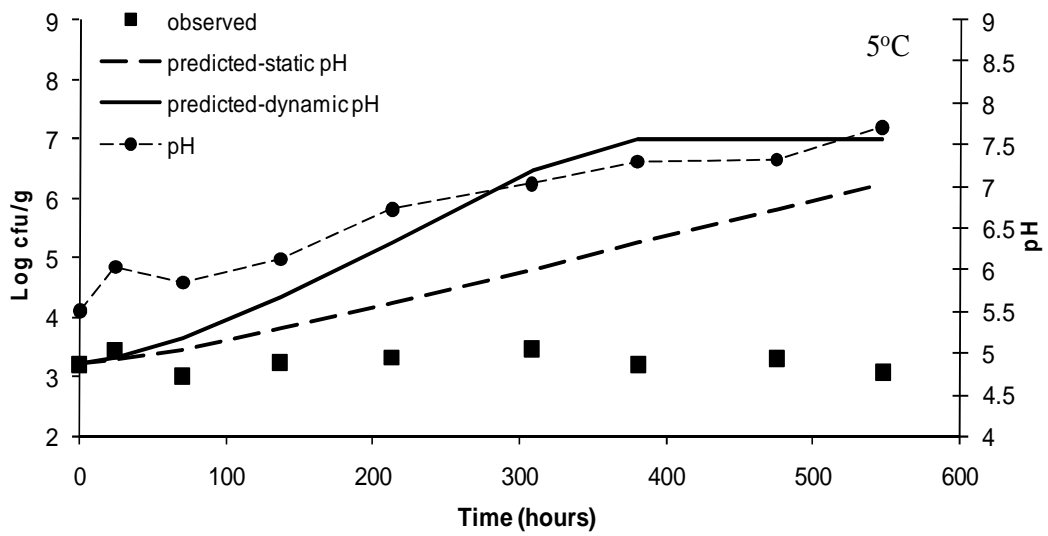


Figure 3.12 Observed and predicted growth of *L. monocytogenes* under static (initial pH value of naturally contaminated minced meat) and dynamic pH in naturally contaminated minced meat-batch 2 stored at 5 and 20°C.

### 3.4 DISCUSSION

Historically, food safety has been assessed using conventional microbiological methods. However, the food industry and authorities are looking for alternative advantageous techniques that will rapidly predict microbial growth and therefore eliminate the time-consuming and retrospective microbiological methods. Over the last years a plethora of mathematical models have emerged to quantitatively estimate the nature and extent of microbial growth under a range of environmental conditions, known to be key determinants of growth. Notwithstanding this progress, there is a wide criticism on whether models, developed in experimental liquid systems, can reliably predict the growth of microorganisms in real foods. Poor prediction performance of mathematical models termed as “completeness error” is attributed to the effect of factors (variables) in a food that are not explicitly considered or yet acknowledged (Ross et al., 2000).

In the present study the growth responses of *L. monocytogenes* were evaluated in laboratory growth media (broth and agar) and in real foods (naturally contaminated minced meat and meat blocks, and sterile meat blocks). The results showed significant differences in the behaviour of the pathogen at the different growth media and food products tested especially at low storage temperatures (Figure 3.1). On the basis that predictive models are developed in experimental liquid systems, a broth-based model was generated and validated against observed growth in other structured growth substrates to account for potential prediction errors. The calculation of bias and accuracy factors indicated that the sources of prediction error may be due to the increase in complexity of the food (model) system as proposed by Pin et al. (1999). The internal validation of the developed model estimated the primary error, i.e. the difference between growth rates predicted by the model under evaluation and the growth rates estimated from growth curves in broth (in this study used as well to create the model) which was found to be within the satisfactory Bf limits ( $0.7 < Bf < 1.15$ ) proposed for models describing pathogens growth rate (Ross et al., 2000). The comparison of the growth rates predicted from the broth model to those observed in other structured laboratory and real food substrates indicated the overall error of the broth model attributed to factors such as the food (model) structure, composition and microbial

interactions, or a combination thereof (Table 3.1). Furthermore, the extent of prediction error was analysed as a function of temperature by calculating the percent relative error (%RE). The observed discrepancies were found to be more pronounced at stressful environmental conditions, i.e. low storage temperatures.

One of the main reasons for this observed behavior of *L. monocytogenes* is the different structure of the growth substrate and consequently the different type of microbial growth. It is well known that microbial growth in foods takes place in the aqueous phase and is affected by the microstructural features of this phase. Aqueous phase can be uniform throughout the food or particular food regions may appear different structures. The variety in structures is defined as micro-architecture and is the reason for the two types of microbial growth, i.e. planktonic and immobilized (Robins and Wilson, 1994; Wilson et al., 2002). The effect of substrate microstructure on the growth responses of *L. monocytogenes* was further isolated and the observed maximum specific growth rate of the pathogen on sterile meat blocks and agar inoculated using two different techniques (spread and pour) was compared to that predicted in broth (Figure 3.4). Under all conditions tested, growth in broth was found to be higher than growth in the bulk or on the surface of the agar medium or sterile meat blocks, a behaviour often referred to as “fail-safe”. In liquid laboratory media, which are commonly used to describe microbial evolution and referred to as homogeneous, growth is typically planktonic and is often accompanied by motility. Accordingly, taxis to preferred regions of the medium is feasible. Conversely, on solid substrates or foods that are referred to as structured (e.g water-in-oil emulsions), cells are immobilized and constrained to form microcolonies or ‘nests’ (Brocklehurst et al., 1995; Wimpenny et al., 1995; Wilson et al., 2002).

Immobilized cultures demonstrate a slower growth rate than planktonic ones and narrower growth yields, especially when growth is combined with stressful environmental conditions (Robins and Wilson, 1994; Brocklehurst et al., 1997; Skandamis et al., 2000; Wilson et al., 2002; Meldrum, et al., 2003; Koutsoumanis et al., 2004). For example, Brocklehurst et al. (1997) reported growth rate of planktonic cells to be faster than that of immersed colonies and even faster than that of surface colonies

while studying the effect of sucrose and sodium chloride on the growth rate of *Salmonella* Typhimurium in broth and on gelatin gel (bulk and surface). Similarly, Koutsoumanis et al. (2004) postulated a constant difference in the growth/no growth responses of *L. monocytogenes* quantified in broth and agar under a wide range of temperature, pH and water activity combination treatments. These findings are consistent with the results of this study as growth of *L. monocytogenes* in broth was faster than in structured laboratory or sterile meat samples in which growing cells were immobilized especially at low temperatures (Figures 3.1 and 3.4). The slower growth rate of colonial growth has been attributed to a variety of reasons, including the supply of nutrients and oxygen to the cells (Wimpenny et al., 1995; Skandamis et al. 2000), the production and diffusion of their metabolic end-products away from the site of growth (Wimpenny et al., 1995; Walker et al., 1997; Skandamis et al. 2000) and the capacity for physical expansion of the colonies (Robins and Wilson, 1994; Stecchini et al., 1998; Antwi et al., 2006).

Apart from the food structure, another considerable difference between laboratory and real food conditions is the complex microbial association that develops in food products. To eliminate the contribution of the former and to evaluate the influence of the endogenous meat flora on the growth of *L. monocytogenes*, a model based on data from the agar-spread experiments was developed, and its performance was evaluated against data obtained from meat products. While at 15 and 20°C a good concordance was obtained between the model predictions and the experimental results, at all temperatures below 10°C the agar-model was found to significantly underestimate the growth of *L. monocytogenes* in naturally contaminated meat blocks and minced meat-batch 1, and thus, leading to “fail-dangerous” predictions (Figure 3.6). The relatively poor performance of models generated in laboratory growth media as compared to data from studies on meat or other food products at unfavorable for the growth of microorganisms conditions has also been reported (Mellefont et al., 2003).

Foods, and in particular raw meat products, are accompanied with changes in their local chemical properties (e.g. pH) through the proliferation of the endogenous meat microflora (Nychas et al., 1998, 2007). Such changes may significantly vary with time

and storage temperature. This was also the case in naturally contaminated meat, where growth of *L. monocytogenes* until stationary phase at 20°C was concomitant with a slight pH increase (0.5 units), whereas, at 5°C an increase of 2.8 units was attained (Figure 3.9). The pronounced pH increase at the low storage temperature could be explained by the dominance of the fast growing (compared to the rest of the members of the meat microbial association) psychrotrophic pseudomonads that induced a rapid shift in the pH due to the catabolism of meat creatine, creatinine and other amino acids (Drosinos and Board, 1994; Nychas et al., 2007). Therefore, the above results suggest that the use of a model that neglects the dynamic pH conditions of the meat environment would not accurately predict the growth responses of the pathogen under stressful storage temperatures.

To support this statement the model of Buchanan and Philips (2000) was used and the growth of *L. monocytogenes* in meat blocks was compared to that predicted under static (initial pH of meat; 5.34) or dynamic pH conditions (pH changes observed during storage of meat blocks). Indeed, the model was found to perform well at both storage temperatures only when the dynamic pH conditions were considered (Figure 3.10). However, that was not the case in naturally contaminated minced meat-batch 2 as the model overestimated the growth of *L. monocytogenes* under all conditions tested (Figure 3.12). In this case it is possible that LAB, initially present in raw meat at population levels comparable to that of total aerobic counts, produced lactic acid and/or other antimicrobial metabolic end-products (e.g. bacteriocins) that retarded (20°C) or inhibited (5°C) the growth of *L. monocytogenes*. As raw meat is characterized by a high buffering capacity, the concentration of produced lactate, which does not completely dissociate, was not reflected in significant changes in the pH of minced meat. In other words, the presence of undissociated lactic acid could have caused an additional inhibitory effect on the pathogens' growth separate from but linked with the effect of pH (Presser et al., 1997). The inclusion of both pH and the undissociated form of lactic has been shown to give rise to more realistic predictions of pathogenic growth (Presser et al., 1997; Antwi et al., 2007).

The complexity of microbial interactions, in terms of initial microbial numbers and types in a given or changing environment, and their affect on the growth or survival of *L. monocytogenes* illustrated in this study has been reported elsewhere (Buchanan and Bagi, 1999; Tsigarida et al., 2000; Gnanou Besse et al., 2006). Buchanan and Bagi (1999) demonstrated, for example, that *L. monocytogenes* growth in co-culture with *Pseudomonas fluorescens* could attain maximum population densities that were lower, higher, or the same compared to levels of the pathogen monoculture, depending on the temperature, acidity, and availability of water in the surrounding environment.

In conclusion, this study demonstrates the need for reevaluating the importance of the above underestimated factors in quantitative microbiology, as they can significantly affect the performance of predictive models especially under stressful environmental conditions. Moreover, the necessity for generating “ecological” predictive models that would take into account not only the environmental factors that affect microbial growth (e.g. temperature, aw and pH) but also a detailed knowledge of the food (i.e. structure, chemical and physicochemical parameters), its complex microbial community dynamics (i.e. ephemeral (specific) spoilage organisms and spoilage level) and microbial metabolites as the consequence of microbial growth, is indicated.





## **CHAPTER 4**

# **FORMATION AND CONTROL OF *Escherichia coli* O157:H7 BIOFILMS ON FOOD-CONTACT SURFACES AND BEHAVIOR OF PATHOGEN CELLS, SURVIVING IN BIOFILMS AFTER SANITATION, IN RAW MEAT**



## Chapter 4

### **Formation and control of *Escherichia coli* O157:H7 biofilms on food-contact surfaces and behaviour of pathogen cells, surviving in biofilms after sanitation, in raw meat**

#### **ABSTRACT**

Contamination of foods by *Escherichia coli* O157:H7 may occur through biofilms formed on stainless steel and other food-contact surfaces. Therefore, it is of primary importance to investigate its potential for biofilm formation, removal and destruction. The first goal of this study was to evaluate the attachment and subsequent biofilm formation on stainless steel surfaces by each of nine different strains of *E. coli* O157:H7. Further, the attachment, survival and growth of a six-strain composite of *E. coli* O157:H7 on food-contact surfaces under conditions encountered in meat processing was investigated. Stainless steel and high-density polyethylene surfaces were inoculated with *E. coli* O157:H7 (6 log CFU/ml or g) suspended in tryptic soy broth, fat-lean homogenate and ground beef and incubated statically at 4 and 15°C. As a final component of this study, the efficiency of various sanitizers to control biofilm formation and to investigate the potential for pathogenic cells, surviving in biofilms after sanitation, to survive in cross-contaminated ground beef was evaluated. The results demonstrated the need for using composites of selected biofilm-forming *E. coli* O157:H7 strains in studies concerning attachment and biofilm formation, and destruction/removal of these biofilms from food-contact surfaces as strains varied both in their ability to attach on stainless steel coupons and on the maximum adhesion levels attained at the end of incubation period. It is also suggested that studying biofilm formation in a single laboratory medium may impose a significant risk of obtaining biased results, especially under inimical environmental conditions, as the influence of organic soil and background microflora is neglected. Biofilm and planktonic cells differ in their susceptibility to sanitation treatments and survivors may grow differently in cross-contaminated minced meat.

## 4.1 INTRODUCTION

*Escherichia coli* O157:H7 was first recognized as a foodborne pathogen in 1982 (Riley et al., 1983). However, it was not until 1993 that it achieved notoriety in the United States, where consumption of lightly-cooked contaminated hamburger led to more than 700 illness cases (primarily children) and four deaths (Bell et al., 1994). Each year in the United States, *E. coli* O157:H7 causes about 73,480 illnesses out of which, over 2000 require hospitalization and 61 lead to death. Moreover, it has been estimated that the average medical costs and lost productivity caused by *E. coli* O157:H7 infections varies from \$5,862 to \$10,533 per patient (Buzby et al., 2004), totaling to an annual cost of \$301,8 to \$726,0 million (USDA/ERS, 1996). Most *E. coli* O157:H7 infections are mild and do not require medical care; however, these infections can result in bloody diarrhoea as well as haemorrhagic colitis (HC), haemorrhagic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) which have been associated with significant morbidity and mortality in humans (Riley et al., 1983; Coia, 1998).

Ruminants and in particular cattle are considered to be the major reservoir of *E. coli* O157:H7. Many outbreaks of *E. coli* O157:H7 have been linked to beef, and analyses of sporadic cases of infection have identified unpasteurized milk and raw or undercooked beef as important risk factors (Mead et al., 1997; Mainil and Daube, 2005). Contamination of food animal carcasses by microorganisms, including spoilage and pathogenic bacteria, can be introduced from hide, pelt, fleece, mouth, viscera or gastrointestinal tract during slaughtering and processing (Koutsoumanis and Sofos, 2004; Koutsoumanis et al., 2005). As a consequence, the resident flora of carcasses surface may transfer to the surface of equipment associated with slaughter and fabrication and lead to cross-contamination of both carcasses and fresh meat products (Gill et al., 2001).

The potential of these surfaces to harbor *E. coli* O157:H7 and serve as sources of contamination has been discussed (Gill and McGinnis, 2000; Gun et al., 2003). Barkocy-Gallagher et al. (2001) recovered *E. coli* O157:H7 and *E. coli* O157 isolates from feces, hides and carcasses at four different beef processing plants. However, only

65.3% (post-evisceration) and 66.7% (in the cooler) of individual isolates from carcasses matched those recovered pre-evisceration. Tutenel et al., (2003) also showed that types of *E. coli* O157:H7 in a cattle slaughterhouse were different than those on the hides of the slaughtered animals.

The presence of *E. coli* O157:H7 in meat fabrication and food-contact surfaces and equipment has been associated with the ability of the organism to attach, colonize and form biofilms on these surfaces (Carpentier and Cerf, 1993; Dewanti and Wong., 1995; Hood and Zottola, 1995, 1997a). Biofilms are microbial communities of immobilized cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a self-produced matrix of extracellular polymeric substances and exhibit an altered phenotype with respect to growth rate and gene transcription than the corresponding planktonic cells (Donlan and Costerton, 2002). Biofilm formation is a dynamic process and when bacteria come in contact with surfaces they may attach readily.

The attachment of bacterial cells on abiotic and biotic surfaces is affected by several factors, including the presence of organic material (i.e. conditioning film), the growth medium and the availability of nutrients, temperature, pH, production of extracellular polysaccharides, presence of cellular structures, physicochemical (hydrophobicity and surface charge) properties of both food-contact and cell surfaces, hydrodynamics and cell-to-cell communication (Hood and Zottola, 1995; Davies et al., 1998; Sharma and Anand, 2002; Chmielewski and Frank, 2003).

Food processors have relied on physical and chemical methods to remove or eliminate microorganisms from food-contact surfaces and equipment. However, the attached microorganisms have been shown to be more resistant to sanitizing compounds than their planktonic counterparts (Costerton et al., 1995; Farrell et al., 1998; Kumar and Anand, 1998; Uhlich et al., 2006). The presence of even low populations of *E. coli* O157:H7 (Paton and Paton, 1998) on food-contact surfaces may pose a significant health risk through cross-contamination of foods.

So far, adherence and biofilm formation by *E. coli* O157:H7 has been mostly studied in well-defined and controlled laboratory media, under environmental factors that are favourable for the growth of the organism and utilizing single strain cultures of the pathogen rather than multi-strain composites (Ryu et al, 2004; Dewanti and Wong, 1995; Uhlich et al. 2006; Rivas et al., 2007a). However, these conditions do not represent the microenvironment encountered in food processing facilities. Knowledge on the attachment and biofilm formation by multi-strain composites of *E. coli* O157:H7 under inimical conditions encountered in processing plants such as cold and moderately cold temperatures, a wide range of soiling substrates and food-contact surfaces and natural microflora is of relevance to meat safety.

Considering the above, this study was conducted to: i) to compare the potential for attachment and biofilm formation of nine *E. coli* O157:H7 strains on stainless steel surfaces, using two methods of inoculation, ii) to evaluate the effect of substrate and storage temperature on attachment and biofilm formation by *E. coli* O157:H7 on stainless steel and high-density polyethylene surfaces, and ili) to estimate the efficacy of sanitizers to control biofilm formation on acetal surfaces and to evaluate the behavior of biofilm and planktonic cells of *E. coli* O157:H7, surviving after exposure to sublethal concentrations of various sanitizers, in ground meat.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Bacterial strains and inocula preparation

Nine rifampicin-resistant (100 µg/ml) derivatives of *Escherichia coli* O157:H7, including ATCC 51657 (clinical isolate), ATCC 51658 (clinical isolate), ATCC 43895 (raw hamburger meat implicated in hemorrhagic colitis outbreak), ATCC 43895 ISEH/GFP (Noah et. al., 2005) and five strains isolated from cattle feces (F284, F289, F460, F468 and F469) were used in this study.

Stock cultures of each strain were maintained at -70°C in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) supplemented with 100 µg/ml of rifampicin (Sigma, St. Louis, MO) and 20% glycerol (Mallinckrodt Baker, Inc., Paris, KY). Working cultures were stored at 4°C on tryptic soy agar (TSA, Difco) slants supplemented with 100 µg/ml rifampicin (TSA + rif) and were renewed monthly. Strains were activated by transferring a loopful of each individual strain from TSA + rif slants to TSB containing 100 µg/ml rifampicin (TSB + rif) and incubating at 35°C for 24 h. The activated strains were subcultured in TSB + rif to ensure that cultures retain their antibiotic resistance and incubated at 35°C for 24 h. Each culture was centrifuged at 4,629×g (Eppendorf model 5810 R; Brinkmann Instruments Inc., Westbury, NY) for 15 min at 4°C. The resulting pellets were washed twice with 10ml of sterile phosphate-buffered saline (PBS; pH 7.4, 0.2 g/L KH<sub>2</sub>PO<sub>4</sub>, 1.5 g/L Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 8.0 g/L NaCl and 0.2 g/L KCl) and cell pellets were resuspended in 10ml TSB to provide working cultures of bacterial concentration of ca. 10<sup>9</sup> CFU/ml for each strain.

In experiments where a composite of selected *E. coli* O157:H7 (ATCC 51657, ATCC 51658, ATCC 43895, ATCC 43895 ISEH/GFP, F284 and F469) strains was used, equal volumes (10ml) of each bacterial culture were combined and cells were harvested by centrifugation. The resulting pellet was washed twice with 60ml of sterile PBS and cells pellet was resuspended in 60 ml PBS or filter-sterilized beef homogenate to provide working cultures of bacterial concentration of ca. 10<sup>9</sup> CFU/ml. For preparation of filtered-sterilized beef homogenate ground beef was 1:10 diluted with sterile distilled water in a sterile WhirlPak bag (Nasco, Modesto, CA), masticated for 2 min

(Masticator, IUL Instruments, Barcelona, Spain), passed through sterile cheesecloth to retain pieces of fat and lean and then passed through a 0.22 µm filter (Millipore).

#### **4.2.2 Test surface**

Stainless steel (type 304, no. 2b finish), high-density polyethylene (HDPE) and acetal surfaces, extensively used in the manufacturing of food-processing equipment, were used in this study. All types of material surfaces (2 by 5 by 0.08 cm) were soaked overnight in commercial detergent solution, degreased with 70% ethanol, thoroughly washed in tap water and subsequently in sterile distilled water, and allowed to air dry. Cleaned coupons were finally autoclaved at 121°C for 20 min prior to use.

#### **4.2.3 Inoculation and biofilm formation**

The substrates used in this study included TSB (pH 7.28), filter-sterilized beef homogenate (pH 5.93), naturally contaminated ground beef (pH 5.75) and naturally contaminated beef fat-lean (1:1) homogenate (pH 5.64). For preparation of fat-lean homogenate, equal portions of fat and lean tissue were obtained, diluted 1:10 with sterile distilled water in a sterile WhirlPak bag, masticated for 2 min and passed through sterile cheesecloth to retain pieces of fat and lean.

Aliquots (2 ml) of the mono- or multi-strain working cultures were then inoculated into 2 l or Kg of each substrate, and a target inoculum of ca.  $10^6$  CFU/ml or g was obtained. Inoculation of material surfaces with cells of *E. coli* O157:H7 was performed as follows.

Stainless steel coupons were placed individually in an upright position into sterile polypropylene tubes (50 ml, Fisher Scientific, Hanover Park, IL) containing TSB (20 ml) inoculated with each strain of *E. coli* O157:H7. Cells were allowed to attach to the coupons for 30 min at ambient temperature ( $22 \pm 1^\circ\text{C}$ ). Afterwards, coupons were either rinsed with 10 ml of sterile distilled water and transferred into 20 ml of fresh TSB (inoculation A) or were left (without being rinsed) in the original inoculated broth throughout the incubation period (inoculation B). Samples were incubated under static conditions at 15°C for up to 10 days.



In a second set of experiments, stainless steel and HDPE surfaces were individually placed into tubes containing TSB, fat-lean homogenate and/or ground beef inoculated with the six-strain composite of *E. coli* O157:H7 and immediately incubated at 4 and 15°C for up to 168 h (7 days).

Acetal surfaces were contaminated by immersion into sterile beef homogenate inoculated with the six-strain composite of *E. coli* O157:H7 for 30 min at 15°C. Following the attachment time, coupons were removed, rinsed with 20 ml sterile distilled water to remove unattached/loosely attached cells, and transferred into tubes containing 20 ml of fresh sterile beef homogenate (pH 5.78). Samples were incubated under static conditions for 7 days at 15°C prior to other treatment (essentially described in section 4.2.5). Storage temperatures (4 and 15°C) were selected to represent the cold and moderately cold conditions often encountered in meat processing plants.

#### **4.2.4 Quantification of biofilm and pH analyses**

To evaluate the initial attachment and biofilm formation at appropriate days of incubation, individual coupons were removed with sterile forceps, rinsed with 20 ml sterile distilled water using a sterile plastic pipette to remove unattached or loosely attached cells, and transferred into tubes (85 ml, Oakridge, Nalgene, Nalge Nunc International, Rochester, NY) containing 40 ml of maximum recovery diluent (MRD; 0.85% NaCl and 0.1% peptone) plus 10 glass beads (4 mm; Kimble Glass Inc., Germany). Biofilm cells were detached from the coupons by vortexing (3200 rpm; Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY) the tubes for 2 min.

Quantification of biofilm formation was performed using the plate count method. Appropriate ten-fold serial dilutions (0.1% buffered peptone water; Difco) were surface plated onto TSA to determine total bacterial counts (when natural flora was present in the substrates) and/or TSA + rif for the selective enumeration of *E. coli* O157:H7. TSA plates were incubated for 48 h at 25°C and TSA + rif plates for 24 h at 35°C. Cells suspended in the substrates (i.e., planktonic cells) were also enumerated as described above. The pH of suspensions after plate culture was measured using a digital pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

#### **4.2.5 Inoculation of ground beef with biofilm and planktonic cells of *E. coli* O157:H7 surviving exposure to sublethal sanitation**

*Exposure of biofilm-derived and planktonic cells to sanitizer treatment and inoculum preparation for contamination of ground beef*

Individual acetal coupons bearing attached cells were removed from beef homogenate on day-7 of storage, rinsed with 20ml of sterile distilled water to remove loosely attached cells and immersed in 30 ml of different sanitizer solutions at ambient temperature ( $22 \pm 1^\circ\text{C}$ ). Sanitizers, concentrations and exposure times (minimum recommended concentrations and exposure times by the manufacturers) tested included:

- i) Quaternary ammonium compound (QUAT; Oasis 146 Multi Quat Sanitizer; Ecolab); 150 ppm (pH  $7.69 \pm 0.22$ ), 1 min and
- ii) Peracetic/octanoic acid mixture (Vortexx, Ecolab); 0.13% (pH  $4.66 \pm 0.67$ ), 1 min.

Both sanitizer solutions were prepared using sterile distilled water.

At the end of the exposure time, coupons were immediately rinsed with 20 ml sterile distilled water and were placed individually in tubes containing 40 ml of D/E neutralizing broth (Difco) and 10 glass beads. Control (untreated) coupons were placed directly in D/E neutralizing broth without applying a sanitizer treatment. Cells attached to the coupons were dislodged, as described previously (section 4.2.4). The detached cells were then concentrated by centrifugation ( $4,629 \times g$ ,  $4^\circ\text{C}$ , 15min) and used to inoculate ground beef. Planktonic cells in the beef homogenate were centrifuged ( $4,629 \times g$ ,  $4^\circ\text{C}$ , 15min) and pelleted cells were resuspended in fresh sterile beef homogenate prior to the application of sanitizers. After exposure to sanitizers, 20 ml of D/E neutralizing broth was added to 20 ml of each solution to neutralize the effect of sanitizers. Planktonic cells surviving sanitation were concentrated by centrifugation, and also used to inoculate ground beef. Levels of surviving cells were determined prior and after the application of sanitizers.

### *Inoculation of ground beef, storage and analysis*

Concentrated biofilm- and planktonic-derived cell suspensions of *E. coli* O157:H7 surviving sanitation treatments were appropriately diluted and 1 ml was added to 1 kg of ground beef. The inoculum and ground beef were mixed for 2 min using a bowl-lift stand mixer (KitchenAid Professional 600, St. Joseph, MI) at a speed setting of 2. Patties (100 g) were formed and were placed onto meat retail foam trays (14×21 cm; Koch) containing a soaker pad (12× 8 cm, Koch) and overwrapped with air permeable plastic film (Omnifilm, Pliant Corp., Uniontown, OH). Packaged samples were stored at 4 and 12°C. After 0, 3, 6 and 9 days, individual samples were analyzed by placing each sample in a sterile WhirlPak bag, adding 40-ml portions of MRD, and masticating (Masticator, IUL Instruments, Barcelona, Spain) the sample for 2 min. Slurries were then serially diluted with 0.1% BPW. For the enumeration of total bacterial populations appropriate dilutions were surface plated on TSA (25°C, 48 h) whereas *E. coli* O157:H7 was enumerated on TSA+rif (24 h at 35°C followed by 12 h at 25°C).

#### **4.2.6 Data analysis**

Experiments were repeated twice with three samples analyzed at each sampling interval. Microbial counts were transformed to logarithms before means and standard deviations were computed, and counts were reported in terms of log CFU/cm<sup>2</sup>, ml or g for attached cells or cells in suspension. Data analysis was performed using the GLIMMIX (generalized linear mixed model) procedure of SAS (SAS Institute version of 9.1). Least squares means were calculated and separated by the least significant difference procedure at a significance level of 95%.

## 4.3 RESULTS

### 4.3.1 Attachment and subsequent biofilm formation on stainless steel surfaces by nine strains of *Escherichia coli* O157:H7 under two inoculation scenarios

The initial attachment and subsequent biofilm formation of nine different strains of *E. coli* O157:H7 on stainless steel under the two inoculation scenarios (A and B) are presented in Table 4.1. The results showed that all strains of *E. coli* O157:H7 attached to stainless steel coupons with populations varying from 0.4 to 3.5 and 1.1 to 3.5 log CFU/cm<sup>2</sup> for inoculation scenario A and B, respectively. The attachment ability among the strains tested was further evaluated by estimating the relative attachment percentage based on the initial culture inoculum and the counts of attached on coupons cells immediately after each inoculation (Figure 4.1). The results showed a wide range ( $p < 0.05$ ) of initial attachment ability among the strains of *E. coli* O157:H7, with ATCC 43895 and F468 presenting the highest and lowest attachment, respectively.

For the majority of strains tested, the inoculation scenario (A or B) did not seem to affect the initial adherent populations and biofilm formed after 10 days of incubation (Table 4.1). However, during storage cells attached at different rates on stainless steel coupons and maximum counts were obtained after 6 and 3 days of storage for inoculation A and B, respectively. This could possibly be explained by the differences in the populations of relevant planktonic cells growing in suspensions at the beginning of storage. Indeed, counts of *E. coli* O157:H7 in suspension on day 0 using inoculation scenarios A and B varied from 0.5 to 2.8 log CFU/ml and 6.0 to 6.4 log CFU/ml, respectively (Table 4.2).

Moreover, on the first day of storage, a reduction on the populations of ATCC 51658, F284, ATCC 43895 ISEH/GFP, ATCC 43895, F460 and F469 was observed for coupons immediately transferred in fresh TSB after their inoculation. That was not the case, however, for coupons left in the original inoculated broth.

Contrary to what was expected, biofilm formation after 10 days of storage was not dependent on initial attachment. Biofilm growth, for example, for *E. coli* 43895

ISEH/GFP was significantly higher than for ATCC 51658, F284, ATCC 51657 and F289, even though the latter strains had comparable ( $p \geq 0.05$ ) relative attachment percentages to ATCC 43895 ISEH/GFP strain (Table 4.1; Figure 4.1). Similarly, biofilm growth for *E. coli* O157:H7 43895 and 43895 ISEH/GFP was similar, although strain 43895 had a higher relative attachment percentage. These differences in biofilm formation at the end of storage could not be correlated with any differences in the counts of *E. coli* O157:H7 in suspension since similar growth performances were observed for suspended cells (Table 4.2). The pH of suspensions inoculated with each of nine *E. coli* O157:H7 strains decreased by ca. 1 unit during storage (Table 4.3).

