Challenge Testing of Microbiological Safety of Raw Milk Cheeses: The Challenge Trial Toolkit

MAF Technical Paper No: 2011/51

Prepared for the Ministry of Agriculture and Forestry
by Tom Ross, PhD
Food Safety Centre, University of Tasmania

ISBN No: 978-0-478-38437-6 (online)
ISSN No: 2230-2794 (online)

10 May 2011

Ministry of Agriculture and Forestry
Te Manatū Ahuwhenua, Ngāherehere

BIOSECURITY NEW ZEALAND

NEW ZEALAND FOOD SAFETY AUTHORITY

Disclaimer

The information in this publication is not government policy. While every effort has been made to ensure the information is accurate, the Ministry of Agriculture and Forestry does not accept any responsibility or liability for error of fact, omission, interpretation or opinion that may be present, nor for the consequences of any decisions based on this information. Any view or opinion expressed does not necessarily represent the view of the Ministry of Agriculture and Forestry.

Requests for further copies should be directed to:

Science, Information & Risk
Ministry of Agriculture and Forestry
P O Box 2526
WELLINGTON

Telephone: 0800 00 83 33

This publication is also available on MAF's Food Safety website at
http://www.foodsafety.govt.nz/science/research-projects/reports-projects/

Food Safety Centre

University of Tasmania

(UTas Ref: ROCU 2689)

Challenge Testing of Microbiological Safety of Raw Milk Cheeses

("Challenge Trial Tool Kit")

(Final Report: 10 May 2011)

prepared by

Tom Ross, PhD

TIAR is a joint venture between the University of Tasmania and the Tasmanian Department of Primary Industries, Parks, Water and Environment

This page left blank deliberately.

Summary

Safety of Raw Milk Cheeses

That raw milk can harbour pathogenic bacteria is well-documented, and cheeses made from raw milk are significantly *over*-represented in outbreaks reported for cheese products. This suggests that raw milk/raw milk products currently *are* a higher public health risk than analogous pasteurized milk products on a risk-per-serving basis.

Despite this, there is increasing consumer demand for raw milk cheese, *prima facie* for reasons of wholesomeness and sensory quality, and the perception of proponents of raw milk cheese that inherent antimicrobial properties of raw milk, and fermentation and maturation of cheese, can reliably eliminate pathogens potentially present in raw milk before consumption of the cheese.

New Zealand Food Safety Authority (NZFSA) has developed a regulatory framework to allow certain unpasteurised milk products that can be produced to an acceptable level of safety[1] to be produced, sold and imported into New Zealand and also exported. One of the means of demonstrating the microbiological safety of food products and processes is to conduct microbiological challenge trials.

Utility of Challenge Trials

Various expert groups that have considered the utility and methodology of challenge trials have concluded that challenge trials are a 'last resort' strategy for assessing the safety of specific food products and processes. Other approaches include consideration of the history of analogous products, consideration of the microbial ecology of cheese production and relevant pathogens, use of predictive microbiology models etc. Guidance on combinations of physicochemical factors in foods that preclude the growth of relevant pathogens is given. When these methods are inadequate a challenge trial may be warranted but, for such challenge trials to be reliable, there are many technical aspects that must be addressed and satisfied.

Well-designed challenge studies can provide critical information on the microbiological safety and stability of a food formulation. They are also invaluable in validating key lethality or microbiological control points in a process. NZFSA commissioned the current report to develop guidelines for the conduct of challenge trials relevant to cheese manufacture from raw milk. The intention is to assist in determination of the safety of raw milk cheeses. Recommendations of the study are summarised overleaf.

[1] An "acceptable level of safety" is one that poses a low level of risk to the general population.

Basic Principles of Challenge Trials

The most fundamental requirement of a challenge trial of a food/food process is that it accurately mimics the conditions that the challenge organisms would experience in the actual food during all stages of processing. A second fundamental requirement is that the variability in the process is also characterized so that the *range* of process efficacy can be determined and appropriate decisions made. Outside of Europe, including in New Zealand, raw milk cheeses are usually produced by small manufacturers and, accordingly, there is likely to be more variability in the process between batches. Accordingly, full characterization of the variability in microbiological efficacy of the cheese-making process will often be impractically expensive and very time consuming. A more common approach is to characterize the variability in conditions of the process so as to characterize a "worst-case" process that will be used for the challenge test. From the results of such "worst-case" trials, it should be possible to develop conservative interpretations of the efficacy of the process. This approach overcomes some of the statistical considerations, mentioned above, but some replication of trials is still necessary.

Challenge Organisms and Location of Challenge Trial

From consideration of the endemicity of certain pathogens in New Zealand, the likelihood of survival of growth of pathogens in cheese, reported outbreaks from cheese, likelihood of contamination of milk, and disease severity, it was concluded that challenge trials considering the fate of:

- *Listeria monocytogenes,*
- *Escherichia coli*,
- *Staphylococcus aureus* and
- *Salmonellae*

are potentially most useful and most relevant in establishing the safety or otherwise of raw milk cheeses made in New Zealand. Ideally, pathogenic strains would be used in the challenge but this will usually preclude undertaking the challenge trial in the actual manufacturing facility. Conducting the trials in the actual processing plant is, *prima facie*, desirable because it will facilitate accurate reproduction of the cheese-making process, but introducing pathogenic bacteria to a few processing plant is unacceptable for public health reasons. Accordingly, well-characterized surrogate strains that are non-pathogenic/non-toxigenic may be used. Alternatively pathogens may be used in challenge trials conducted in appropriate research facilities under the direct supervision of the cheese maker to ensure that the process is mimicked as fully as possible. Recommendations for specific challenge strains are given.

Specific Considerations for Cheese Process Challenge Trials

Cheese processes are complex and may provide opportunities for pathogen growth, for concentration and for inactivation. Accordingly, the design of the challenge trial has to include, and accurately mimic, all of the conditions during milk warming, fermentation/curd formation, curd handling and maturation that could affect pathogen numbers. In the case of surface ripened cheeses, this could include potential for growth after the pH rises during the maturation. The complexity of the microbial ecology of cheeses is the reason that the process will be best evaluated in cheese made by, or under the very close supervision of, the cheese maker. Before undertaking the challenge trial, considerable analysis of the cheese making process will be needed to characterize the set of 'worst-case' conditions that will be used for the challenge trial, i.e., so as to generate conservative estimates of the safety of the process. Additionally, post-processing contamination can also occur and, while not unique to raw-milk cheeses, a second contamination process (i.e., other than that due to the use of contaminated milk) may have to be mimicked as part of a challenge trial.

Challenge Organism Levels and Inoculum Preparation

Milk can become contaminated with pathogens due to infections of the udder, from organisms present on the outside of the teat, from accidental faecal contamination by animals during milking, from the dairy environment and from equipment used in milking. From

i) review of the available literature about pathogen levels in mastitic animals and in bulk raw milk, and
ii) practical considerations concerning expected changes in levels of pathogens in the cheese during processing and limits of detection of common microbiological enumeration methods

an inoculum level of ~10^5 cfu.ml^{-1} of milk is recommended. This both allows for some pathogen growth, or concentration of challenge organisms into the curd, and also for substantial inactivation to be measured by conventional microbiological enumeration methods, and is also a realistically 'high' contamination level.

Challenge organisms introduced to milk should be prepared to be in the exponential phase of growth, having been cultured in milk, or a media closely resembling milk, prior to inoculation. This represents a worst-case assumption, namely that the organisms will be able to grow exponentially as soon as the milk becomes warm enough (i.e., prior to addition of the starter culture) to allow their (potential) growth.

Methods of Analysis and Data Interpretation

In general, officially endorsed methods for enumeration of pathogens should be used and, if relevant, methods specifically relevant for detection and enumeration of pathogens in cheese. If other methods are proposed for use (either with greater detection sensitivity or to reduce costs/labour) strong evidence to demonstrate the equivalence or superiority of those methods compared to standard methods is required.

Given the variability in analytical methods, batch-to-batch variability and heterogeneity in product characteristics combined with the random distribution of the challenge organisms within the cheese, replication of trials will be necessary. Additionally, within trials replicate samples should be analysed. Where many samples are taken over the course of the process, regression can reduce the need for replication, provided that the relationship between pathogens levels at different time points in the process is reasonably well understood. Given the complexity of the cheese-making process, and the resultant microbial ecology of the product that can lead to increases or decreases in challenge organism levels at different stages of the process, samples should be taken in each of these stages.

A longer-term aim of the challenge trial is to develop a quantitative understanding of the factors relevant to cheese-making that determine the survival of pathogens. As such, in addition to testing the levels of the challenge organisms over time during the cheese process, it is useful to measure physico-chemical properties of the cheese (e.g., pH, organic acid levels, a_w, etc.) and levels of other microorganisms in the cheese so as to be able to determine whether correlations exist between these factors and the survival of the challenge organisms.

Expert groups considering the conduct and interpretation of food safety challenge trials have noted that *universally acceptable rules for interpreting test results are not available*. This highlights the need for the expertise of a food microbiologist as well as rigorous statistical analysis to correctly interpret challenge trial results as indicating the safety, or otherwise, of a cheese making process involving raw milk.

About this report

This report presents detailed information about the conduct of challenge trials to assess the microbiological safety of raw milk cheeses made in New Zealand and explanation for those recommended procedures and strategies. Given the complexity of design and interpretation of challenge trials relevant to raw milk cheeses, a number of example challenge trial protocols are also included as an appendix to this report.

Acknowledgements

The patience of the New Zealand Food Safety Authority during the long delays in completion of this report is gratefully acknowledged.

Assoc Prof John Bowman gave useful advice in selection of strains for challenge organisms. Dr. David Ratkowsky provided statistical advice. Their assistance is also gratefully acknowledged.

Contents

1 Introduction

While France, Italy, and Switzerland have long histories of production of cheeses from raw milk, in many other developed nations cheeses are required to be made from pasteurized milk, or milk that has received a heat treatment to inactivate vegetative pathogenic bacteria. Nonetheless, even in those latter nations, some specific types of cheese are permitted to be produce from raw milk (i.e., milk that has not been pasteurized or otherwise heat-treated to inactivate vegetative pathogenic bacteria). This is because of their long maturation time and physico-chemical composition, especially their acidic pH and low water activity (a_w), which are considered to reduce the probability of survival of pathogens to an acceptably low level. There is increasing interest in various nations, however, in the production of other types of cheeses made from raw milk. Proponents argue that pasteurization reduces health-promoting properties of milk and that cheeses based on raw milk are of superior sensorial quality.

Pathogenic bacteria that come to contaminate raw milk can arise from soil in the vicinity of the milking area, from the skin/hide of the animal being milked, from infections of the cow's udder, and from indirect faecal contamination from the animals themselves. Those pathogens include bacteria that can cause foodborne gastro-intestinal illness but milk can also transmit other zoonoses, including systemic salmonelloses, brucellosis and tuberculosis. Additionally, there is increasing concern about the possibility of transmission of *Mycobacterium avium* subsp. *paratuberculosis* from domestic animals to humans through meat and other animal products, including milk.

While the reasons for introduction of pasteurisation of milk are disputed by some, in United States of America it was ostensibly for reasons of protection of public health, and statistics since the introduction of pasteurisation suggest that it has been effective in that regard (Potter *et al*., 1984; Leedom, 2006). Arguments against the original introduction of pasteurization included that it was being used as a way to "clean up" poor quality milk produced under sub-standard conditions: increasing urbanization and industrialization of dairying in the early 20th century had led to a decline in hygiene in dairies, and also to increased production costs due to measures (other than pasteurization) to counter transmission of tuberculosis to humans (Weisbacker, 2007). Furthermore, even for good dairies, in the absence of refrigeration the increased distance (and hence time) between farms and consumers also contributed to increased milk-borne human illness (Czaplicki, 2007) presumably due to increased potential for growth of pathogens. In the United Kingdom, the original reasons for introduction of pasteurization by the dairy industry in the 1890s were shelf life extension (for the same reasons as noted above for USA) but the health benefits were soon realized (Adams and Moss, 2008). Campbell *et al*. (1996) asserted that since

the introduction of compulsory pasteurization of cows' milk in Scotland in 1983 "outbreaks associated with milk … have ended".

Animal health has improved generally due to improved animal husbandry practices and due to improvements in (dairy) farm hygiene. This has also enabled better microbiological quality of raw milk and some sectors of the community have begun to question the need for pasteurization, particularly the requirement for pasteurized milk in the preparation of cheese. It is argued that, due to the acidification and drying that occur during fermentation and maturation of cheese, the process will eliminate any pathogenic bacteria initially present.

1.1 Association of Raw Milk and Raw Milk Cheese with Microbial Foodborne Disease

That raw milk can harbour pathogenic bacteria is well-documented (Donnelly, 2004), and cheeses made from raw milk – particularly soft, surface-ripened cheeses, and fresh[2] cheeses (IFST, 2000) – have been implicated in foodborne outbreaks. The association of raw milk, compared to pasteurized milk, with these outbreaks seems to vary by country or region (de Buyser *et al*., 2001). Nonetheless, there are well-documented outbreaks involving raw milk cheese and many countries prohibit the production of raw milk cheeses, often with the exception of very hard, grating-type cheeses (e.g., parmesan or Parmigiano-Reggiano, Grana, Romano, Sbrinz, etc.,) or cheeses that have been matured for prescribed periods[3]. However, several reports (de Buyser et al., 2001; Donnelly, 2004; Johnson *et al*., 1990; Hages, 2010) conclude that there is not a clear distinction between the incidence of illness from raw milk products compared to those from pasteurised milk products. A possible confounding factor in these analyses, however, is that most dairy products are pasteurized or based on pasteurized milk, i.e., so that even though the *absolute* incidence of illness from raw milk, or pasteurized milk products is similar, the *proportion* of raw milk products involved in outbreaks is higher.

Marler (2010) also reviewed relevant literature and databases concerning the incidence of foodborne illness from dairy products. They did this specifically to compare the incidence of illness from raw milk and raw milk products to that from pasteurized milk products. They

---

2 For categorization/nomenclature of cheeses *see* Appendix 1.

3 North America has adopted a policy that cheese matured for longer than 60 days can be made from raw milk (Boor, 2005; D'Amico *et al.*, 2008), apparently based on empirical evidence that cheeses matured for this time are very rarely involved in outbreaks. Recently, the so-called "60 day rule" has come under scrutiny because of increasing evidence that some pathogens of concern can survive in cheeses beyond sixty days (*see* e.g., Altekruse *et al*., 1998; Boor, 2005). Also, as a result of the rule, some cheese makers are varying production methods for cheeses that previously had shorter maturation periods so as to exploit the rule to avoid having to use pasteurised milk. In Australia, Standard 4.2.4 of the Food Standards Code specifies maturation duration and conditions for specific types of cheese that may be manufactured with milk that has not been pasteurized, but has been 'thermised' (62°C x 15 s) or curd cooked (i.e., ≥ 48°C).

concluded that dairy products were much less frequently involved in foodborne illness than other food commodity groups, supporting the general perception that cheese is, in general, a relatively safe food (Lund *et al*., 1995; FSANZ, 2009). Raw milk dairy products, however, were significantly *over*-represented in outbreaks reported for dairy products suggesting that raw milk/raw milk products *are* a higher public health risk than pasteurized milk products on a risk-per-serving basis. FSANZ (2009) reviewed available literature on foodborne outbreaks related to cheese and concluded that 70% of outbreaks were attributed to raw milk cheeses despite that raw milk represents only about 10% of all cheeses sold.

New Zealand Food Safety Authority (NZFSA) has developed a regulatory framework to allow certain unpasteurised milk products that can be produced to an acceptable level of safety[4] to be produced, sold and imported in New Zealand and also exported. Given the increasing consumer demand for raw milk cheese, *prima facie* for reasons of wholesomeness and sensory quality, and the perception of proponents of raw milk cheese that fermentation and maturation of cheese can reliably eliminate pathogens potentially present in raw milk before consumption (as discussed above), NZFSA commissioned the current report to develop guidelines for the conduct of challenge trials relevant to cheese manufacture. The intention is to assist in determination of the safety of raw milk cheeses. As discussed below, however, the general applicability of the results of challenge studies can be limited and the results of challenge studies are only one of a range of types of evidence that should be used to evaluate the safety of the process. Other considerations include the record of safety of the particular style of cheese, hygiene of the process, diligence of the manufacturer, degree of reproducibility of the process, and scientific knowledge of the microbial ecology of the process and product, e.g., as may be inferred from the predictive microbiology literature and models.

1.2 Challenge Trials

1.2.1 Overview

Challenge trials may be undertaken for a number of reasons such as to characterize the shelf life of a product with respect to quality, or for validation of processing conditions, or for the ability of a process to satisfy a performance standard, e.g. a 5-log reduction in the load of a specific organisms of concern. They are also used to assess the safety of a product or process with respect to growth and/or survival of pathogens. The design of the challenge study will vary according to the question being asked, e.g., to answer the question "can pathogen 'X' grow in this product

[4] An "acceptable level of safety" is one that poses a low level of risk to the general population.

under normal conditions and times of storage?" requires less samples to be tested than to answer the question: "how fast does pathogen 'X' grow in this product?".

Irrespective of the specific question to be resolved by the challenge test, the common requirements of challenge trials are that they mimic as closely as possible the mode of contamination of a product of interest with the relevant organism(s) of concern, and the subsequent fate of that organism(s) between the point of contamination and point of consumption. Numerous factors can affect the fate of the organism and these, collectively, make up the microbial ecology of the product with respect to the organism(s) of concern. Those factors include:

i) the physico-chemical properties of the foods,
ii) other micro-organisms in the food,
iii) the conditions (temperature, gaseous atmosphere, packaging type) under which the product is processed, distributed, stored and displayed and
iv) the properties of the organism(s) of concern (e.g., environmental limits to growth, responses to environmental conditions singly and in combination)

These factors will govern *rates* of growth, or inactivation, of the organism(s) in the food. Thus, the amount of time for which each set of conditions is experienced is also an important variable affecting the fate of the organism(s)of concern.

Accordingly, to design and conduct a challenge trial that will generate reliable and representative results about the safety of a product/process, it is necessary to understand:

i) pathogen(s) that could contaminate the product,
ii) the manner in which they come to contaminate the product,
iii) the load of pathogens (i.e., number of cells) that could contaminate the product,
iv) the conditions (temperature, pH, water activity, organics acids, other microorganisms, etc.) and duration of steps in the process, and subsequent handling of the product prior to consumption,
v) type of packaging,
vi) conditions and duration of storage and distribution, and
vii) intrinsic characteristics of the products itself.

All of these factors can affect the potential presence and number of the pathogen of interest in the product at the time of consumption. EFSA (2008) note that the level of contamination, the heterogeneity of the contamination and the physiological state of the bacteria may be difficult to mimic.

1.2.2 Variability

When designing challenge trials it is also necessary to consider the influence of *variations* in the factors described above, and particularly the extent to which those variations could lead to higher than average risk. Such variability could relate to variations in:

- product formulation from batch to batch,
- the virulence of the pathogens of concern,
- milk quality
- processing conditions or,
- conditions and duration of storage etc.

Challenge trials are usually quite costly in terms of time or money or both, and for small producers, or for low-volume products, it is usually not practical to conduct multiple trials to assess the effects of variability. Instead, the trial is often designed to represent a worst-case scenario and, as such, is a compromise compared to the ideal of a series of trial that are truly representative of the range of variability of the fate of the pathogen in the food. For practical reasons, other compromises may also have to be made, e.g. to use non-pathogenic strains or surrogates for pathogens of concern if the product is to be prepared in the food processing plant, to modify the size of the product to facilitate sampling, to use unrealistically high levels of the target pathogen so that it can be reliably enumerated during inactivation processes, etc. These topics are discussed in greater detail in Sections 4.2 and 4.3, below.

There are a number of detailed reviews on the planning and conduct of challenge trials that are to be used to evaluate the safety of specific foods/food processes with respect to pathogenic microorganisms. These include Notermans *et al.* (1993), IFTS&T (2003a), EFSA (2008), NACMSF (2010) and ICMSF (*in press*). The guidance provided in those documents is essentially consistent and this report is largely based on them. Accordingly, further specific reference to those guideline documents will only be made where important differences exist between them. Material derived from other sources will be identified as appropriate.

1.2.3 Is a Challenge Study Needed or Useful?

As noted above, the general applicability of the results of challenge studies can be limited because of the extent of variability in real cheese production processes, both within and between batches, the difficulty in mimicking the actual processes and extent of contamination, and the time and financial cost of rigorous challenge trials. As such, the need for a challenge trial to make a decision about the safety of the product should be carefully evaluated to ensure that the safety of the cheese cannot be resolved in other ways. As described below, the results of challenge studies

are only one of a range of types of evidence that can be used to evaluate the safety of the process. *In essence, challenge studies may be considered as a "last resort" when the safety and stability of a cheese cannot be unambiguously determined by reference to the existing body of knowledge or, perhaps, to confirm an inference based on existing knowledge.*

NACMSF (2010) and Notermans *et al*. (1993) provide practical advice on deciding whether a challenge study is needed. NACMSF (2010) recommend that when the conditions of a food are consistent with parameters that are well recognized as controlling the growth of a pathogen, microbiological challenge studies are not needed. Cheese, however, may be a more challenging commodity because the processing and maturation of cheese involve a sequence of steps that may allow microbial growth, or concentrate microbial cells, or inactivate them and possibly allow them to resume growth (in the case of surface ripened cheeses). Moreover, because of the great diversity of cheeses and the current lack of understanding of the mechanisms and kinetics of inactivation, a product's record of safety is only relevant to identical products, i.e., if all conditions remain the same, even apparently innocuous changes to the cheese, its packaging or processing can affect its relative safety.

Where there is uncertainty about the safety of the product, the first step is to conduct a hazard assessment, akin to the first step of a HACCP planning procedure. This process can consider i) the likelihood of hazards being present in the raw ingredients, and specifically which hazards might be expected, ii) the effects of the process on the likelihood of survival or growth of the pathogens of interest, iii) the potential for re-contamination after processing, iv) the survival or growth of the pathogen during storage, distribution, and preparation (if any) etc. prior to consumption, v) levels of the pathogen that would be expected to cause illness, vi) whether the expected consumers are likely to have increased susceptibility to the pathogens that might be present. Answers to these questions may be available from various sources including scientific knowledge of the microbial ecology of the process and product, e.g., as may be inferred from the predictive microbiology literature and models, epidemiological data concerning the record of safety of the particular style of cheese, the hygiene of the process and diligence of the manufacturer (including the degree of reproducibility/control of the process), etc.

Some of the information relevant to conduct of hazard analysis in relation to cheese is presented in Sections 2, 3 and 4.1.1, below.

1.2.4 Expertise Needed to Conducting a Challenge Trial

In addition to the technical aspects, commentaries on challenge studies emphasise that they are complex tasks in terms of planning, execution and interpretation and that an expert (food) microbiologist is needed to assist in the planning and interpretation stages. NACMSF (2010)

provide detailed recommendations concerning the qualifications and experience of those who design, conduct and/or evaluate microbiological challenge trials in terms of knowledge and skills, education and training, experience and abilities. In essence, they suggest that those involved in design and evaluation should have a PhD in food microbiology or related discipline, knowledge of pathogens that are likely to be encountered in different foods, and at least two years of experience in the independent design, conduct and evaluation of challenge trials. To some extent, this report aims to document and make more readily accessible some of the required knowledge of the expert microbiologist that is of relevance to challenge testing of raw milk cheeses.

2 Cheese Processing

This section describes the general steps of cheese making and relates those to potential for pathogen growth, removal, or inactivation.

Milk is a colloid of fat globules and micelles of the protein casein. In essence, cheese is coagulated milk protein, predominantly the casein. As the casein coagulates to form the curd it also entraps the fat globules, and expels water and other soluble components of the milk (whey). The coagulation can be achieved by pH reduction alone, but more complete coagulation of the protein occurs if the enzyme rennin[5] (= "chamois") is used. This enzyme, traditionally derived from the stomach of baby cows or other suckling mammals, cleaves the casein molecule between specific amino-acid residues, which has the effect of removing a long side-chain on the casein molecule[6]. Removal of that side chain allows the casein molecules to aggregate and to form the curd. As the casein molecules coagulate and the curd contracts (a process called 'syneresis') water and solutes (including soluble proteins, minerals, vitamins and some lactose) are expelled. The tighter the curd, the more water is expelled and the drier, and firmer, the cheese becomes. Curd density can be increased by external compression, chopping the curd into smaller particles to aid syneresis, and/or gentle heating.

While there are many different types of cheese, the main differences between cheeses arise during the ripening process, or from post-fermentation processes involving curd handling, not during the fermentation. Prior to that, the processes undertaken are common to almost all types of cheese. They include:

i.) milk treatment (checking for antibiotic residues, filtering out foreign particles, testing and adjusting fat levels may be performed but are perhaps less likely for artisanal-type cheese production),

ii.) for non-raw milk cheeses, heat treatment (e.g., pasteurization) to minimise presence of pathogens,

iii.) addition of starter culture[7] (starter cultures aren't essential to manufacture of cheese – *see* point iv, below) - but are the traditional method of acidification which is essential for stabilising the product and for optimising the activity of the rennet; enzymes from starter cultures may also contribute to final product characteristics),

---

5 Rennin (chymosin) is a specific enzyme that is a component of rennet. Rennet is a mixture of mammalian enzymes from the stomach of suckling animals. Other sources of chymosin are now available, including those produced in genetically modified organisms, or from plant extracts.

6 Specifically, chymosin attacks the peptide bond between (the amino acid) Phenylalanine (at position 105 on the κ-casein molecule) and Methionine (at position 106).