**Table 4.1 Counts (mean  $\pm$  standard deviation; log CFU/cm<sup>2</sup>) of nine *E. coli* O157:H7 strains attached to stainless steel surfaces during storage in tryptic soy broth (TSB) at 15°C, under two inoculation scenarios**

Inoculation scenario <sup>a</sup>	Storage time (days)	<i>Escherichia coli</i> O157:H7 strain								
		ATCC 51658	F284	ATCC 43895 ISEH/GFP	ATCC 43895	F460	ATCC 51657	F289	F469	F468
A	0	1.9 $\pm$ 0.4	2.0 $\pm$ 0.1	1.8 $\pm$ 0.4	3.5 $\pm$ 0.1	1.2 $\pm$ 0.3	1.5 $\pm$ 0.2	1.5 $\pm$ 0.1	1.1 $\pm$ 0.6	0.4 $\pm$ 0.0
	1	$\leq$ 0.4 <sup>b</sup>	1.6 $\pm$ 1.0	0.5 $\pm$ 0.2	2.5 $\pm$ 0.3	$\leq$ 0.4	1.0 $\pm$ 0.3	1.3 $\pm$ 0.2	$\leq$ 0.4	0.5 $\pm$ 0.2
	3	1.9 $\pm$ 0.2	2.8 $\pm$ 0.0	3.6 $\pm$ 0.1	4.7 $\pm$ 0.5	1.6 $\pm$ 0.2	1.8 $\pm$ 0.1	3.1 $\pm$ 0.4	1.1 $\pm$ 0.2	1.0 $\pm$ 0.5
	6	3.8 $\pm$ 0.2	4.1 $\pm$ 0.3	7.3 $\pm$ 0.2	7.3 $\pm$ 0.2	3.0 $\pm$ 0.5	4.0 $\pm$ 0.8	4.0 $\pm$ 0.7	3.8 $\pm$ 0.1	3.4 $\pm$ 0.3
	10	4.6 $\pm$ 0.4	3.8 $\pm$ 0.2	7.1 $\pm$ 0.2	7.8 $\pm$ 0.4	3.3 $\pm$ 0.2	3.5 $\pm$ 0.0	3.2 $\pm$ 0.4	4.4 $\pm$ 0.8	3.4 $\pm$ 0.3
B	0	2.4 $\pm$ 0.2	2.3 $\pm$ 0.2	2.1 $\pm$ 0.3	3.5 $\pm$ 0.1	1.3 $\pm$ 0.4	2.4 $\pm$ 0.3	2.2 $\pm$ 0.2	1.6 $\pm$ 0.3	1.1 $\pm$ 0.2
	1	2.7 $\pm$ 0.6	3.4 $\pm$ 0.3	3.3 $\pm$ 0.4	3.2 $\pm$ 0.3	2.5 $\pm$ 0.2	3.0 $\pm$ 0.3	3.1 $\pm$ 0.6	2.8 $\pm$ 0.2	3.4 $\pm$ 0.8
	3	3.4 $\pm$ 0.5	3.8 $\pm$ 0.8	6.6 $\pm$ 0.1	6.4 $\pm$ 0.2	3.3 $\pm$ 0.2	3.2 $\pm$ 0.4	3.6 $\pm$ 0.1	3.7 $\pm$ 0.7	3.5 $\pm$ 0.5
	6	4.3 $\pm$ 0.4	3.7 $\pm$ 0.4	6.6 $\pm$ 0.2	7.7 $\pm$ 0.3	3.5 $\pm$ 0.8	4.5 $\pm$ 0.4	4.2 $\pm$ 0.2	4.2 $\pm$ 0.4	3.4 $\pm$ 0.5
	10	4.8 $\pm$ 0.1	4.1 $\pm$ 0.4	7.3 $\pm$ 0.5	7.6 $\pm$ 0.2	3.2 $\pm$ 0.3	4.2 $\pm$ 0.4	3.6 $\pm$ 0.4	4.2 $\pm$ 0.4	4.0 $\pm$ 0.7

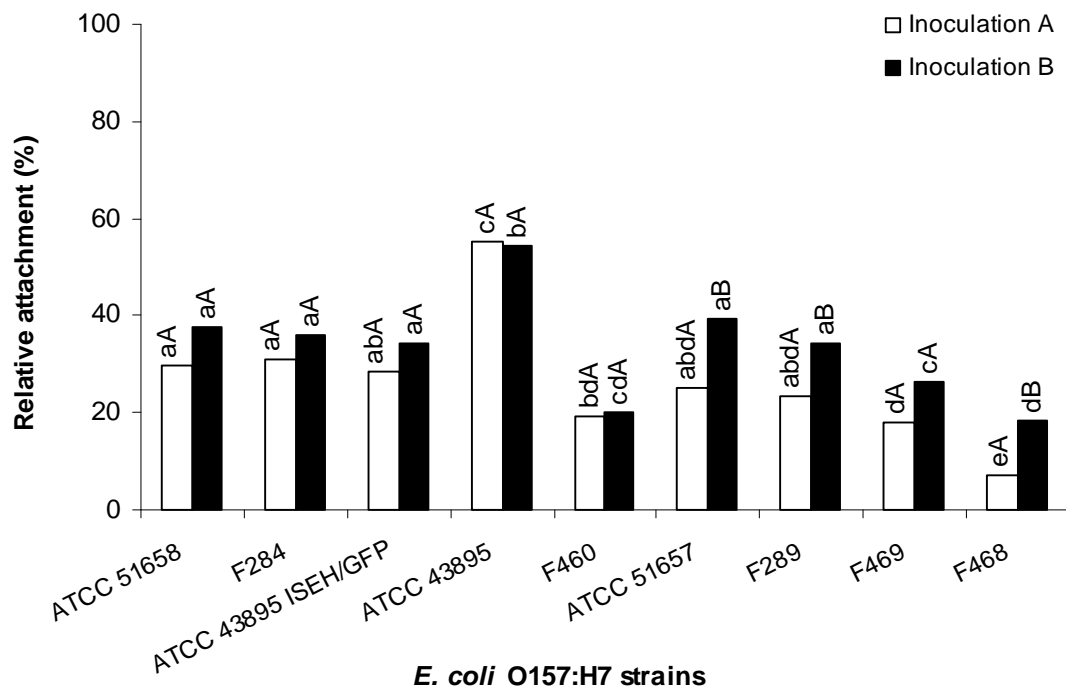
<sup>a</sup> Stainless steel surfaces were placed into 20 ml of TSB inoculated with each of nine strains of *E. coli* O157:H7 for 30 min at 22°C to initiate attachment and then were either transferred into 20 ml of fresh TSB (Inoculation A) or were left in the original inoculated TSB (Inoculation B).

<sup>b</sup> Detection limit: 0.4 log CFU/cm<sup>2</sup>

**Table 4.2 Counts (mean  $\pm$  standard deviation; log CFU/ml) of *E. coli* O157:H7 suspended in tryptic soy broth (TSB) during storage at 15°C, under two inoculation scenarios**

Inoculation Scenario	Storage time (days)	<i>Escherichia coli</i> O157:H7 strain								
		ATCC 51658	F284	ATCC 43895 ISEH/GFP	ATCC 43895	F460	ATCC 51657	F289	F469	F468
A	0	1.7 $\pm$ 0.4	1.4 $\pm$ 0.2	1.8 $\pm$ 0.1	2.8 $\pm$ 0.1	0.7 $\pm$ 0.3	1.3 $\pm$ 0.3	1.6 $\pm$ 0.6	0.9 $\pm$ 0.4	0.5 $\pm$ 0.3
	1	2.7 $\pm$ 0.2	3.5 $\pm$ 0.7	2.3 $\pm$ 0.3	4.1 $\pm$ 0.2	2.0 $\pm$ 0.2	2.7 $\pm$ 0.1	3.1 $\pm$ 0.2	2.2 $\pm$ 0.3	2.2 $\pm$ 0.3
	3	5.2 $\pm$ 0.2	6.2 $\pm$ 0.5	6.3 $\pm$ 0.1	7.7 $\pm$ 0.2	3.3 $\pm$ 0.3	6.2 $\pm$ 0.3	6.1 $\pm$ 0.3	4.8 $\pm$ 0.6	4.5 $\pm$ 0.3
	6	8.6 $\pm$ 0.2	8.9 $\pm$ 0.1	8.9 $\pm$ 0.5	9.0 $\pm$ 0.2	6.9 $\pm$ 0.4	9.0 $\pm$ 0.2	8.9 $\pm$ 0.1	8.8 $\pm$ 0.2	8.3 $\pm$ 0.1
	10	8.9 $\pm$ 0.1	8.9 $\pm$ 0.3	8.9 $\pm$ 0.3	9.0 $\pm$ 0.2	8.9 $\pm$ 0.6	9.1 $\pm$ 0.2	9.3 $\pm$ 0.1	9.3 $\pm$ 0.2	9.1 $\pm$ 0.2
B	0	6.4 $\pm$ 0.1	6.3 $\pm$ 0.1	6.2 $\pm$ 0.0	6.4 $\pm$ 0.2	6.3 $\pm$ 0.1	6.2 $\pm$ 0.1	6.3 $\pm$ 0.1	6.0 $\pm$ 0.1	6.0 $\pm$ 0.0
	1	6.8 $\pm$ 0.2	7.1 $\pm$ 0.1	6.7 $\pm$ 0.1	6.6 $\pm$ 0.3	6.2 $\pm$ 0.2	7.0 $\pm$ 0.4	7.1 $\pm$ 0.1	7.3 $\pm$ 0.3	7.2 $\pm$ 0.1
	3	8.7 $\pm$ 0.1	8.8 $\pm$ 0.1	8.6 $\pm$ 0.2	8.7 $\pm$ 0.2	8.4 $\pm$ 0.2	9.2 $\pm$ 0.6	8.8 $\pm$ 0.1	8.8 $\pm$ 0.1	8.7 $\pm$ 0.0
	6	8.9 $\pm$ 0.2	9.0 $\pm$ 0.1	8.6 $\pm$ 0.1	8.7 $\pm$ 0.2	9.1 $\pm$ 0.4	8.9 $\pm$ 0.0	9.3 $\pm$ 0.3	9.1 $\pm$ 0.4	9.5 $\pm$ 0.1
	10	9.0 $\pm$ 0.3	9.0 $\pm$ 0.1	8.9 $\pm$ 0.2	8.9 $\pm$ 0.2	8.7 $\pm$ 0.3	8.7 $\pm$ 0.4	9.0 $\pm$ 0.3	8.7 $\pm$ 0.4	9.1 $\pm$ 0.0

<sup>a</sup>Stainless steel surfaces were placed into 20 ml of TSB inoculated with each of nine strains of *E. coli* O157:H7 for 30 min at 22°C to initiate attachment and then were either transferred into 20 ml of fresh TSB (Inoculation A) or were left in the original inoculated TSB (Inoculation B).



**Figure 4.1** Relative attachment (%) of nine strains of *E. coli* O157:H7 on stainless steel coupons immediately after inoculations A and B. Means within inoculation scenario with different lowercase letter are significantly different ( $p < 0.05$ ). Means within strain with different uppercase letter are significantly different ( $p < 0.05$ ).



**Table 4.3 pH values (mean  $\pm$  standard deviation) of tryptic soy broth (TSB) inoculated with *E. coli* O157:H7 under two inoculation scenarios during storage at 15°C**

Inoculation scenario	Storage time (days)	<i>Escherichia coli</i> O157:H7 strain								
		ATCC 51658	F284	ATCC 43895 ISEH/GFP	ATCC 43895	F460	ATCC 51657	F289	F469	F468
A	0	7.37 $\pm$ 0.01	7.36 $\pm$ 0.01	7.31 $\pm$ 0.04	7.40 $\pm$ 0.03	7.40 $\pm$ 0.04	7.36 $\pm$ 0.02	7.34 $\pm$ 0.02	7.36 $\pm$ 0.01	7.43 $\pm$ 0.02
	1	7.38 $\pm$ 0.03	7.39 $\pm$ 0.03	7.38 $\pm$ 0.05	7.43 $\pm$ 0.03	7.40 $\pm$ 0.01	7.39 $\pm$ 0.02	7.38 $\pm$ 0.01	7.41 $\pm$ 0.05	7.39 $\pm$ 0.02
	3	7.28 $\pm$ 0.09	7.30 $\pm$ 0.18	7.45 $\pm$ 0.02	7.25 $\pm$ 0.12	7.44 $\pm$ 0.01	7.42 $\pm$ 0.01	7.40 $\pm$ 0.01	7.41 $\pm$ 0.04	7.42 $\pm$ 0.01
	6	5.62 $\pm$ 0.09	5.78 $\pm$ 0.04	5.87 $\pm$ 0.11	6.14 $\pm$ 0.02	7.36 $\pm$ 0.06	5.81 $\pm$ 0.08	5.89 $\pm$ 0.06	5.83 $\pm$ 0.04	6.88 $\pm$ 0.32
	10	6.10 $\pm$ 0.13	6.05 $\pm$ 0.11	6.16 $\pm$ 0.07	6.33 $\pm$ 0.08	5.99 $\pm$ 0.06	6.09 $\pm$ 0.03	5.98 $\pm$ 0.05	6.13 $\pm$ 0.02	6.06 $\pm$ 0.03
B	0	7.07 $\pm$ 0.05	7.24 $\pm$ 0.03	7.15 $\pm$ 0.03	7.20 $\pm$ 0.04	7.38 $\pm$ 0.02	7.34 $\pm$ 0.01	7.33 $\pm$ 0.02	7.35 $\pm$ 0.00	7.46 $\pm$ 0.03
	1	6.78 $\pm$ 0.05	6.93 $\pm$ 0.08	7.01 $\pm$ 0.08	6.79 $\pm$ 0.07	7.20 $\pm$ 0.06	6.31 $\pm$ 0.09	6.74 $\pm$ 0.22	6.13 $\pm$ 0.23	6.72 $\pm$ 0.11
	3	6.05 $\pm$ 0.06	6.22 $\pm$ 0.06	6.17 $\pm$ 0.09	5.76 $\pm$ 0.07	6.47 $\pm$ 0.28	5.71 $\pm$ 0.03	6.01 $\pm$ 0.01	6.14 $\pm$ 0.59	5.80 $\pm$ 0.12
	6	5.92 $\pm$ 0.03	5.91 $\pm$ 0.03	6.06 $\pm$ 0.07	6.37 $\pm$ 0.07	5.95 $\pm$ 0.01	6.02 $\pm$ 0.02	5.91 $\pm$ 0.04	6.04 $\pm$ 0.02	6.00 $\pm$ 0.02
	10	6.15 $\pm$ 0.02	6.26 $\pm$ 0.07	6.42 $\pm$ 0.03	6.52 $\pm$ 0.06	6.17 $\pm$ 0.02	6.23 $\pm$ 0.05	6.09 $\pm$ 0.03	6.19 $\pm$ 0.02	6.23 $\pm$ 0.01

<sup>a</sup>Stainless steel surfaces were placed into 20 ml of TSB inoculated with each of nine strains of *E. coli* O157:H7 for 30 min at 22°C to initiate attachment and then were either transferred into 20 ml of fresh TSB (Inoculation A) or were left in the original inoculated TSB (Inoculation B).

#### **4.3.2 Effect of substrate and storage temperature on biofilm formation by *Escherichia coli* O157:H7 on various food-contact surfaces**

The initial attachment and subsequent biofilm formation of a six-strain composite of *E. coli* O157:H7 on stainless steel and high-density polyethylene (HDPE) submerged into inoculated TSB, fat-lean homogenate and ground beef during storage under static conditions at 4 and 15°C was examined. Counts of attached cells of *E. coli* O157:H7 and total bacterial populations on material surfaces submerged into each one of the substrates during storage are shown in Figure 4.2. Corresponding counts of suspended cells are presented in Figure 4.3.

The results showed that both *E. coli* O157:H7 and total bacterial populations attached, survived and/or formed biofilm on the surfaces and storage temperatures tested. In all cases, attached and suspended *E. coli* O157:H7 and total flora populations were dependent ( $p < 0.05$ ) on temperature (4 or 15°C), on growth substrate (TSB, fat-lean homogenate or ground beef), on time of analysis (0.5, 4, 72 or 168 h), but not ( $p \geq 0.05$ ) on the type of surface (stainless steel and HDPE) tested (microbial counts corresponding to HDPE surfaces and pH changes in the substrates are provided in the appendix; Figures A.4 and A.5, Table A.1).

At 4°C initial attachment (after 30 min of exposure) of *E. coli* O157:H7 to stainless steel was greater in ground beef (2.4 log CFU/cm<sup>2</sup>) followed by fat-lean homogenate (1.8 log CFU/cm<sup>2</sup>) and TSB (0.9 log CFU/cm<sup>2</sup>). During prolonged storage for 168 h (7days) numbers of attached *E. coli* O157:H7 cells remained unchanged in ground beef, and increased to 1.8 and 3.8 log CFU/cm<sup>2</sup> in TSB and fat-lean homogenate, respectively (Figure 4.2). Inoculated *E. coli* O157:H7 populations in the substrates (ca. 6.0 log CFU/ml or g) did not change considerably during storage.

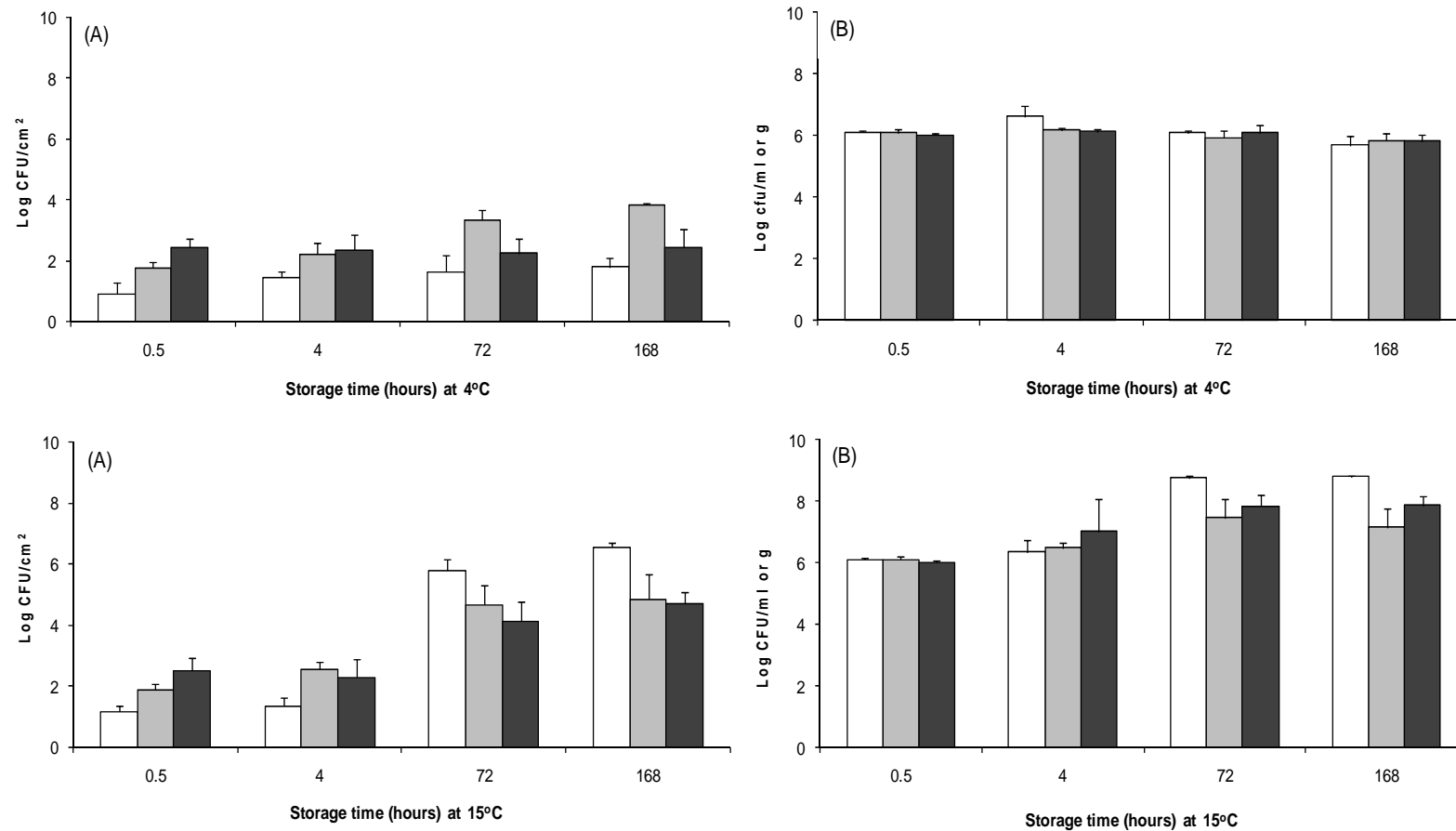
Comparatively, at 15°C initial attachment of the pathogen was greater in ground beef (2.5 log CFU/cm<sup>2</sup>) followed by fat-lean homogenate (1.9 log CFU/cm<sup>2</sup>) and TSB (1.2 log CFU/cm<sup>2</sup>). However, during subsequent storage, counts of attached *E. coli* O157:H7 cells increased by 5.4 log CFU/cm<sup>2</sup> in TSB, 2.9 log CFU/cm<sup>2</sup> in fat-lean homogenate and 2.2 log CFU/cm<sup>2</sup> in ground beef. Suspended *E. coli* O157:H7 populations

increased during storage with maximum cell densities, being higher in the case of TSB compared to the rest of the soiling substrates.

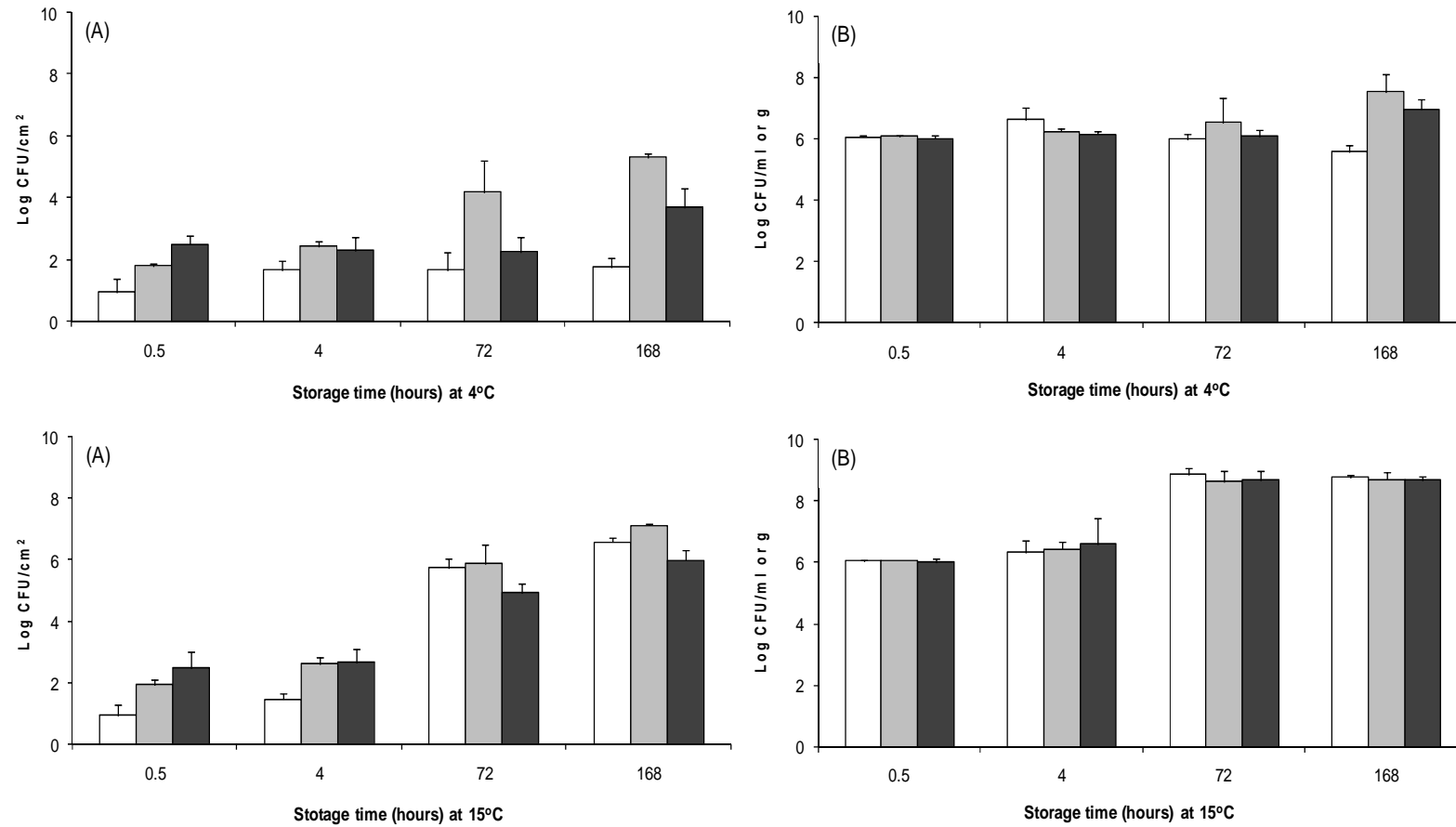
The responses of attached total bacterial populations on stainless steel and HDPE surfaces followed similar trends to those of *E. coli* O157:H7 with greater attachment in ground beef (2.5 log CFU/cm<sup>2</sup>) followed by fat-lean homogenate (1.8 log CFU/cm<sup>2</sup>) and TSB (0.9 log CFU/cm<sup>2</sup>) for both storage temperatures (Figure 4.3). Subsequent storage at 4°C resulted in better attachment and/or biofilm formation when coupons were submerged in fat-lean homogenate (5.3 log CFU/ml) followed by ground beef (3.7 log CFU/g) and TSB (1.7 log CFU/ml). A slight decrease of 0.5 log CFU/ml in TSB and an increase of 1.4 and 1.0 log CFU/ml or g in fat-lean homogenate and ground beef, respectively, was observed for suspended cells (Figure 4.3). At 15°C, no obvious difference on the counts of total suspended bacteria was observed among the different substrates at the end of storage. In the case of attached cells, however, better growth was observed in fat-lean homogenate (7.1 log CFU/ml), followed by TSB (6.6 log CFU/ml) and ground beef (6.0 log CFU/g).

The pH of all substrates remained constant at 4°C, whereas, at 15°C decreased by 1.16 units in TSB, increased by 0.72 units in fat-lean homogenate and remained relatively unchanged in ground beef (Table 4.4).

Notably, attachment and/or biofilm formation showed a temperature dependent response only in the late stages of storage (by 72 h and until the end of storage) with counts of attached *E. coli* O157:H7 cells being significantly higher at 15°C compared to 4°C. The same response was observed for cells in suspensions (Figure 4.2).



**Figure 4.2 Growth/survival of attached (A) or suspended (B) *E. coli* O157:H7 cells (mean log CFU/cm<sup>2</sup>, ml or g  $\pm$  standard deviation; N=2, n=3) after the submersion of stainless steel coupons in TSB (○), fat-lean homogenate (●) and ground beef (●), and storage at 4 and 15°C.**



**Figure 4.3** Growth/survival of attached (A) or suspended (B) total bacterial populations (mean log CFU/cm<sup>2</sup>, ml or g ± standard deviation; N=2, n=3) after the submersion of stainless steel coupons in TSB (○), fat-lean homogenate (●) and ground beef (●), and storage at 4 and 15°C.

**Table 4.4 pH values (mean  $\pm$  standard deviation; N=2, n=3) of TSB, fat-lean homogenate and ground beef inoculated with *Escherichia coli* O157:H7 and stored at 4 and 15°C for 168 h**

Temperature	Storage time (h)	Substrate*		
		TSB	Fat-lean homogenate	Ground beef
4°C	0.5	7.28 $\pm$ 0.05	5.64 $\pm$ 0.12	5.72 $\pm$ 0.05
	4	7.34 $\pm$ 0.15	5.56 $\pm$ 0.05	5.77 $\pm$ 0.01
	72	7.37 $\pm$ 0.08	5.47 $\pm$ 0.15	5.73 $\pm$ 0.03
	168	7.46 $\pm$ 0.05	5.76 $\pm$ 0.07	5.65 $\pm$ 0.16
15°C	0.5	7.28 $\pm$ 0.05	5.64 $\pm$ 0.12	5.72 $\pm$ 0.05
	4	7.39 $\pm$ 0.14	5.61 $\pm$ 0.14	5.76 $\pm$ 0.02
	72	5.69 $\pm$ 0.07	6.05 $\pm$ 0.14	5.44 $\pm$ 0.04
	168	6.12 $\pm$ 0.05	6.39 $\pm$ 0.14	5.57 $\pm$ 0.12

\*Stainless steel surfaces were submerged into substrates during storage

#### **4.3.3 Survival/growth of *E. coli* O157:H7 in ground meat cross-contaminated with biofilm and planktonic cells surviving exposure to sublethal sanitation**

The behavior of *E. coli* O157:H7 in ground beef cross-contaminated with biofilm and planktonic cells surviving exposure to sublethal concentrations of various sanitizers during aerobic storage at 4 and 12°C was evaluated.

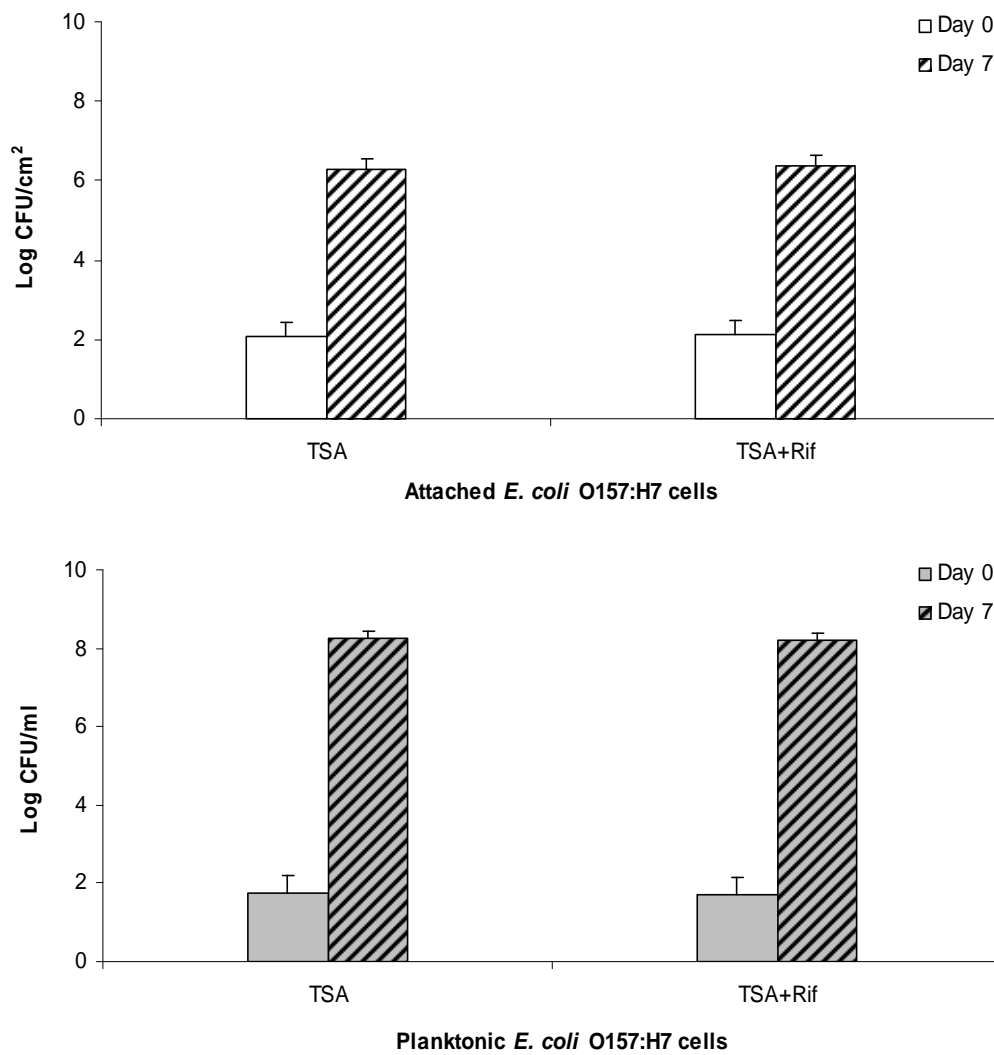
In order to form biofilm of *E. coli* O157:H7, acetal coupons were submerged for 30 min in a six-strain composite inoculum (6 log CFU/ml) of *E. coli* O157:H7 in beef homogenate. Following inoculation coupons were rinsed and immersed in fresh sterile beef homogenate (day 0) and stored at 15 °C for up to 7 days (Figure 4.4). The pathogen was able to attach to acetal at levels of 2 log CFU/cm<sup>2</sup> and form biofilm of 6.3 log CFU/cm<sup>2</sup> at the end of the incubation period. With the submersion of inoculated coupons in the fresh sterile beef homogenate, attached pathogenic cells were dispersed in the substrate at levels of 1.7 log CFU/ml, increasing to 8.1 CFU/ml by day 7 (Figure 4.4). As beef homogenate was sterilized prior to inoculation and no cross-contamination

was allowed or observed during storage, *E. coli* O157:H7 counts on TSA and TSA + rif were similar.

Exposure of biofilm cells attached to the acetal surfaces and relevant planktonic counterparts suspended in beef homogenate to the two sanitizers (minimum recommended exposure times and concentrations) resulted in decreases on the pathogens' population for all sanitizers tested (Figure 4.5). Vortexx was found to be more effective than QUAT against both planktonic (reduction of 5.2 log CFU/ml) and biofilm cells (reduction of 1.7 log CFU/cm<sup>2</sup>). It was also observed that sanitizers were in general more effective against planktonic cells compared to biofilm cells (incubation at 15°C) as recovered with two different growth media (TSA and TSA + rif).

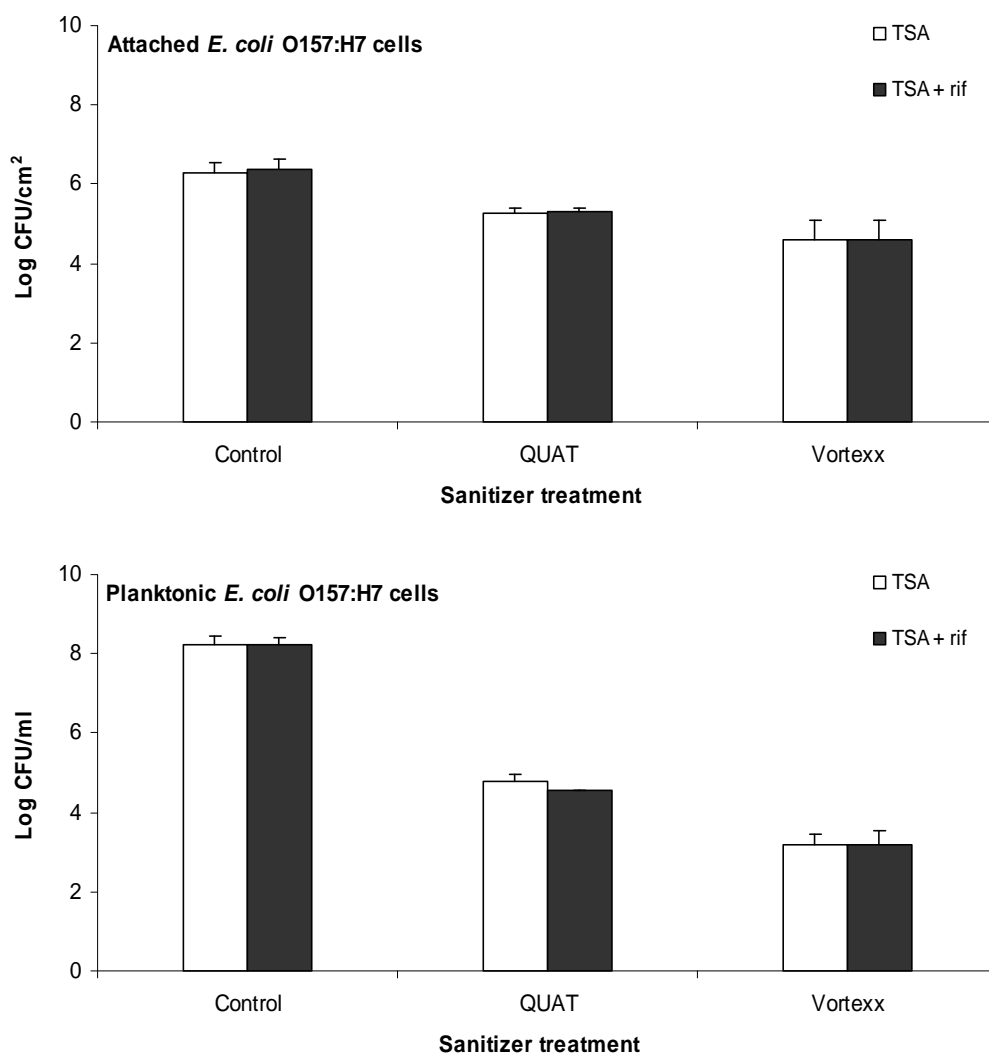
Counts of natural flora of ground beef (6.0 log CFU/g) stored aerobically at 4 and 12°C increased by ca. 2 and 3.5 log CFU/g, respectively, after storage for 9 days regardless of the inoculum cell type and sanitizer treatment applied to cells prior to inoculation (Figure 4.6). The pH of samples did not change considerably during storage and presented a slight decrease or increase at 4 and 12°C, respectively (Table 4.5). *E. coli* counts on samples stored at 4°C remained unchanged during storage (Figure 4.7). At 12°C only slight increases on pathogens' counts were observed during storage varying from 0.4 to 1.3 and 0.3 to 1.0 log CFU/g in samples inoculated with biofilm- and planktonic- derived inocula (Figure 4.8).

Biofilm cells surviving sanitation presented slightly higher increases during storage than corresponding planktonic cells in ground beef. However, this trend was not observed for cells not treated with sanitizers (control) (Figure 4.8).

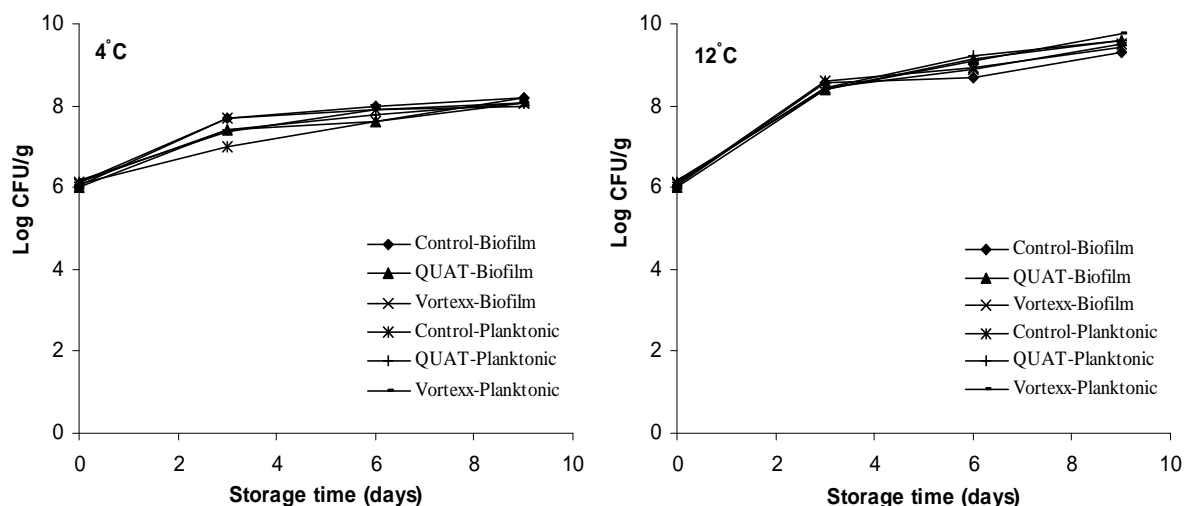


**Figure 4.4** Counts (mean log CFU/cm<sup>2</sup> or ml ± standard deviation; N=2, n=3) of attached and planktonic *E. coli* O157:H7 cells under storage in fresh sterile beef homogenate at 15°C for 7 days. Initial population of planktonic and attached cells was assessed immediately after the submersion of the inoculated acetal surfaces to the fresh sterile beef homogenate.

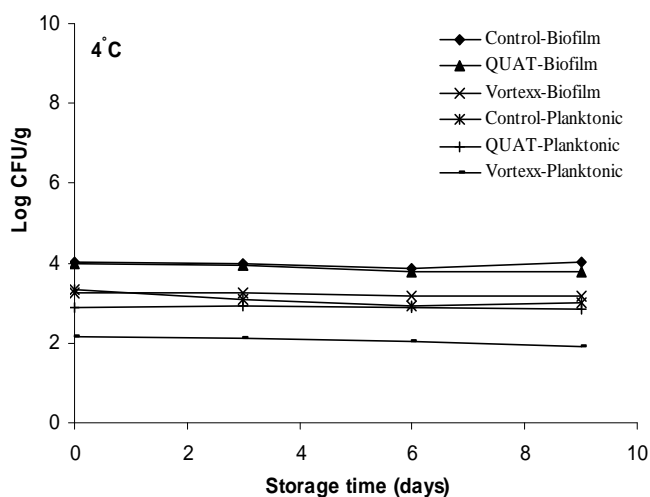




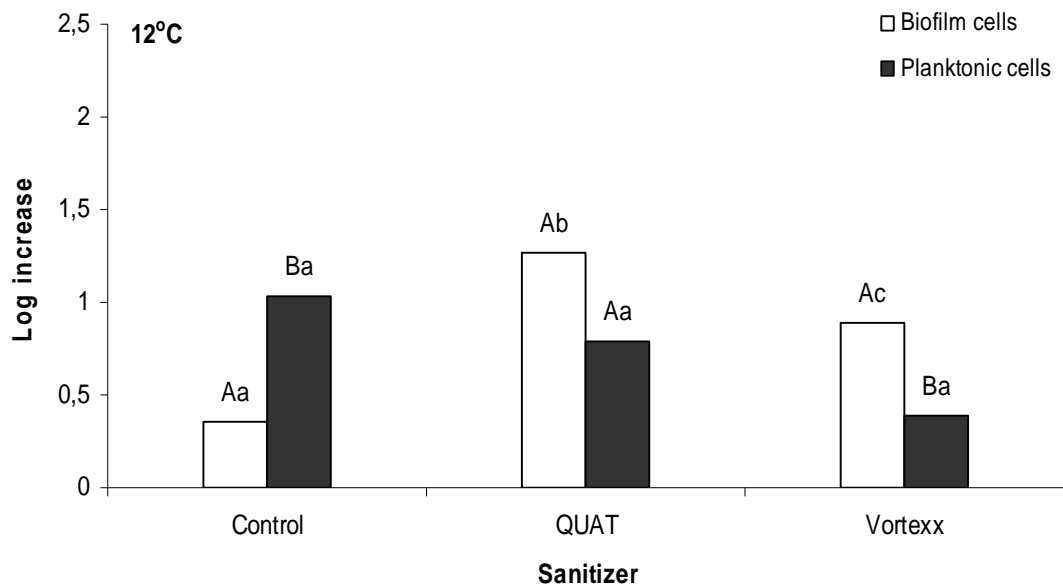
**Figure 4.5** Counts (mean log CFU/cm<sup>2</sup> or ml  $\pm$  standard deviation; N=2, n=3) of *E. coli* O157:H7 cells in suspension or attached to acetal surfaces exposed to the minimum recommended concentrations of QUAT and Vortexx (at the seventh day of incubation at 15°C) as recovered with two different growth media (TSA and TSA + rif).



**Figure 4.6 Growth of total bacterial populations (mean log CFU/g; N=2, n=3) in ground beef inoculated with biofilm or planktonic cells of *E. coli* O157:H7 surviving treatment with sanitizers (QUAT, Vortexx and control -no treatment-), during storage at 4 and 12°C.**



**Figure 4.7 Survival of *E. coli* O157:H7 populations (mean log CFU/g; N=2, n=3) in ground beef inoculated with biofilm or planktonic cells of *E. coli* O157:H7 surviving treatment with sanitizers (QUAT, Vortexx and control -no treatment-), during storage at 4 °C.**



**Figure 4.8 Log increases (mean log CFU/g; N=2, n=3) of *E. coli* O157:H7 in ground beef inoculated with biofilm or planktonic cells of *E. coli* O157:H7 surviving treatment with sanitizers (QUAT, Vortexx and control -no treatment-), during storage at 12°C for 9 days. Means with different uppercase letters for each sanitizer are statistically different ( $p < 0.05$ ). Means with different lowercase letters for each type of cell (biofilm or planktonic) are statistically different ( $p < 0.05$ ).**

**Table 4.5 pH values (mean  $\pm$  standard deviation) of ground beef samples inoculated with planktonic or biofilm cells of *E. coli* O157:H7 surviving after treatment with sanitizers during storage at 4 and 12°C**

Temperature	Cell type	Treatment	Storage time (days)			
			0	3	6	9
4°C	Biofilm	Control	5.82 $\pm$ 0.10	5.50 $\pm$ 0.07	5.44 $\pm$ 0.10	5.50 $\pm$ 0.23
		QUAT	5.73 $\pm$ 0.04	5.47 $\pm$ 0.14	5.43 $\pm$ 0.14	5.46 $\pm$ 0.25
		Vortexx	5.73 $\pm$ 0.07	5.47 $\pm$ 0.10	5.41 $\pm$ 0.13	5.49 $\pm$ 0.21
	Planktonic	Control	5.72 $\pm$ 0.06	5.59 $\pm$ 0.08	5.53 $\pm$ 0.01	5.72 $\pm$ 0.03
		QUAT	5.65 $\pm$ 0.02	5.49 $\pm$ 0.09	5.44 $\pm$ 0.15	5.57 $\pm$ 0.26
		Vortexx	5.66 $\pm$ 0.05	5.55 $\pm$ 0.07	5.41 $\pm$ 0.13	5.47 $\pm$ 0.22
12°C	Biofilm	Control	5.82 $\pm$ 0.10	5.29 $\pm$ 0.03	5.47 $\pm$ 0.08	5.85 $\pm$ 0.15
		QUAT	5.73 $\pm$ 0.04	5.30 $\pm$ 0.01	5.57 $\pm$ 0.05	6.27 $\pm$ 0.27
		Vortexx	5.73 $\pm$ 0.07	5.29 $\pm$ 0.04	5.51 $\pm$ 0.12	6.15 $\pm$ 0.28
	Planktonic	Control	5.72 $\pm$ 0.06	5.28 $\pm$ 0.04	5.55 $\pm$ 0.19	6.14 $\pm$ 0.21
		QUAT	5.65 $\pm$ 0.02	5.34 $\pm$ 0.10	5.61 $\pm$ 0.04	6.24 $\pm$ 0.32
		Vortexx	5.66 $\pm$ 0.05	5.27 $\pm$ 0.08	5.51 $\pm$ 0.03	6.17 $\pm$ 0.25

#### 4.4 DISCUSSION

Attachment and biofilm formation of pathogenic microorganisms on food-contact surfaces and equipment in food processing environments is of major public health and economic concern due to the potential for cross-contamination of otherwise unadulterated food products. *E. coli* O157:H7 has been found to colonise food-contact surfaces and equipment encountered in food processing and, specifically, in meat fabrication (Barkocy-Gallagher et al., 2001; Gun et al., 2003).

The potential of nine individual *E. coli* O157:H7 strains for attachment and biofilm formation was initially evaluated, as subsequent studies would utilize a mixture of selected strains with high attachment and/or biofilm forming ability for inoculation of various food-contact surfaces. Stainless steel coupons were contaminated with each one of nine *E. coli* O157:H7 strains under two inoculation scenarios representing persistent (inoculation B; coupons remained in the original inoculated TSB) and non persistent contamination, i.e. contamination that could be removed from surfaces with regular cleaning process before being well established (inoculation A; coupons after inoculation were rinsed and transferred in fresh sterile TSB).

The inoculation scenario did not seem to significantly affect the levels of initial (day-0) and final (day-10) bacterial populations attached to coupons. However, during the first days of incubation, strains were found to be attached at higher levels in the case of inoculation B as compared to inoculation A. This could probably be attributed to the higher suspended populations in the former case that resulted in faster biofilm formation (maximum attached populations were obtained after 3 and 6 days, respectively). The above results indicate that frequent cleaning operations within a working shift could potentially retard but not completely prevent the formation of a mature biofilm.

The initial attachment of *E. coli* O157:H7 to stainless steel varied significantly among strains (Table 4.1). Surprisingly, some non high-adherent strains were observed to form biofilms of the same density as high-adherent strains. Although studies involving foodborne pathogens, such as *L. monocytogenes* (Norwood and Gilmour, 1999; Chavant

et al., 2002) have found a relationship between initial attachment and biofilm formation, our results are not consistent with other studies that suggested no correlation between attachment and subsequent biofilm formation (Kalmokoff et al., 2001; Chae et al., 2006).

The different extent of adhesion and biofilm formation among the strains tested could not be explained in terms of growth medium or physicochemical properties of the food-contact surface tested. Moreover, the physicochemical properties of *E. coli* O157:H7 cells such as cell surface charge, hydrophobicity, cell surfaces structures (type I fimbriae and flagella) and cell surface free energy have been reported to have no influence on attachment to stainless steel (Rivas et al., 2007a). Production of exopolysaccharide (Ryu et al., 2004) and expression of curli (red-dry-rough phenotype) (Uhlich et al., 2006; Rivas et al., 2007b) have been suggested to account for inter-strain differences for attachment. Production of curli by *E. coli* O157:H7 is uncommon, but it can occur in association with *csgD* promoter point mutations (Uhlich et al., 2001). It could be postulated that in addition to the above mentioned factors other strain-intrinsic factors, may have modulated the extent of colonization.

In the light of the above results, a multi-strain composite of selected *E. coli* O157:H7 strains (i.e. strains presenting good attachment and/or biofilm forming ability) was subsequently utilized to evaluate the effect of storage temperature (4 and 15°C) and growth substrate on attachment and biofilm formation on stainless steel and HDPE surfaces. In order to simulate the range of soiling substrates encountered in meat processing, fat-lean homogenate (liquid residue) and ground beef (solid residue) were utilised. These substrates were not sterilized prior to use as it was important to evaluate the effect of natural contamination on *E. coli* O157:H7 biofilm formation. A well defined laboratory medium (TSB) was additionally used to assess potential discrepancies in attachment as compared to the real food substrates.

The pathogen was found to attach readily to both stainless steel and HDPE surfaces submerged in TSB and real food residues (i.e. fat-lean homogenate and ground beef). Controversial results on the correlation between the physicochemical properties (e.g.

hydrophobicity, surface energy, surface roughness and micro-topography) of different types of food-contact surfaces and bacterial colonization have been reported in the literature (Flint et al., 2000; Allan et al., 2004; Di Bonaventura et al., 2008; Rondriguez et al., 2008). Although no effort was made in this study to investigate the surface properties of stainless steel and HDPE, under the conditions tested, the type of material surface was not found to affect the extent of attachment or biofilm formation of *E. coli* O157:H7.

The extent of attachment, however, was found to be significantly affected by the different soiling substrates used. More specifically, initial cell attachment/transfer was found to be optimised in the present of ground beef followed by fat-lean homogenate and TSB (Figure 4.2). Soil in a processing environment is composed of both inorganic and organic matter, the latter in the form of proteins, fat or carbohydrates. The conditioning of food-contact surfaces with food residues (e.g. meat and fat) has been reported to stimulate attachment as bacterial cells attach to the meat residue which builds up over time or become trapped in fat that is strongly affiliated with contact surfaces (Hood and Zotolla, 1995). This could probably explain the initially higher numbers of bacterial cells attached (or transferred) on the coupons submerged into ground beef as compared to fat-lean homogenate and TSB or fat-lean homogenate as compared to TSB. The above results also confirm previously published reports showing that adherence and biofilm formation increases under low nutrient conditions (Dewanti and Wong, 1995; Oh et al., 2007).

The growth/no growth temperature boundary of *E. coli* O157:H7 is approximately 10°C (Palumbo et al., 1995). Thus, as expected, at 4°C planktonic *E. coli* O157:H7 cells in all substrates remained relatively unchanged during storage. Surprisingly, cells of the pathogen recovered from the coupons were found at higher levels than the initial attached populations by ca. 1.3 and 2 log CFU/cm<sup>2</sup> in TSB and fat-lean homogenate, respectively. It is possible that, with prolonged storage, suspended *E. coli* O157:H7 cells attached further on acetal surfaces through migration towards the surface. Indeed, in *E. coli* flagellar-mediated motility and Brownian motion has been reported to contribute to attachment processes (Van Houdt and Michiels, 2005). In case of fat-lean homogenate,

the presence of the endogenous microflora and potentially of exopolysaccharide-producing microorganisms may have also led to entrapment of pathogenic cells within the matrix of the exopolymers, and thus, to enhanced attachment (Castonguay et al., 2006). Pseudomonads, for example, which are commonly found in meat processing environments (Hood and Zottola, 1997b) have been previously documented as good producers of extracellular polymeric substances (Das, et al., 1998). Members of the meat microflora have also been reported to have no influence or reduce adherence and biofilm growth of pathogens (Norwood and Gilmour, 2001). This could explain the maintenance of attached *E. coli* O157:H7 at the initial population levels when ground beef was used. Ground beef microstructure might have also minimised the swimming ability of *E. coli*, and thus, migration phenomena towards the acetal surface.

At 15°C, populations of attached *E. coli* O157:H7 were found at higher populations in TSB compared to fat-lean homogenate and ground beef. Growth of bacterium to higher levels in the substrate could account for the higher biofilm growth.

The above results suggest that studying attachment and biofilm formation in a laboratory medium may impose a significant risk of obtaining biased results as the type and composition of the growth substrate, and the presence of natural flora may affect the responses of the pathogen differently. Moreover, it was shown that at cold temperatures *E. coli* is not only able to attach and survive on food-contact surfaces, but most importantly, to increase in population due to further attachment if contamination in the surrounding environment persists.

The sanitation of food-processing equipment is important for the control of cross-contamination during production. Despite being carried out regularly it can only eliminate some microorganisms from the equipment (Carpentier and Cerf, 1993). Knowledge on the efficacy of most sanitation products in the market has been obtained from tests against planktonic (suspended) cells (Broomfield et al., 1993). Suspension tests, however, may provide little information on how products perform on contaminated surfaces (Carpentier and Cerf, 1993; Hood and Zottola, 1995).



During the last few years there has been an increased interest on the efficacy of sanitizers on biofilms of *E. coli* O157:H7 formed on food-contact surfaces; however, most studies have been conducted in laboratory media neglecting the influence of soil present on equipment surfaces (Ryu and Beuchat, 2005; Uhlich et al., 2006). Considering the above, the effect of two commercial sanitizers, QUAT and Vortexx, on biofilm of *E. coli* O157:H7 formed on acetal surfaces submerged in beef-homogenate was evaluated.

*E. coli* O157:H7 was found to attach readily to acetal surfaces and form biofilm of 6.3 log CFU/cm<sup>2</sup> within 7 days of storage. Exposure of cells in biofilms and in suspensions to the sanitizers resulted in decreases for both attached and suspended cells (Figure 4.5). None of the tested sanitizers could meet the criterion of EN 13697 (i.e. >4 log reduction on clean and soiled surfaces; Anonymous, 2001) for effective sanitation of acetal surfaces. This was somewhat expected as in order to simulate insufficient cleansing conditions in a processing environment, surfaces were not cleaned prior to sanitation treatments.

Of the sanitizers tested, Vortexx, was found to be the most efficient towards both planktonic and biofilm *E. coli* O157:H7 cells. In agreement with previous studies, sanitizers were more effective against planktonic cells compared to biofilm cells (Eginton et al., 1998; Ryu and Beuchat, 2005). Uhlich et al. (2006) showed that biofilm associated cells of two different variants of *E. coli* ATCC 43895 formed on glass were more resistant to low hydrogen peroxide (5%; 10 min exposure) and quaternary ammonium compounds (1:64 dilution in distilled water; 1 or 2 min exposure) than their respective planktonic counterparts. The mechanisms affording protection of cells in biofilms against antimicrobial agents have not been yet fully elucidated. Possible reasons for increased resistance maybe the delayed penetration of the antimicrobial agent through the matrix of the biofilm, the altered (slower) growth rate of biofilm organisms and other physiological changes due to the biofilm mode of growth (Donlan and Costerton, 2002).

Biofilm cells treated with sanitizers can be more easily removed from the test surface as compared to untreated cells and consequently serve as sources of cross-contamination of food products (Eginton et al., 1998; Midelet and Carpentier, 2004). Thus, the growth responses of biofilm and planktonic cells of *E. coli* O157:H7 surviving sanitation treatments and inoculated into ground beef were additionally evaluated during storage at refrigeration and abuse temperatures.

Planktonic- and biofilm-derived cells of the pathogen exhibited a prolonged survival during storage at 4°C. At 12°C, the presence of the competitive meat microflora at relatively high population in combination with the stressful temperature for the growth of *E. coli* O157:H7, allowed in general only slight increases of the pathogen (Figure 4.8). However, it was noticed that biofilm cells surviving sanitation presented slightly higher increases than relevant planktonic cells in ground beef by the end of storage (Figure 4.8). A possible explanation could be that biofilm cells are susceptible to the sanitizers from only the outer layer of the biofilm matrix, while suspended cells are susceptible to the sanitizers from all sides of the cell (Bower and Daeschel, 1999). Thus, it is likely that biofilm-derived cells needed less time to repair damages and initiate growth than corresponding planktonic-derived cells in ground beef. This trend, however, was not obtained for cells not treated with the sanitizers. In this case, it is possible that untreated biofilm-derived cells retained their biofilm phenotype after being detached from the surfaces (Donlan, 2002) and thus presented a slower growth than untreated planktonic-derived cells in ground beef. Further studies are needed to support the above results at less adverse (i.e. higher storage temperatures and lower levels of competitive microflora) for the growth of *E. coli* O157:H7 conditions.

In conclusion, the high variability observed in the attachment and biofilm formation ability among nine *E. coli* O157:H7 strains demonstrated that caution should be exercised when drawing conclusions from single strain studies and accentuated the necessity for using composites of the pathogen when studying attachment or biofilm formation, or when developing sanitation programs for food-contact surfaces. Moreover, the obtained results suggested that studying biofilm formation in a single laboratory medium imposes a significant risk of obtaining biased results especially at

inimical growth conditions (e.g. 4°C) as they may allow *E. coli* O157:H7 to reach high populations in sites within facilities where naturally contaminated food residues remain for long time periods. Further studies are required to elucidate potential differences between planktonic and biofilm modes of growth of the pathogen in raw meat products, especially after exposure to sanitation stresses.



## **CHAPTER 5**

# **GROWTH OF *Salmonella* Enteritidis AND *Salmonella* Typhimurium IN THE PRESENCE OF QUORUM SENSING SIGNALLING COMPOUNDS PRODUCED BY SPOILAGE AND PATHOGENIC BACTERIA**



## Chapter 5

### **Growth of *Salmonella* Enteritidis and *Salmonella* Typhimurium in the presence of quorum sensing signalling compounds produced by spoilage and pathogenic bacteria**

#### **ABSTRACT**

The effect of acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2) signalling molecules produced by *Pseudomonas aeruginosa* 108928, *Yersinia enterocolitica*-like GTE 112, *Serratia proteamaculans* 00612, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844 on the growth kinetic parameters (i.e. lag phase and growth rate) of two *Salmonella* Enteritidis and *S. Typhimurium* strains, respectively, has been assessed by using conductance measurements. AHLs and AI-2 in the cell-free supernatants (CFS) of these microorganisms were assayed using different bacterial biosensors and/or thin layer chromatography (TLC). Except from *P. aeruginosa*108928, which was not found to produce AI-2, all other strains produced both AHLs and AI-2. Thereafter, aliquots (20% in the final volume) of these CFS were transferred in NZ Amine broth inoculated with  $10^3$ - $10^4$  CFU/ml of 18h cultures of *S. Enteritidis* and *S. Typhimurium* strains, respectively. Changes in conductance of the medium were monitored and detection time (Tdet) was recorded. While *P. aeruginosa* 108928 induced a shorter detection time, i.e. start of the metabolic activity, the other microorganisms increased the detection time of *Salmonella* serotypes compared to control samples. Results obtained provided evidence of the effects of QS signalling compounds from other bacterial species on the growth of *Salmonella* and confirmed the complexity of bacterial interaction and communication in food spoilage or poisoning processes.

## 5.1 INTRODUCTION

*Salmonella* is one of the most prevalent pathogens associated with foodborne illness, worldwide. It is the second in the list of human zoonotic diseases across the EU with 160,649 people infected in 2006 (EFSA-ECDC, 2007). In the USA, salmonellosis is a leading cause of foodborne illness, resulting in an estimated 1.4 million infections, with more than 16,000 hospitalizations and nearly 600 deaths each year (Lynch et al., 2006). *Salmonella* infections can cause diarrhea, fever, vomiting, and abdominal cramps. *S. enterica* serotype Typhimurium and *S. enterica* serotype Enteritidis are among the serotypes most commonly associated with human disease (Galanis et al., 2006).

It is increasingly apparent that, in nature, bacteria function less as individuals and more as coherent groups that are able to inhabit multiple ecological niches. The role of cell-cell communication, i.e. quorum sensing (QS) in food ecological niches has recently received attention from food microbiologists and a growing body of evidence is suggesting that bacterial food spoilage and poisoning could be regulated by QS (for review see (Smith et al., 2004; Ammor et al., 2008)).

Quorum sensing involves the production of diffusible low-molecular-weight signalling molecules called autoinducers, which have been referred to as bacterial pheromones. When a critical level of such molecules is reached, signal-recognizing microorganisms sense that a sufficient level or “quorum” of bacteria is present and consequently respond through a coordinated expression of certain genes allowing them to mount a unified response favourable to population survival (Smith et al., 2004).

In many Gram-negative bacteria, quorum sensing is mediated by acyl-homoserine lactone molecules (AHLs) that are synthesized and recognized by QS circuits composed of LuxI and LuxR homologs (Miller and Bassler, 2001). *Salmonella* do not have a *luxI* gene that codes for AHL synthetase and thus do not produce AHLs; however, this organism does have a LuxR homolog, known as *SdiA*, that enables detecting signals produced by other bacteria (Michael et al., 2001; Smith and Ahmer, 2003). In addition, *Salmonella* use two others QS systems, the *luxS/AI-2* (Taga et al., 2001; Taga et al.,



2003) and the AI-3/epinephrine/norepinephrine (Walters and Sperandio, 2006) to achieve intercellular signaling. The latter is also involved in interkingdom communication. These three QS systems have been shown to modulate the physiology, colonization and virulence of *Salmonella* (for review see (Walters and Sperandio, 2006; Kendall and Sperandio, 2007; Hughes and Sperandio, 2008)).

The increased awareness of the role of cell-to-cell communication in the ecology of *Salmonella* has to be matched by an understanding of both the physiology and the molecular biology that underlie this process. So far, the majority of studies have focused on the molecular aspects of this phenomenon and much less attention has been paid to the physiological aspects (i.e., metabolism, initiation of growth and growth rate).

Thus, the goal of this study was to investigate the effect of quorum sensing compounds produced by different environmental microorganisms potentially associated with food spoilage or poisoning on the growth kinetics parameters of two *S. Enteritidis* and two *S. Typhimurium* strains as assayed by changes in conductance measurements.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Bacterial strains and culture conditions

*Pseudomonas aeruginosa* 108928, *Serratia proteamaculans* 00612 (maintained in Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, GR), *Yersinia enterocolitica* CITY650, *Y. enterocolitica* CITY844 and *Yersinia enterocolitica*-like GTE 112, were propagated and subcultured in brain heart infusion (BHI; LAB M, Lancashire, UK) broth following incubation at 28°C (*Yersinia enterocolitica*-like GTE 112, *S. proteamaculans* 00612) or 37°C (*P. aeruginosa* 108928, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844) for 24 h prior to use. The identity of these strains was confirmed using sequencing of a 640-bp internal fragment of the 16S rRNA gene.