7 Starter cultures refer to microorganisms deliberately added to the milk to convert the lactose (sugar) present in the milk into lactic acid and other products by their metabolism. This process is called 'fermentation'. The lactic acid produced decreases the pH of the milk which begins the process of curd formation, and increases the activity of rennet.

iv.) curd formation, which is usually brought about by protease enzymes, e.g., rennet, *after* acidification, that modify casein so that it cannot remain in colloidal suspension and starts to clot, i.e., to form a curd. (Acidification alone can induce curd formation but the curd is much looser, such as in 'fresh' cheese, e.g., cottage cheese)

v.) cutting of the curd (one of the processes in which the characteristics of the final cheese can be influenced; more cutting lets more whey escape and leads to a drier, harder, cheese)

vi.) cooking the curd in the whey (optional; can be undertaken to increase syneresis and leads to a drier, firmer, cheese)

vii.) curd handling, whey removal – (additional whey removal can be encouraged by compressing the cheese, e.g., by stacking, or further cutting, of the whey; by salting etc.)

viii.) moulding (cheese shape is one of the characteristics of different varieties of cheese, and can affect the ripening process because the surface area to volume ratio can affect the migration of enzymes, e.g., in surface ripened cheeses such as Brie, red smear, etc., that are essential to the final texture and flavour of the cheese)

ix.) maturation–is possibly the most complex part of cheese-making and also the one in which the characteristic flavours, odours and textures of the cheese develop. It is dictated by the metabolism of microorganisms in the cheese, and activity of residual microbial enzymes in the cheese. That metabolism/catalytic activity can be manipulated by control of temperature and humidity over time. In many cheeses this process is dominated by the addition of a well-defined microbial cultures but in other cheeses the process can be relatively uncontrolled because the microbes that are present are those that were present in the milk initially, or were 'adventitious' contaminants from the processing and maturing environment. Even without microbial growth in this stage, enzymes from either live or dead cells will continue to be active and to develop the characteristics of the cheese.

2.1 Critical Control Points in Cheese Making for Control of Microbial Pathogens

Several steps in the manufacture of cheese that are control points for microbial food safety can be discerned from the above discussion. A useful review of the Critical Control Points for a number of different cheese styles is presented in Sandroua and Arvanitoyannisa (2000).

2.1.1 Milk hygiene

The first control point is the use of good quality milk that has had minimal opportunity to become contaminated. All microbial inactivation processes are time-dependent and are usually

characterized by a *rate* of inactivation under specific conditions. Consequently, inactivation processes can be 'overwhelmed' and fail to eliminate all pathogens if the initial pathogen load in the product is too high or the time is too short. Thus, in addition to inactivation steps, actions to minimise contamination of milk are an essential element of the microbiological safety of cheeses. This is particularly important for raw milk products, i.e., those in which indigenous enzymes in the milk (e.g., lysozyme, lactoferrin, the lacto-peroxidase system) and the fermentation and maturation steps provide the only subsequent means of eliminating pathogens that might be present in the raw milk. Pasteurisation/thermisation of milk, if used, is a key critical control point for the removal of most pathogenic microorganisms, although spore-forming bacteria can survive pasteurization. Thus, if pasteurization is not used, other controls assume much greater importance.

Raw milk is a very suitable medium for microorganism growth, but refrigeration can minimize microbial growth between the time of milking and cheese-making. The extent of microbial growth will also depend on time, so that reducing the time between milking and cheese production will also contribute to risk minimisation. Microbial contaminants can be derived from the udder interior, the teat exterior, the environment, the milk-handling equipment, and personnel (Mossel *et al*., 1995; Adams and Moss, 2008). Thus, general hygiene of the udder and milking area, personal hygiene of staff, avoidance of milking of animals with mastitis, and cold storage of the milk prior to its use in cheese-making contribute to minimisation of microbial contamination of raw milk. Typical microbial levels in milk from healthy animals are 10^2 – 10^3 cfu.mL^{-1} but milk from sub-clinically infected mastitic animals can harbour up to 10^5 cfu.mL^{-1} and in early, acute, stages of clinical infections can be as high as 10^8 cfu.mL^{-1} (Adams and Moss, 2008; *see also* Section 4.3.3).

2.1.2 Role of Fermentation

The fermentation and salting of cheeses is important for the establishment of pH and water activity conditions that will prevent pathogen growth. Additionally, provided that the combination of physico-chemical conditions in the cheese will preclude pathogen growth (*see* Tables 2, 3) the pathogen will usually be inactivated *over time*. The rate of inactivation in such environments is strongly affected by temperature (McQuestin *et al*., 2009). In this regard, the presence of antibiotic residues in milk after therapeutic administration to cows can inhibit starter cultures resulting in slow acidification and, potentially providing more time before the potential growth of pathogens is prevented (Fox *et al*., 2000). Starter culture activity can also be reduced due to contamination with bacteriophages.

Milk must be warmed prior to cheese making to promote the rapid growth and metabolism of starter cultures, i.e., so as to acidify the milk prior to addition of rennin and the subsequent curd

formation. This warming will also promote the growth (i.e., multiplication) of pathogens that may be present in the milk, potentially leading to an *increased* risk. Thus, because reduction in pH and water activity are key factors in the microbiological stability of cheeses and both depend on fermentation, appropriate *progress* of the fermentation may be considered as a control point in the safety of cheeses and slow fermentation has been shown to be the cause of increased growth of pathogens in milk during cheese making (Schoder *et al.*, 2003) and cheese-borne food poisoning outbreaks (Fox *et al*., 2000). Even without pH monitoring, starter culture failure might be expected to be evident due to poor, or slow, curd formation.

2.1.3 Physico-chemical Parameters of Cheese

Given the diversity of cheeses, and the attendant hurdles to growth and survival of pathogens, and the diversity of environmental tolerances of pathogens of relevance it is difficult to specify combinations of factors that will preclude pathogen growth in cheeses. However, as a rule of thumb, the Institute of Food Technologists (USA)[8] have developed matrices of pH and water activity combinations that are known to prevent growth of bacterial pathogens. In the case of non-heat treated foods, the matrix is shown in Table 1, below.

.

Table 1: Combinations of water activity and pH that confer shelf-stability ('non-TCS') to food products that have not been heat-treated or that have been heat-treated but are not protected against recontamination (*after* IFTS&T, 2003b)

Critical a_w values	Critical pH values			
	< 4.2	**4.2 to 4.6**	**> 4.6 to 5.0**	**>5.0**
< 0.88	Non-TCS	Non-TCS	Non-TCS	Non-TCS
0.88 to 0.90	Non-TCS	Non-TCS	Non-TCS	further evaluation
> 0.90 to 0.92	Non-TCS	Non-TCS	further evaluation	further evaluation
> 0.92	Non-TCS	further evaluation	further evaluation	further evaluation

In the Table 'non-TCS' denotes 'temperature control not required for safety', i.e., that the product is microbiologically shelf-stable. The categories in Table 1 represent extreme limits where pH and water activity are the only hurdles to microbial growth for *any* microorganism. If other hurdles are present, e.g., organic acids, bacteriocins, etc. the food may still be shelf-stable at less

[8] That report was prepared as part of a contract with United States' Food and Drug Administration (USFDA) and the findings now form part of USFDA policy.

extreme pH/a_w combinations but more evidence, such as reference to appropriate predictive models, or the results of a challenge trial, is required to make that decision.

A more complete table of combinations of growth-preventing pH and a_w conditions *for selected foodborne bacterial pathogens* was compiled by NACMSF (2010) and is presented in Table 2, overleaf. As with Table 1, the combinations presented are worst-case combinations, i.e. when all other factors are optimal for growth. Where pH and a_w alone do not preclude growth of the specific organism, other factors may act as hurdles to growth. Thus, other approaches as discussed above, may be required to determine whether growth is, or is not, possible.

To put the information in Tables1 and 2 into context, it is useful to have information concerning the pH and a_w levels found in different styles of cheese. Table 3, below, presents such information for a variety of cheeses available in Switzerland. From the data in Table 3 it is also apparent that there is much variability in pH/a_w combinations for apparently similar styles of cheese (*see also* Appendix 1), and accentuates that it is necessary to have this information specifically for each cheese of interest.

Many investigators (Buazzi *et al.*, 1992; Spahr and Schafroth, 2001; Schlesser *et al*., 2006; Hages, 2010; Schvartzman *et al.,* 2010) have observed during challenge trials that the concentration of pathogens can increase approximately five to ten-fold during curd formation. While some of this increase may be due to growth, several reports indicate that this increase may be due mainly to the preferential partitioning of bacterial cells into the curd, rather than remaining in the whey (Ryser, 1997; Fox *et al*., 2000; Donaghy *et al*., 2004; Schlesser *et al*., 2006; Hages, 2010; Schvartzman *et al.,* 2010). Sung and Collins (2000) note that the concentration effect is common but that the extent of concentration may be strain dependent.

The data in Table 3 can be somewhat misleading. Cheese is a dynamic environment and continues to change throughout its life, in response to storage conditions. This is because of the residual activity of enzymes that metabolise the proteins, fat and other compounds present in cheese. Thus, over time, attributes of the cheese can change. The pH of surface-ripened cheese, in particular, can change during maturation and become growth-permitting for some pathogens. This results from proteolysis and metabolism of amino acids and their de-amination (release of amine groups). The amino groups increase pH. Fox *et al.* (2000) have reviewed literature on pH changes in cheese and the effects of those changes on growth potential of a variety of microorganisms, showing that as pH increases growth of some pathogens can occur.

Table 2. Growth preventing combinations of pH and water activity for selected foodborne bacterial pathogens (after NACMSF 2010)

a_w value	pH level					
	< 3.9	3.9 to < 4.2	4.2 to 4.6	> 4.6 to 5.0	> 5.0 to 5.4	> 5.4
<0.88	NG[c]	NG	NG	NG	NG	NG
0.88–0.90	NG	NG	NG	NG	*S. aureus*	*S. aureus*
>0.90–0.92	NG	NG	NG	*S. aureus*	*S. aureus*	*L. monocytogenes* *S. aureus*
>0.92–0.94	NG	NG	*L. monocytogenes* *Salmonella*	*B. cereus* *C. botulinum* *L. monocytogenes* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* *Salmonella* *S. aureus*
>0.94–0.96	NG	NG	*L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus*	*B. cereus* *C. botulinum* *C. perfringens* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus*
> 0.96	NG	*Salmonella*	*L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus* *V. vulnificus*	*B. cereus* *C. botulinum* *C. perfringens* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus* *V. vulnificus*

Table 3. Examples of pH and water activities of a range of cheese styles available in Switzerland (*after* Lund *et al.*, 1995, based on data of Rüegg and Blanc, 1977).

Cheese	Water (%)	NaCl (%)	pH	a_w
Hard Cheeses				
Bergkäse	23.5	3.1	5.6	0.89
Emmentaler	35.2	1.1	5.6	0.97
Greyerzer	36.3	2.3	5.3	0.96
Parmesan	28.9	3.5	5.3	0.89
Sbrinz	28.8	2.9	5.5	0.90
Semi-Hard Cheeses				
Appenzeller	40.6	3.1	6.3	0.97
Bel Paese	46.1	4.9	5.1	0.96
Cheddar	34.5	3.1	5.2	0.96
Edamer	45.1	5.9	5.3	0.95
Fontal	42.0	4.1	5.3	0.96
Gouda	39.6	3.8	5.1	0.95
St. Paulin	45.8	3.4	5.3	0.97
Tete de Moine	39.0	3.5	5.5	0.96
Tilsiter	38.5	3.3	5.7	0.96
Raclette	39.3	4.2	5.6	0.96
Belle de Chamos	50.7	3.9	5.7	0.99
Brie Suisse	48.7	3.7	5.6	0.97
Camembert Suisse	50.4	5.4	7.4	0.99
Soft Cheeses with Red Smear				
Limburger	54.9	6.5	6.8	0.98
Münster	46.2	2.3	5.3	0.97
Vacherin Mon d'Or	53.4	1.8	5.1	0.99
Fresh Cheese				
Cottage Cheese	80.6	4.5	4.9	0.98
Speisequark	70.5	0.8	4.7	0.99

3 Pathogens of Concern

While many pathogens can be associated with raw milk and raw milk cheeses (Lund *et al*., 1995; Rampling, 1996; Altekruse *et al*., 1998; IFST, 1998; Fox *et al*., 2000; Donnelly, 2004; FSANZ, 2009; Marler, 2010), those that are most frequently associated with illness appear to be *Brucella melitensis* and *B. abortus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and gram negative enteric pathogens such as Salmonellae, *Escherichia coli* and *Campylobacter jejuni* and *C. coli*. Of that group, *L. monocytogenes, Salmonellae*, and pathogenic *E. coli* are generally considered (*see* e.g., Johnson *et al*., 1990; de Buyser *et al*., 2001; IFTS&T, 2003a) to represent the greatest public health risk in raw milk cheeses due either to the severity of infection and/or epidemiological evidence of frequency of cases. *Staphylococcus aureus* intoxications from cheese, while causing similar levels of outbreaks and cases to *L. monocytogenes* and pathogenic *E. coli* (de Buyser *et al*., 2001; FSANZ, 2009; Figure 1) cause only mild disease and may be considered to be a lower risk (Lund *et al*., 1995; Hill and Warriner, 2010). Nonetheless, there are microbiological limits for Salmonellae, *L. monocytogenes, E. coli* and *S. aureus* in EU legislation (EFSA, 2005). In New Zealand, the Animal Products Act (Raw Milk Products Specifications Notice 2009) articulates required raw milk quality at the commencement of processing and for Salmonellae, *L. monocytogenes,* and *S. aureus* enterotoxin in finished product. IFTS&T (2003a) nominated those four organisms in their list of appropriate challenge organisms for evaluation of microbiological safety of dairy products, but also included *Clostridium botulinum* as a potential hazard in dairy products. *C. botulinum* was also noted by IFST (1998) to have caused an outbreak and to be a potential hazard in raw milk products. From analysis of cheese-related outbreaks in USA from 1973 to 1992 Altekruse *et al.* (1998) observed that maturation alone may not be sufficient to eliminate *Salmonella*, *Listeria* or *E. coli* O157:H7 from cheese, noting also the significant involvement of cheese that included raw or improperly pasteurized milk in the eleven largest outbreaks among the 32 outbreaks in the study period.

EpiCentre (2008), based on available epidemiological outbreak data and reports, concluded that there is only moderate evidence of a causal link between consumption of raw milk/raw milk products and illness from *Campylobacter* spp., *E. coli* spp., *L. monocytogenes* and *Salmonella* serovars. That analysis also concluded that there was some evidence of association between *Brucella* infections and consumption of raw milk/raw milk products. According to the rigorous quality criteria adopted in the EpiCentre (2008) review, it was not possible to objectively evaluate whether there is a causal link between exposure to raw milk/raw milk products and Staphylococcal intoxications.

While Fox *et al*. (2000) considered that the main pathogens of concern in cheese currently are pathogenic *E. coli* and *L. monocytogenes,* there has also been much interest in the role of

Mycobacterium species in farmed animals as possible causes of dairy product-borne human illness.

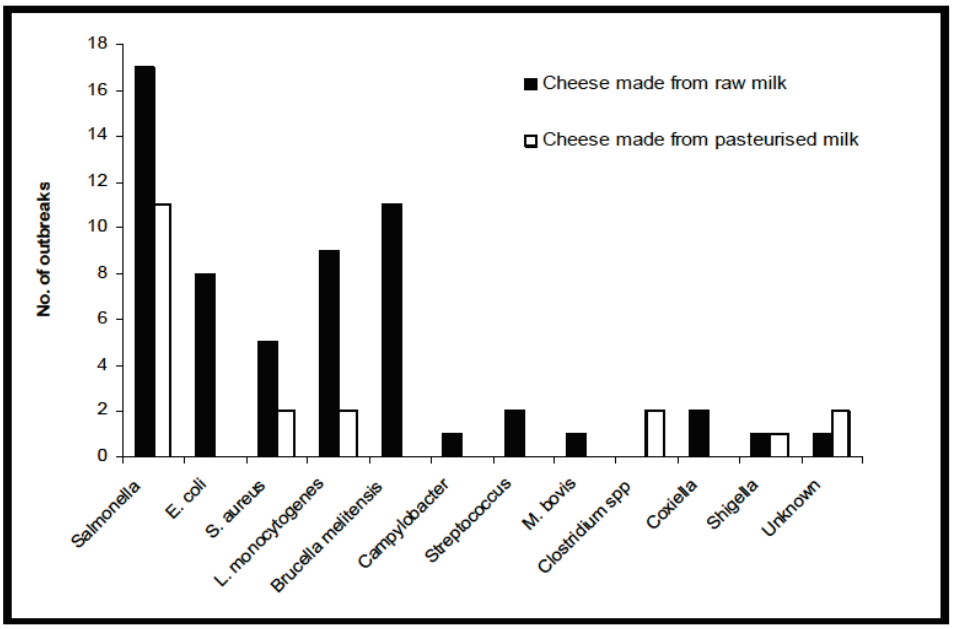

Figure 1. Numbers of reported outbreaks of foodborne infections from raw and pasteurised milk products (reproduced from FSANZ, 2009). (Note, however, that most cheeses are made from pasteurized milk).

4 Challenge Testing of Cheese

As noted above, challenge trials need to mimic as closely as possible the processes of contamination of the product, its processing, packaging, storage and distribution and end use, so as to evaluate the fate of pathogenic contaminants and consequent risk to public health. As such, challenge trials need to consider the following:

i.) the type of study (i.e., whether pathogen growth, or inactivation, or both are expected) so as to be able to correctly design the experiment to answer the specific question,

ii.) the organism(s) of interest,

iii.) factors related to the product of interest that will affect the fate of the challenge organism, including product preparation (process steps, particularly Critical Control Points), variability in product and process characteristics, types of packaging, the presence of competitive flora,

iv.) the natural mode(s) of contamination of the product (i.e., stage of processing, how transferred), including

  a.) levels of the organism(s) of interest that could be encountered in the food in "real world" situations

  b.) the physiological state of natural contaminants (e.g., whether stationary or exponential phase, spores or vegetative cells, etc)[9],

v.) storage duration and conditions (e.g., temperature, packaging type),

vi.) variability (e.g., in pathogen response, in product or process characteristics, storage duration and conditions, etc., and including potential for product mishandling by others in the chain)

and, from a more pragmatic perspective:

vii.) number of samples and frequency of sampling,

viii.) sampling method and analytical methods.

[9] Bacterial cells in exponential phase are more likely to be inactivated by sudden changes in environment, e.g., sudden osmotic (salt, drying) stress, sudden reduction in temperature or pH etc., that require the cell to change its physiology. Conversely, cells that have already experienced stress will probably have induced physiological changes to ameliorate the effect of those stresses. Conditions leading to stationary phase also cause physiological stress that leads to induction of physiological changes that increase the resistance of bacterial cells to a range of environmental stresses. Thus, while exponential phase cells are preferable to reflect a 'worst case' for conditions that are near-to-optimal for the growth of the specific challenge organisms, cells in stationary phase will already have induced those changes in physiology that render them more resistant (and that are manifest as slower death rates) and are more appropriate for inocula that will be subject highly stressful or lethal conditions.

NACMSF (2010) note that because the results of challenge studies will be used to make decisions that could affect public health, food safety managers need assurance that the challenge studies are conducted rigorously. While this document and those that it is based on give technical and philosophical advice for design, conduct, and analysis of challenge studies, skilled technicians and experienced scientists should also be involved in the planning and conduct of the work and interpretation of the results. There are a number of important microbiological considerations that need to be followed to generate reliable, representative, results and this document is intended to make more accessible knowledge required for planning of experiments and analysis/interpretation of results. Nonetheless, challenge trials will involve working with high numbers of pathogens, and microbiological expertise is required to protect those working on the project, and the working and external environment from accidental release of those pathogens. Similarly, full interpretation of all nuances of the results will be more likely if a microbiologist(s) skilled in microbiological challenge trials is involved.

4.1 Selection of Challenge Organisms (Organisms of Interest)

According to NACMSF (2010) the first steps after determining that a challenge study is needed are to conduct a hazard analysis for the product/process to determine significant biological hazards, including potential routes of contamination (*discussed in* Section 4.3.2), and to assess what is known about their growth or inactivation in the product, due in turn to product formulation parameters such as pH, water activity, organic acid levels, etc. Determining whether only inactivation will occur, or whether growth is possible, may affect the choice of inoculum concentration, further discussed in Section 4.3.3. Given that this report is solely concerned with raw milk cheese, other than the passive inactivation that happens during fermentation and maturation there no other processing steps envisaged that would reliably eliminate pathogens that may be present. Thus, all pathogens that could realistically come to contaminate the raw milk should be considered when planning challenge tests for raw milk cheese production. Accordingly, this section extends the discussion presented in Section 3 and is specifically concerned with identification of pathogens of specific relevance to raw milk cheeses made in New Zealand.

4.1.1 Microbiological Hazards in Cheese

From the discussion presented in Section 3, a number of pathogens were identified as potential hazards in cheese. These were:

- pathogenic *E. coli*
- *Listeria monocytogenes*
- Salmonellae

- *Staphylococcus aureus*
- *Campylobacter* spp.
- *Brucella* spp
- *Clostridium botulinum*
- *Mycobacterium spp.*

As discussed above, microbiological hazards in cheese can arise as contaminants from the milking and cheese factory environment, from infections of the teat and udder, from organisms on the skin of the animal, and from pathogens that might be present in the faeces of the cow and that accidently contaminate the milk.

Contamination of itself does not necessarily constitute a risk to consumers of cheese. The risk will also be a function of i) the number of pathogen cells ingested at the time of consumption, ii) the dose typically required to cause infections, and iii) the severity of the resulting infection. In turn, the dose consumed will depend on the initial level of contamination and the fate of the pathogens in the cheese after the contamination occurs, i.e., whether growth is possible, whether death ensues, or whether both growth and death occur at different stages in the cheese's 'life'. This, in turn, depends on the physico-chemical characteristics of the cheese, (due to the formulation of the cheese itself and the action of microbial and indigenous enzymes in the cheese), the time and conditions of processing, storage, distribution etc., and the biology of the organisms itself, e.g. its limits to growth, rate of growth (if possible), and ability to resist inimical conditions in the cheese (as appropriate). These aspects constitute the "microbial ecology" of the product.

Knowledge of the microbial ecology of cheeses is incomplete, but information concerning the microbial ecology of foodborne pathogens in general terms is available in the scientific literature and can, in principle, be matched to the physicochemical conditions in the cheese to adjudge the likelihood for pathogen growth and/or inactivation. An overview of the pertinent knowledge was presented in Section 2.

The potential for, and rate of, growth of a wide range of pathogens in response to combinations of temperature, water activity, pH, various organic acids, and other inhibitory compounds is available in predictive microbiology models (*for review see* Ross and Dalgaard, 2004), some of which have been incorporated into web-based software including the USDA's Pathogen Modeling Program (http://pmp.arserrc.gov/PMPHome.aspx), the international food microbial ecology database 'ComBase' (http://www.combase.cc/) and Seafood Spoilage and Safety Predictor (http://sssp.dtuaqua.dk/). ICMSF (1996) presents a collation of information available until that time on the growth rate, growth limits and inactivation rates of pathogenic bacteria in foods. These resources can be used to help assess the risk that could arise from pathogens in cheese.

Additionally, the scientific literature contains information about contamination levels and frequencies of pathogens in foods, including different varieties of cheeses, as do reports of government and industry surveys. Food product 'recall' notices can also provide insights concerning the frequency of contamination of different foods with pathogens. Similarly, epidemiology literature and reports of organizations (e.g., OzFoodNet, USA's FoodNet, PHLS, Eurosurveillance, etc.), and the scientific literature itself, though imperfect and incomplete, can provide information about the frequency of outbreaks. This information, identifying the observed incidence of cheese-borne microbial illness, can be used to "ground truth" predictions of risk potential derived from the microbial ecology of foods and cheese making process conditions.

The following section aims to assess the relevance of potential microbial hazards in raw milk cheese based on the above-mentioned sources and strategies.

Pathogenic *Escherichia coli*.

Escherichia coli is a species of Gram-negative bacteria that is a normal, and beneficial, resident of the intestinal tracts of mammals and birds. Non-pathogenic strains are often termed "generic *E. coli'*. Some strains, however, can cause disease and several sub-groups of pathogenic *E. coli* are distinguished based on the severity and type of disease caused. These include:

- Enteropathogenic *E. coli* (EPEC)
- Enteroaggregative *E. coli* (EAEC)
- Enteroinvasive *E. coli* (EIEC)
- Enterotoxigenic *E. coli* (ETEC)
- Shiga-toxin producing *E. coli* (STEC)
- Enteroahaemorrhagic *E. coli* (EHEC)

Summaries of the disease syndromes caused by these groups can be found in basic food microbiology texts and FSANZ (2004) also provides a useful summary. Most research attention has been given to EHEC, particularly strain *E. coli* O157:H7, due to the severity of the disease caused which can be fatal or lead to serious life-long sequelae.

Due to the potential presence of these types of *E. coli* in the intestine of cows, sheep, goats, etc. they can contaminate milk, and foodborne outbreaks involving both raw and pasteurized milk, and cheese products have occurred, and caused at least one death (*see* Ammon, 1997; D'Amico *et al*., 2010; Marler-Clark, 2010). While pathogenic strains in cheese appear to be uncommon, high counts of generic *E. coli* were found to be associated with raw milk cheeses in Italy (Civera *et al.,* 2007), and Rosengren *et al.* (2010) detected generic *E. coli* in 34% of raw milk cheeses and 3% of

cheese made with pasteurized milk from Swedish farm-dairies. Most (52% of samples) of the Swedish cheeses were fresh, 24% were Camembert and 15% were Chevre. Civera *et al.* (2007) noted the presence of *E. coli* in cheeses that had been ripened for at least 60 days. Several other studies (Reitsma and Henning, 1996; Schlesser *et al*., 2007; D'Amico *et al*., 2010) have indicated that *E. coli*, even at relatively low levels, can survive the processing of harder style cheeses, such as chedder, or Gouda, even beyondthe60 day maturation time mandated for raw milk cheeses in USA.