For the AI-1 bioassays, the AHL reporter strain *Agrobacterium tumefaciens* A136 (pCF218, pCF372) and the AHL producing strain *A. tumefaciens* KYC6 (pCF28; a 3-oxo-C8 HSL overproducer) (Fuqua and Winans, 1996), kindly donated by Prof. Robert McLean (Department of Biology, Texas State University), were activated and subcultured in luria bertani (LB; Bertani, 1951) medium (supplemented with 4.5 µg/mL tetracycline and 50 µg/mL spectinomycin for *A. tumefaciens* A136) at 28°C for 28 h with agitation (160 rpm). *Hafnia alvei* 718 (a 3-oxo-C6 HSL producing strain) (Bruhn et al., 2004), kindly donated by Dr. L. Gram (Danish Institute for Fisheries Research, DK) was activated and subcultured in BHI broth following incubation at 37°C for 24 h.

The reporter strain *Vibrio harveyi* BAA-1117<sup>TM</sup> (BB170 *luxN*::Tn5, sensor 1<sup>-</sup>, sensor 2<sup>+</sup>), and the AI-2-producing strain *V. harveyi* BAA-1119<sup>TM</sup> (BB152 *luxL*::Tn5, autoinducer-1<sup>-</sup>, autoinducer-2<sup>+</sup>), purchased from the American Type Culture Collection (LGC Promochem), were used for the AI-2 bioassays (Surette and Bassler 1998). These strains were grown in the autoinducer bioassay (AB) medium at 28°C for 24 h with agitation (160 rpm). AB medium was prepared according to Greenberg et al. (1979). The basal medium contained NaCl (17.5 g/l; Merck), MgSO<sub>4</sub> (12.3 g/l; Merck) and casamino acids (2 g/l; Sigma). After adjusting the pH to pH 7 with KOH, the solution was sterilised by autoclaving (15 min, 121°C, 15 psi). After cooling, the following

sterile compounds were added: 1 M potassium phosphate (pH 7.0; 10 ml/l), 50 % glycerol (20 ml/l; Carlo Erba), and filter-sterilized 0.1 M L-arginine (10 ml/l; Sigma).

For conductance experiments, *S. enterica* serovar Enteritidis strains PT4 and PT7 (kindly provided by School of Biomedical and Molecular Sciences, Surrey University, UK), and *S. enterica* serovar Typhimurium strains DT193 (maintained in Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, GR) and DSM554 (kindly provided by Dr E. Smid, ATO-DLO, The Netherlands) were activated and subcultured in tryptone soy broth (TSB; LAB M) following incubation at 37°C for 24 and 18 h, respectively.

### **5.2.2 Cell-free Supernatant (CFS) preparation**

*P. aeruginosa* 108928, *S. proteamaculans* 00612, *Yersinia enterocolitica*-like GTE 112, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844, herein after called tester strains, were grown (ca.  $10^8$ - $10^9$  CFU/ml) in 30 ml of BHI broth under conditions previously described. Cultures (25 ml) were then centrifuged at 8000 rpm for 15 min at 4°C and filtered-sterilized through a 0.22 µm pore-size cellulose acetate filter (Millipore) to obtain CFS. For screening for AHL signalling molecules and monitoring the effect of the CFS on *Salmonella* strains growth, a mixture (1:1, v/v) of CFS of *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844 was utilised. The remaining 5 ml from each culture were used to enumerate bacterial cells population. CFSs were immediately screened for the presence of QS signalling molecules as essentially described below. In parallel the pH of the CFS was recorded (Metrohm 691 pH meter).

### **5.2.3 Screening for AHL signalling molecules**

*A. tumefaciens* A136 reporter strain was used for the screening of AI-1 like signalling molecules in CFS using a well diffusion assay. Briefly, 1 ml of the culture was inoculated into 50 ml of melted ABT agar (1.5 % agar; ABT per liter: 0.4 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6 g Na<sub>2</sub>HPO<sub>4</sub>, 0.3 g KH<sub>2</sub>PO<sub>4</sub>, 0.3 g NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 0.01 mM FeCl<sub>3</sub>, 2.5 mg thiamine supplemented with 0.5% glucose and 0.5% casamino acids) supplemented with the relevant antibiotics and 50 µg/mL X-Gal (AppliChem GmbH, Darmstadt, Germany) which was then immediately poured into 5.0 cm diameter Petri

dishes. A portion (150 µl) of the tester strain CFS or the tester strain 20% (80% BHI) CFS (i.e. amount used in the conductance experiments) was pipetted into wells (diameter 6.0mm) punched in the solidified agar using a sterile Pasteur pipette. The plates were incubated at 28°C for 48 h. CFS of the AHL-producing strain *A. tumefaciens* KYC6 (donor) and the reporter strain were used as positive and negative controls, respectively, in the above assay.

The development of blue colour (catabolism of X-Gal) in the plates indicated presence of AI-1 like substances. The blue coloured area and the total plate area were measured using the Image J software (Wayne Rasband, NIH, Bethesda, Maryland, USA). The ratio of the coloration area per plate area was used to estimate the amount of AHL production as compared to the donor strain *A. tumefaciens* KYC6. Two independent assays were performed.

#### **5.2.4 Thin-layer chromatography (TLC)**

Extracts for TLC were prepared from 5 ml cultures of the tester strains, *Hafnia alvei* 718 and *A. tumefaciens* KYC6 grown as essentially described in section 5.2.1. Bacteria were removed by centrifugation and the supernatants were extracted twice with equal volumes of ethyl acetate acidified with 0.1 % acetic acid. The combined extracts were filtered, and evaporated to dryness. Residues from the cultures were dissolved in 100 µl of HPLC-grade ethyl acetate.

Synthetic N-hexanoyl-DL homoserine lactone (C6-HSL; Fluka, Biochemica) or extracts dissolved in HPLC-grade ethyl acetate were applied (0.5-80 µl) onto C18 reversed-phase TLC plate (Merck) and the chromatograph was developed with methanol/water (60:40 v/v) until the elution top reached the top of the plate (approximately 3 h). After development, the TLC plate was allowed to air dry. 50 ml of a late exponential phase culture of *A. tumefaciens* A136 reporter strain was then used to inoculate 100 ml of melted (ca. 46°C) ABT agar supplemented with 150 µl X-Gal. After gentle mixing, the culture was poured over the surface of the developed plate and the agar was left to solidify. The TLC-plate was incubated overnight at 28°C in a sterile metallic container. AHLs were visualised as blue spots on the TLC chromatographs.

### 5.2.5 Screening for AI-2 signalling molecules

The *luxCDABE*-encoded luminescence response of the reporter strain *V. harveyi* BB170 was used as the basis for determining AI-2 activity in CFS of tester strains. The assay was performed as follows. An overnight culture of *V. harveyi* BB170 was diluted 4-fold with ¼ strength Ringer's solution containing 1.5 % NaCl. This cell suspension was then centrifuged for 10 min (10,000 x g at 4°C) and the supernatant was discarded. The pellet (10<sup>4</sup> CFU) was then resuspended in 0.8 ml of AB medium and mixed with 0.2 ml of the tested CFS. Each solution mixture served to load (200 µl) 5 different wells in a 96-well polystyrene microplate (µClear, Greiner Bio-one). The CFS (0.2 ml) of *V. harveyi* BB152 strain, prepared exactly as described above for the tester strains, was used as the positive control and AB medium (0.2 ml) was used as the negative control. The microplates were incubated at 28°C and luminescence was measured every 20 min by a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT 05404, USA), until the negative control exhibited an increase in luminescence (7h).

AI-2 activity was calculated as the ratio of luminescence of the test sample to the negative control (i.e. AB medium) sample. The induction of light production by the addition of *V. harveyi* BB152 CFS to *V. harveyi* reporter strain BB170 was normalised to 100 % activity. Results of duplicate assays were considered as positive when the light induction reaches 10 % of the *V. harveyi* BB152 stimulation.

### 5.2.6 Monitoring the effect of the CFS on *Salmonella* strains growth

*Salmonella* strain growth was indirectly monitored by conductance measurements using the Malthus 2000 instrument (Radiometer International, Copenhagen, Denmark). Conductance measurements were performed by dispensing 3.5 ml from a stock solution of NZ Amine broth (NZA) (containing per litre: 20 g NZ amine A (Sheffield Chemical Co. Norwich, N.Y, USA) and 20 g proteose peptone No.3 (Difco Laboratories, Detroit, MI)) supplemented with 20 % CFS of each tester strain or with 20 % BHI broth (control) and inoculated with the *Salmonella* (10<sup>3</sup>-10<sup>4</sup> CFU/ml) into each of 12 sterile reaction Malthus tubes. The pH of BHI broth used was adjusted to the same value recorded on the corresponding CFS. Three other non-inoculated with *Salmonella* tubes (3.5 ml NZA, supplemented or not with CFS) were used as an additional control.

Malthus tubes were then incubated into the Malthus apparatus for 24 h at 37°C while the analyzer was adjusted to measure conductivity changes every 6 min. Malthus system detects changes in conductance caused by bacterial metabolism in the growth medium in the reaction tubes which contain platinum electrodes (Firstenberg-Eden and Eden, 1984). Conductance changes are expressed in microsiemens ( $\mu\text{S}$ ).

The Detection Time ( $T_{\text{det}}$ ; h) signal appears when three consecutive measurements exceed the minimum threshold of detection criteria ( $5 \mu\text{S}$ ), and is defined as the time interval between the start of conductance monitoring and the beginning of the acceleration phase of the signal (Firstenberg-Eden and Eden, 1984; Silley and Forsythe, 1996). It is apparent that  $T_{\text{det}}$  depends on the bacterial population into the Malthus tubes ( $\log \text{CFU/ml}$ ), the growth kinetics of microorganism and the properties of the test medium.

#### **5.2.7. Data analysis**

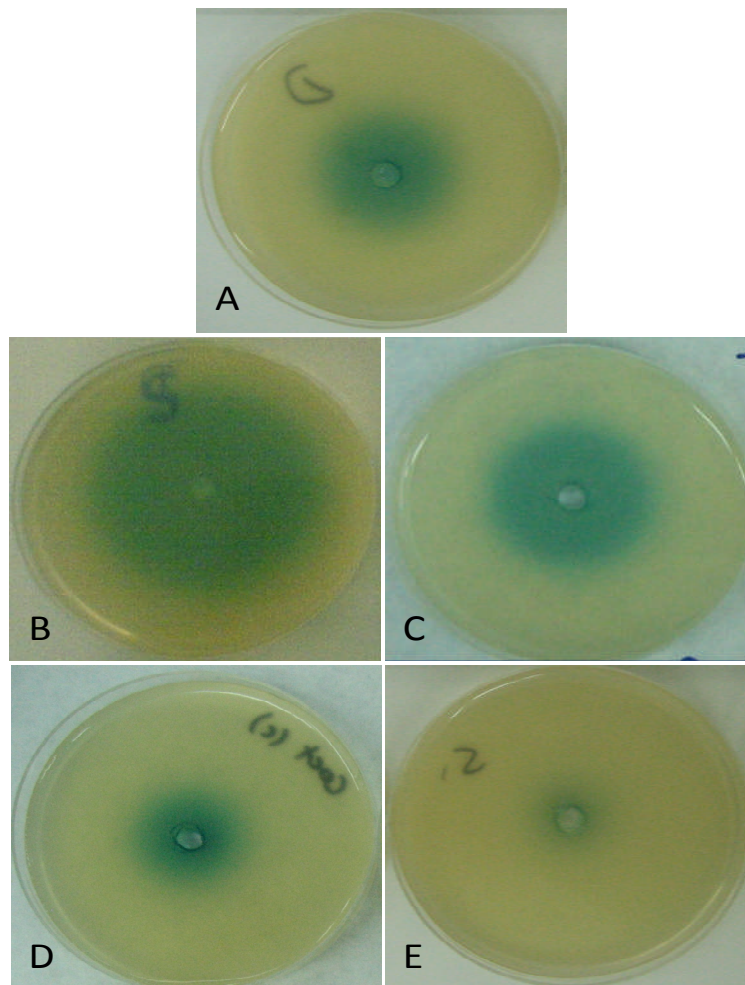
Growth data of *Salmonella* strains (values of  $\mu\text{S}$  for 24 h incubation) in NZA, were fitted to the model of Baranyi and Roberts (1994). This primary model was used to estimate the kinetic parameters: (i) lag phase (LP; min) represented by the detection time and (ii) the rate parameter [represented by the maximum slope (rate) of conductance changes ( $\text{MSrCC}$ );  $\mu\text{Smin}^{-1}$ ] or the slope of the curve in which the maximum conductance change was observed. For curve fitting, the in-house programme DMFit (Institute of Food Research, Norwich, United Kingdom) was used. The microbial activity was monitored indirectly by calculating the area under the conductance/time curves using the trapezoidal rule. Thus, the effect of CFS on microbial activity was also manifested by a reduction or increase in the area under the conductance/time curve of the tests relative to the control at any specified time (Lambert and Pearson, 2000; Chorianopoulos et al., 2006). For the estimation of the effect of CFS on the growth of *Salmonella* strains two independent trials were performed.

## 5.3 RESULTS

### 5.3.1. Screening for QS signalling molecules

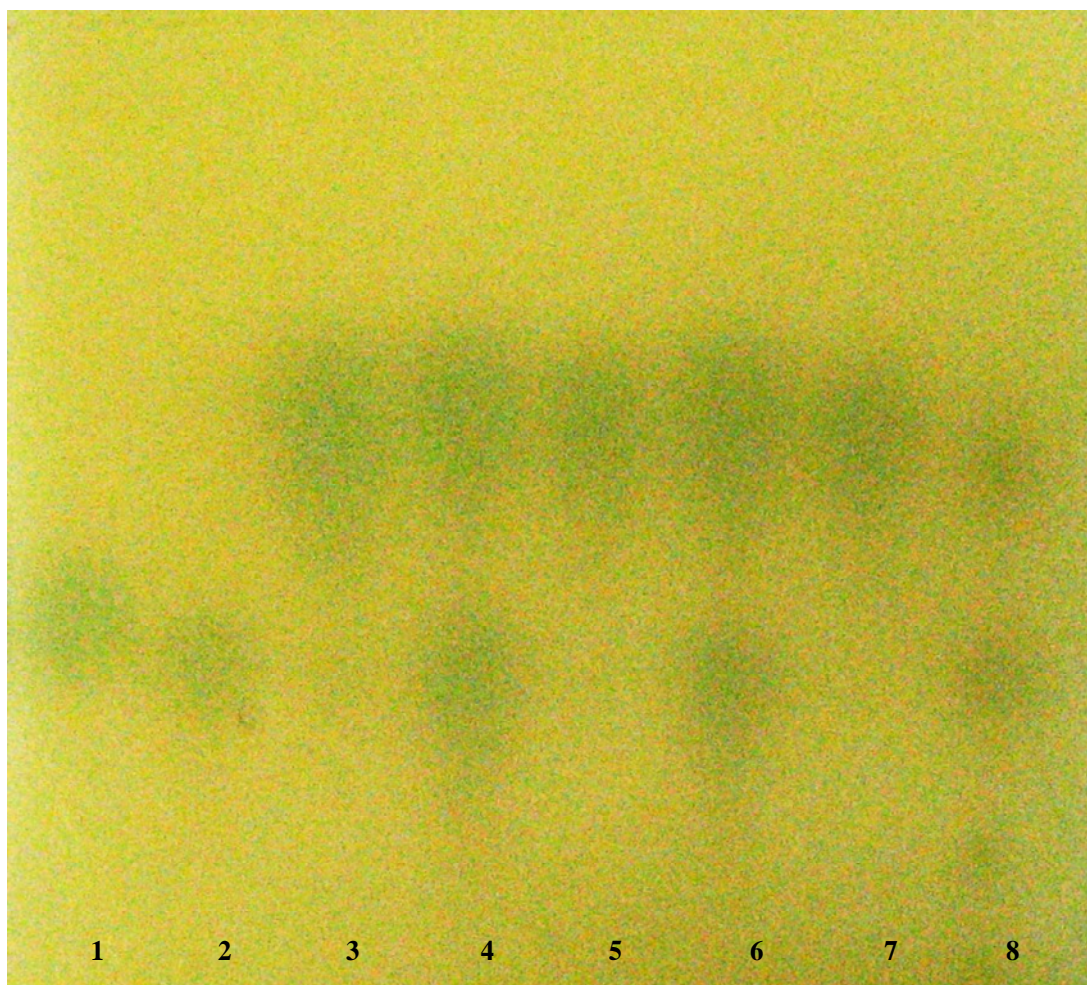
AHL production was screened using the *A. tumefaciens* A136 well diffusion assay and image analysis of the plates allowed to estimate the amounts of AHLs produced as compared to the *A. tumefaciens* KYC6 strain (donor). Figure 5.1 and Table 5.1 summarize obtained results. It was shown that all tester strains produced AHLs, with *Yersinia enterocolitica*-like GTE 112 being the most producing, followed by *S. proteamaculans* 00612, *P. aeruginosa* 108928, and *Y. enterocolitica* CITY650 and CITY844. The presence of AHL compounds was additionally confirmed performing TLC (Figure 5.2). The TLC results indicated the presence of at least one type of AHL in the CFS of the tester strains visualized using *A. tumefaciens* A136.

AI-2-like activity was assessed using the *V. harveyi* bioluminescence assay. With the exception of *P. aeruginosa*, all strains displayed an AI-2 activity. *Yersinia enterocolitica*-like GTE 112 stimulated the highest light induction in the reporter strain *V. harveyi* BB170 (56 %), followed by *S. proteamaculans* 00612 (26 %) (Table 5.2).



**Figure 5.1** Presence of acylated homoserine lactones (AHLs) in the cell-free supernatants (CFS) of *A. tumefaciens* KYC6 (donor) (A), *Yersinia enterocolitica*-like GTE 112 (B), *S. proteamaculans* 00612 (C), *Y. enterocolitica* CITY650 and CITY844\* (D) and *P. aeruginosa* 108928 (E). Cell-free supernatants (CFS) of the tester strains were added to wells in agar containing *A. tumefaciens* A136. Zones of AHL(s)-induced blue colour production are seen surrounding the wells. \* Mixture (1:1, v/v) of CFS of the two *Y. enterocolitica* strains.





**Figure 5.2** Thin layer chromatogram profiles of the AHLs present in the cell-free supernatants (CFS) of the tester strains used herein in this study. Samples were chromatographed on c18 reversed-phase TLC plates, developed with methanol/water (60:40, v/v) and the spots were visualised by *A. tumefaciens* A136 reporter strain. Lanes: (1) synthetic C6-AHL; (2) *A. tumefaciens* KYC6 (donor); (3) *H. alvei* 718; (4) *Yersinia enterocolitica*-like GTE 112; (5) *S. proteamaculans* 00612; (6) *Y. enterocolitica* CITY650; (7) *Y. enterocolitica* CITY844; (8) *Ps. aeruginosa* 108928.

**Table 5.1 Autoinducer-1 activity of tester strains cell-free supernatant (CFS) and 20% CFS as compared to the AHL producing *A. tumefaciens* KYC6 (donor)**

	Tester strain							
	<i>Y. enterocolitica</i> -like GTE 112		<i>P. aeruginosa</i> 108928		<i>S. proteamaculans</i> 00612		<i>Y. enterocolitica</i> CITY650 and CITY844*	
	1 <sup>st</sup> assay	2 <sup>nd</sup> assay	1 <sup>st</sup> assay	2 <sup>nd</sup> assay	1 <sup>st</sup> assay	2 <sup>nd</sup> assay	1 <sup>st</sup> assay	2 <sup>nd</sup> assay
Donor	0.45*	0.37	0.24	0.30	0.34	NT <sup>†</sup>	0.14	NT
CFS	0.70	0.59	0.09	0.14	0.31	NT	0.13	NT
20% CFS	0.53	0.41	0.05	0.05	0.17	NT	0.06	NT

\*Results are displayed as the mean value of the ratio of colorized area per plate area of two plates in each assay. Values superior to 0 mean production of AI-1 compounds

\*\*Mixture (1:1, v/v) of CFS of the two *Y. enterocolitica* strains.

<sup>†</sup>NT; Not tested-test was not performed.

**Table 5.2 Induction of luminescence in *V. harveyi* BB170 reporter strain by cell-free supernatants (CFS) from *V. harveyi* BB152 (positive control) and tester strains**

Tester strain	Induction of luminescence* (%)
<i>V. harveyi</i> BB152	100**
<i>Y. enterocolitica</i> -like GTE 112	56
<i>P. aeruginosa</i> 108928	4
<i>S. proteamaculans</i> 00612	26
<i>Y. enterocolitica</i> CITY650	12
<i>Y. enterocolitica</i> CITY844	13

\*Induction of luminescence was calculated as the ratio of luminescence of the CFS to the negative control (AB medium).

\*\*Results represent mean fold induction values of two independent assays.

### 5.3.2. *Salmonella* kinetics with and without tester strains CFS

*Salmonella* strains were inoculated (ca.  $10^3$  -  $10^4$  CFU/ml) into NZA supplemented with 20% of the tester strain CFS or 20% of sterile BHI broth (control). The pH of BHI was adjusted to the same value recorded on the corresponding CFS with the purpose to rule out the influence of pH on the observed results.

Conductance changes followed a sigmoid curve typical of microbial growth (Koutsoumanis et al., 1998), and thus, the calculation of the lag phases and growth rates of *Salmonella* strains was allowed. The putative effect of signalling compounds present in CFS on  $T_{det}$  provided by the instrument, on LP and MSrCC calculated using the Baranyi and Roberts model (1994) as well as on the area of the conductance/time curves is shown in Tables 5.3-5.6. Tubes containing only BHI broth (with/without addition of CFS and non-inoculated with *Salmonella*) did not exhibit any change in conductance during incubation, suggesting the absence of any metabolic activity (data not shown).

In comparison to control samples, tester strains CFS proved to influence ( $p < 0.05$ ) the growth parameters of all *Salmonella* strains but with different trends. Specifically, the addition of CFS of *Yersinia enterocolitica*-like GTE 112 or *S. proteamaculans* 00612

inoculated with each *Salmonella* strain NZA broth reduced the MSrCC and enlarged the LP of *S. Enteritidis* PT4, *S. Enteritidis* PT7, *S. Typhimurium* DSM554 and *S. Typhimurium* DT193, respectively (Tables 5.3 and 5.4). As expected, Tdet obtained from the instrument was influenced accordingly with LP. The area of the conductance/time curves of the tubes containing CFS was found to be lower than that of the control samples. Similar differences were observed when the CFS of *Y. enterocolitica* CITY650 and CITY844 was used with the exception of MSrCC of both *S. Enteritidis* strains which did not present any significant difference compared to control samples (Table 5.5).

When the CFS of *P. aeruginosa* 108928 was used, however, the values of MSrCC and area of the conductance/time curves appeared to be significantly higher than in control samples for all *Salmonella* strains. In contrast, the Tdet and in most cases the LP of *Salmonella* was lower when the Malthus tubes were supplemented with the CFS (Table 5.6).

**Table 5.3 Effect of addition of 20% in the final volume of cell-free supernatant (CFS) of *Yersinia enterocolitica*-like GTE 112, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)**

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	8.85 ± 0.37a	7.64 ± 0.46b	9.50 ± 0.34a	8.25 ± 0.14b
	LP	699.36 ± 21.72a	613.39 ± 18.90b	844.79 ± 19.97a	663.30 ± 19.96b
	MSrCC	2.10 ± 0.23a	2.02 ± 0.21a	2.13 ± 0.19a	2.69 ± 0.16b
	Area	13907 ± 3064a	21422 ± 3704b	14557 ± 2138a	30175 ± 642b
<i>S. Enteritidis</i> PT7	Tdet	11.89 ± 0.48a	11.19 ± 0.18b	10.69 ± 0.72a	9.78 ± 0.32b
	LP	938.64 ± 24.99a	859.84 ± 24.99b	1001.15 ± 0.22a	818.34 ± 66.41b
	MSrCC	1.23 ± 0.20a	1.50 ± 0.20b	1.72 ± 0.22a	2.09 ± 0.18b
	Area	7703 ± 1977a	13219 ± 3108b	10943 ± 2975a	23562 ± 3183b
<i>S. Typhimurium</i> DSM554	Tdet	9.74 ± 0.23a	9.28 ± 0.04b	9.78 ± 0.34a	8.41 ± 0.32b
	LP	799.23 ± 14.58a	743.80 ± 18.34b	805.04 ± 20.66a	681.43 ± 26.93b
	MSrCC	2.21 ± 0.29a	2.45 ± 0.22b	2.35 ± 0.37a	2.84 ± 0.31b
	Area	13154 ± 3463a	23542 ± 2374b	15793 ± 2023a	28548 ± 2371b
<i>S. Typhimurium</i> DT193	Tdet	9.43 ± 0.20a	8.77 ± 0.60b	8.21 ± 0.65a	7.63 ± 0.46b
	LP	748.54 ± 8.91a	699.23 ± 9.68b	740.76 ± 23.41a	666.14 ± 13.20b
	MSrCC	2.13 ± 0.31a	2.57 ± 0.53b	2.38 ± 0.19a	2.84 ± 0.47b
	Area	5462 ± 856a	7807 ± 2337b	5346 ± 911a	14909 ± 2637b

\* Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test, p < 0.05).

**Table 5.4 Effect of addition of 20% in the final volume of cell-free supernatant (CFS) of *S. proteamaculans* 00612, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)**

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	9.45 ± 0.27a**	9.31 ± 0.40a	9.62 ± 0.39a	9.09 ± 0.39b
	LP	789.70 ± 23.42a	714.45 ± 19.03b	792.10 ± 17.92a	695.50 ± 30.22b
	MSrCC	1.68 ± 0.34a	1.97 ± 0.24b	1.74 ± 0.19a	2.00 ± 0.26b
	Area	27757 ± 3356a	31850 ± 2865b	11801 ± 2657a	19796 ± 3521b
<i>S. Enteritidis</i> PT7	Tdet	13.20 ± 0.48a	12.34 ± 0.25b	12.47 ± 0.25a	11.08 ± 0.11b
	LP	1035.00 ± 20.53a	959.68 ± 24.93b	962.08 ± 16.21a	843.37 ± 27.39b
	MSrCC	1.00 ± 0.25a	1.61 ± 0.17b	1.26 ± 0.30a	1.59 ± 0.14b
	Area	32482 ± 3123a	45869 ± 4650b	6501 ± 1505a	21014 ± 2602b
<i>S. Typhimurium</i> DSM554	Tdet	9.89 ± 0.27a	9.62 ± 0.23b	10.01 ± 0.10a	9.53 ± 0.16b
	LP	793.15 ± 15.50a	680.60 ± 71.67b	799.04 ± 12.69a	769.56 ± 13.54b
	MSrCC	1.66 ± 0.19a	2.07 ± 0.23b	1.87 ± 0.36a	2.63 ± 0.25b
	Area	28925 ± 3401a	36807 ± 4863b	12554 ± 3045a	18004 ± 2109
<i>S. Typhimurium</i> DT193	Tdet	9.30 ± 0.24a	8.94 ± 0.40b	9.11 ± 0.16a	8.60 ± 0.35b
	LP	743.06 ± 11.61a	732.94 ± 6.17b	727.82 ± 12.53a	703.15 ± 19.88b
	MSrCC	1.83 ± 0.27a	2.61 ± 0.23b	1.76 ± 0.41a	2.73 ± 0.24b
	Area	27563 ± 2691a	31686 ± 2970b	9289 ± 3455a	16161 ± 3016b

\*Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test,  $p < 0.05$ ).

**Table 5.5 Effect of addition of 20% in the final volume of cell-free supernatant (CFS)<sup>†</sup> of *Y. enterocolitica* CITY650 & CITY844, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)**

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	8.93 ± 0.63a	8.18 ± 0.68b	10.81 ± 0.14a	9.47 ± 0.09b
	LP	753.85 ± 12.56a	678.32 ± 45.92b	822.40 ± 18.70a	748.50 ± 26.57b
	MSrCC	1.83 ± 0.21a	1.56 ± 0.31a	2.05 ± 0.40a	2.22 ± 0.33a
	Area	14217 ± 1725a	19443 ± 6552b	15879 ± 2312a	25002 ± 4722b
<i>S. Enteritidis</i> PT7	Tdet	11.33 ± 0.47a	10.93 ± 0.28b	12.13 ± 0.39a	11.55 ± 0.19b
	LP	899.88 ± 15.54a	837.35 ± 33.44b	958.09 ± 23.34a	894.54 ± 16.23b
	MSrCC	1.85 ± 0.14a	1.74 ± 0.23a	1.39 ± 0.14a	1.50 ± 0.30a
	Area	18260 ± 2609a	29931 ± 4602b	15000 ± 1520a	22263 ± 3056b
<i>S. Typhimurium</i> DSM554	Tdet	9.57 ± 0.32a	9.11 ± 0.27b	10.22 ± 0.16a	9.34 ± 0.29b
	LP	780.99 ± 17.32a	709.41 ± 22.79b	817.97 ± 9.24a	754.02 ± 10.14b
	MSrCC	2.08 ± 0.15a	2.63 ± 0.20b	2.03 ± 0.30a	2.36 ± 0.30b
	Area	30889 ± 4441a	40207 ± 4432b	15917 ± 1912a	27289 ± 3674b
<i>S. Typhimurium</i> DT193	Tdet	8.73 ± 0.53a	8.03 ± 0.45b	9.33 ± 0.14a	8.80 ± 0.14b
	LP	715.37 ± 9.49a	657.85 ± 26.65b	594.19 ± 13.28a	560.85 ± 5.59b
	MSrCC	1.92 ± 0.36a	2.35 ± 0.20b	1.59 ± 0.17a	1.75 ± 0.04b
	Area	14898 ± 1433a	19066 ± 3337b	13017 ± 1415a	17933 ± 1691b

\*Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test,  $p < 0.05$ ). <sup>†</sup>Mixture (1:1, v/v) of CFS of the two *Y. enterocolitica* strains.

**Table 5.6 Effect of addition of 20% in the final volume of cell-free supernatant (CFS) of *P. aeruginosa* 108928, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)**

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	7.07 ± 0.05a**	7.30 ± 0.05b	6.34 ± 0.39a	6.88 ± 0.63b
	LP	599.86 ± 5.95a	613.01 ± 9.68b	473.79 ± 23.18a	494.72 ± 20.94b
	MSrCC	3.19 ± 0.23a	2.79 ± 0.361b	3.27 ± 0.36a	2.65 ± 0.41b
	Area	19532 ± 1991a	14579 ± 1240b	31578 ± 2285a	27279 ± 2674b
<i>S. Enteritidis</i> PT7	Tdet	7.72 ± 0.08a	7.88 ± 0.16b	7.97 ± 0.67a	8.56 ± 0.24b
	LP	627.62 ± 7.10a	637.79 ± 10.70a	728.22 ± 34.93a	725.02 ± 13.80a
	MSrCC	2.28 ± 0.23a	1.89 ± 0.27b	2.51 ± 0.27a	2.22 ± 0.33b
	Area	13723 ± 2485a	10910 ± 1837b	30206 ± 4806a	27443 ± 1864b
<i>S. Typhimurium</i> DSM554	Tdet	7.17 ± 0.08a	7.40 ± 0.05b	7.16 ± 0.45a	7.53 ± 0.05b
	LP	597.61 ± 7.37a	613.61 ± 12.14b	657.97 ± 14.79a	683.21 ± 24.05b
	MSrCC	3.13 ± 0.43a	2.76 ± 0.54a	3.66 ± 0.38a	3.24 ± 0.32b
	Area	18603 ± 2446a	14606 ± 2842b	30645 ± 3727a	26762 ± 1841b
<i>S. Typhimurium</i> DT193	Tdet	6.63 ± 0.05a	6.79 ± 0.07b	7.01 ± 0.39a	7.40 ± 0.17b
	LP	548.89 ± 6.53a	561.30 ± 6.25b	662.53 ± 40.76a	636.18 ± 18.98a
	MSrCC	3.47 ± 0.23a	3.22 ± 0.12b	3.52 ± 0.41a	3.15 ± 0.29b
	Area	20369 ± 1461a	18418 ± 1037b	27014 ± 2771a	24180 ± 1500b

\* Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test, p < 0.05)



## 5.4 DISCUSSION

Most of the studies in the area of cell-to-cell communication (quorum sensing) are focused on the molecular aspects of this phenomenon; however, much less attention has been paid to the possible influence of QS signalling compounds on microbial growth characteristics. In the present study, the potential role of AHL and AI-2 molecules produced by spoilage and pathogenic bacteria on the microbial activity and growth of two *Salmonella* serotypes was assessed by using conductance measurements.