Bayliss (2009) reviewed the potential for raw milk and raw milk cheeses to transmit toxigenic *E. coli.* He identified multiple outbreaks due to toxigenic *E. coli* strains in USA, Scotland, England, France and Canada due to raw milk cheeses. Investigations of the outbreaks in Scotland revealed thermal abuse of the milk prior to fermentation in two outbreaks and another in which no starter culture was used, and another in which the processing times time was too short for adequate acid development and inactivation of the pathogen. More recently, a 2010 outbreak in USA involving raw milk Gouda cheese contaminated with *E. coli* O157:H7, caused at least 38 cases of illness (CDC, 2010).

E. coli grows over a temperature range of ~7 to 50°C, at a_w~≥ 0.95 and at pH ~≥4. When growth is prevented, death ensues at a rate largely governed by temperature, but there is considerable variability in the rate of inactivation between strains (McQuestin *et al*., 2009). Some strains have been recognized as being acid tolerant and surviving for long times in fermented foods. Organic acids are more inhibitory to microbial growth at any given pH, than are mineral acids. This is believed to be due largely to the lipophilicity of the undissociated form of the organics acid. Microbial tolerance of acidic conditions can also be induced by various stressful environments, including growth in a sub-lethally acidic environment, entry into stationary phase, starvation etc. Variation in acid resistance appears to be similar among both pathogenic and 'generic' strains (Duffy *et al*., 2000).

The infectious doses (ID_{50})[10] of EPEC, ETEC and EIEC have been estimated to be in the range 10^8 – 10^{10} cells based on human volunteer trials, but can be 100-fold less if stomach acidity is compromised. Much lower doses can induce illness in children. Children less than five years old and the elderly are particularly at risk from infection with EHEC, which can cause haemolytic uraemic syndrome (HUS), leading to kidney dysfunction and permanent kidney damage. Shiga-toxins produced by the EHECs bind to and interfere with the function of kidney cells, leading to formation of blockages. Impaired kidney function leads to other serious symptoms. In the United States, haemolytic uremic syndrome is the principal cause of acute kidney failure in children, and most cases of HUS are caused by *E. coli* O157:H7.

[10] The ID_{50} is that dose of the pathogen that will lead to illness in 50% of a population.

The dose of EHEC required to cause illness has received more research attention than other sub-groups of *E. coli* because of the severity of HUS, and the related thrombocytopaenic purpura (TTP). From outbreak data the $ID_{50}$for EHEC is estimated to be in the range 3×10^5 to 3×10^6 cells. Other estimates (FDA, 2003) suggest that as few as 1 – 700 cells can cause illness, but this may represent exposure of susceptible populations.

Limited data on levels of generic *E. coli* or pathogenic sub-groups in raw milk, or raw milk cheese, is available in the public domain. In a survey in Queensland, Australia (Eglezos *et al*., 2008), no samples (n= 74) of raw goat's milk cheeses were found to contain any *E. coli* and total aerobic counts in 214 samples never exceeded $10^{4.9}$ CFU/g. In USA a survey (D'Amico et al., 2008) of raw milk for farmstead cheese-making revealed that 98.6% of samples had Total Viable Counts (TVC)<10^5CFU/mL. In Portugal, levels of Enterobacteriaceae (a group of bacteria including *E. coli*) ranged from 5.9 to 7 logCFU.mL^{-1} in raw milk, but between 1.3 logCFU.mL^{-1} and <1 cell.g^{-1} in 4-month old cheeses (Kongo *et al.,* 2008). In Switzerland 407 samples of goat's and sheep milk were examined for a range of bacterial pathogens, including EHEC. DNA from EHECs was detected in 16.3% of goat's milk and 12.3 % of ewe's milk samples (Muehlherr *et al.*, 2003). In that study Enterobacteriaceae levels in goat's milk had an average concentration of 5.72 (95% confidence interval 5.26 – 5.94) logCFU.mL^{-1} and in ewe's milk were 3.93 (95% confidence interval 2.97 – 4.20) logCFU.mL^{-1}.

Other reports (Reitsma and Henning, 1996; Maher *et al*., 2001; Amer and Nabila, 2004; Vernozy-Rozand *et al*., 2005; Schlesser et al., 2006; D'Amico *et al*., 2010) indicate that *E. coli* in milk can survive the processes involved in production of a variety of cheeses. O'Brien *et al*. (2009) examined 351 Irish farmhouse cheeses, including raw milk cheeses, and found that *E. coli* counts in 79% of the raw milk cheeses were <10 CFU/g (the limit of detection), and none had levels >10,000 CUF/g while no pasteurised milk cheeses had levels >250CFU/g.

Salmonellae

Salmonellae are also Gram-negative bacteria in the Enterobacteriaceae group. They are similar in their growth range to *E. coli*, being able to grow at temperatures from 7 to 47°C, pH from 3,8 to 9.5, and water activity in the range > 0.94. Salmonellae can cause typical gastro-enteritis with symptoms of abdominal pain, nausea, diarrhoea, mild fever, vomitting and headache. Symptoms typically last for two to three days and resolve without medical intervention. In a few case, more severe disease can result, leading to septicaemia, and long-term sequelae including arthritis, endocarditis, local abscesses, peritonitis, and urinary tract infection. Salmonellosis is the most commonly reported foodborne illness in many industrialized nations, after Campylobacteriosis.

The primary source of Salmonellae is the gastrointestinal tract of mammals and birds, though Salmonella can also be recovered from the gastro-intestinal tracts of reptiles also.

Raw milk can be contaminated by faecal matter from animals being milked and, as such, the principal reservoir of *Salmonella* spp. in the dairy industry is raw milk itself (i.e., there are no other probable sources of contamination). Given this contamination, products derived from raw milk might also be at risk of contamination with salmonellae if there is no reliable inactivation process. According to FSIA (2004) the reported prevalence (1985-1996) of salmonellae in raw milk is 0%- 8.9% (based on data from 7 countries: Canada, England, Wales, France, India, Ireland & United States). In a more recent European report (EC, 2002) incidence in ready-to-eat dairy products was reported at 0 -0.25%. That report indicated that low contamination levels are usual. Conversely, during 2001 a contamination rate of 2.95% was observed in Spanish samples (n = 237).

Historically, consumption of unpasteurised milk *has* led to large outbreaks of salmonellosis, though they are not common. During the various steps in cheese production, the physico-chemical parameters of the products are expected to challenge the survival of any Salmonella that come to contaminate the samples. However, as noted earlier, survival is a function of time, and cheeses matured for shorter times are expected to be more susceptible to survival of pathogenic bacteria, including Salmonellae, and there are many reports of Salmonellosis outbreaks from raw milk fresh-style cheeses in Europe and USA (see e.g., *Morbidity and Mortality Weekly Reports*, or *Eurosurveilance*). However, outbreaks from hard-style cheeses have also recently occurred (CDC, 2008; van Duynhoven *et al.*, 2009). De Buyser *et al*. (2001) reported that of all foodborne outbreaks due to milk and dairy products, Salmonella was the most frequently reported aetiological agent in the international literature, and that most outbreaks involved raw or unpasteurized milk.

There are thousands of strains of Salmonella. They vary in virulence and regions of endemicity are distinguishable by serotyping. Molecular taxonomy methods have enabled a more consistent categorization of Salmonella and they are now classified as two species: *S. enterica* with six main subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) and *S. bongori*, Historically, serotype (V) was *bongori*, but this is now considered as a distinct species. This nomenclature is now formalized but, because it is not in harmony with the traditional usage of many workers, the traditional nomenclature is still common.

A “dose-response” relationship for foodborne Salmonellosis was developed by FAO/WHO (2002) and is illustrated in Figure 2. From the figure it is apparent that there is variability, or uncertainty, concerning the probability of infection for a given dose, and this presumably arises from imperfect epidemiological data, variability in virulence between strains and variability in

the susceptibility of hosts. Taking this into account, an ID_{50} in the range of 10^3 to $10^{5.5}$ CFU is suggested.

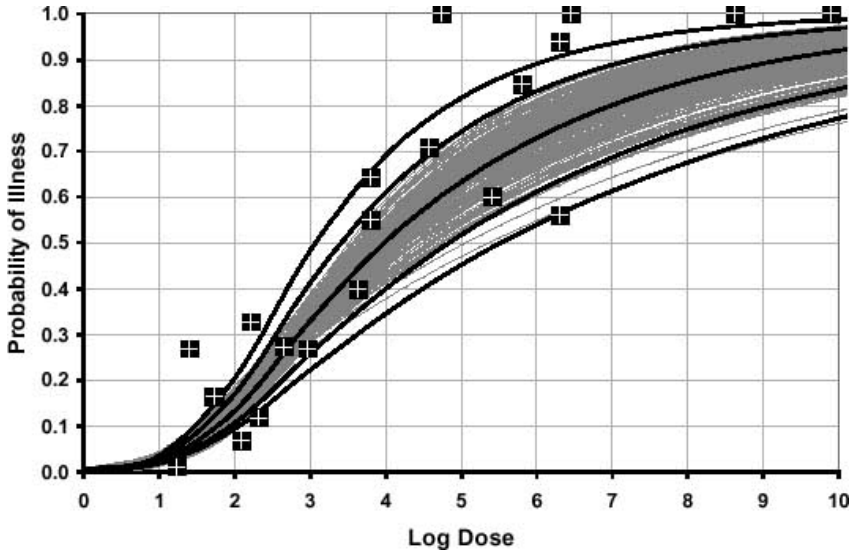

Figure 2. A dose-*vs*-probabilty of infection relationship for all strains of *Salmonella* derived by FAO/WHO (2002). The figure shows the variability in the predicted relationship. Plots symbols represent outbreak data that the analysis was based on.

Staphylococcus aureus

S. aureus is Gram-positive bacterium that is a common resident of the skin, glands and mucous membranes of humans and animals. *S. aureus* produces a range of toxins including enterotoxins that are released into food as they grow in those foods. If *S. aureus* cannot grow toxins will not be produced.

The toxins are heat stable and, once formed, are unlikely to be eliminated by other food processes. The toxins induce vomiting by a specific association with the vagus nerve, i.e., the principal effects are neurological rather than against the cells of the intestine. Nausea and stomach cramps are related symptoms and victims may also experience diarrhoea. In general the symptoms, though unpleasant, are self-limiting, and resolve with 12 – 48 hours. Consequently, it is considered that only a low proportion of cases are ever reported.

Being a Gram-positive bacterium, *S. aureus* is relatively hardy, and survives well in the environment, even dry environments. However, they are relatively slow growing compared to many other bacteria, and are more often associated with foods in which other microorganisms have been inactivated or inhibited (e.g., by cooking, high levels of salt) and which have been

subject to much manual handling. They are the most salt tolerant of the known food-poisoning bacteria, and can grow at salt levels of ~15% (a_w ~0.86). They can grow at temperatures from 7 – 48°C, pH range 4 – 10. Growth is faster under aerobic conditions.

S. aureus often cause minor skin infections, e.g., of scratches and sores, and these can serve as concentrated reservoirs of the organism from whence they can cross-contaminate foods. *S. aureus* is the most important cause of mastitis in milk-producing animals (Little *et al.,* 2008) and it appears to be more common in ewe's and goat's milk, than cows. In a study of the microbiological status of cheese at retail in the United Kingdom, 2% of goat's milk cheese contained *S. aureus* at levels ≥ 10^4 CFU.g^{-1} while 1% of cow's milk cheese contained *S. aureus* at those levels. In sub-clinical animals, levels in the secreted milk can be up to 10^4 CFU.mL^{-1}. Levels in bulk milk tanks (where milk is pooled and held on farms prior to pick-up by tankers) appear to be typically a factor of ten lower than the milk taken directly from the animal, irrespective of herd size. Howard (2006) reported that *S. aureus* was repeatedly isolated from bulk tank milk (BTM) in Waikato, New Zealand, in four out of seven farms. Levels in the BTM were from 0 to 1000 CFU.mL^{-1}. Mahony and Austin (1991) reported that 27% of samples of bulk milk in Ireland contained mastitis pathogens at levels >10^4 CFU.mL^{-1}, and 11% of samples had levels of mastitis pathogens >20,000 CFU.mL^{-1}. Rysanek *et al*. (2009) examined BTM from the Czech Republic. Among 36 samples containing *S. aureus*, the mean logCFU.mL^{-1}was 2.532 ± 0.316. Murdough *et al*. (1996) using naturally contaminated milk from sub-clinically mastitic cows, found an average *S. aureus* level of $10^{4.1}$ CFU.mL^{-1}. Rosengren *et al.* (2010) found that 69%of raw milk cheeses in Sweden were positive for *S. aureus* and 16% of all raw milk cheese samples had levels > 10^5 CFU.g^{-1}. In that study, the highest level was 6.56 log(CFU.g^{-1})in a raw milk cheese made without a starter culture and those authors observed that levels in raw milk fresh cheeses were significantly lower when starter cultures were used. *S. aureus* is generally regarded to be a 'poor competitor' in mixed cultures, and this observation may be an example of the Jameson Effect (Cornu *et al*., 2010).

S. aureus is found on the skin of animals and people. Perhaps not surprisingly, then, *S. aureus* is a common contaminant of artisanal raw milk cheeses and is a common cause of cheese-borne disease outbreaks. In France it is by far the most frequently identified cause of foodborne outbreaks linked to milk products (de Buyser *et al*., 2001) and Kerouanton *et al.* (2007) further report that of 31 well-investigated *S. aureus* outbreaks in France from 1981 to 2002, 13 were due to raw milk cheeses, including semi-hard cheeses. In 2009 further *S. aureus* outbreaks in France were linked to raw milk cheeses. In all cases where samples were available for microbiological analysis, counts of *S. aureus* were >10^5 CFU.g^{-1}. In Brazil, Moraes *et al*. (2009) found that 30.9% of raw milk soft cheeses contained >10^4 CFU.g^{-1} of *S. aureus.* To place this in to perspective, Irish regulations tolerate up to 10^5 CFU.g^{-1} in raw milk cheeses, though only 1000 CFU.g^{-1} in

pasteurized milk cheeses. Nonetheless, despite this greater tolerance 14.3% of raw milk cheeses samples exceeded this limit, whereas only 3.7 % of pasteurized milk cheeses exceeded the relevant limit.

Listeria monocytogenes

L. monocytogenes causes rare, but frequently fatal, systemic infections. It is a common inhabitant of wet sites where decaying organic material is available including decaying vegetation in natural environments or in soils, or filth or food residues in food processing plants or dairies. *L. monocytogenes* is also carried asymptomatically in 5 to 10% of food animals, and humans. Listeriosis, the infection caused by *L. monocytogenes*, usually only affects consumers with known predisposing conditions that reduce immune function. Such consumers include the very young (unborn and neonates up to ~30 days after birth), the elderly (because immune function begins to decline progressively from about age 60 onwards), pregnant women, and people with diseases (e.g., AIDS, alcoholism) or undergoing therapies (e.g., after organ transplants or auto-immune diseases, anti-cancer therapies) that compromise immune function. These people are sometimes described as the YOPI (**Y**oung, **O**ld, **P**regnant, **I**mmunocompromised) group. Members of the YOPI group are between 10 and 1000 times more susceptible to listeriosis than otherwise healthy adults.

Listeria can cause mastitis in milk-producing animals and the organism first came to prominence as a foodborne pathogen due to an outbreak involving nearly 300 people and 85 deaths that occurred in Los Angeles in 1985. The outbreak was traced back to a fresh-style cheese, common in the latin-American community, and to which unpasteurized milk was added. Another major outbreak occurred in Switzerland, also linked to raw milk cheese, but these may have been contaminated after processing.

Ryser (1999) comments on the feasibility of making cheese that is free from *L. monocytogenes* from raw milk. He notes that in USA, according to FDA regulations, 16 styles of cheese are not permitted to be able to be made from unpasteurised (or equivalently treated) milk. These styles all have short maturation times, or have low acidity and/or high moisture levels (high water activity). Thirty-four styles of cheese are permitted to be made from unpasteurized milk, provided that they are held for a minimum of 60 days at a temperature ≥ 1.7°C (35°F). However, it has been shown that *L. monocytogenes* can survive these conditions in a variety of cheeses (Ryser, 1998, Table 15), and that inactivation during cheese processing is typically from 1 to 3 $\log CFU.g^{-1}$.

This accentuates the need to use milk of very high microbiological quality for manufacture of cheeses from raw milk. Ryser notes that ~4% of raw milk in USA is contaminated with

L. monocytogenes. A review by Lake *et al.*(2005) of international literature also suggests a mean frequency of contamination of raw milk of ~4 – 5%, but also as high as 12% in two reports in United States of America.

Essentially, many commentators and authorities (Ryser, 1998; Bemrah *et al*. 1998) have concluded that, for safe production of raw milk cheeses, Listeria-free milk is required, particularly for cheeses that could allow the growth of *L. monocytogenes*. However, for foods, including cheeses in which growth is not possible, improved understanding of the ID_{50} of *L. monocytogenes* has resulted in both Codex Alimentarius Commission and EFSA to propose that levels of up to 100cfu/g at the point of consumption, are tolerable and do not pose a risk to public health.

This has arisen because recent risk assessment and animal model studies (FDA/USDA/CDC, 2003; Chen *et al.*, 2003; FAO/WHO, 2004; Williams *et al*., 2009) suggest that the ID_{50} (dose required to cause infection in 50% of cases) for *L. monocytogenes* is of the order of hundreds of millions of cells, even among the immunocompromised population. Wide variability in ID_{50}s are inferred from animal studiesand variation in human susceptibility (particularly among severely immuno-compromised people) ranging over seven orders of magnitude (*see* Table 2.11 in FAO/WHO, 2004). The studies referred to above have suggested that, even for the YOPI group the dose of *L. monocytogenes* required to cause illness is much greater than 10,000 cells, so that a level of up to 100 cells per gram in foods does not pose an unacceptable public health risk.

Campylobacter species

While they are often found to contaminate raw milk, there is little evidence that *Campylobacter* spp. can survive cheese-making processes. Figure 1 indicates that *Campylobacter* spp. are, at present, an infrequent cause of outbreaks due to raw milk cheeses. In dairy product-related outbreaks of Campylobacteriosis, raw milk is the most common vehicle of transmission. Studies in Canada, Germany and Italy did not find *Campylobacter* in cheese at retail, but other studies *have* recovered these organisms from cheese products and at least two outbreaks related to very fresh cheeses in USA have been documented (FSNet, 2006; CDC, 2009).

IFTS&T (2003a) recommends against specific challenge studies with *Campylobacter* spp. because other organisms, *e.g.* Salmonella, have similar routes of contamination, are more hardy in foods, and are easier to culture. Furthermore, *Campylobacter* spp. do not grow at temperatures less than ~30 – 32°C nor at water activity <0.99 (i.e., greater than 2% NaCl) and beyond these levels inactivation ensues.

Campylobacter spp. can grow at pH in the range 4.9 – 9.5 (FSANZ, 2009).

Campylobacter spp. are intolerant of osmotic stress in comparison to other enteric foodborne pathogens possibly due to poor capacity for uptake or synthesis of compatible solutes (Park, 2002). Thus, while *Campylobacter* spp. are contaminants of raw milk and have been associated with outbreaks from raw milk, and rarely from fresh cheeses, they are unlikely to survive in cheese and therefore are not considered a relevant hazard. The virtual absence of cases from ripened cheeses provides support for IFTS&T's conclusions and recommendations.

Brucella spp.

New Zealand is currently considered to be free from species of *Brucella* that are known to be able to cause human illness. While *Brucella ovis* is endemic in New Zealand sheep (MAFNZ, 2010) it is not regarded as a human pathogen (eMedicine, 2010). NZMOH (2010) reports that since 1997 there have 12 cases of Brucellosis in New Zealand but all of them were acquired outside of New Zealand. NZMOH (1997) further notes that there has been no evidence of New Zealand-acquired cases of Brucellosis since cattle in New Zealand were declared free of Brucellosis in 1998.As such, cheese-borne transmission of *Brucella* spp. does not appear to be a realistic risk in New Zealand at present.

Clostridium botulinum

C. botulinum is a spore-forming, Gram-positive bacterium. It is a strict anaerobe and will not grow unless oxygen levels are very low. It produces the most potent naturally occurring neurotoxin currently known which is produced during growth of the organism. As such, prevention of growth of the organism renders products safe. Levels of $>10^5$ cfu.g^{-1} are usually required to produce sufficient toxin to cause illness[11]. The toxin, called botulin, blocks transmission of acetylcholine, which is the compound responsible for transmission of nerve impulses between nerve cells. As such, ingestion of botulin causes paralysis (described as 'flaccid paralysis') that eventually leads to respiratory and heart failure. With prompt diagnosis and treatment (passive immunization with anti-botulinum antibodies; respiration support) the fatality rate has been reduced but is still ~50%. The toxin can remain active in the body for months to years and during that time patients often requires intensive medical support (e.g., mechanical respiration, intravenous feeding) and physiotherapy to restore normal function.

[11] An exception to this general conclusion is infant botulism, in which *C. botulinum* spores ingested are able to germinate and establish infection in the gut of infants without well-developed indigenous gastrointestinal microbiota. The spores can be ingested *via* application of honey to the teat of a baby's bottle, used to encourage the infant to suckle.

Two main types of *C. botulinum* are distinguished, based on their proteolytic capacity, i.e., 'proteolytic' or 'non-proteolytic' strains. The significance is that proteolytic strains can cause observable degradation of the food they contaminate, thus providing some warning that the food may be unsafe. Non-proteolytic strains do not affect the taste, odour or appearance of food even if lethal levels of botulin are present. Botulin is heat labile and is usually denatured by normal cooking, so that botulism most often is caused by foods that are not thoroughly (re)heated prior to consumption.

There are seven types of *Clostridium botulinum* which are usually considered as four groups. Only Groups I and II include types known to cause human foodborne botulism. Group I strains are characterised by being proteolytic, having a minimum growth temperature of 10 - 12°C and being able to grow in the presence of 10% NaCl. Types A, B and F are in group I. Group II comprises non-proteolytic strains including type E, and variants of type F and type B. They are able to grow at temperatures as low as 3.3˚C but have less heat resistant spores ($D_{100°C}$<0.1 min) than type A strains ($D_{100°C}$ 25 min). *C. botulinum* type E, and Group II types B and F cannot grow in the presence of > 5% salt (a_w 0.970 - 0.971) whereas other strains can grow in the presence of 8 - 9 % salt (a_w ~0.94). The lower pH limit for growth of proteolytic *C. botulinum* is generally considered to be ~4.7 while non-proteolytic strains are unable to grow at pH<5.0 – 5.2 (Adams and Moss, 2008).

The incidence of botulism appears to be higher in some regions than others, and is known to be associated with specific ethnic foods. Contamination often arises from soil or sediments. Most cases arise from home prepared canned, or bottled, vegetables that are of low acidity, and that are inadequately heated during the bottling process, so that viable spores remain, germinate and grow and are then eaten without further cooking.

C. botulinum is a rare cause of cheese-borne disease, but thermal processed cheese spreads are known to be at risk from contamination with, and growth of, this organism. A case occurred in Australia in 2007 involving a 25 year old man who ate cheese spread included as part of a snack meal. Processed cheeses, being less acidic and having no other microbiota (processed cheese is pasteurized during the processing), rely essentially on salt level and moisture control to prevent *C. botulinum* outgrowth. The risk of botulism from cheese spread received much research attention in the 1980s (Tanaka *et al.*, 1982; Tanaka *et al*., 1986) and models were developed from those studies for cheese spread formulations that would prevent growth of *C. botulinum* and those models are still in use today.

In 1914 home made cottage cheese was implicated in a small outbreak (that caused three deaths) and in 1993 in Georgia, USA, an outbreak involving 5 people, one of whom died, was traced to a pasteurized cheese sauce used in a delicatessen that also served food to patrons (Townes *et al.*,

1996). Aureli *et al.* (2000) reported an outbreak of botulism due to mascarpone cheese used in a dessert. That outbreak occurred in various parts of southern Italy and involved eight young people all of whom consumed desserts incorporating mascarpone produced commercially by one company. A15 year old boy died from the intoxication.

Fortunately botulism remains a rare disease and in New Zealand there have been no cases reported since the first report in 1985 (Flacks, 1985; NZMOH, 2010), which may suggest that *C. botulinum* is not prevalent in New Zealand environments. Gilbert *et al*. (2006) reviewed data from surveys for C*. botulinum* in New Zealand habitats. No *C. botulinum* was found in 498 New Zealand coastal sediment samples. Of 250 strains *C. botulinum* isolated from meat samples, meat processing plants, animal hides, soil and vegetation in New Zealand, all were non-proteolytic (Group II) types B, E or F. However, none of the isolates carried botulinal neurotoxin genes, Based on further analysis of DNA sequence homology it was concluded that some New Zealand isolates are true non-toxigenic *C. botulinum* while others need to be classified as different species. An environmental study of the presence of *C. botulinum* in New Zealand ponds and waterway sediments detected types C and D in 11 of 20 sites in the Auckland area. Samples from other urban North Island sediments were negative. It was concluded that, although the survey in Auckland was limited, the results indicate the rarity of *C. botulinum* in New Zealand.

Despite the alert of IFST (1998; *see* Section 3) given the information presented above it appears that *C. botulinum* is most likely to cause a problem in cheese in which other organisms have been eliminated (e.g., by post-fermentation pasteurization/sterilization), thereby giving *C. botulinum* (which, being a spore-former, can survive pasteurisation) an opportunity, after germination, to grow. Furthermore, given that toxigenic *C. botulinum* appears to be a rare organism in New Zealand, it appears to represent a low risk to consumers of New Zealand-produced raw milk cheeses.

Mycobacterium spp.