The metabolic activity of a culture can be inferred from (i) the detection time (Tdet) provided by the impedimetric instrument and (ii) the kinetic parameters calculated indirectly from the conductance curves (Fistenberg-Eden and Eden, 1984; Tranter et al., 1993; Tassou and Nychas, 1994; Koutsoumanis et al., 1998; Koutsoumanis and Nychas, 2000; Giaouris et al., 2005; Chorianopoulos et al., 2006, 2008; Nychas et al., 2009). In the first case, the Tdet corresponds to the period where there appears to be no multiplication, defined as the "lag phase". In this period, the organisms are metabolizing, but there is no cell division and the population remains relatively 'constant' whilst individual cells adapt to their new environment. In the later case, activity of a culture can be calculated indirectly from curve parameters such as slope, represented by the maximum slope of conductance changes (MSrCC) or area i.e. comparison of the area under the conductance/time curve provided by the instrument with or without the examined factors (Chorianopoulos et al. 2006).

These characteristics e.g. Lag phase, MSrCC and area, are associated with biochemical (metabolic) changes taking place in the medium of the reaction tube of the impedimetric instrument. As microorganisms metabolize uncharged or weakly charged substrates (e.g. sugars and proteins) are utilized and highly charged end-products (amino acids, lactate etc) are produced, which increase the conductivity of the solution. Thus, the metabolic activity of a culture can be inferred from the steepness of the slope of the conductance curve after detection. The greater the activity of the culture, the steeper is the slope or the greater the area. It needs to be stressed that microbial metabolic activity and generation times are not always simply related.

In the present study the growth and/or microbial activity parameters (LP, MSrCC and area) as well as the parameter provided from the instrument (Tdec) of the both *Salmonella* serotype were found to be affected significantly in the presence of these QS signals extracted from different producer (tester) bacteria (Tables 5.3- 5.6). In all cases, the area of the conductance/time curve, which represents the effect of the quorum sensing compounds on both initiation of growth and metabolic activity, was significantly affected in their presence. However, it needs to be noted that this parameter was always significantly lower in samples supplemented with CFS produced from *Yersinia enterocolitica-like* GTE 112, *S. proteamaculans* 00612 and *Y. enterocolitica* CITY650 and CITY844, while the CFE from *P. aeruginosa* 108928 affected this characteristic in the opposite way (significantly higher). The other parameters (e.g. Tdec, MSrCC, and LP) examined in the four *Salmonella* strains were also significantly affected but this effect varied and depended on the strain of the *Salmonella* serotype or the QS compounds produced by the tester strain(s). These findings suggested a diverse effect of the QS signalling molecules produced from the different tester strains towards kinetic and/or microbial activity of *Salmonella*.

The production of different QS compounds may have also contributed to the explanation of this phenomenon. The four tester strains have been tested for AHL and AI-2 production using different bacterial bioassays. The use of reporter bacteria in which the *luxI* or *luxS* homologue genes responsible for AHL and AI-2 synthesis, respectively, have been inactivated, has shown to be a valuable system for detection of autoinducers production (Surette and Bassler, 1998; Ravn et al., 2001). It was found that all strains synthesized AHLs (Table 5.1). The presence of at least one kind of AHL in the CFS of each tester strain was additionally confirmed performing TLC (Figure 5.1). Possibly, not all AI-1 signals produced by the tester organisms were detected due to the detection limits of the bioreporter *A. tumefaciens* A136 with regard to the type (number of carbons in the acyl chain and substitution of the C3 of the acyl chain) and the concentration of the signal molecule (Shaw et al., 1997).

These findings are in agreement with the literature since it has already been shown that *Y. enterocolitica*, *P. aeruginosa*, and *S. proteamaculans* synthesize and recognize AHLs

by QS circuits composed of LuxI and LuxR homologs (Pearson et al., 1994, 1995; Throup et al., 1995; Christensen et al., 2003). *Y. enterocolitica* contains the LuxR-I pair (YenRI). YenI is responsible for the synthesis of two short chain AHLs, N-hexanoyl-homoserine lactone (C6-HSL) and N-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL) (Throup et al., 1995) and also for directing the synthesis of three long chain AHLs, N-(3-oxotetradecanoyl)-homoserine lactone, N-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL), and N-(3-oxotetradecanoyl)-homoserine lactone (Atkinson et al., 2006a). This QS system modulates swimming and swarming motility in this microorganism (Atkinson et al., 2006a). AHL production by *Y. enterocolitica* has been already detected in food matrices (Medina-Martinez et al., 2007). A set of *luxI/luxR* homologs, SprI/SprR, encoding a putative quorum sensor was also identified in the N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL)-producing *S. proteamaculans* strain B5a (Gram et al., 1999). This AHL has been suggested to regulate the production of extracellular lipolytic and proteolytic exoenzymes responsible for food spoilage (Christensen et al., 2003). *P. aeruginosa* contains two AHL-dependent QS systems, the LasI/LasR and RhlI/RhlR, which direct the biosynthesis of N-(3-oxo-dodecanoyl)-homoserine lactone (OdDHL) and N-butanoyl-homoserine lactone (C4-HSL), respectively (Pearson et al. 1994, 1995). The *las* and *rhl* QS signalling networks have been reported to regulate biofilm development and to control the production of numerous virulence factors such as exoenzymes (elastase, protease, alkaline protease and phospholipase), secondary metabolites (pyocyanin, haemolysins, hydrogen cyanide, rhamnolipid and pyoverdine) and toxins (exotoxin A) (Passador et al., 1993; Davies et al., 1998; Williams, 2002; Hentzer et al., 2003).

With the exception of *P. aeruginosa* 108928, all tester strains were shown to produce AI-2 (Table 5.2), hence confirming results published elsewhere. In fact, while *Pseudomonas* spp. lack *luxS* and thus do not produce AI-2 (Winzer et al., 2002), *Yersinia* and *Serratia* species possess a *luxS* gene and produce AI-2 (Atkinson et al., 2006b; Van Houdt et al., 2007), enabling therefore a semi-quantitative indication of the AI-2 production from each tester strains. The role of AI-2 in these species is still unclear and requires further investigation.

Similar variations in the effects of synthetic AHLs or cell-free culture fluids of microorganisms and cell-free meat extracts containing AI-1 signals on the growth kinetics parameters of other pathogenic or spoilage bacteria have been suggested. Indeed, Dunstall et al. (2005), Zhao et al. (2006) and Nychas et al. (2009), have reported various results in the synergistic or competitive effects of these signals on the evolution of microorganisms. Such variations could be possibly explained by the different sources of QS signals and/or the different bacterial strains assayed.

In addition to the function of QS signalling compounds to communicate cell density, these compounds have been also reported to act as proxies that provide individual cells with information on the diffusion and flow properties of their environment preventing the wasteful synthesis of “expensive” extracellular substances, such as exoenzymes, bacteriocins, siderophores and other effectors. Provided that they remain in the cells immediate environment, these metabolites, increase nutrient availability and ultimately benefit the fitness of their producers (Redfield, 2002). This concept could explain the results of this study. Indeed with the addition in the reaction tubes of the QS signalling compounds and/or other novel signals existing in CFS, these were rapidly mixed and diffused into the pathogens’ microenvironment altering *Salmonella* activity possibly through an over- or under- production of necessary for its growth requirements (e.g. enzymes, metabolites etc.) (Redfield, 2002). In all cases, production of AI-2 signals by *Salmonella* and potential interference with AI-1 and/or AI-2 of the tester strains was ruled out by inoculation of *Salmonella* in NZA, a glucose or other carbohydrate free medium, the presence of which has been reported to be necessary for the production of the signaling factor (Surette and Bassler, 1998).

In conclusion, findings reported in this study suggest that cell-to-cell communication between spoilage or pathogenic flora and *Salmonella* exist, as the growth kinetics parameters of all *S. Enteritidis* and Typhimurium strains were affected by the presence of QS signalling molecules. The response seems to be mostly affected by the type of QS compound. Further investigations are required in order to discriminate the effect of each type of signalling molecule on the growth kinetics parameters and more specifically on the growth determinants of *Salmonella* (i.e. target genes and phenotypes). Such

approaches could potentially lead to the exploitation of these autoinducers as novel antimicrobial agents and compounds to control microbial growth, survival and virulence in foods.



**CHAPTER 6**  
**GENERAL CONCLUSIONS / FUTURE WORK**





## Chapter 6

### General conclusions

#### Chapter 2

- Teewurst, a traditional/ethnic raw spreadable sausage which is typically consumed raw, does not provide a favorable environment for the outgrowth of *E. coli* O157:H7, *L. monocytogenes* and *S. Typhimurium*.
- With prolonged storage the growth of indigenous lactic acid bacteria and the concomitant pH decrease resulted in the reduction of pathogenic populations.
- Differences in the extent and rate of pathogen inactivation shown in the trials were probably due to batch-to-batch variations in the initial numbers of lactic acid bacteria and the associated microbial interactions.

#### Chapter 3

- *L. monocytogenes* growth in laboratory media and real food substrates was similar at high storage temperatures (i.e. 15 and 20°C).
- At low (more stressful) temperatures the pathogen grew significantly faster in broth than on agar (agar-spread and agar-pour plate technique), whereas, in the case of real food substrates, the pathogen grew faster in naturally contaminated meat (minced and tissue) as compared to laboratory media.
- A broth model generated to describe the effect of temperature on the growth kinetics of *L. monocytogenes* led to significant over-predictions of growth in the laboratory media (agar spread- and pour-plate plate technique) and sterile meat blocks at low storage temperatures. This indicated the importance of taking food structure into account while generating predictive models.
- Similarly, at low storage temperatures the agar model significantly underestimated the growth of *L. monocytogenes* in naturally contaminated minced meat and meat blocks highlighting the influence of the natural microflora and associated microbial interactions on the growth of the pathogen.

#### Chapter 4

- *E. coli* O157:H7 ability to attach and form biofilms on food-contact surfaces was strain-dependent.
- The type of growth substrate (laboratory or real food) influenced attachment and biofilm formation on food-contact surfaces.
- Temperature affected biofilm formation but not initial attachment on a food-contact surface.
- Biofilm cells were more resistant to sanitation treatments as compared to planktonic cells.
- Pathogenic cells, surviving in biofilms after exposure to sanitizers, were able to survive in cross-contaminated ground meat and presented slightly higher increases ( $p < 0.05$ ) as compared to relevant planktonic counterparts.

#### Chapter 5

- *S. proteamaculans*, *Y. enterocolitica* strains and *P. aeruginosa* produced both AHLs and/or AI-2 signalling compounds.
- The growth kinetics (lag phase and growth rate) of *Salmonella* Enteritidis and *Salmonella* Typhimurium strains were significantly affected in the presence of these quorum sensing signals.
- The effect of quorum signalling compounds varied and was dependent on the strain of *Salmonella* serotype and/or quorum sensing signal.

## **FUTURE WORK**

- Conditions under which adherent and biofilm cells may dislodge from food contact surfaces and cross-contaminate foods.
- Behaviour of biofilm cells surviving stress conditions (e.g. sanitation treatments) in cross-contaminated foods.
- Biofilm formation by foodborne pathogenic bacteria in the presence of food natural microflora.
- Contribution of quorum sensing signalling compounds in attachment and biofilm formation of foodborne pathogenic bacteria.
- Effect of purified/chemically synthesised AHLs and AI-2 signalling compounds on microbial growth.
- Cell-to-cell communication between spoilage microflora and pathogenic bacteria in foods.



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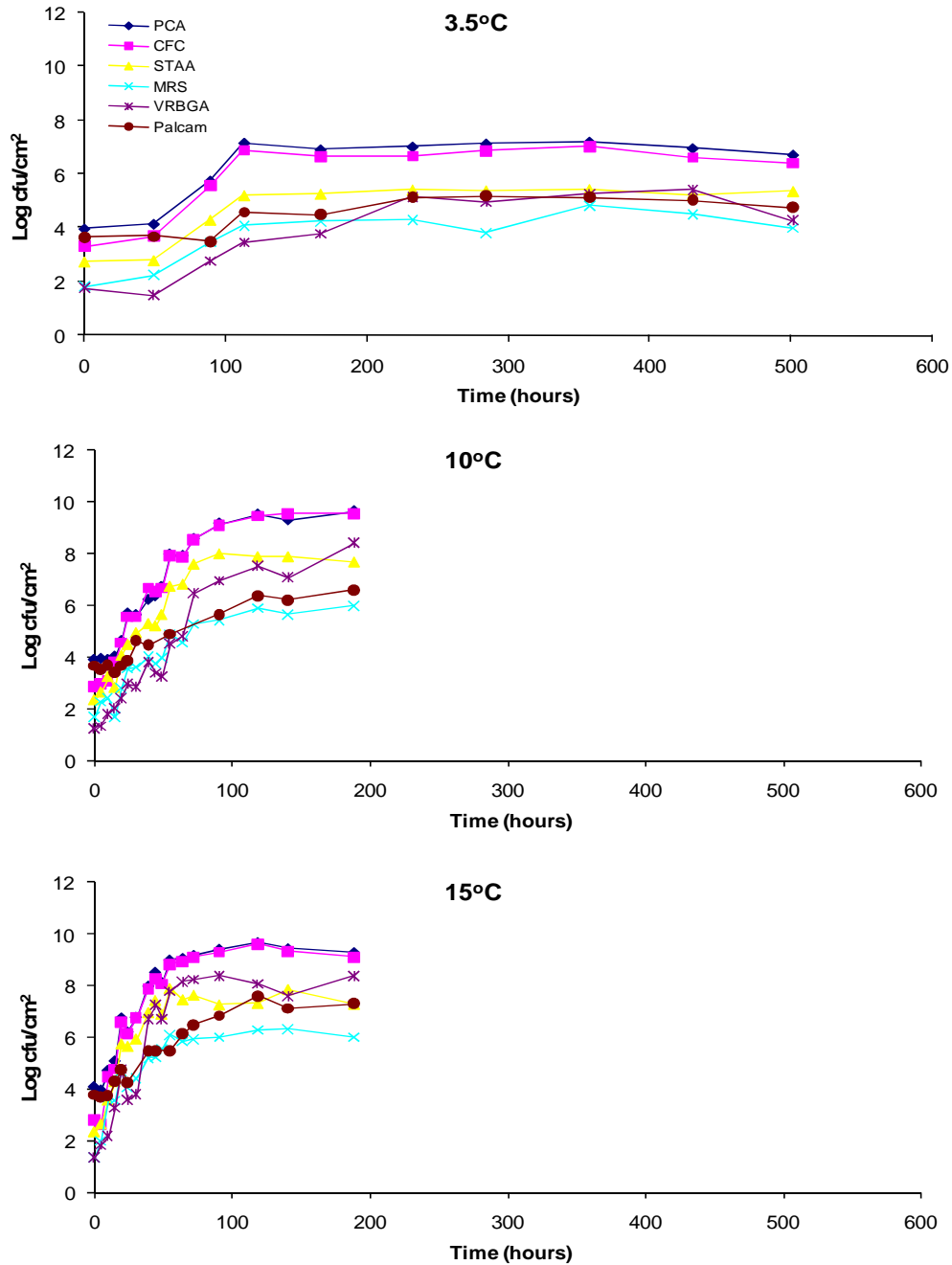




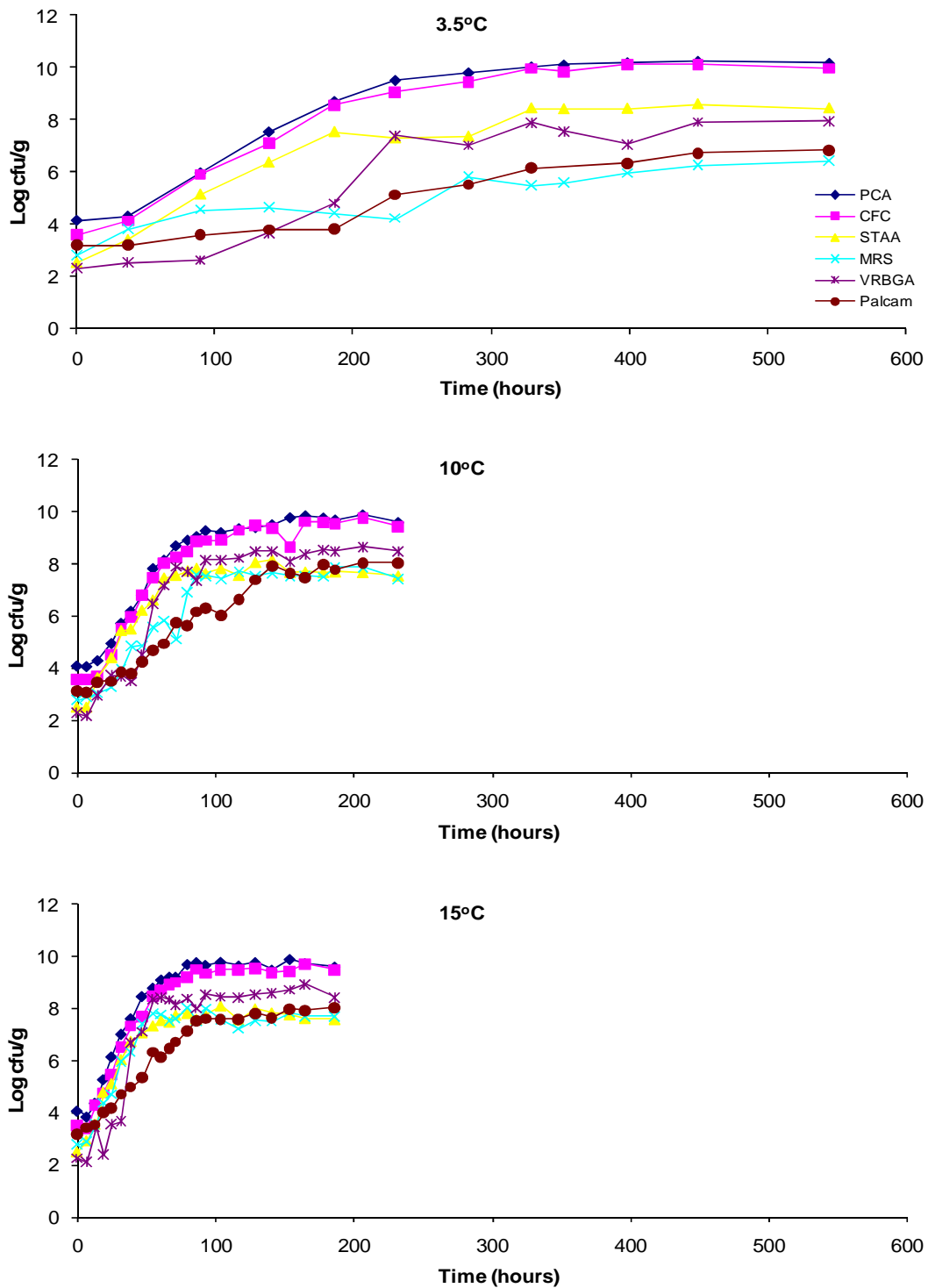
## **APPENDIX**



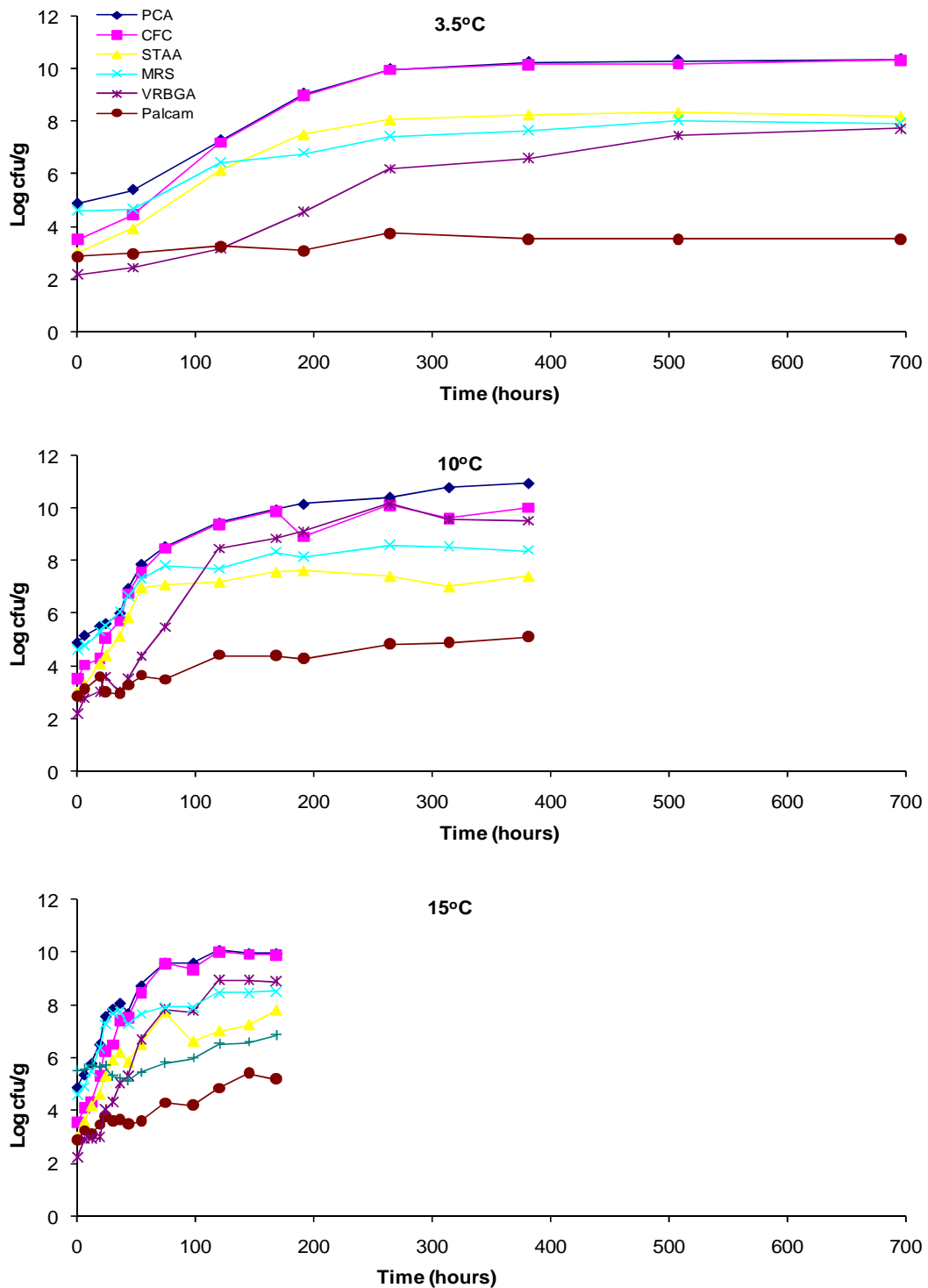
**APPENDIX A  
FIGURES AND TABLES**



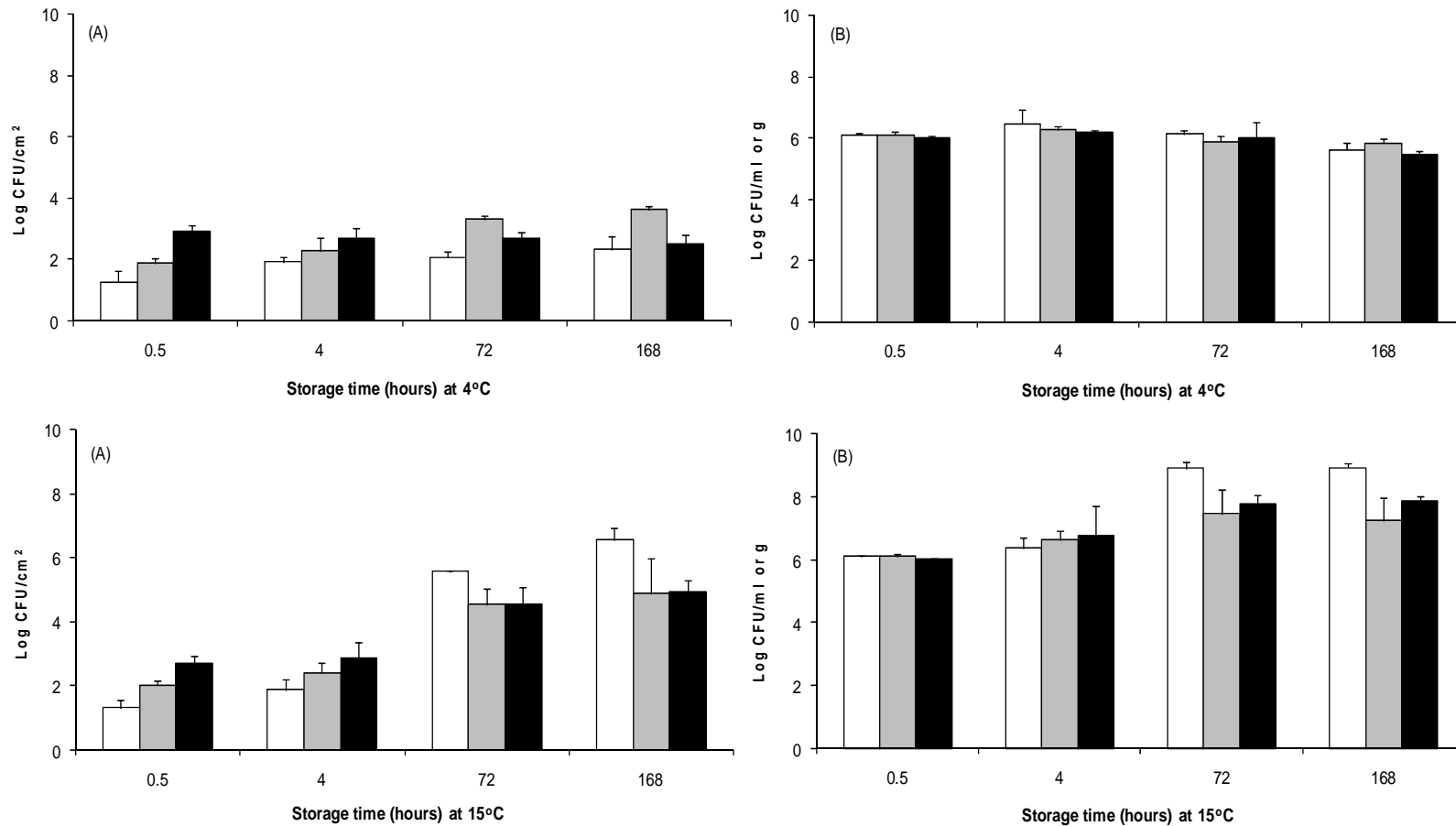
**Figure A.1** Growth curves of the spoilage microflora and *L. monocytogenes* on naturally contaminated meat blocks stored aerobically at 3.5, 10 and 15°C. Media: PCA, plate count agar (total aerobic populations); CFC, cetrimide fusidin cephaloridine (pseudomonads); STAA, streptomycin-thallos acetate (actidionine) agar (*B. thermosphacta*); MRS, Man Rogosa Sharp (lactic acid bacteria); VRBGA, violet red bile glucose agar (*Enterobacteriaceae*); Palcam, Palcam Listeria Selective Agar (*L. monocytogenes*).



**Figure A.2** Growth curves of the spoilage microflora and *L. monocytogenes* on naturally contaminated minced meat-batch 1 stored aerobically at 3.5, 10 and 15°C. Media: PCA, plate count agar (total aerobic populations); CFC, cetrimide fusidin cephaloridine (pseudomonads); STAA, streptomycin-thallos acetate (actidionine) agar (*B. thermosphacta*); MRS, Man Rogosa Sharp (lactic acid bacteria); VRBGA, violet red bile glucose agar (*Enterobacteriaceae*); Palcam, Palcam *Listeria* Selective Agar (*L. monocytogenes*).



**Figure A.3** Growth/survival curves of the spoilage microflora and *L. monocytogenes* and pH changes on naturally contaminated minced meat-batch 2 stored aerobically at 3.5, 10 and 15°C. Media: PCA, plate count agar (total aerobic populations); CFC, cetrimide fusidin cephaloridine (pseudomonads); STAA, streptomycin-thallosus acetate (actidionine) agar (*B. thermosphacta*); MRS, Man Rogosa Sharp (lactic acid bacteria); VRBGA, violet red bile glucose agar (*Enterobacteriaceae*); Palcam, Palcam *Listeria* Selective Agar (*L. monocytogenes*).



**Figure A.4** Growth/survival of attached (A) or suspended (B) *E. coli* O157:H7 cells (mean log CFU/cm<sup>2</sup>, ml or g ± standard deviation; N=2, n=3) after the submersion of high-density polyethylene coupons in TSB (○), fat-lean homogenate (●) and ground beef (●), and storage at 4 and 15°C.

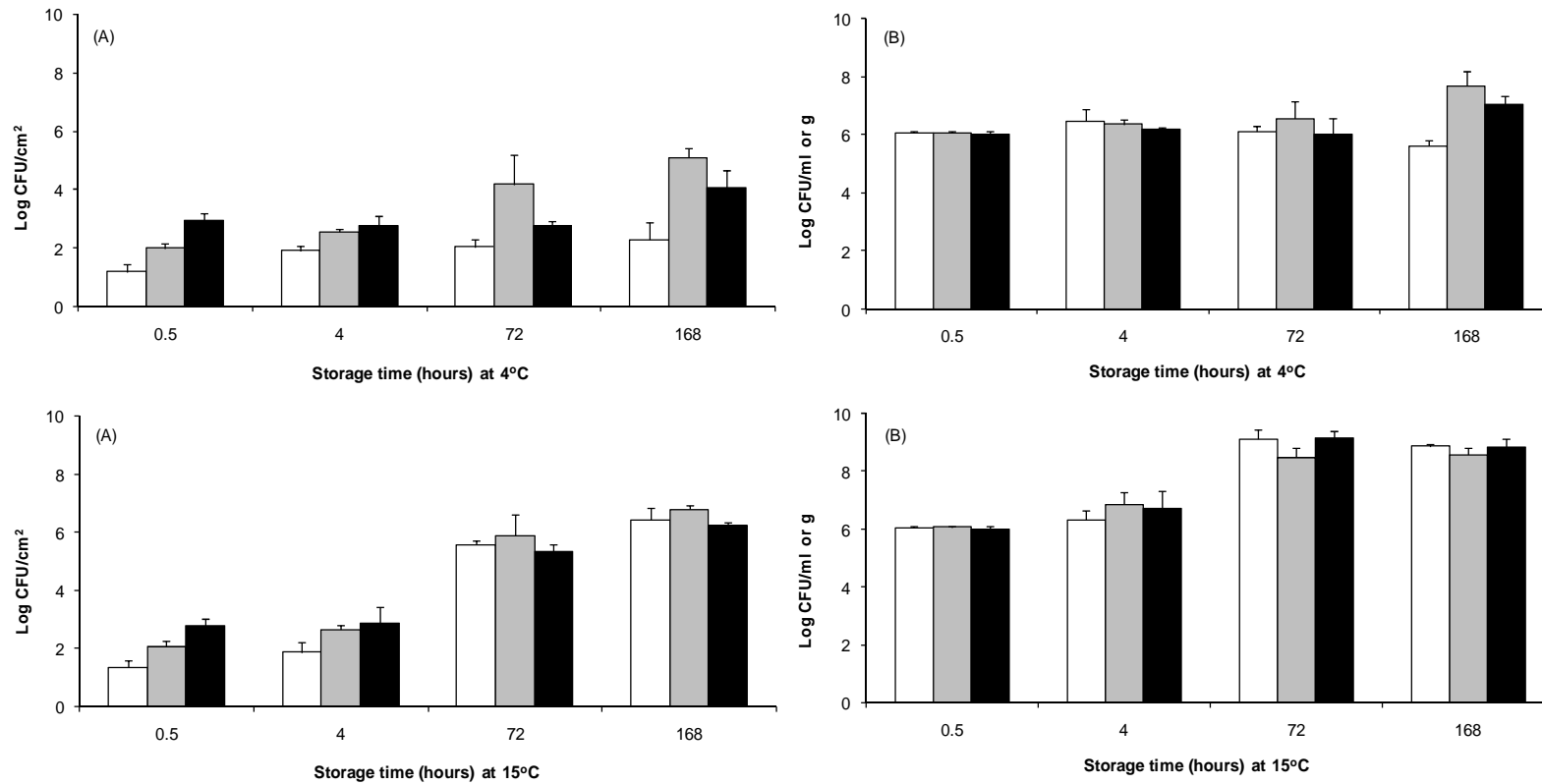


Figure A.5 Growth/survival of attached (A) or suspended (B) total bacterial populations (mean log CFU/cm<sup>2</sup>, ml or g ± standard deviation; N=2, n=3) after the submersion of high-density polyethylene coupons in TSB (○), fat-lean homogenate (●) and ground beef (●), and storage at 4 and 15°C.