Tuberculosis is a serious infection in humans, characteristically of the lungs, caused by the bacterium *Mycobacterium tuberculosis.* The virtually indistinguishable *Mycobacterium bovis* causes tuberculosis in cattle but can also be transmitted to humans. Children, in particular, are more susceptible. *M. bovis* can be acquired from dairy products, and the association between bovine tuberculosis and tuberculosis in children was used as an argument for pasteurization of milk in many nations in the early 20th Century. *M. bovis* is also present in farmed and native animal populations in New Zealand (Baker *et al*., 2006), being most prevalent in the introduced Australian brushtail possum from whence farm animals can become infected, albeit infrequently

(Baker *et al*., 2006). Tuberculosis in New Zealand is usually found in immigrants from regions of high endemicity of tuberculosis, and compounded by poverty (Das *et al*., 2006).

The incidence of tuberculosis in New Zealand was 6.9 cases per 100,000 (in 2007), of which 3% are due to *Mycobacterium bovis*. In the period 1992 – 2007, this incidence translated to 54 cases of human tuberculosis due to *M. bovis*. Of those cases, most were associated with contact with wild animals or among men who worked on farms or on abattoirs suggesting occupational, rather than milk-borne, exposure.

While raw milk consumption in rural areas may be relatively common (Lake *et al*., 2009) raw milk consumption is likely to represent only a very low proportion of total milk consumption in New Zealand (Lake *et al*., 2009). As such, the absence of evidence for tuberculosis from consumption of raw milk or raw milk-based products, such as raw milk cheeses cannot be interpreted as evidence that there is no risk, especially as there is strong historical evidence of the association of raw milk consumption with human tuberculosis. However, it should also be noted that, due to recognition of this association, considerable improvements have occurred in animal husbandry practices to reduce the incidence of infected animals contributing to the milk supply. Consequently the overall risk of exposure to *M. bovis* from milk is now much lower than at the beginning of the 20th century (MWLR, 2011). This is due to testing of dairy cows for evidence of infection, and control of native animal populations that are reservoirs of *M. bovis* infection, so as to minimise the level of infected animals.

Lake *et al*. (2009) considered the risk from *M. bovis* from unpasteurized milk in New Zealand. They concluded that the risk would remain low due to the low prevalence in dairy cattle (demonstrated by the Animal Health board testing) and "the indications that the dose-response relationship for ingestion is markedly lower than for the "respiratory route" . They suggested that additional controls be imposed on raw milk and milk products from *M. bovis* positive herds and the *Animal Products (Raw Milk Cheese Specifications) Notice 2009* requires that animal health, including the tuberculosis statusof herds that produce milk destined for raw milk product manufacture, is managed. This measure is intended to reduce the likelihood that milk contaminated with *M. bovis* will enter the food chain.

It is also pertinent to consider the fate of *M. bovis* during cheesemaking, but both Rowe and Donaghy (2008) and Lake *et al.* (2009) observed that the lack of such data limits the ability to assess the risk from *M. bovis* in cheese. Rowe and Donahgy observed that the risk is potentially greater in cheese due to the concentration of bacterial cells in the curd (*see* Section 2.1.3). Trials have now been undertaken, however, by Rowe and colleagues in Ireland (M.T. Rowe, *pers. comm*., 2011) for the UK Food Standards Agency. From those studies, both in model cheese systems and cheese challenge trials with cheddar and caerphilly-style cheeses, inactivation of *M. bovis* was

observed in all trials and typically followed log-linear inactivation kinetics. Differences in inactivation were observed between strains of *M. bovis,* and also cheese type, with D_{10} values usually in the range 30 to 50 days, but up to 130 days in one trial. Given the maturation times for those cheeses, inactivation of between 1.5 and 5 logCFU was observed. As such, with respect to *M. bovis*, raw milk chee

se is at least as safe, on a gram-for-gram basis, and potentially *much* safer than the raw milk that it is produced from. Given the above assessment of Lake *et al.* (2009) it is concluded that at the risk from at least some cheeses produced from unpasteurized milk in New Zealand is also low.

Mycobacterium avium subspecies *paratuberculosis* (MAP) is strongly associated with a wasting disease of cattle and sheep termed Johne's Disease. Since the 1990s there has been growing research interest in the relationship between Johne's Disease and an analogous chronic inflammatory bowel disease in humans, called Crohn's Disease. MAP is found in the bowels of Crohn's Disease patients and the similar etiology of Crohn's Disease and Johne's Disease has led to speculation that foodborne MAP is the cause of Crohn's Disease. Numerous government-sponsored reviews of the scientific and epidemiological literature on the association between MAP in farmed animals and humans have been undertaken (FSANZ, 2004; Gould et al., 2004; AAM, 2008) but all have concluded that there is insufficient evidence to confirm or disprove the proposed association. The most recent review (FSIA, 2009) based on 56 publications between 2000 and 2008 concluded that: "the balance of evidence does not supp

ort a causal relationship between MPA and the incidence of Crohn's Disease". AAM (2008), however, were more equivocal in their evaluation.

Recommendation

Given the above discussion a crude ranking of pathogen risk from preparation of raw milk cheeses can be developed, based on evidence of reported outbreaks, likelihood of contamination of raw milk and potential for survival of cheese-making processes, and severity of illnesses caused by the respective pathogens.

The pathogens of most immediate concern are pathogenic *E. coli, L. monocytogenes* and Salmonellae. *S. aureus*, while causing a milder disease, apparently is a common cause of illness from artisanal cheese, particularly in France.

Whilst *Mycobacteria* are also relatively common, and can cause severe, long-term illness, the low incidence of infection in cows and management of dairy herds to minimize infections makes it a relatively low risk from unpasteurized milk. Furthermore, the practicalities of undertaking challenge trials are such that it is unlikely challenge trials will be undertaken outside specialist facilities. At a pragmatic level, *Mycobacteria* are very slow growing and difficult to culture in

matrices with a complex microbial ecology (e.g. cheese), and level three physical containment is required.

Campylobacter spp. whilst common in milk producing animals and common causes of foodborne disease are relatively fastidious in their growth requirements and are unlikely to survive any conditions that do not allow survival of *E. coli* or Salmonellae. Thus, a successful challenge trial with *E. coli* or Salmonellae can be regarded as a conservative proxy for the potential risk from *Campylobacter* spp.

In the context of their (low) prevalence in New Zealand animals and environments, *Brucella* spp. and *C. botulinum* are low risks and low priority for challenge studies of raw milk cheese-making processes.

This ranking also generally agrees with the list presented at the beginning of Section 3, including the conclusions of Epicentre (2008) after taking into account the specific status of New Zealand with respect to some potential pathogens and the potential for *E. coli* or *Salmonella* to be conservative proxies for *Campylobacter*.

4.1.2 Selection of Strains: General Considerations

While certain pathogens may be expected to contaminate specific foods from time-to-time, there is less certainty about which strain of a species of bacterium will contaminate such products. As described earlier, there are many sources of microbial contaminants in milk, and of finished cheese. Within a given species, different strains will have different tolerances to the hurdles imposed either in the environment in which the food is harvested or processed, or in the food itself. Equally, processing conditions vary so that different strains may have an advantage in different batches of product, due to batch-to-batch variability. It is not usually possible to know whether one strain will represent the greatest risk (e.g., of growth, or survival) under all circumstances and, as discussed, it is not practical (either in time or financially) to test every strain against every set of conditions. For this reason most challenge trials involve challenge with a number of strains so as to increase the chances that worst-case scenarios will be assessed. Generally it is suggested that 3 -5 strains should be used, but NACMSF (2010) recommend that when there is little known about variability or the behaviour of the organisms in analogous foods, up to 10 strains may be required.

4.1.3 Cocktails of Strains

When multiple strains are used they could be tested individually but this will be time- and labour-intensive. Thus, a combination or “cocktail” of strains is often used in a single challenge trial instead. In either case, the strains selected should include those believed to be most tolerant

(i.e., slowest inactivation rate, or fastest growth rate) under the conditions expected to occur during the preparation and subsequent storage of the cheese. This includes consideration of tolerance to low water activity, relevant temperature extremes, acidic pH, specific organic acids, *etc*. Ideally, strains selected would be isolated from the product/process under consideration or analogous product and, if possible, the selected strains should be those that are known to be resistant to the conditions imposed during processing and in the product. Inclusion of strains that are known to be virulent and to have caused outbreaks from analogous products is also desirable, as is the inclusion of a strain(s) that is very well characterised from other research studies, and which can then serve as a point of reference for comparison of the challenge trial results to other published studies. Ideally, several of these attributes might be found in single strains, thereby reducing the total number of strains to be included in the cocktail but this will not usually be the case. Fortunately, many of the organisms that would be considered suitable for challenge studies have been characterized and are available from international culture collections.

When cocktails of strains are used it is preferable that the strains can be distinguished from one another. This is useful to be able to determine whether one strain dominates at the end of the process. In that case, the characteristics of that strain can provide additional insights into traits that characterize resistant strains and which may be useful to design steps/processes to better control those organisms in the processing environment and the product itself.

Numerous methods are available for differentiation of strains, most being based on recognition/identification of specific DNA sequences that are present in all strains, but subtly different in the strains of interest. These methods include restriction fragment length polymorphism (RFLP), multilocus sequence typing (MLST) and derivatives, repetitive element polymorphism (REP-PCR) typing, ribotyping, pulsed-filed gel electrophoresis, etc. In general, methods based on PCR (i.e., the polymerase chain reaction) are probably more accessible because of the wide uptake of that technology in both research and diagnostic laboratories. A potential weakness, however, is that those typing schemes are still evolving and lack universal endorsement. Serotyping alone is likely to be of limited values for differentiation of some pathogens of concern in raw milk cheese (e.g., *L. monocytogenes*) where there are limited serotypes differentiated and only some of those are associated with foodborne illness. Serotyping may be more appropriate for Salmonellae where extensive serotyping information is available for over 2000 strains. Various naturally-antibiotic resistant strains can also be useful for differentiation. Strains can also have various genetic markers introduced (by molecular biology techniques), including antibiotic resistance, or green fluorescent protein, etc.

If using genetically-modified or naturally occurring marked strains its important to ensure that they have the same (important) characteristics as the parent organisms, or at least for those traits that are important to growth and/or survival of the organism in the product and this task could prove onerous.

It must also be demonstrated that the marker is stable within the test strain, i.e., that the gene is not lost, and is expressed under the (often stressful) conditions expected to be experienced by the organism during the processing of the cheese.

Another consideration when using cocktails of challenge strains is the need to evaluate whether the strains inhibit one another within the conditions of the experiment. This usually only involves consideration of whether strains actively produce compounds that inhibit the growth of other challenge strains and this can be assessed relatively simply by inoculating strains on agar plates and streaking them at 90° to one another to determine whether there are zones of inhibition.

Suggestions for cocktails of challenge strains relevant to cheese manufacture are presented in Section 4.1.4 below.

4.1.4 Surrogates/Avirulent Strains

As is discussed in Section 4.1.5, the challenge study should be conducted under conditions that reproduce the actual production of the cheese as closely as possible. Thus, ideally, the challenge trial would be conducted in the cheese processing plant, by the cheese maker with all conditions and techniques being those normally employed, except with the introduction of challenge strains to the milk prior to commencement of cheese-making (e.g., the warming of the milk prior to the introduction of the starter culture)[12]. Clearly this will not be possible due to the risks to public health and the difficulty of complete disinfection of the cheese-making equipment and the cheese making plant/room etc. after the challenge trial. Even in food processing research facilities there is some reluctance to introduce live pathogens to equipment for reasons of adequacy/feasibility of disinfection after the processing, and disposal of large volumes of potentially contaminated waste. However, given the potential difficulties of completely replicating a commercial, artisanal cheese-making process in a laboratory, the 'in-plant" challenge trial might be preferable. In these situations, however, non-pathogenic surrogates may be an alternative. As for the selection of pathogenic strains for challenge trials, the response of surrogate organisms or avirulent strains must be representative of the responses of the most tolerant pathogenic strain so that the likelihood of representing the worst-case outcome, leading to conservative ("fail-safe") decisions about the safety of the cheese process and product, is maximized. IFT (2003a) recommend that

[12] As noted, in some challenge trials contaminatnts might be added as post-processing contaminants, e.g. during curd handling, or ripening, etc.

"If a surrogate is to be used it should be well characterized prior to its use in the study…" and add that "The use of surrogates should be limited only to those cases where specific pathogens absolutely cannot be used for product safety or personnel safety reasons".

If the circumstances do require the use of surrogate organisms, IFT (2003a) consider that an ideal surrogate is a strain of the target organism, or a closely related species that retains all characteristics *except* its virulence. NACMSF (2010) add that surrogates should have inactivation characteristics and kinetics that can be used to predict those of the pathogen, have similar susceptibility to injury, be easy to prepare as high density cultures that are stable until used, should be able to be easily enumerated and differentiated, have similar attachment capacities, and be genetically stable.

A number of avirulent strains and species closely related to the pathogens of interest have been used as surrogates in food safety challenge trials.