**Table A.1** pH values (mean  $\pm$  standard deviation) of TSB, fat-lean homogenate and ground beef inoculated with *Escherichia coli* O157:H7 and stored at 4 and 15°C for 168 h.

Temperature	Storage time (h)	Substrate*		
		TSB	Fat-Lean Homogenate	Ground beef
4°C	0.5	7.28 $\pm$ 0.05	5.64 $\pm$ 0.12	5.72 $\pm$ 0.05
	4	7.39 $\pm$ 0.09	5.58 $\pm$ 0.14	5.76 $\pm$ 0.04
	72	7.40 $\pm$ 0.06	5.57 $\pm$ 0.15	5.73 $\pm$ 0.04
	168	7.46 $\pm$ 0.04	5.78 $\pm$ 0.09	5.59 $\pm$ 0.20
15°C	0.5	7.28 $\pm$ 0.05	5.64 $\pm$ 0.12	5.72 $\pm$ 0.05
	4	7.43 $\pm$ 0.06	5.62 $\pm$ 0.16	5.75 $\pm$ 0.03
	72	5.74 $\pm$ 0.09	6.03 $\pm$ 0.10	5.42 $\pm$ 0.02
	168	6.11 $\pm$ 0.05	6.36 $\pm$ 0.16	5.61 $\pm$ 0.13

\*High-density polyethylene surfaces were submerged into substrates during storage.



## **APPENDIX B LIST OF PUBLICATIONS**

### **Papers in international distributed journals with peer-review**

- “Behavior of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium in teewurst, a raw spreadable sausage”  
(Accepted for publication in the International Journal of Food Microbiology)
- “Growth of *Salmonella* Enteritidis and *Salmonella* Typhimurium in the presence of quorum sensing signalling compounds produced by spoilage and pathogenic bacteria”  
(Submitted to Foodborne Pathogens and Disease)
- “Effect of Microbial Cell Free Meat Extract on the Growth of Spoilage Bacteria”  
(Accepted for publication in the Journal of Applied Microbiology)

### **Papers in preparation for submission to scientific journals**

- “Effect of food structure, composition and microbial interactions on the growth kinetics of *Listeria monocytogenes*”
- “Attachment and subsequent biofilm formation on stainless steel surfaces by nine strains of *Escherichia coli* O157:H7 under two inoculation scenarios”
- “Effect of substrate and storage temperature on biofilm formation by *Escherichia coli* O157:H7 on various food-contact surfaces”
- “Survival/growth of *Escherichia coli* O157:H7 in ground beef cross-contaminated with biofilm and planktonic cells surviving exposure to sublethal sanitation”

### **Short papers and abstracts published in conference proceedings**

- Dourou D., A.P. Stamatiou, K. Koutsoumanis, G.-J.E. Nychas. “Effect of ephemeral spoilage microflora of raw pork minced meat on the growth kinetics of *Listeria monocytogenes* stored at 3.5, 5, 10, 15, 20°C”. Proceedings of the 2<sup>nd</sup>

Panhellenic Congress: Biotechnology and Food Technology, Athens 31-2/4/2005, p. 456-461.

- Dourou D., A.P. Stamatiou, K. Koutsoumanis, Stamatiou, G -J. E. Nychas, 2007. “Effect of food structure (type of growth), composition and microbial interaction on the growth kinetics of *Listeria monocytogenes*”. Proceedings of the 5<sup>th</sup> International Conference of Predictive Modelling in Foods, Athens 16-19/9/2007, p. 95-98.
- D. Dourou, C. Michaelidis, V. Stergiou, P. Skandamis and G.-J.E. Nychas. “Quorum Sensing–Can Be a Variable for Modeling Microbial Behavior?”. Proceedings of the 5<sup>th</sup> International Conference of Predictive Modelling in Foods, Athens 16-19/9/2007, p. 371.
- D. Dourou, A. Porto-Fett, B. Shoyer and J. Luchansky. Behavior of Foodborne Pathogens in Teewurst Raw Spreadable Sausage. Proceedings of the 5<sup>th</sup> International Conference of Predictive Modelling in Foods, Athens 16-19/9/2007, p. 427.
- Dimitra Dourou, Catherine A. Simpson, Yohan Yoon, Keith E. Belk, John A. Scanga, Gary C. Smith, Konstantinos Koutsoumanis, George-John E. Nychas and John N. Sofos. “Effect of Temperature, Shear and Substrate on Attachment and Biofilm Formation by *Escherichia coli* O157:H7 on Various Food-Contact Surfaces Encountered in Beef Processing”. Proceedings of the 95th Annual Meeting of the International Association for Food Protection, Columbus, Ohio, 3-6/8/2008, P2-48.
- Cathrine A. Simpson, Dimitra Dourou, Yohan Yoon, Keith E. Belk, John A. Scanga, Gary C. Smith and John N. Sofos. “Effect of Substrate on Attachment and Subsequent Fate of *Escherichia coli* O157:H7 on Meat-Contact Surfaces”. Proceeding of the 95th Annual Meeting of the International Association for Food Protection, Columbus, Ohio, 3-6/8/2008, P2-47.
- Dimitra Dourou, Catherine A. Simpson, Keith E. Belk, John A. Scanga, Gary C. Smith, Konstantinos Koutsoumanis, George-John E. Nychas, and John N. Sofos. “Variation on the attachment and biofilm formation among *Escherichia coli* O157:H7 strains on stainless steel surfaces under two inoculation scenarios”.

Proceeding of the 2008 IFT Annual Meeting + Food Expo, New Orleans, LA, 28/6-1/7/2008, P010-28.

- Catherine A. Simpson, Dimitra Dourou, Keith E. Belk, John A. Scanga, Gary C. Smith, and John N. Sofos. “Effect of beef cutting surface material, previous exposure to beef residues and inoculation method on attachment of *Escherichia coli* O157:H7 and its fate in beef fabrication-floor fluids”. Proceeding of the 2008 IFT Annual Meeting + Food Expo, New Orleans, LA, 28/6-1/7/2008, P045-03.

**“Behavior of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella*  
Typhimurium in teewurst, a raw spreadable sausage”**

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*Running title:* Fate of pathogens in and on teewurst.

*Keywords:* *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium,  
pathogen, food safety, teewurst, meats.

## ABSTRACT

The fate of *Listeria monocytogenes*, *Salmonella* Typhimurium, or *Escherichia coli* O157:H7 were separately monitored both in and on teewurst, a traditional raw and spreadable sausage of Germanic origin. Multi-strain cocktails of each pathogen (ca. 5.0 log CFU/g) were used to separately inoculate teewurst that was subsequently stored at 1.5, 4, 10, and 21°C. When inoculated into commercially-prepared batter just prior to stuffing, in general, the higher the storage temperature, the greater the lethality. Depending on the storage temperature, pathogen levels in the batter decreased by 2.3 to 3.4, ca. 3.8, and 2.2 to 3.6 log CFU/g for *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*, respectively, during storage for 30 days. When inoculated onto both the top and bottom faces of sliced commercially-prepared finished product, the results for all four temperatures showed a decrease of 0.9 to 1.4, 1.4 to 1.8, and 2.2 to 3.0 log CFU/g for *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*, respectively, over the course of 21 days. With the possible exceptions for salt and carbohydrate levels, chemical analyses of teewurst purchased from five commercial manufacturers revealed only subtle differences in proximate composition for this product type. Our data establish that teewurst does not provide a favourable environment for the survival of *E. coli* O157:H7, *S. Typhimurium*, or *L. monocytogenes* inoculated either into or onto the product.

## INTRODUCTION

Teewurst is a traditional sausage of Germanic origin, typically made from pork and beef, that is characterized by a soft spreadable texture. It is usually manufactured by small producers and sold under refrigeration as a raw spreadable meat (USDA, 1993, USDA, 2005). At present, there is a general lack of criteria for both the manufacture and the compositional descriptions for fresh and raw spreadable sausages, including teewurst (Islam and Jockel, 2005). Teewurst is grouped with other meat products such as mettwurst that display a relatively low acid content (e.g., pH 5.3-5.5) and high moisture content (e.g.,  $a_w \geq 0.95$ ) (Brown, 2000). Processing and preparation of this product does not typically include any heat treatment or antimicrobial interventions other than the salts, spices, nitrites, and perhaps phenolics contributed by liquid smoke, that are added directly to the batter (Brown, 2000). The teewurst manufactured in the USA is the same as the teewurst produced in Germany, unless it is cooked, as is practiced by some manufacturers. As USA regulations stipulate, true product names and a “safe handling statement” must be accurately affixed to the label to provide consumers with the

ability to choose between “teewurst uncooked, cured meat spread” or “cooked teewurst”, with the former being “raw”.

The association of teewurst with foodborne illnesses in recent years is well documented (Ammon *et al.*, 1999, Werber *et al.*, 2006). In Germany, consumption of raw spreadable sausages, including teewurst, was identified as a risk factor for sporadic illnesses associated with Shiga toxin producing *E. coli* (STEC) in persons aged 10 years or older (Werber *et al.*, 2006). Similarly, a large outbreak (28 cases, 3 deaths) of haemolytic uremic syndrome (HUS) caused by a sorbitol-fermenting strain of *E. coli* O157:H- was associated with consumption of teewurst, a raw pork product, and mortadella, a cooked product (Ammon *et al.*, 1999). Although teewurst is intended to be cooked by the consumer, its production includes ingredients such as nitrites that cause the raw sausage to appear as a ready-to-eat (RTE) product; therefore, teewurst is notoriously eaten without proper cooking, either by preference or by perception. In a survey conducted in Germany in 2001 related to knowledge and handling of raw meat, and in particular teewurst, ca. 50% of the 510 participants reported eating teewurst and, somewhat surprisingly, only ca. 36% of them recognised it as a raw meat product (Bremer *et al.*, 2005). Thus, this study was conducted to evaluate the behaviour of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* inoculated either into the batter or onto the surface of sliced teewurst that was subsequently stored under aerobic conditions at refrigeration and abuse temperatures. Proximate composition analyses of commercial teewurst produced by five relatively small processors were also conducted to address the potential variety and range of chemical traits, since a standard of identity does not currently exist for this product.

## MATERIALS AND METHODS

**Bacterial strains.** The multi-strain cocktails of *L. monocytogenes* (MFS2, MFS102, MFS104, MFS105, and MFS110), *E. coli* O157:H7 (EC505B, C7927, and SLH21788), and *S. Typhimurium* (H3278, G7601, H3402, H2662, H3380, and G8430) used in this study were confirmed, cultured, combined, and/or maintained as described previously (Porto-Fett *et al.*, 2008a).

**Formulation and manufacture of teewurst.** The formulation of teewurst batter, as purchased from a local manufacturer (Ernst A. Illg Meats, Inc.; Chalfont, PA), consisted of certified pork trimmings (60 lbs; fat-lean ratio 70%-30%), boneless beef plates (40 lbs; fat-lean ratio 70%-30%), and 3.83 lbs of the following non-meat ingredients: seasoning spices (First Spice Mixing Co., Long Island City, NY), sodium nitrite curing salt, liquid smoke flavoring, paprika, cardamom, and sugar. The manufacturing process for this brand of teewurst is shown in Figure 1. Chubs and slices of this brand of finished teewurst are shown in Figure 2.

**Inoculation of teewurst batter.** To simulate contamination at the processing plant, three batches (one batch per trial) of freshly-processed teewurst batter were separately inoculated with ca. 5.2 log CFU/g of each multi-strain pathogen cocktail. After inoculation, the batter was mixed at ambient temperature ( $22^{\circ} \pm 1^{\circ}\text{C}$ ) using a commercial countertop mixer (Univex SRM12; Salem, NH) for ca. 2 min to ensure for relatively even distribution of the inoculum. The batter was stuffed using a commercial (manual) stuffer (D-73779; Dick, Deizisau, Germany) into commercial 4.5 cm diameter artificial “fibrous” casings (F Plus; Walsroder GMBH, Germany) in portions of ca. 100 g. The resulting chubs were stored at 1.5, 4, 10, or 21°C for up to 30 days. In each of the three trials two chubs were sampled at each sampling interval (N = 3 trials; n = 2 replicates/chubs per sampling interval per trial). It should be noted that the terms “batter” and “chub” herein refer to teewurst inoculated prior to stuffing.

**Inoculation of the surface of teewurst slices.** To simulate post-process contamination in the home or in a food service establishment, three batches (one batch per trial) of freshly-processed teewurst were obtained from our collaborating manufacturer as above. Teewurst was transferred aseptically from the original packages onto sterile styrofoam trays (1012S; Genpak,

Glens Falls, NY) and sliced (ca. 20 g each slice, ca. 5 cm diameter) with the aid of an ethanol-sterilized knife. Individual slices were placed onto styrofoam trays (Genpak) and separately inoculated on the top surface of each slice with 50  $\mu$ l of each multi-strain pathogen cocktail. Cells were then distributed with the aid of a sterile plastic cell spreader (Midsci; St. Louis, MO). The trays containing the inoculated teewurst were placed into a biological safety cabinet and held for ca. 15 min at ambient temperature ( $22 \pm 1^\circ\text{C}$ ) to allow for the inocula to better attach to the meat slices. Next, the slices were inverted and the process was repeated on the opposite side. The final concentration of each pathogen was ca. 4.5 log CFU/g. Inoculated slices (one slice per bag) were then placed into sterile polyethylene bags (Ziploc Brand Snack Bags; S.C. Johnson Products, Inc., Racine WI). The bags were stored at 1.5, 4, 10, or 21 $^\circ\text{C}$  for up to 21 days. In each of the three trials two bags/slices were sampled at each sampling interval (N = 3 trials; n = 2 replicates/slices per sampling interval per trial).

**Microbiological analyses.** Initial and final populations of total plate count (TPC) and total lactic acid bacteria (LAB) were enumerated on slices and in chubs as follows. A total of three slices (ca. 20 g each) or three chubs (ca. 5 g each), from each of the three trials/batches tested, were separately transferred into plastic two-chamber filter stomacher bags (Fisherbrand; Fisher Scientific, Pittsburgh, PA) containing 15 or 45 ml of 0.1% sterile peptone water (Difco, Becton, Dickinson and Co., Sparks, MD), respectively, and stomached for ca. 2 min (Stomacher 400; Seward, Cincinnati, OH). The TPC were enumerated by spread-plating 100  $\mu$ l of the resulting slurry, with or without prior dilution in sterile peptone water, onto Brain Heart Infusion agar plates (BHI; Difco,) and aerobic incubation at 30 $^\circ\text{C}$  for 72 h. For enumeration of LAB, appropriate dilutions of the slurry were spread-plated (100  $\mu$ l) onto deMan Rogosa Sharpe agar (MRS; Difco) and incubated anaerobically at 37 $^\circ\text{C}$  for 72 h (10.1% carbon dioxide, 4.38% hydrogen and the balance in nitrogen; Bactron IV Anaerobic/Environmental Chamber; Sheldon Manufacturing Inc., Cornelius, OR).

For enumeration of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* from teewurst, the inoculated slices and chubs were sampled at periodic intervals and treated as above and appropriate dilutions of the resulting slurry were surface-plated (100  $\mu$ l) onto Modified Oxford agar for enumeration of *L. monocytogenes* (MOX; Difco), MacConkey sorbitol agar for enumeration of *E. coli* O157:H7 (SMAC; Difco), and xylose lysine tergitol-4 agar for enumeration of *S. Typhimurium* (XLT4; Difco). Typical colonies of each pathogen were counted after aerobic incubation of plates at 37 $^\circ\text{C}$  for 48 h (MOX) or 24 h (SMAC and XLT4). When pathogen numbers in batter decreased to  $\leq 1.0$  log CFU/g by direct plating their presence or absence were determined by enrichment as described (*Porto-Fett et al., 2008a*).

**Physicochemical analyses.** At both the beginning and at the end of storage, control (non-inoculated) teewurst samples [N = 3 trials; n = 3 slices (ca. 20 g each) or n = 3 chubs (ca. 5 g each) per trial] were analyzed for pH and  $a_w$  by using a model 6000P pH/temperature electrode and a model 5500 pH meter (Daigger, Vernon Hills, IL) and a water activity meter (Decagon Aqualab Model series 3; Decagon Devices, Pullman, WA), respectively, according to the manufacturer's instructions. For the market basket component of this study, two chubs from each brand were analyzed to determine the proximate composition of the teewurst purchased from five commercial processors as determined by a commercial laboratory using methods approved and described by the Association of Official Analytical Chemists (*McNeal, 1990*).

**Statistical analyses.** Microbial counts were transformed to logarithms before means and standard deviations were computed, and counts were reported in terms of log CFU/g. When bacterial counts in teewurst batter decreased to below the threshold of detection ( $\leq 1.0$  log CFU/g) a value of 1 was used for positive samples after enrichment for determination of the arithmetic mean. Statistical analyses were performed using the SPSS 12.0 software program for windows (*SPSS Inc., Chicago, IL*). For each contamination scenario and pathogen an analysis of variance (ANOVA) was performed to evaluate the effect of storage time and temperature on pathogen viability. Differences in the proximate composition of teewurst manufactured by

different processors were also evaluated using ANOVA. Least squares means separation was performed using the Tukey procedure at a significance level of  $p < 0.05$ .

## RESULTS

**Microbiological analyses of teewurst.** Direct plating of samples of control (non-inoculated) teewurst slices or control batter/chubs taken from each of the three trials/batches tested revealed the absence ( $\leq 0.2$  and  $\leq 1.0$  log CFU/g for teewurst slices and batter/chubs, respectively) of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* (data not shown). These samples also tested negative for each of these same three pathogens following enrichment. The average initial TPC and LAB levels were  $7.2 \pm 0.7$  and  $5.7 \pm 0.9$  log CFU/g, respectively, for teewurst batter, whereas for teewurst slices the average initial TPC and LAB levels were  $6.5 \pm 0.7$  and  $5.5 \pm 0.9$  log CFU/g, respectively (Table 1). Average initial values of pH were  $5.87 \pm 0.25$  and  $6.18 \pm 0.19$  for teewurst batter and teewurst slices, respectively, whilst thereafter the pH decreased somewhat to about pH 4.39 and 4.78, respectively, at the end of storage for both batter and slices. Average initial  $a_w$  values were about 0.960 (SD  $\leq 0.005$ ) for both slices and batter, and  $a_w$  changed relatively little over the storage period. For both slices and batter, numbers of TPC and LAB were very similar at the end of the respective storage period (Table 1).

**Viability of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* inoculated into teewurst batter or onto the surface of teewurst slices.** Regardless of the storage temperature, numbers of all three pathogens inoculated into the batter decreased after 30 days of storage (Table 2). With the exception of storage at 21°C which generated the greatest overall lethality, the observed reductions were not appreciably different for the other temperatures tested. More specifically, when chubs inoculated with *E. coli* O157:H7 prior to stuffing were subsequently stored at 1.5, 4, and 10°C pathogen numbers decreased by 2.3, 3.2, and 3.0 log CFU/g, respectively, after 30 days of storage. When chubs inoculated with *L. monocytogenes* prior to stuffing were stored at 1.5, 4, and 10°C pathogen numbers decreased by 2.2, 2.6, and 2.6 log CFU/g, respectively, after 30 days of storage. *E. coli* O157:H7 and *L. monocytogenes* levels decreased to below the level of detection by both direct plating ( $\leq 1.0$  log CFU/g) and enrichment after 25 and 18 days of storage at 21°C, respectively. *S. Typhimurium* levels decreased below detectable levels by direct plating within 15, 18, and 11 days at 1.5, 4, and 10°C, respectively. The absence of *S. Typhimurium* was confirmed by the inability to recover cells of this pathogen even by enrichment after 30 days at 1.5 and 4°C, after 21 days at 10°C, and after 11 days at 21°C. In general, *S. Typhimurium* was inactivated at a greater rate and to a greater extent (absent by enrichment within 11 days at 21°C) than *E. coli* O157:H7 or *L. monocytogenes* when inoculated into batter (Table 2).

Regarding survival on teewurst slices, pathogen numbers remained relatively unchanged after four days of storage for all temperatures tested (Table 3). Storage at 1.5, 4, 10, and 21°C for up to 21 days resulted in reductions of *E. coli* O157:H7 and *S. Typhimurium* from ca. 4.8 log CFU/g to 3.7, 3.7, 3.9, and 3.4 log CFU/g and from ca. 4.3 log CFU/g to 2.5, 2.8, 2.9, and 2.7 log CFU/g, respectively. When slices were inoculated with *L. monocytogenes* and stored at 1.5, 4, 10, and 21°C for up to 21 days, pathogen numbers decreased from ca. 4.5 log CFU/g to 1.8, 2.3, 1.8, and 1.5 log CFU/g, respectively. In general, *L. monocytogenes* was inactivated at a greater rate and to a greater extent than *S. Typhimurium* and *E. coli* O157:H7 at all temperatures tested. Moreover, the decrease in levels of *E. coli* O157:H7, *S. Typhimurium*, or *L. monocytogenes* when inoculated onto slices of teewurst was not appreciably affected by the storage temperature (Table 3), that being, similar reductions in pathogen levels were observed at all temperatures tested for a given pathogen.

**Proximate composition analyses.** With possible exceptions of the carbohydrate levels that were not statistically ( $p \geq 0.05$ ) different among the five brands and the salt level for brand

A that was significantly ( $p \leq 0.05$ ) lower compared to the others four brands, chemical analyses revealed only subtle differences ( $p \leq 0.05$ ) for a given chemical trait among the five commercial brands tested. These findings establish that teewurst displays a range of compositional compounds and characteristics (Table 4).

**Market basket survey.** As a final component of this study, we conducted a market basket survey of commercially available teewurst. With reference to USDA/FSIS directive 7235.1 (USDA, 1994) for raw or partially cooked meat and poultry products, the labels from four of the five brands tested herein declared teewurst as an uncooked product and/or provided safe handling instructions, that being “Keep refrigerated” and/or “Cook thoroughly” (Table 5). A lack of uniformity in the listed ingredients and additives used by these five processors was also observed and subsequently confirmed by proximate composition analyses (Tables 4 and 5). Proximate composition analyses also revealed that teewurst in general has relatively low nitrite and salt levels and a relatively high moisture and high fat content, characteristics that typically do not provide a sufficient barrier to microbial persistence in such products.

## DISCUSSION

Teewurst is a very popular traditional/ethnic sausage, typically consumed raw, that remains in demand, albeit in the face of generally declining sales (Ernst K. Illg, personal communication, 2008). It is produced by a limited number of small plants that are located primarily in the northeast and upper midwest regions of the USA. From a public health perspective spreadable sausages such as teewurst are considered to be higher-risk products, presumably because consumers are not aware of the safe-handling requirements for teewurst as a product that may contain raw meat (Bremer *et al.*, 2005) and/or due to their preference to consume it “as is”. In recent years, consumption of teewurst has caused human illnesses due to its contamination with *E. coli* O157:H7 and *L. monocytogenes* and, therefore, such products may potentially be a vehicle for harborage and/or transmission of foodborne pathogens (Brown, 2000; FAO, 2004; Goulet *et al.*, 2002; Pichner *et al.*, 2004; Timm *et al.*, 1999). If opened/sampled, teewurst has a refrigerated shelf life of ca. 1 (Campbell-Platt, 1995; Ockerman and Basu, 2007) to 5 days (Brown, 2000), whereas if left unopened the shelf life could extend for up to 7 to 21 days at 4°C (Ernst K. Illg, personal communication). In the present study, however, visible mold-like spoilage was evident on teewurst slices within 21 days of refrigerated storage (1.5 and 4°C) or within 5 days of storage at abuse temperatures (10 and 21°C). Regardless, pathogen levels decreased during storage; however, in the event of post-process contamination with relatively high levels of these pathogens, as seen for other meat products, teewurst could possibly expose some consumers to a health risk (Gounadaki *et al.*, 2007; Matargas *et al.*, 20008; Yang *et al.*, 2006).

Levels of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* decreased appreciably in teewurst chubs during storage for 30 days (Table 2). Greater reductions in pathogen numbers were observed at 10 and 21°C as compared to 1.5 and 4°C. However, at the end of storage, with the exception of *S. Typhimurium* for which the most significant lethality was observed, surviving numbers of each pathogen were of similar levels. Moreover, in agreement with related studies conducted on salami and soudjouk (Nightingale *et al.*, 2006; Porto-Fett *et al.*, 2008b), *S. Typhimurium* inoculated into teewurst batter/chubs was less viable than *L. monocytogenes* and *E. coli* O157:H7. Lethality may be attributed to the presence of native LAB in addition to antimicrobial ingredients such as nitrites, since according to Rödel *et al.* (Rödel and Scheuer, 2006) inhibition of *E. coli* in short fermented raw spreadable sausages was enhanced due to the acidification of the product by LAB and ensuing reduction of  $a_w$ , whereas the presence of sodium nitrite had only a weak effect. Similar findings were reported by Birzele *et al.* (Birzele *et al.*, 2005), who found that nitrite at levels of 0.5 or 0.9% incorporated into fresh spreadable ham and onion sausage inhibited growth of *Salmonella* Enteritidis, *E. coli*, and *Staphylococcus aureus*, as well as partially inhibited *L. monocytogenes*.



The proliferation and metabolic activity of LAB are known to inhibit undesirable bacteria, mainly through the production of lactic acid and the subsequent pH reduction of foods, but also by the production of CO<sub>2</sub>, hydrogen peroxide, ethanol, diacetyl, and/or bacteriocins (Hugas, 1998). The batch-to-batch levels and diversity of LAB naturally present in raw meat and associated microbial interactions (i.e. chemical changes in product) could possibly explain the observed variability in lethality for each pathogen among trials and between chubs and slices (Comi et al., 2005; Kaya et al., 2004; Skandamis et al., 2007).

The findings of the present study suggest that inclusion of a defined lactic starter culture(s) and perhaps a limited/controlled fermentation during manufacture would improve the reproducibility from batch-to-batch and enhance both the quality and safety of the finished product (Calicioglu et al., 2001; Lucke, 2000). As previously reported, fermentation of a German-style uncooked sausage (24°C/24 h) followed by smoking (22°C/20 h) resulted in a 2.0- to 3.0-log reduction of *L. monocytogenes* (Farber et al., 1993). In fact, fermentation of some spreadable raw sausages in Germany constitutes a critical element of the manufacturing process so as to insure that the final product is characterised by an appropriate flavour, colour, texture, and acidification level ( $\leq$ pH 5.6; D-lactic acid  $\geq$  0.2 g/100 g; Islam and Jockel, 2005). In the case of teewurst, however, the addition of a starter culture and the ensuing production of organic acid(s) and other compounds could possibly have an untoward effect on product taste, that being too sour, and on product texture, that being too firm and, as such, less spreadable (Ernst K. Illg, personal communication, 2008). Thus, it may be prudent to consider adding food grade chemicals as an ingredient to further enhance the wholesomeness of teewurst. In fact, in prefatory studies we observed an immediate decrease of ca. 1.6 log CFU/g of *L. monocytogenes* in the presence of 5.5 ppm of nisin added directly to the teewurst batter; however, no further decrease in pathogen levels was observed during storage at 4 or 10°C over 10 days of storage (data not shown). Regardless, the need for a more precise standard of identity was evident from the differences among brands in the various physicochemical traits measured, as well as from differences in the information included on product labels (Table 4 and 5). In the absence of any readily accessible and/or published information, the data in Tables 4 and 5 may serve as a starting point for assisting in the development of a list of ingredients and range of attendant concentrations for defining a standard of identity for teewurst.

To our knowledge, there is limited scientific literature on the fate of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* either “on” or “in” teewurst. This study provides valuable information to small and very small plants producing teewurst and to regulatory authorities overseeing its production for assessing product safety from these foodborne pathogens. The need to establish both a standard of identity and guidelines for its manufacture are critical given that teewurst is typically a raw rather than RTE product, as well as given that despite labeling instructions to the contrary, this product is commonly/openly ingested as raw without cooking. The data herein also highlight the need to educate both producers and consumers as to the appropriate manner to produce/handle and store teewurst so as not to introduce pathogens at any point from production through to consumption.

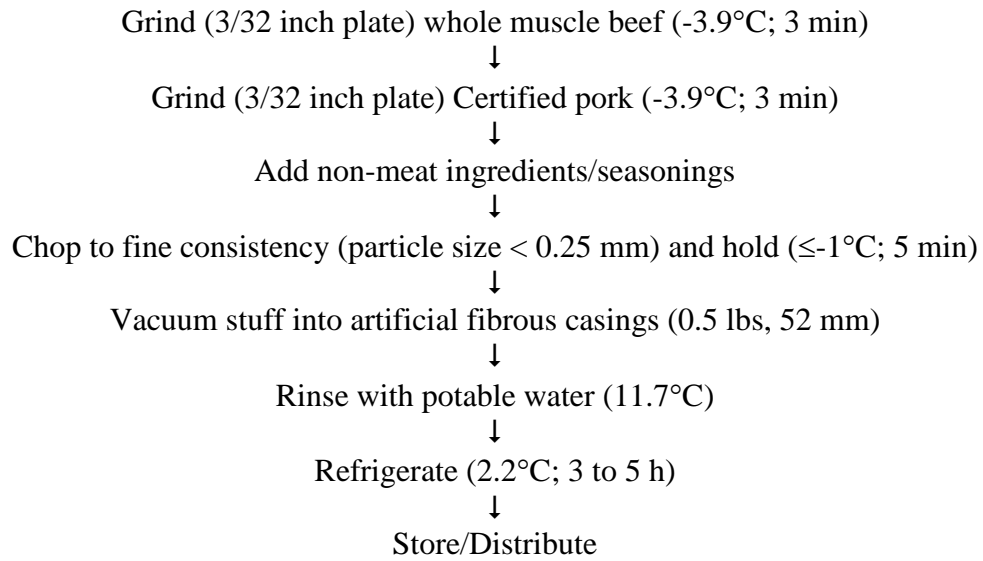
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**Figure 1** Flow diagram describing the teewurst manufacturing process used in this study.



**Figure 2** Teewurst, a raw spreadable sausage.

**Table 1** Evaluation of native biota, pH, and  $a_w$  of non-inoculated teewurst before and after storage.

Product Type	Storage time (days)	Temperature (°C)	TPC	LAB	pH	$a_w$
Teewurst chubs	0		$7.2 \pm 0.7^a$	$5.7 \pm 0.9$	$6.18 \pm 0.19$	$0.957 \pm 0.004$
	30	1.5	$6.5 \pm 1.0$	$7.0 \pm 1.5$	$4.85 \pm 0.19$	$0.955 \pm 0.002$
		4	$6.3 \pm 0.7$	$6.6 \pm 1.2$	$4.58 \pm 0.15$	$0.953 \pm 0.002$
		10	$6.6 \pm 0.6$	$6.8 \pm 0.5$	$4.45 \pm 0.15$	$0.953 \pm 0.003$
		21	$6.7 \pm 0.3$	$6.6 \pm 0.5$	$4.39 \pm 0.10$	$0.945 \pm 0.003$
Teewurst slices	0		$6.5 \pm 0.7$	$5.5 \pm 0.9$	$5.87 \pm 0.25$	$0.960 \pm 0.005$
	21	1.5	$7.0 \pm 1.1$	$6.4 \pm 1.2$	$4.66 \pm 0.48$	$0.958 \pm 0.004$
		4	$7.6 \pm 0.7$	$7.2 \pm 0.7$	$5.13 \pm 0.81$	$0.958 \pm 0.003$
		10	$8.3 \pm 0.3$	$8.0 \pm 0.2$	$5.51 \pm 0.40$	$0.956 \pm 0.003$
		21	$8.1 \pm 0.1$	$8.0 \pm 0.1$	$4.78 \pm 0.76$	$0.952 \pm 0.002$

<sup>a</sup>Values are mean log CFU/g  $\pm$  standard deviation (N = 3, n = 3).

**Table 2** Counts (mean log CFU/g  $\pm$  standard deviation; n = 6 chubs for each sampling interval) of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* inoculated into teewurst batter<sup>1</sup>.

Microorganism/ Temperature (°C)	Storage time (days)								
	0	3	8	11	15	18	21	25	30
<i>E. coli</i> O157:H7									
1.5	5.3 $\pm$ 0.0 <sup>aA</sup>	4.9 $\pm$ 0.3 <sup>abAB</sup>	4.9 $\pm$ 0.1 <sup>abA</sup>	4.8 $\pm$ 0.1 <sup>abA</sup>	4.3 $\pm$ 0.6 <sup>bcA</sup>	3.6 $\pm$ 0.6 <sup>cdA</sup>	3.5 $\pm$ 0.4 <sup>dA</sup>	3.4 $\pm$ 0.5 <sup>dA</sup>	3.0 $\pm$ 0.6 <sup>dA</sup>
4	5.3 $\pm$ 0.0 <sup>aA</sup>	5.1 $\pm$ 0.1 <sup>aAB</sup>	5.0 $\pm$ 0.1 <sup>aA</sup>	4.7 $\pm$ 0.3 <sup>aA</sup>	4.0 $\pm$ 0.3 <sup>bA</sup>	3.7 $\pm$ 0.5 <sup>bcA</sup>	3.6 $\pm$ 0.3 <sup>bcA</sup>	3.3 $\pm$ 0.4 <sup>cAB</sup>	2.1 $\pm$ 0.7 <sup>dA</sup>
10	5.3 $\pm$ 0.0 <sup>aA</sup>	5.2 $\pm$ 0.1 <sup>aA</sup>	4.6 $\pm$ 0.4 <sup>aA</sup>	4.5 $\pm$ 0.7 <sup>aA</sup>	2.9 $\pm$ 0.7 <sup>bB</sup>	2.7 $\pm$ 0.4 <sup>bAB</sup>	2.3 $\pm$ 1.1 <sup>bAB</sup>	2.5 $\pm$ 0.8 <sup>bbB</sup>	2.3 $\pm$ 1.1 <sup>bA</sup>
21	5.3 $\pm$ 0.0 <sup>aA</sup>	4.8 $\pm$ 0.3 <sup>abB</sup>	3.5 $\pm$ 0.4 <sup>bcB</sup>	2.4 $\pm$ 1.1 <sup>cdB</sup>	1.7 $\pm$ 0.6 <sup>dC</sup>	2.3 $\pm$ 1.4 <sup>cdB</sup>	1.9 $\pm$ 0.9 <sup>cdB</sup>	ND <sup>2</sup>	ND
<i>S. Typhimurium</i>									
1.5	4.9 $\pm$ 0.1 <sup>aA</sup>	4.2 $\pm$ 0.5 <sup>aA</sup>	3.2 $\pm$ 1.6 <sup>abA</sup>	2.0 $\pm$ 1.5 <sup>bcA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	ND
4	4.9 $\pm$ 0.1 <sup>aA</sup>	4.3 $\pm$ 0.3 <sup>aA</sup>	3.1 $\pm$ 0.7 <sup>bA</sup>	2.5 $\pm$ 1.2 <sup>bA</sup>	1.2 $\pm$ 0.3 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	ND
10	4.9 $\pm$ 0.1 <sup>aA</sup>	3.8 $\pm$ 1.0 <sup>bAB</sup>	1.6 $\pm$ 0.5 <sup>cB</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	$\leq$ 1.0 $\pm$ 0.0 <sup>cA</sup>	ND	ND	ND
21	4.9 $\pm$ 0.1 <sup>aA</sup>	2.6 $\pm$ 1.6 <sup>bbB</sup>	1.1 $\pm$ 0.2 <sup>cB</sup>	ND	ND	ND	ND	ND	ND
<i>L. monocytogenes</i>									
1.5	5.4 $\pm$ 0.2 <sup>aA</sup>	5.3 $\pm$ 0.1 <sup>aA</sup>	4.5 $\pm$ 0.9 <sup>abA</sup>	4.2 $\pm$ 0.9 <sup>abcA</sup>	4.1 $\pm$ 0.8 <sup>bcA</sup>	3.5 $\pm$ 1.0 <sup>bcA</sup>	3.8 $\pm$ 0.3 <sup>bcA</sup>	3.6 $\pm$ 0.6 <sup>bcA</sup>	3.2 $\pm$ 0.5 <sup>cA</sup>
4	5.4 $\pm$ 0.2 <sup>aA</sup>	5.2 $\pm$ 0.1 <sup>abA</sup>	4.4 $\pm$ 0.7 <sup>bcA</sup>	4.6 $\pm$ 0.1 <sup>bA</sup>	3.7 $\pm$ 0.2 <sup>cdAB</sup>	3.5 $\pm$ 0.3 <sup>dA</sup>	3.1 $\pm$ 0.8 <sup>dA</sup>	2.9 $\pm$ 0.2 <sup>dA</sup>	2.8 $\pm$ 0.6 <sup>dA</sup>
10	5.4 $\pm$ 0.2 <sup>aA</sup>	5.3 $\pm$ 0.1 <sup>aA</sup>	3.9 $\pm$ 0.4 <sup>bAB</sup>	3.5 $\pm$ 0.6 <sup>bcAB</sup>	3.1 $\pm$ 0.3 <sup>cdB</sup>	3.4 $\pm$ 0.3 <sup>bcdA</sup>	3.1 $\pm$ 0.4 <sup>cdA</sup>	3.2 $\pm$ 0.4 <sup>bcdA</sup>	2.8 $\pm$ 0.6 <sup>dA</sup>
21	5.4 $\pm$ 0.2 <sup>aA</sup>	3.9 $\pm$ 0.9 <sup>bbB</sup>	2.6 $\pm$ 1.0 <sup>cB</sup>	2.2 $\pm$ 1.4 <sup>cB</sup>	1.8 $\pm$ 0.6 <sup>ccC</sup>	ND	ND	ND	ND

<sup>1</sup>Means with different lowercase letters within a row are significantly different ( $p < 0.05$ ). Means with different uppercase letters within a column for each organism are significantly different ( $p < 0.05$ ).

<sup>2</sup>ND; not detected by either direct plating or by enrichment.

**Table 3** Counts (mean log CFU/g  $\pm$  standard deviation; n = 6 slices for each sampling interval) of *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* inoculated onto teewurst slices<sup>1</sup>.

Organism/ Temperature (°C)	Storage time (days)									
	0	1	2	4	6	8	11	13	18	21
<i>E. coli</i> O157:H7										
1.5	4.8 $\pm$ 0.2 <sup>aA</sup>	4.4 $\pm$ 0.2 <sup>abA</sup>	4.5 $\pm$ 0.2 <sup>abA</sup>	4.5 $\pm$ 0.1 <sup>abA</sup>	4.4 $\pm$ 0.1 <sup>abA</sup>	4.1 $\pm$ 0.3 <sup>abcA</sup>	4.4 $\pm$ 0.1 <sup>abcA</sup>	3.9 $\pm$ 0.7 <sup>bcAB</sup>	3.7 $\pm$ 0.4 <sup>cA</sup>	3.7 $\pm$ 0.5 <sup>cA</sup>
4	4.8 $\pm$ 0.2 <sup>aA</sup>	4.4 $\pm$ 0.3 <sup>abA</sup>	4.5 $\pm$ 0.1 <sup>abA</sup>	4.5 $\pm$ 0.1 <sup>abA</sup>	4.1 $\pm$ 0.2 <sup>bcdA</sup>	4.1 $\pm$ 0.2 <sup>bcdA</sup>	4.2 $\pm$ 0.3 <sup>bcA</sup>	4.1 $\pm$ 0.5 <sup>bcdA</sup>	3.7 $\pm$ 0.1 <sup>cdA</sup>	3.7 $\pm$ 0.3 <sup>dA</sup>
10	4.8 $\pm$ 0.2 <sup>aA</sup>	4.5 $\pm$ 0.3 <sup>abA</sup>	4.5 $\pm$ 0.2 <sup>abA</sup>	4.4 $\pm$ 0.2 <sup>abA</sup>	4.1 $\pm$ 0.1 <sup>abA</sup>	4.0 $\pm$ 0.3 <sup>bA</sup>	4.2 $\pm$ 0.3 <sup>abA</sup>	3.8 $\pm$ 0.0 <sup>bAB</sup>	4.5 $\pm$ 0.8 <sup>abA</sup>	3.9 $\pm$ 0.2 <sup>bA</sup>
21	4.8 $\pm$ 0.2 <sup>aA</sup>	4.6 $\pm$ 0.1 <sup>abA</sup>	4.6 $\pm$ 0.3 <sup>abA</sup>	4.2 $\pm$ 0.1 <sup>abcB</sup>	4.1 $\pm$ 0.2 <sup>abcA</sup>	3.8 $\pm$ 0.5 <sup>bcdA</sup>	3.7 $\pm$ 0.4 <sup>bcdA</sup>	3.0 $\pm$ 0.2 <sup>dB</sup>	3.4 $\pm$ 1.1 <sup>cdA</sup>	3.4 $\pm$ 0.5 <sup>cdA</sup>
<i>S. Typhimurium</i>										
1.5	4.3 $\pm$ 0.2 <sup>aA</sup>	4.1 $\pm$ 0.2 <sup>abAB</sup>	3.7 $\pm$ 0.2 <sup>abcA</sup>	3.7 $\pm$ 0.2 <sup>abcA</sup>	3.4 $\pm$ 0.4 <sup>bcA</sup>	3.2 $\pm$ 0.2 <sup>cA</sup>	3.1 $\pm$ 0.4 <sup>cdA</sup>	3.1 $\pm$ 0.2 <sup>cA</sup>	2.5 $\pm$ 0.4 <sup>dA</sup>	2.5 $\pm$ 0.5 <sup>dA</sup>
4	4.3 $\pm$ 0.2 <sup>aA</sup>	4.1 $\pm$ 0.2 <sup>abAB</sup>	4.0 $\pm$ 0.2 <sup>abA</sup>	3.6 $\pm$ 0.2 <sup>abcA</sup>	3.5 $\pm$ 0.2 <sup>bcdA</sup>	3.2 $\pm$ 0.1 <sup>cdeA</sup>	2.7 $\pm$ 0.3 <sup>eA</sup>	2.5 $\pm$ 0.2 <sup>eAB</sup>	2.9 $\pm$ 0.5 <sup>deA</sup>	2.8 $\pm$ 0.8 <sup>deA</sup>
10	4.3 $\pm$ 0.2 <sup>aA</sup>	3.9 $\pm$ 0.1 <sup>abcB</sup>	4.0 $\pm$ 0.1 <sup>abA</sup>	3.5 $\pm$ 0.3 <sup>abcA</sup>	2.9 $\pm$ 0.1 <sup>cB</sup>	2.9 $\pm$ 0.5 <sup>cA</sup>	3.1 $\pm$ 0.4 <sup>bcA</sup>	3.2 $\pm$ 0.1 <sup>bcA</sup>	3.1 $\pm$ 0.3 <sup>bcA</sup>	2.9 $\pm$ 0.6 <sup>cA</sup>
21	4.3 $\pm$ 0.2 <sup>aA</sup>	4.2 $\pm$ 0.2 <sup>aA</sup>	4.0 $\pm$ 0.3 <sup>abA</sup>	3.5 $\pm$ 0.1 <sup>abA</sup>	3.5 $\pm$ 0.5 <sup>abA</sup>	3.2 $\pm$ 0.5 <sup>abcA</sup>	2.6 $\pm$ 1.0 <sup>bcA</sup>	2.0 $\pm$ 0.6 <sup>CB</sup>	3.0 $\pm$ 1.4 <sup>abcA</sup>	2.7 $\pm$ 0.8 <sup>bcA</sup>
<i>L. monocytogenes</i>										
1.5	4.5 $\pm$ 0.1 <sup>aA</sup>	4.3 $\pm$ 0.5 <sup>aA</sup>	4.6 $\pm$ 0.2 <sup>aA</sup>	4.3 $\pm$ 0.3 <sup>aA</sup>	4.3 $\pm$ 0.2 <sup>aA</sup>	4.0 $\pm$ 0.8 <sup>abA</sup>	3.1 $\pm$ 1.3 <sup>abcA</sup>	2.5 $\pm$ 1.6 <sup>bcA</sup>	1.9 $\pm$ 1.1 <sup>cA</sup>	1.8 $\pm$ 0.8 <sup>cA</sup>
4	4.5 $\pm$ 0.1 <sup>aA</sup>	4.3 $\pm$ 0.5 <sup>abA</sup>	4.6 $\pm$ 0.1 <sup>aA</sup>	3.8 $\pm$ 0.8 <sup>abcA</sup>	3.9 $\pm$ 0.9 <sup>abcAB</sup>	3.5 $\pm$ 1.4 <sup>abcA</sup>	2.7 $\pm$ 1.1 <sup>bcA</sup>	2.6 $\pm$ 1.3 <sup>bcA</sup>	2.2 $\pm$ 1.2 <sup>cA</sup>	2.3 $\pm$ 0.9 <sup>cA</sup>
10	4.5 $\pm$ 0.1 <sup>aA</sup>	4.4 $\pm$ 0.5 <sup>aA</sup>	4.6 $\pm$ 0.5 <sup>aA</sup>	3.4 $\pm$ 1.2 <sup>abA</sup>	3.1 $\pm$ 1.2 <sup>abAB</sup>	3.0 $\pm$ 1.3 <sup>abA</sup>	3.7 $\pm$ 0.9 <sup>abA</sup>	2.2 $\pm$ 1.3 <sup>abA</sup>	2.4 $\pm$ 1.9 <sup>abA</sup>	1.8 $\pm$ 1.7 <sup>bA</sup>
21	4.5 $\pm$ 0.1 <sup>abA</sup>	4.3 $\pm$ 0.6 <sup>abcA</sup>	4.8 $\pm$ 0.5 <sup>aA</sup>	4.0 $\pm$ 0.3 <sup>abcA</sup>	2.3 $\pm$ 1.4 <sup>cdB</sup>	2.8 $\pm$ 1.0 <sup>bcdA</sup>	3.0 $\pm$ 1.7 <sup>abcdA</sup>	2.4 $\pm$ 0.1 <sup>cdA</sup>	1.8 $\pm$ 1.0 <sup>dA</sup>	1.5 $\pm$ 1.1 <sup>dA</sup>

<sup>1</sup>Means with different lowercase letters within a row are significantly different (p < 0.05). Means with different uppercase letters within a column for each organism are significantly different (p < 0.05).



**Table 4** Proximate composition analyses of five brands of commercial teewurst<sup>1</sup>.

	Teewurst A <sup>2</sup>	Teewurst B	Teewurst C	Teewurst D	Teewurst E
Phenolics(g/100g)	0.07 ± 0.00 <sup>ab3</sup>	0.07 ± 0.01 <sup>ab</sup>	0.09 ± 0.00 <sup>bc</sup>	0.05 ± 0.00 <sup>a</sup>	0.11 ± 0.01 <sup>c</sup>
Salt (g/100g)	1.26 ± 0.16 <sup>a</sup>	2.10 ± 0.22 <sup>b</sup>	2.12 ± 0.10 <sup>b</sup>	2.16 ± 0.00 <sup>b</sup>	2.34 ± 0.00 <sup>b</sup>
Nitrite (mcg/g)	<1.00 <sup>a</sup>	4.03 ± 0.07 <sup>b</sup>	1.19 ± 0.26 <sup>a</sup>	1.61 ± 0.24 <sup>a</sup>	<0.10 <sup>c</sup>
Moisture (g/100g)	44.35 ± 0.21 <sup>ab</sup>	40.65 ± 0.07 <sup>a</sup>	52.60 ± 0.99 <sup>c</sup>	51.90 ± 0.14 <sup>c</sup>	50.10 ± 3.11 <sup>bc</sup>
Protein (g/100g)	12.35 ± 0.64 <sup>a</sup>	12.95 ± 0.21 <sup>ab</sup>	15.60 ± 0.71 <sup>bc</sup>	15.00 ± 0.28 <sup>abc</sup>	16.20 ± 1.13 <sup>c</sup>
Fat (g/100g)	39.00 ± 0.42 <sup>a</sup>	41.60 ± 1.84 <sup>a</sup>	26.15 ± 0.21 <sup>b</sup>	25.85 ± 0.21 <sup>b</sup>	28.80 ± 0.57 <sup>b</sup>
Acidity <sup>4</sup> (%)	0.35 ± 0.06 <sup>a</sup>	0.40 ± 0.13 <sup>a</sup>	0.67 ± 0.11 <sup>ab</sup>	0.94 ± 0.04 <sup>b</sup>	0.65 ± 0.06 <sup>ab</sup>
CHO <sup>5</sup> (g/100g)	1.77 ± 0.11 <sup>a</sup>	1.27 ± 1.65 <sup>a</sup>	2.75 ± 0.15 <sup>a</sup>	4.17 ± 0.75 <sup>a</sup>	1.70 ± 2.40 <sup>a</sup>
Ash (g/100g)	2.54 ± 0.11 <sup>a</sup>	3.57 ± 0.00 <sup>b</sup>	2.91 ± 0.22 <sup>ac</sup>	3.08 ± 0.11 <sup>bc</sup>	3.38 ± 0.10 <sup>bc</sup>
pH	6.11 ± 0.01 <sup>a</sup>	NT <sup>6</sup>	NT	5.69 ± 0.04 <sup>b</sup>	6.09 ± 0.03 <sup>a</sup>
a <sub>w</sub>	0.956 ± 0.004 <sup>a</sup>	NT	NT	0.973 ± 0.001 <sup>b</sup>	0.967 ± 0.001 <sup>b</sup>

<sup>1</sup>Proximate analyses were performed on two samples from each processor (mean values ± standard deviation).

<sup>2</sup>Teewurst A (Ernst A. Illg Meats Inc.) product was utilized in all challenge experiments conducted in this study.

<sup>3</sup> Means with different letter within a row are significantly different (p < 0.05).

<sup>4</sup>Acidity titratable as acetic acid.

<sup>5</sup>CHO; carbohydrates.

<sup>6</sup>NT; not tested.

**Table 5** Labeling information from 5 brands of commercial teewurst<sup>a</sup>

Ingredients/Other information	Teewurst A <sup>b</sup>	Teewurst B	Teewurst C	Teewurst D	Teewurst E
Pork	x	x	x	x	x
Beef	x	x	x		
Salt	x	x	x	x	x
Carbohydrates	x		x		x
Spices	x	x		x	x
Paprika	x	x	x	x	x
Oleoresin of paprika				x	
Rum		x		x	
Smoke flavor/natural smoke	x			x	x
Flavoring			x		x
Sodium nitrite	x	x	x	x	x
Sodium erythorbate			x	x	x
Sodium acetate			x		
“Uncooked product”	x	x	x		x
“Cook thoroughly”	x				x
“Keep refrigerated”	x		x	x	x

<sup>a</sup>According to the labeling information declared from processor.

<sup>b</sup>Teewurst A (Ernst A. Illg Meats Inc.) product was utilized in all c

## “Growth of *Salmonella* Enteritidis and *Salmonella* Typhimurium in the presence of quorum sensing signalling compounds produced by spoilage and pathogenic bacteria”

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Key words: *Salmonella enterica*; quorum sensing; acylated homoserine lactones; autoinducer-2; conductance.

Running title: *Salmonella* senses other quorated bacteria.

### ABSTRACT

The effect of acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2) signalling molecules produced by *Pseudomonas aeruginosa* 108928, *Yersinia enterocolitica*-like GTE 112, *Serratia proteamaculans* 00612, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844 on the growth kinetic parameters (i.e. lag phase and growth rate) of two *Salmonella* Enteritidis and *S. Typhimurium* strains, respectively, has been assessed by using conductance measurements. AHLs and AI-2 in the cell-free supernatants (CFS) of these microorganisms were assayed using different bacterial biosensors and/or thin layer chromatography (TLC). Except from *P. aeruginosa*108928, which was not found to produce AI-2, all other strains produced both AHLs and AI-2. Thereafter, aliquots (20% in the final volume) of these CFS were transferred in NZ Amine broth inoculated with 10<sup>3</sup>-10<sup>4</sup> CFU/ml of 18h cultures of *S. Enteritidis* and *S. Typhimurium* strains, respectively. Changes in conductance of the medium were monitored and detection time (Tdet) was recorded. While *P. aeruginosa* 108928 induced a shorter detection time, i.e. start of the metabolic activity, the other microorganisms enlarged the detection time of *Salmonella* serotypes compared to control samples. Results suggest that the growth of *Salmonella* may be affected by the presence of QS signalling compounds produced by other bacterial species and confirm the complexity of bacterial communication.

### INTRODUCTION

The role of cell–cell communication in food ecological niches has recently received an attention from food microbiologists and a growing body of evidence is suggesting that bacterial food spoilage and poisoning could be regulated by QS (for review see Smith *et al.*, 2004; Ammor *et al.*, 2008).

*Salmonella* is one of the most prevalent pathogens associated with foodborne illness, worldwide. It is the second in the list of human zoonotic diseases across the EU (EFSA-ECDC, 2007), while in the USA, is a leading cause of foodborne illness resulting in an estimated 1.4 million infections, with more than 16,000 hospitalizations and nearly 600 deaths each year (Lynch *et al.*, 2006). *Salmonella* infections can cause diarrhoea, fever, vomiting, and abdominal cramps. *Salmonella enterica* serotype Typhimurium and *Salmonella enterica* serotype Enteritidis are among the serotypes most commonly associated with human salmonellosis.

Studies with Gram-negative bacteria (e.g. *Y. enterocolitica*, *P. aeruginosa*, *S. proteamaculans*), have been shown to synthesize and recognize various types of AHLs by QS circuits composed of LuxI and LuxR homologs (Pearson et al., 1994, 1995; Trhoup et al., 1995, Gram et al., 1999; Miller and Bassler, 2001; Christensen et al., 2003; Bruhn et al., 2004, Atkinson et al., 2006). With the exception of *P. aeruginosa*, the above mentioned strains were shown to produce AI-2. In fact while *Pseudomonas* spp. lack *luxS* and thus do not produce AI-2 (Winzer et al., 2002), *Yersinia* and *Serratia* species possess a *luxS* gene and synthesize the AI-2 signal (Schauder and Bassler, 2001; Van Houdt et al., 2007).

*Salmonella* do not possess a *luxI* gene that codes for AHL synthetase and thus do not produce AHLs; however, this organism does have a LuxR homolog, known as *SdiA*, that enables detecting signals produced by other bacteria (Michael et al., 2001; Smith and Ahmer, 2003). In addition, *Salmonella* use two other QS systems, the *luxS*/AI-2 (Taga et al., 2001; Taga et al., 2003) and the AI-3/epinephrine/norepinephrine (Walters et al., 2006) to achieve intercellular signaling. The latter is also involved in interkingdom communication. These three QS systems have been shown to modulate the physiology, colonization and virulence of *Salmonella* (for review see (Walters and Sperandio, 2006; Kendall and Sperandio, 2007; Hughes and Sperandio, 2008)).

The increased awareness of the role of cell-to-cell communication in the ecology of *Salmonella* has to be matched by an understanding of both the physiology and the molecular biology that underlie this process. So far, the majority of studies have focused on the molecular aspects of this phenomenon and much less attention has been paid to the physiological aspects (i.e., expression of metabolism and kinetic characteristics such as lag phase and growth rate).

Thus, the aim of this study was to investigate the effect of QS signalling compounds produced by different microorganisms potentially associated with food poisoning on the growth kinetics parameters of two *S. Enteritidis* and two *S. Typhimurium* strains. *Salmonella* growth was assessed by conductance measurements, a well established methodology which allows monitoring of bacterial activity and kinetic characteristics (Firstenberg-Eden and Eden, 1984; Silley and Forsythe, 1996).

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *Pseudomonas aeruginosa* 108928, *Serratia proteamaculans* 00612, *Yersinia enterocolitica* CITY650, *Y. enterocolitica* CITY844 and *Yersinia enterocolitica*-like GTE 112, were propagated and subcultured in brain heart infusion (BHI; LAB M, Lancashire, UK) broth following incubation at 28°C (*Yersinia enterocolitica*-like GTE 112, *S. proteamaculans* 00612) or 37°C (*P. aeruginosa* 108928, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844) for 24 h prior to use. The identity of these strains was confirmed using sequencing of a 640-bp internal fragment of the 16S rRNA gene.

For the AI-1 bioassays, the AHL reporter strain *Agrobacterium tumefaciens* A136 (pCF218, pCF372) and the AHL producing strain *A. tumefaciens* KYC6 (pCF28; a 3-oxo-C8 HSL overproducer) (Fuqua and Winans, 1996), kindly donated by Prof. Robert McLean (Department of Biology, Texas State University), were activated and subcultured in luria bertani (LB; Bertani, 1951) medium (supplemented with 4.5 µg/mL tetracycline and 50 µg/mL spectinomycin for *A. tumefaciens* A136) at 28°C for 28 h with agitation (160 rpm). *Hafnia alvei* 718 (a 3-oxo-C6 HSL producing strain) (Bruhn et al., 2004), kindly donated by Dr. L. Gram (Danish Institute for Fisheries Research, DK) was activated and subcultured in BHI broth following incubation at 37°C for 24 h.

The reporter strain *Vibrio harveyi* BAA-1117<sup>TM</sup> (BB170 *luxN*::Tn5, sensor 1<sup>-</sup>, sensor 2<sup>+</sup>), and the AI-2-producing strain *V. harveyi* BAA-1119<sup>TM</sup> (BB152 *luxL*::Tn5, autoinducer-1<sup>-</sup>, autoinducer-2<sup>+</sup>), purchased from the American Type Culture Collection (LGC Promochem),

were used for the AI-2 bioassays (Surette and Bassler 1998). These strains were grown in the autoinducer bioassay (AB) medium at 28°C for 24 h with agitation (160 rpm). AB medium was prepared according to Greenberg et al. (1979). The basal medium contained NaCl (17.5 g/l; Merck), MgSO<sub>4</sub> (12.3 g/l; Merck) and casamino acids (2 g/l; Sigma). After adjusting the pH to pH 7 with KOH, the solution was sterilised by autoclaving (15 min, 121°C, 15 psi). After cooling, the following sterile compounds were added: 1 M potassium phosphate (pH 7.0; 10 ml/l), 50 % glycerol (20 ml/l; Carlo Erba), and filter-sterilized 0.1 M L-arginine (10 ml/l; Sigma).

For conductance experiments, *S. enterica* serovar Enteritidis strains PT4 and PT7 (kindly provided by School of Biomedical and Molecular Sciences, Surrey University, UK), and *S. enterica* serovar Typhimurium strains DT193 (maintained in Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, GR) and DSM554 (kindly provided by Dr E. Smid, ATO-DLO, The Netherlands) were activated and subcultured in tryptone soy broth (TSB; LAB M) following incubation at 37°C for 24 and 18 h, respectively.

**Cell-free Supernatant (CFS) preparation.** *P. aeruginosa* 108928, *S. proteamaculans* 00612, *Yersinia enterocolitica*-like GTE 112, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844, herein after called tester strains, were grown (ca. 10<sup>8</sup>-10<sup>9</sup> CFU/ml) in 30 ml of BHI broth under conditions previously described. Cultures (25 ml) were then centrifuged at 8000 rpm for 15 min at 4°C and filtered-sterilized through a 0.22 µm pore-size cellulose acetate filter (Millipore) to obtain CFS. In the case of *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844 a mixture (1:1, v/v) of both CFS was prepared (equal populations) and utilized (unless otherwise stated) in all experiments of this study). The remaining 5 ml from each culture were used to enumerate bacterial cells population. CFS were immediately screened for the presence of QS signalling molecules as essentially described below. In parallel the pH of the CFS was recorded (Metrohm 691 pH meter).

**Screening for AHL signalling molecules.** *A. tumefaciens* A136 reporter strain was used for the screening of AI-1 like signalling molecules in CFS using a well diffusion assay. Briefly, 1 ml of the culture was inoculated into 50 ml of melted ABT agar (1.5 % agar; ABT per liter: 0.4 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6 g Na<sub>2</sub>HPO<sub>4</sub>, 0.3 g KH<sub>2</sub>PO<sub>4</sub>, 0.3 g NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 0.01 mM FeCl<sub>3</sub>, 2.5 mg thiamine supplemented with 0.5% glucose and 0.5% casamino acids) supplemented with the relevant antibiotics and 50 µg/mL X-Gal (AppliChem GmbH, Darmstadt, Germany) which was then immediately poured into 5.0 cm diameter Petri dishes. A portion (150 µl) of the tester strain CFS or the tester strain 20% (80% BHI) CFS (i.e. amount used in the conductance experiments) was pipetted into wells (diameter 6.0mm) punched in the solidified agar using a sterile Pasteur pipette. The plates were incubated at 28°C for 48 h. CFS of the AHL-producing strain *A. tumefaciens* KYC6 (donor) and the reporter strain were used as positive and negative controls, respectively, in the above assay.

The development of blue colour (catabolism of X-Gal) in the plates indicated presence of AI-1 like substances. The blue coloured area and the total plate area were measured using the Image J software (Wayne Rasband, NIH, Bethesda, Maryland, USA). The ratio of the coloration area per plate area was used to estimate the amount of AHL production as compared to the donor strain *A. tumefaciens* KYC6. Two independent assays were performed.

**Thin-layer chromatography (TLC).** Extracts for TLC were prepared from 5 ml cultures of the tester strains, *Hafnia alvei* 718 and *A. tumefaciens* KYC6 grown as essentially described above. Bacteria were removed by centrifugation and the supernatants were extracted twice with equal volumes of ethyl acetate acidified with 0.1 % acetic acid. The combined extracts were dried, filtered, and evaporated to dryness. Residues from the cultures were dissolved in 100 µl of HPLC-grade ethyl acetate.

Synthetic N-hexanoyl-DL homoserine lactone (C6-HSL; Fluka, Biochemica) or extracts dissolved in HPLC-grade ethyl acetate were applied (0.5-80 µl) onto C18 reversed-phase TLC

plate (Merck) and the chromatograph was developed with methanol/water (60:40 v/v) until the elution top reached the top of the plate (approximately 3 h). After development, the TLC plate was allowed to air dry. A 50 ml of a late exponential phase culture of *A. tumefaciens* A136 reporter strain was then used to inoculate 100 ml of melted (ca. 46°C) ABT agar supplemented with 150 µl X-Gal. After gentle mixing, the culture was poured over the surface of the developed plate and the agar was left to solidify. The TLC-plate was incubated overnight at 28°C in a sterile metallic container. AHLs were visualised as blue spots on the TLC chromatographs.

**Screening for AI-2 signalling molecules.** The *luxCDABE*-encoded luminescence response of the reporter strain *V. harveyi* BB170 was used as the basis for determining AI-2 activity in CFS of tester strains. The assay was performed as follows. An overnight culture of *V. harveyi* BB170 was diluted 4-folds with ¼ strength Ringer's solution containing 1.5% NaCl. This cell suspension was then centrifuged for 10 min (10,000 x g at 4°C) and the supernatant was discarded. The pellet (10<sup>4</sup> CFU) was then resuspended in 0.8 ml of AB medium and mixed with 0.2 ml of the tested CFS. Each solution mixture served to load (200 µl) 5 different wells in a 96-well polystyrene microplate (µClear; Greiner Bio-One). The CFS (0.2ml) of *V. harveyi* BB152 strain, prepared exactly as described above for the tester strains, was used as the positive control and AB medium (0.2 ml) was used as the negative control. The microplates were incubated at 28°C and luminescence was measured every 20 min by a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT 05404, USA) until the negative control exhibited an increase in luminescence (7 h).

AI-2 activity was calculated as the ratio of luminescence of the test sample to the negative control (AB medium) sample. The induction of light production by the addition of *V. harveyi* BB152 CFS to *V. harveyi* reporter strain BB170 was normalized to 100% activity. Results of duplicate assays were considered as positive when light production was more than 10% of the *V. harveyi* BB152 stimulation (Bassler et al., 1997).

**Monitoring the effect of the CFS on *Salmonella* strains growth.** *Salmonella* strains growth was indirectly monitored by conductance measurements using the Malthus 2000 instrument (Radiometer International, Copenhagen, Denmark). Conductance measurements were performed by dispensing 3.5 ml from a stock solution of NZ Amine broth (NZA) (containing per litre: 20 g NZ amine A (Sheffield Chemical Co. Norwich, N.Y, USA) and 20 g proteose peptone No.3 (Difco Laboratories, Detroit, MI)) supplemented with 20 % CFS of each tester strain or with 20 % BHI broth (control) and inoculated with the *Salmonella* (10<sup>3</sup>-10<sup>4</sup> CFU/ml) into each of 12 sterile reaction Malthus tubes. The pH of BHI broth used was adjusted to the same value recorded on the corresponding CFS. Three other non-inoculated with *Salmonella* tubes (3.5 ml NZA) were used as an additional control.

Malthus tubes were then incubated into the Malthus apparatus for 24 h at 37°C while the analyzer was adjusted to measure conductivity changes every 6 min. Malthus system detects changes in conductance caused by bacterial metabolism in the growth medium in the reaction tubes which contain platinum electrodes (Firstenberg-Eden and Eden, 1984). Conductance changes are expressed in microsiemens (µS).

The Detection Time (T<sub>det</sub>; h) signal appears when three consecutive measurements exceed the minimum threshold of detection criteria (5 µS), and is defined as the time interval between the start of conductance monitoring and the beginning of the acceleration phase of the signal (Firstenberg-Eden and Eden, 1984; Silley and Forsythe, 1996). It is apparent that T<sub>det</sub> depends on the bacterial population into the Malthus tubes (log CFU/ml), the growth kinetics of microorganism and the properties of the test medium.

**Data analysis.** Growth data of *Salmonella* strains (values of  $\mu\text{S}$  for 24 h incubation) in NZA, were fitted to the model of Baranyi and Roberts (1994). The Baranyi model (Baranyi and Roberts, 1994) is based on four parameters. A parameter expressing the lag phase (LP), (presented as detection time (DT));  $\mu$ , the growth rate (presented as the maximum slope (rate) in conductance curve (MSrCC));  $y_0$  representing the initial bacterial counts (presented as initial value of  $\mu\text{S}$ ); and  $y_{\text{end}}$  representing the final conductance values (presented as final value of  $\mu\text{S}$ ). For curve fitting, the in-house programme DMFit (Institute of Food Research, Norwich, United Kingdom) was used. The area under the conductance/time curves was calculated using the trapezoidal rule. Thus, the effect of CFS on microbial growth was also manifested by a reduction or increase in the area under the conductance/time curve of the tests relative to the control at any specified time (Lambert and Pearson, 2000; Chorianopoulos et al., 2006). For the estimation of the effect of CFS on the growth of *Salmonella* strains two independent trials were performed.

## RESULTS

**Screening for QS signalling molecules.** AHL production was screened using the *A. tumefaciens* A136 well diffusion assay and image analysis of the plates allowed to estimate the amounts of AHLs produced as compared to the *A. tumefaciens* KYC6 strain (donor). Figure 1 and Table 1 summarize obtained results. It was shown that all tester strains produced AHLs, with *Yersinia enterocolitica*-like GTE 112 being the most producing, followed by *S. proteamaculans* 00612, *P. aeruginosa* 108928, and *Y. enterocolitica* CITY650 and CITY844. The presence of AHL compounds was additionally confirmed performing TLC (Figure 2). The TLC results indicated the presence of at least one type of AHL in the CFS of the tester strains visualized using *A. tumefaciens* A136.

AI-2-like activity was assessed using the *V. harveyi* bioluminescence assay. With the exception of *P. aeruginosa*, all strains displayed an AI-2 activity. *Y. enterocolitica*- like GTE112 stimulated the highest light production in the reporter strain *V. harveyi* BB170 (56%) followed by *S. proteamaculans* 00612 (26%) (Table 2).

**Salmonella kinetics with and without tester strains CFS.** *Salmonella* strains were inoculated (ca.  $10^3 - 10^4$  CFU/ml) into NZA supplemented with 20% of the tester strain CFS or 20% of sterile BHI broth (control). The pH of BHI was adjusted to the same value recorded on the corresponding CFS with the purpose to rule out the influence of pH on the observed results.

Conductance changes followed a sigmoid curve typical of microbial growth, and thus, the calculation of the lag phases and growth rates of *Salmonella* strains was allowed. The putative effect of signalling compounds present in CFS on  $T_{\text{det}}$  provided by the instrument, on LP and MSrCC calculated using the Baranyi and Roberts model (1994) as well as on the area of the conductance/time curves is shown in Tables 3-6. Tubes containing only BHI broth (without addition of CFS and non-inoculated with *Salmonella*) did not exhibit any change in conductance during incubation, suggesting the absence of any metabolic activity (data not shown).

In comparison to control samples, tester strains CFS proved to influence ( $p < 0.05$ ) the growth parameters of all *Salmonella* strains but with different trends. Specifically, the addition of CFS of *Yersinia enterocolitica*-like GTE 112 or *S. proteamaculans* 00612 into NZA broth inoculated with each *Salmonella* strain reduced the MSrCC and enlarged the LP of *S. Enteritidis* PT4, *S. Enteritidis* PT7, *S. Typhimurium* DSM554 and *S. Typhimurium* DT193, respectively (Tables 3 and 4). As expected,  $T_{\text{det}}$  obtained from the instrument was influenced accordingly with LP. The area of the conductance/time curves of the tubes containing CFS was found to be lower than that of the control samples. Similar differences were observed when the CFS of *Y. enterocolitica* CITY650 and CITY844 was used with the exception of MSrCC of both *S. Enteritidis* strains which did not present any significant difference compared to control samples (Table 5).

When the CFS of *P. aeruginosa* 108928 was used, however, the values of MSrCC and area of the conductance/time curves appeared to be significantly higher than in control samples for all *Salmonella* strains. In contrast, the T<sub>det</sub> and in most cases the LP of *Salmonella* was lower when the Malthus tubes were supplemented with the CFS (Table 6).

## DISCUSSION

During the last few years, a tremendous research effort has been made to increase our understanding on the genetic processes that regulate the synthesis, release and detection of diffusible signal molecules employed on bacterial communication. However, much less consideration has been given on understanding the ecological context inherent to such communication, i.e. why bacteria produce signalling molecules and what is their role in conflicts or collaboration within or between species in natural and/or engineered environments (Keller and Surette, 2006).

In this study, we evaluated the growth characteristics of four *Salmonella* strains in the presence of QS signalling compounds produced by various Gram-negative pathogenic bacteria using a conductance-based method. The use of conductance (e.g. Malthus instrument) to obtain a better understanding of the microbial growth kinetics and activity in complex systems has been widely reported in the literature (Fistenberg-Eden and Eden, 1984; Tranter *et al.* 1993; Tassou and Nychas 1995; Silley and Forsythe, 1996; Skandamis *et al.* 2001; Giaouris *et al.* 2005; Chorianopoulos *et al.* 2006).

Indeed, the metabolic activity of a culture can be inferred from (i) the detection time (T<sub>det</sub>) provided by the impedimetric instrument and (ii) the kinetic parameters calculated indirectly from the conductance curves (Tassou and Nychas 1994; Koutsoumanis *et al.* 1998, 2002; Koutsoumanis and Nychas 2000; Chorianopoulos *et al.* 2008). In the first case, the T<sub>det</sub> corresponds to the period where appears to be no multiplication and is defined as the "lag phase". In this period, the organisms are metabolizing, but there is no cell division and the population remains relative 'constant' whilst individual cells adapt to their new environment. In the later case, the curve parameters such as slope, represented by the maximum slope of conductance changes (MSrCC) or area under the conductance/time curve are associated with biochemical (metabolic) changes taking place in the medium of the reaction tube of the impedimetric instrument. As microorganisms metabolize, uncharged or weakly charged substrates (proteins and carbohydrates) are converted into highly charged end-products (amino acids, lactate etc), thus increasing the conductivity of the growth medium. Thus, the greater the activity of the culture, the steeper is the slope or the greater is the area.

Under the conditions of this study, the growth and/or microbial activity parameters (lag phase, MSrCC and area) as well as the parameter provided from the instrument (T<sub>det</sub>) of both *Salmonella* serotype were found to be significantly affected in the presence of QS signals extracted from different pathogenic bacteria (Tables 3-6). It should be noted that production of AI-2 signals by *Salmonella* and potential interference with AHLs and/or AI-2 of the tester strains was ruled out by inoculation of *Salmonella* in a free of glucose medium (i.e. NZA broth) the presence of which has been reported to be necessary for the production of the signaling factor (Surette and Bassler, 1998).

In all cases, the area of the conductance/time curve, which represents the influence of the QS signalling compounds on both initiation of growth and metabolic activity, was



significantly affected in their presence. However, it needs to be noted that this parameter was always significant lower in samples supplemented with CFS produced from *Y. enterocolitica*-like GTE112, *S. proteamaculans* 00612 and mixture CFS from *Y. enterocolitica* CITY650 and CITY844, while the CFS from *Pseudomonas aeruginosa* 108928 influenced this characteristic with opposite way (significant higher). The other parameters (e.g.  $T_{det}$ , MSrCC, and LP) examined were also significantly affected but this effect varied and was depended on the *Salmonella* strain and/or the QS signalling molecule(s) in the CFS of the tester strains

The presence of at least one type of AHLs and AI-2 signalling molecules in the CFS was confirmed using different bacterial bioassays (Ravn *et al.*, 2001; Surette and Bassler, 1998) and/or TLC analysis (Shaw *et al.*, 1997). These findings are in agreement with other studies as *Y. enterocolitica*, *P. aeruginosa*, and *S. proteamaculans* have been shown to synthesize and recognize various types of AHLs by QS circuits composed of LuxI and LuxR homologs (Pearson *et al.*, 1994; Pearson *et al.*, 1995; Throup *et al.*, 1995; Gram *et al.*, 1999; Atkinson *et al.*, 2006). With the exception of *P. aeruginosa*, all other tester strains were shown to produce AI-2. In fact, while *Pseudomonas* spp. lack *luxS* and thus do not produce AI-2 (Winzer *et al.*, 2002), *Yersinia* and *Serratia* species possess a *luxS* gene and synthesize the AI-2 signal (Schauder and Bassler, 2001; Van Houdt *et al.*, 2007).

Similar variations in the rate of conductance changes and  $T_{det}$  have been reported when synthetic AHLs or cell-free culture fluids of microorganisms and cell-free meat extracts, containing AI-1 and AI-2 signals, have been used to evaluate the synergistic or competitive effect of these signals on the evolution of other pathogenic or spoilage bacteria (Whan *et al.* 2003; Dunstall *et al.* 2005; Zhao *et al.* 2006; Nychas *et al.* 2009). Such variations could be possibly explained by the different sources of QS signals and/or the different bacterial strains assayed.

In addition to the function of QS signalling compounds to communicate cell density, these compounds have also been proposed to act as proxies that provide individual cells with information on the diffusion and flow properties of their environment preventing the wasteful synthesis of “expensive” extracellular substances, such as exoenzymes, bacteriocins, siderophores and other effectors (Redfield, 2002; Keller and Surette, 2006; Hense *et al.*, 2007). Provided that they remain in the cells immediate environment, these metabolites, increase nutrient availability and ultimately benefit the fitness of their producers (Redfield, 2002). This concept could explain the results of this study as with the addition of the CFS in the reaction tubes the QS signalling compounds and/or other novel signals produced by the tester strains were rapidly mixed and diffused into the pathogens’ microenvironment altering *Salmonella* activity possibly through an over- or under- production of necessary for its growth requirements (e.g. enzymes, metabolites etc).

Findings reported in this study suggest that (i) the growth kinetic parameters as well as the microbial activity of four *Salmonella* strains were affected by the addition of CFS produced by other pathogenic bacteria and (ii) there was not a similar response in all bacterial strains tested. This response seems to be mostly affected by the type of QS compound. Further investigations are required in order to discriminate the effect of each type of signalling molecule on the growth kinetics parameters and more specifically on the growth determinants of *Salmonella* (i.e. target genes and phenotypes). Such

approach could potentially lead to the exploitation of these autoinducers as novel antimicrobial agents and compounds to control microbial growth, survival and virulence in foods.

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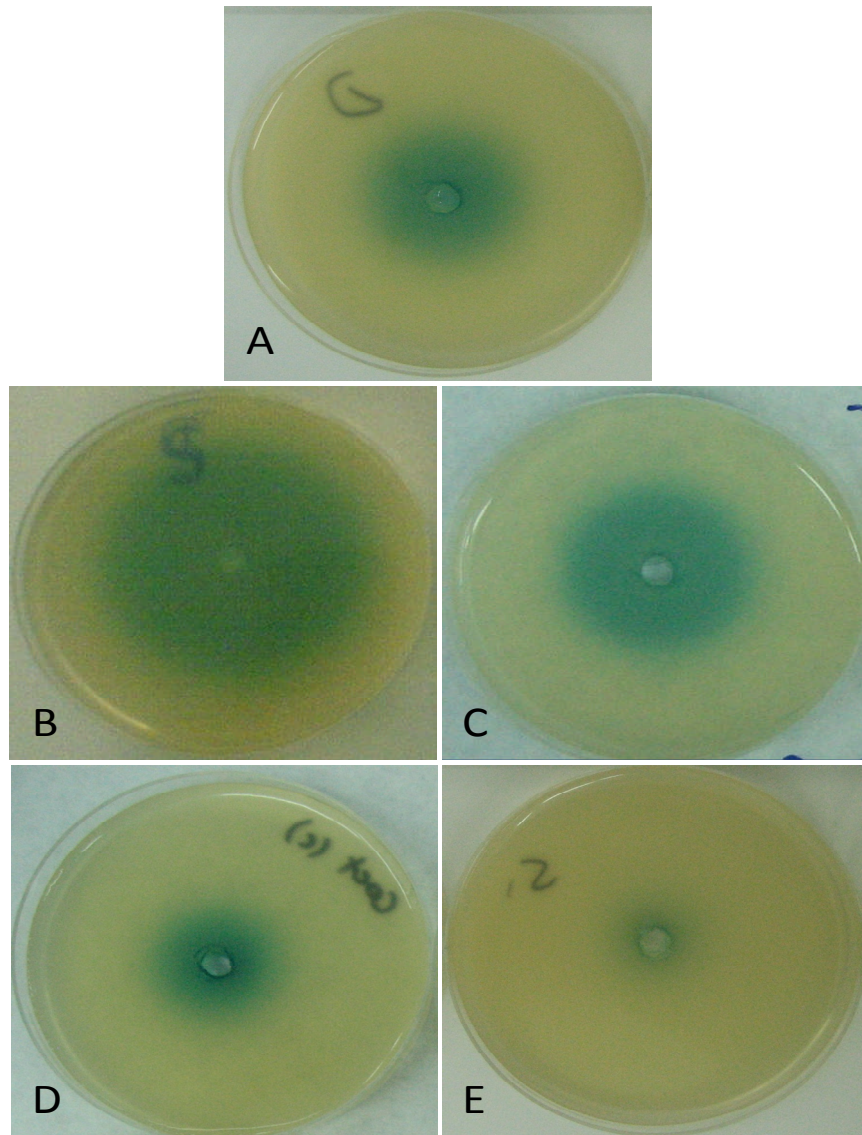
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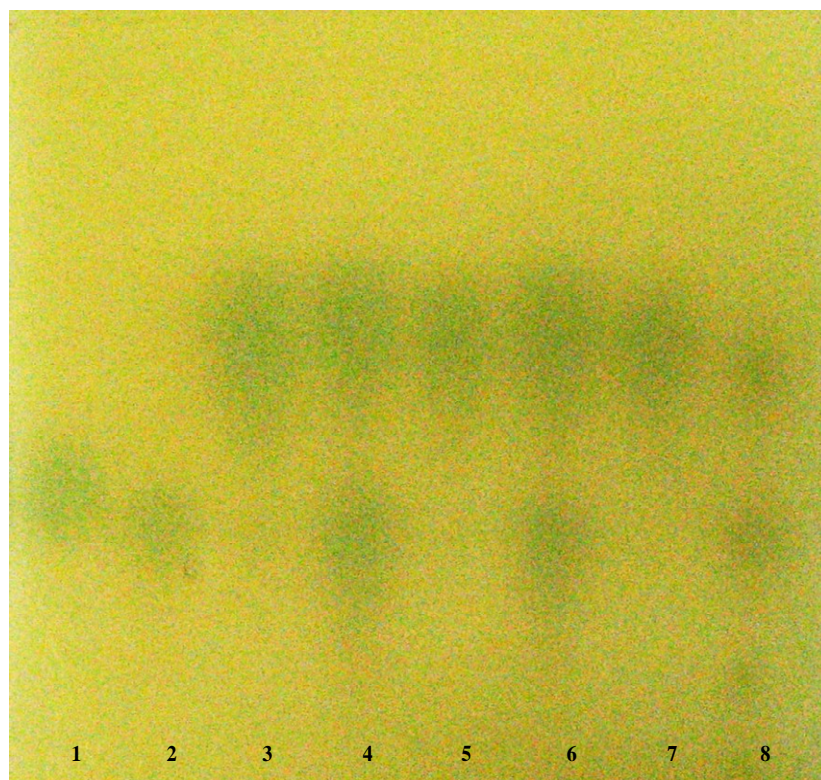
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**Figure 1** Presence of acylated homoserine lactones (AHLs) in the cell-free supernatants (CFS) of *A. tumefaciens* KYC6 (donor) (A), *Yersinia enterocolitica*-like GTE 112 (B), *S. proteamaculans* 00612 (C), *Y. enterocolitica* CITY650 and CITY844\* (D) and *P. aeruginosa* 108928 (E). Cell-free supernatants (CFS) of the tester strains were added to wells in agar containing *A. tumefaciens* A136. Zones of AHL(s)-induced blue colour production are seen surrounding the wells. \* Mixture (1:1, v/v) of CFS of the two *Y. enterocolitica* strains.



**Figure 2** Thin layer chromatogram profiles of the AHLs present in the cell-free supernatants (CFS) of the tester strains used herein in this study. Samples were chromatographed on c18 reversed-phase TLC plates, developed with methanol/water (60:40, v/v) and the spots were visualised by *A. tumefaciens* A136 reporter strain. Lanes: (1) synthetic C6-AHL; (2) *A. tumefaciens* KYC6 (donor); (3) *H. alvei* 718; (4) *Yersinia enterocolitica*-like GTE 112; (5) *S. proteamaculans* 00612; (6) *Y. enterocolitica* CITY650; (7) *Y. enterocolitica* CITY844; (8) *Ps. Aeruginosa* 108928.



**Table 1** Autoinducer-1 activity of tester strains cell-free supernatant (CFS) in the well diffusion assay using the *A. tumefaciens* A136 reporter strain

Tester strain	Assay	AI-1 activity (%)	
		CFS	20% CFS <sup>a</sup>
<i>A. tumefaciens</i> KYC6	1	100 <sup>b</sup>	nt <sup>c</sup>
	2	100	nt
<i>Y. enterocolitica</i> - like GTE112	1	159	108
	2	154	118
<i>P. aeruginosa</i> 108928	1	47	18
	2	37	23
<i>S. proteamaculans</i> 00612	1	92	50
	2	98	47
<i>Y. enterocolitica</i> CITY650 and	1	94	39
	2	nt	nt

<sup>a</sup> 20% CFS; the concentration of CFS used in conductance experiments.

<sup>b</sup> Results are displayed as the mean value (n=2) of the ratio of induction zone (area) of CFS of the tester strain to the induction zone (area) of the AHL producing *A. tumefaciens* KYC6.

<sup>c</sup> nt; not tested.

<sup>d</sup> Mixture (1:1, v/v) of the CFS of the two *Y. enterocolitica* strains.

**Table 2** Induction of luminescence in *V. harveyi* BB170 reporter strain by cell-free supernatants (CFS) from *V. harveyi* BB152 (positive control) and tester strains

Tester strain	Induction of luminescence (%)
<i>V. harveyi</i> BB152	100*
<i>Yersinia enterocolitica</i> -like GTE 112	56
<i>P. aeruginosa</i> 108928	4
<i>S. proteamaculans</i> 00612	26
<i>Y. enterocolitica</i> CITY650	12
<i>Y. enterocolitica</i> CITY844	13

\* AI-2 activity was calculated as the ratio of luminescence of each CFS to the negative control (AB medium). The induction of luminescence (%) by the addition of *V. harveyi* BB152 CFS to *V. harveyi* reporter strain BB170 was normalised to 100% activity. Results (mean values of two independent assays) were considered as positive when light production was more than 10% of the *V. harveyi* BB152 stimulation.

**Table 3** Effect of addition of 20% in the final volume of cell-free supernatant (CFS) of *Yersinia enterocolitica*-like GTE 112, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	8.85 ± 0.37a	7.64 ± 0.46b	9.50 ± 0.34a	8.25 ± 0.14b
	LP	699.36 ± 21.72a	613.39 ± 18.90b	844.79 ± 19.97a	663.30 ± 19.96b
	MSrCC	2.10 ± 0.23a	2.02 ± 0.21a	2.13 ± 0.19a	2.69 ± 0.16b
	Area	13907 ± 3064a	21422 ± 3704b	14557 ± 2138a	30175 ± 642b
<i>S. Enteritidis</i> PT7	Tdet	11.89 ± 0.48a	11.19 ± 0.18b	10.69 ± 0.72a	9.78 ± 0.32b
	LP	938.64 ± 24.99a	859.84 ± 24.99b	1001.15 ± 0.22a	818.34 ± 66.41b
	MSrCC	1.23 ± 0.20a	1.50 ± 0.20b	1.72 ± 0.22a	2.09 ± 0.18b
	Area	7703 ± 1977a	13219 ± 3108b	10943 ± 2975a	23562 ± 3183b
<i>S. Typhimurium</i> DSM554	Tdet	9.74 ± 0.23a	9.28 ± 0.04b	9.78 ± 0.34a	8.41 ± 0.32b
	LP	799.23 ± 14.58a	743.80 ± 18.34b	805.04 ± 20.66a	681.43 ± 26.93b
	MSrCC	2.21 ± 0.29a	2.45 ± 0.22b	2.35 ± 0.37a	2.84 ± 0.31b
	Area	13154 ± 3463a	23542 ± 2374b	15793 ± 2023a	28548 ± 2371b
<i>S. Typhimurium</i> DT193	Tdet	9.43 ± 0.20a	8.77 ± 0.60b	8.21 ± 0.65a	7.63 ± 0.46b
	LP	748.54 ± 8.91a	699.23 ± 9.68b	740.76 ± 23.41a	666.14 ± 13.20b
	MSrCC	2.13 ± 0.31a	2.57 ± 0.53b	2.38 ± 0.19a	2.84 ± 0.47b
	Area	5462 ± 856a	7807 ± 2337b	5346 ± 911a	14909 ± 2637b

\* Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test, p<0.05).

**Table 4** Effect of addition of 20% in the final volume of cell-free supernatant (CFS) of *S. proteamaculans* 00612, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	9.45 ± 0.27a**	9.31 ± 0.40a	9.62 ± 0.39a	9.09 ± 0.39b
	LP	789.70 ± 23.42a	714.45 ± 19.03b	792.10 ± 17.92a	695.50 ± 30.22b
	MSrCC	1.68 ± 0.34a	1.97 ± 0.24b	1.74 ± 0.19a	2.00 ± 0.26b
	Area	27757 ± 3356a	31850 ± 2865b	11801 ± 2657a	19796 ± 3521b
<i>S. Enteritidis</i> PT7	Tdet	13.20 ± 0.48a	12.34 ± 0.25b	12.47 ± 0.25a	11.08 ± 0.11b
	LP	1035.00 ± 20.53a	959.68 ± 24.93b	962.08 ± 16.21a	843.37 ± 27.39b
	MSrCC	1.00 ± 0.25a	1.61 ± 0.17b	1.26 ± 0.30a	1.59 ± 0.14b
	Area	32482 ± 3123a	45869 ± 4650b	6501 ± 1505a	21014 ± 2602b
<i>S. Typhimurium</i> DSM554	Tdet	9.89 ± 0.27a	9.62 ± 0.23b	10.01 ± 0.10a	9.53 ± 0.16b
	LP	793.15 ± 15.50a	680.60 ± 71.67b	799.04 ± 12.69a	769.56 ± 13.54b
	MSrCC	1.66 ± 0.19a	2.07 ± 0.23b	1.87 ± 0.36a	2.63 ± 0.25b
	Area	28925 ± 3401a	36807 ± 4863b	12554 ± 3045a	18004 ± 2109
<i>S. Typhimurium</i> DT193	Tdet	9.30 ± 0.24a	8.94 ± 0.40b	9.11 ± 0.16a	8.60 ± 0.35b
	LP	743.06 ± 11.61a	732.94 ± 6.17b	727.82 ± 12.53a	703.15 ± 19.88b
	MSrCC	1.83 ± 0.27a	2.61 ± 0.23b	1.76 ± 0.41a	2.73 ± 0.24b
	Area	27563 ± 2691a	31686 ± 2970b	9289 ± 3455a	16161 ± 3016b

\*Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test, p < 0.05).

**Table 5** Effect of addition of 20% in the final volume of cell-free supernatant (CFS)<sup>†</sup> of *Y. enterocolitica* CITY650 & CITY844, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	8.93 ± 0.63a	8.18 ± 0.68b	10.81 ± 0.14a	9.47 ± 0.09b
	LP	753.85 ± 12.56a	678.32 ± 45.92b	822.40 ± 18.70a	748.50 ± 26.57b
	MSrCC	1.83 ± 0.21a	1.56 ± 0.31a	2.05 ± 0.40a	2.22 ± 0.33a
	Area	14217 ± 1725a	19443 ± 6552b	15879 ± 2312a	25002 ± 4722b
<i>S. Enteritidis</i> PT7	Tdet	11.33 ± 0.47a	10.93 ± 0.28b	12.13 ± 0.39a	11.55 ± 0.19b
	LP	899.88 ± 15.54a	837.35 ± 33.44b	958.09 ± 23.34a	894.54 ± 16.23b
	MSrCC	1.85 ± 0.14a	1.74 ± 0.23a	1.39 ± 0.14a	1.50 ± 0.30a
	Area	18260 ± 2609a	29931 ± 4602b	15000 ± 1520a	22263 ± 3056b
<i>S. Typhimurium</i> DSM554	Tdet	9.57 ± 0.32a	9.11 ± 0.27b	10.22 ± 0.16a	9.34 ± 0.29b
	LP	780.99 ± 17.32a	709.41 ± 22.79b	817.97 ± 9.24a	754.02 ± 10.14b
	MSrCC	2.08 ± 0.15a	2.63 ± 0.20b	2.03 ± 0.30a	2.36 ± 0.30b
	Area	30889 ± 4441a	40207 ± 4432b	15917 ± 1912a	27289 ± 3674b
<i>S. Typhimurium</i> DT193	Tdet	8.73 ± 0.53a	8.03 ± 0.45b	9.33 ± 0.14a	8.80 ± 0.14b
	LP	715.37 ± 9.49a	657.85 ± 26.65b	594.19 ± 13.28a	560.85 ± 5.59b
	MSrCC	1.92 ± 0.36a	2.35 ± 0.20b	1.59 ± 0.17a	1.75 ± 0.04b
	Area	14898 ± 1433a	19066 ± 3337b	13017 ± 1415a	17933 ± 1691b

\*Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test,  $p < 0.05$ ). <sup>†</sup>Mixture (1:1, v/v) of CFS of the two *Y. enterocolitica* strains.

**Table 6** Effect of addition of 20% in the final volume of cell-free supernatant (CFS) of *P. aeruginosa* 108928, in the growth medium, on the kinetics of *S. Enteritidis* (strains PT4, PT7) and *S. Typhimurium* (strains DSM554, DT193)

Microorganism	Kinetic parameters*	Trial 1		Trial 2	
		CFS	Control	CFS	Control
<i>S. Enteritidis</i> PT4	Tdet	7.07 ± 0.05a**	7.30 ± 0.05b	6.34 ± 0.39a	6.88 ± 0.63b
	LP	599.86 ± 5.95a	613.01 ± 9.68b	473.79 ± 23.18a	494.72 ± 20.94b
	MSrCC	3.19 ± 0.23a	2.79 ± 0.361b	3.27 ± 0.36a	2.65 ± 0.41b
	Area	19532 ± 1991a	14579 ± 1240b	31578 ± 2285a	27279 ± 2674b
<i>S. Enteritidis</i> PT7	Tdet	7.72 ± 0.08a	7.88 ± 0.16b	7.97 ± 0.67a	8.56 ± 0.24b
	LP	627.62 ± 7.10a	637.79 ± 10.70a	728.22 ± 34.93a	725.02 ± 13.80a
	MSrCC	2.28 ± 0.23a	1.89 ± 0.27b	2.51 ± 0.27a	2.22 ± 0.33b
	Area	13723 ± 2485a	10910 ± 1837b	30206 ± 4806a	27443 ± 1864b
<i>S. Typhimurium</i> DSM554	Tdet	7.17 ± 0.08a	7.40 ± 0.05b	7.16 ± 0.45a	7.53 ± 0.05b
	LP	597.61 ± 7.37a	613.61 ± 12.14b	657.97 ± 14.79a	683.21 ± 24.05b
	MSrCC	3.13 ± 0.43a	2.76 ± 0.54a	3.66 ± 0.38a	3.24 ± 0.32b
	Area	18603 ± 2446a	14606 ± 2842b	30645 ± 3727a	26762 ± 1841b
<i>S. Typhimurium</i> DT193	Tdet	6.63 ± 0.05a	6.79 ± 0.07b	7.01 ± 0.39a	7.40 ± 0.17b
	LP	548.89 ± 6.53a	561.30 ± 6.25b	662.53 ± 40.76a	636.18 ± 18.98a
	MSrCC	3.47 ± 0.23a	3.22 ± 0.12b	3.52 ± 0.41a	3.15 ± 0.29b
	Area	20369 ± 1461a	18418 ± 1037b	27014 ± 2771a	24180 ± 1500b

\* Tdet: Detection Time (h) as provided from Malthus instrument; LP: Lag Phase (min) and MSrCC: Maximum Slope (rate) of Conductance Changes as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

\*\*Mean values with different letters (a, b) within rows for each experiment are significant different (t-test, p < 0.05)