Generic strains of *E. coli* are often used as substitutes for *E. coli* O157:H7. According to IFT (2003a), however, caution must be used with the latter however, since generic strains do not have the same level of acid resistance as *E. coli* O157:H7. In fact, this statement is now known to be untrue. Generic strains *do* display the same range of acid tolerance as EHEC or other pathogenic strains (Duffy *et al*., 2000). The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia developed a set of five strains of non-pathogenic acid tolerant *E. coli* that were to be used for challenge trials in fermented meat products, a somewhat analogous system to cheese. The strains were selected to be surrogates for EHEC strains based on acid tolerance. Those strains can be obtained from CSIRO's Division of Food Science and Applied Nutrition, Cooper's Plains Laboratories in Queensland, Australia.

Listeria innocua is very closely related to *L. monocytogenes,* and differs essentially only in that it does not contain a 10-kb virulence locus, i.e., a cluster of genes that engenders pathogenicity to *L. monocytogenes*. For this reason, *L. innocua* has been used as a surrogate for *L. monocytogenes* in some studies. *L. innocua* M1 was recommended by Friedly *et al*. (2008) as the principal surrogate for *L. monocytogenes* in thermal inactivation studies. *L. innocua* also has similar inactivation and growth profiles to *L. monocytogenes* in response to ultraviolet light, flash pasteurization, ionizing radiation, and organic acid salts (Sommers *et al*., 2008; Sommers *et al*., 2009). Salts of organic acids dissociate and behave *in vivo* just as the acid species, but do not acidify the product. Thus salts of organic acids (primarily lactate or diacetate) are used in non-acidic products. As such, the above lists of conditions under which *L. innocua* responds similarly to *L. monocytogenes* may not be relevant to fermented foods, such as cheese, in which acidification and desiccation are the main hurdles to microbial growth. Avirulent strains of *L. monocytogenes* are also known for which the lack of virulence relates to faulty internalin proteins. The fault renders the strains

incapable of invading host cells and derives from premature stop codons on the internalin gene that encodes a truncated internalin protein that is dysfunctional. Such avirulent strains might also be developed from known parent strains.

NACMSF (2010) notes that *Clostridium sporogenes* PA3679 has proven to be an excellent surrogate for *C. botulinum* in studies of low acid canned food. They add, however, that properties of *C. sporogenes* PA3679 match those of *C. botulinum* in thermal processes, but not in high pressure treatments, citing this as an example that if no directly relevant studies are available to justify the choice of the surrogate, studies need to be conducted to establish the validity of that surrogate for a specific pathogen-product-process combination.

4.1.5 Recommended Strains

The following discussion identifies sets of strains that are intended to satisfy the above criteria for selection of challenge strains. Where relevant, a strain originally isolated from the product under challenge, or the processing environment, or involved in an outbreak in New Zealand, could be substituted for one of the nominated strains.

Escherichia coli

The ecology and responses of *Escherichia coli* in various foods have been extensively studied both due to the traditional use of a *E. coli* as a 'model' species and its increasing importance as a foodborne pathogen. Due to the number of studies it is difficult to nominate the 'best' set of strains for challenge studies. Some strains, however, have been extensively studied in response to growth rates, limits and rates of inactivation under non-thermal inimical conditions. The University of Tasmania as been very active in this area and the results have been published in the international refereed literature. Particularly well-studied strains from that group include strains M23, R31, MG1655 and SB1 (Salter *et al.*, 1998; Ross *et al.*, 2003; Ross *et al.*, 2004; Hages, 2010). M23 is non-pathogenic but has been shown to be as acid-tolerant as other enterohaemorrhagic strains. Despite that it is non-pathogenic the large amount of information about it, including challenge studies in cheese make it a good candidate for inclusion in challenge studies. Strain M23 was also used in the development of a model for nonthermal inactivation of *E. coli* in fermented meats, an environment somewhat analogous to cheese. The data from *E. coli* M23 were representative of a wide variety of other strains (Ross *et al.*, 2003; Ross *et al.*, 2004), including pathogenic strains (Salter *et al.*, 1998). The University of Tasmania has undertaken numerous studies with this organism, often in parallel with other *E. coli* strains including the

enterohaemorrhagic strains R31 and SB1, the reference strain MG 1655 and, latterly, the full genome-sequenced enterohaemorrhagic strain Sakei (Kocharnuchitt *et al,* submitted).

Recommended surrogates

In response to the need to undertake challenge tests of fermented meat processes, in which enterohaeomorrhagic *E. coli* are the most serious hazards, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia undertook studies to develop a suite of non-pathogenic strains that could be used for 'in-house' challenge trials (Duffy et al., 2000). These strains can be obtained by contacting Ms. Lesley Duffy, at:

CSIRO Food and Nutritional Sciences - Coopers Plains
PO Box 745
Archerfield BC QLD 4108
Australia

Phone: 1300 363 400*
Alt Phone: 61 3 9545 2176

Email: Enquiries@csiro.au

Tan *et al.* (2008) also used a set of non-pathogenic *E. coli* as surrogates in challenge studies in soft cheese in Victoria, Australia (*see below for contact details*).

Listeria monocytogenes

All strains of *L. monocytogenes* are considered to be pathogenic (FAO/WHO, 2004; EFSA, 2007) although strains of 'lineage 1' (serotypes: 1/2b, 3b, 4b, c, d) are more likely to be involved in human infections and outbreaks. The University of Tasmania has also conducted and published the results of many studies on the ecology of *L. monocytogenes* in foods and studies to assess the tolerance of a large number of strains to acid, organic acid, and high salt levels (Shabala *et al*., 2008; Bowman *et al.*, 2011). Additionally, that group have conducted numerous challenge trials with *Listeria monocytgenes* in the presence of salts of organic acids in processed meats (Mellefont and Ross, 2007; Mellefont *et al*., 2008) and also in cheeses (Hages, 2010) and mapped the growth boundaries in response to pH, water activity and lactic acid (Tienungoon *et al.*, 2000). In those studies the reference strain Scott A (serotype 4b) was extensively used, as was the fully sequenced strain EGD (ATCC BAA-679, serotype 1/2a). In cheese challenge studies (Hages, 2010) strains 79-2759 (serotype 4a) and 79-0430 (serotype 4a) of ovine origin were used because of their high pH and salt tolerance respectively. However, other strains described in Shabala *et al.* (2008) may be more relevant by virtue of serotype, resistance to salt and acidity and originally isolated from cows, sheep or goats. Included in this category would be strain 80-

2901 (min pH 4.1; max NaCl 12.1%; ovine origin; serotype 4b) and strain 77-2294 (min pH 4.2; max NaCl 13.9 %; ovine origin; serotype 4b). Buchanan *et al.* (1997) developed models for non-thermal inactivation of *L. monocytogenes* using a cocktail of strains including Scott A, HO-V-5 and V-7. Other recent challenge studies in cheese include Angelidis *et al.* (2010), Samelis *et al.* (2009), Kagkli *et al.* (2009) all of whom included Scott A, among other strains in their set of challenge organisms.

Recommended surrogates

Several authors have considered the use of *L. innocua* as a surrogate for *L. monocytogenes* in studies of inactivation/survival in cheese. Samelis *et al*. (2009) used both *L. innocua* and avirulent strains of *L. monocytogenes* and found no difference in survival in the cheese they challenged with those strains. Liu *et al.* (2009) specifically considered the utility of *L. innocua* as a surrogate for *L. monocytogenes* in the challenge trials for camembert cheese. Their study involved assessing *L. innocua* survival in parallel with that of *L. monocytogenes* at different time and at different locations with the developing cheeses (i.e., centre, or side, or rind etc). They found growth did occur, and was faster near the surface of the cheese compared to the centre, but found no significant difference in the trends of survival and growth of *L. monocytogenes* and *L. innocua* in the camembert cheeses. They concluded that *L. innocua* was a suitable surrogate for *L. monocytogenes* challenge trials during ripening of soft cheese.

Salmonellae

There was a spate of Salmonellosis outbreaks implicating cheese products in the 1970 and 1980s. These led to a series of research papers to understand the ecology of Salmonella in cheeses, but since the mid-1980s there has been little published research on Salmonella in cheese. Among those studies (e.g., Kasrazadeh and Genigeorgis, 1994; Glass *et al*., 1998; Linton and Harper, 2008) there seems little coherence in the strains used for the challenge, with some studies apparently not even involving strains of relevance to cheese (Glass *et al*., 1998). Salmonella is a diverse genus, with over 2500 different serovars now recognized and may explain why there appear to be no obvious universal candidates for challenge studies nor strains common to the studies that have been published in the last decade. Using the criteria identified above, the cocktail should include a widely used or well- characterized strain. In the absence of an obvious experimental strain, the type strain (*Salmonella enterica* subsp. Enterica strain LT2T (wild); ATCC Number: 15277) might be considered. This strain has been sequenced (McClelland *et al*., 2001), and compared to other strains, further supporting its inclusion. Other strains should be selected according to previous use in cheese challenge trials, and implication in outbreaks involving cheese.

Staphylococcus aureus

Tan *et al.* (2008) at the University of Melbourne, Australia, presented results of challenge trials involving three pathogens (*L. monocytogenes, S. aureus, E. coli*) in soft cheeses. Those workers assembled a set of strains of *S. aureus* that included isolates from dairy products and strains from food poisoning cases and a strain from a recognised culture collection. Given that this set of strains satisfies the criteria set out above, offers a set of strains whose survival in a cheese has already been determined, and offers convenience, it is suggested that those strains, or a subset of them, be obtained from those workers. Contact details are:

Ms. Agnes Tan

Microbiological Diagnostic Unit Public Health Laboratory

University of Melbourne

Victoria 3010, Australia.

Fax: +61 3 8344 7833 or e-mail: agct@unimelb.edu.au

If a greater diversity of strains is required, Kérouanton (2007) described cheese-related outbreak strains of *S. aureus* from France. That strain collection represents a useful resource for development of a challenge strain collection in New Zealand.

Recommended surrogates

Given the mild nature of illness due to *S. aureus,* and that it is not an infection but an intoxication resulting from ingestion of highly contaminated foods, the risk involved in using *S. aureus* in a challenge trial is considered low. The affected product would have to be segregated and clearly marked during the trial to avoid accidental ingestion, and destroyed after the trial for the same reasons. Accordingly, surrogates are not proposed.

4.1.6 Selection of Challenge Conditions

As for the selection of challenge strains, the selection of challenge conditions should match the normal processing of the cheese as closely as possible. This should include explicit consideration of the effects of variability (e.g. due to strain variation, variability in product formulation, variation in raw milk quality and composition, etc.) so as to be able to mimic a worst-case situation. Thus, the times and temperatures of process steps must follow the normal production process the normal ratio of starter culture and rennet to the volume of milk, the normal pH at which fermentation is concluded, the normal amount of salt absorbed during brining, etc., and the

time, temperature and relative humidity of maturation must all mimic the normal process as closely as possible. Rather than normal conditions, however, conditions should be more representative of the 'worst-case' scenario by determining conditions that represent the upper range of potential growth when growth is possible, and that represent the lower extreme of inactivation during inactivation steps (e.g., higher than average levels of pH, higher than average levels of water activity, lower temperatures during inactivation steps, higher than average temperatures during growth permitting steps, high levels of pathogens, etc.).

Given the desire to use pathogens directly, rather than surrogates, it is likely that the challenge trial will not be conducted in the normal production facilities nor with the normal production equipment. In this case the design of the challenge trial must seek to identify and reproduce as closely as possible those factors likely to affect the fate of pathogens in the cheese. This will require detailed characterization of the process and the variability within the process before an "off-site" challenge trial can be conducted with confidence.

Whilst milk volumes and additives can be balanced to mimic the commercial processes, and while times and temperatures and relative humidity during maturation can also be reproduced, some, potentially influential, factors may not be able to be replicated in a research laboratory. This reinforces the need to characterise the conditions of those processes before conducting a challenge trial "off-site" so that they can be reproduced as closely as possible in the laboratory.

The normal microbiota of the milk that is normally used could affect the development of acidity, as well as generating inhibitory compounds (e.g., siderophores, reactive oxygen species, organic acids, bacteriocins, etc.) in addition to those produced by the starter culture. The complexity of microbial interactions in cheese has been described by others (Irlinger and Mounier, 2009; Pereira *et al*., 2009). Those interactions could be difficult to mimic if the actual milk used for cheese making is not available, e.g., if the challenge trial were undertaken at a site physically distant from the dairy supplying milk to the cheese-maker and, accordingly, wherever possible the same milk as is used by the cheese-maker should be used if at all possible. When conducting challenge trials the significance of this possibility could be assessed by using multiple sources of raw milk to make several batches of the same style of cheese and conducting challenge trials on each of them.

Similarly, it may be desirable for ease of sampling, and minimization of costs and space needs to produce smaller cheeses than those that are produced commercially. A potential problem with this strategy is that the processes of ripening may be affected by surface area: volume ratios of cheese, particularly with regard to desiccation/water activity in the cheese, thereby leading to faster drying or a lower ultimate water activity. These differences could affect the fate of pathogens, e.g. their rate of inactivation, in the cheese. Similarly any packaging should match

exactly that of the commercial product. Thus, if cheeses are matured in packaging it is usually best obtained directly from the stocks held by the cheese manufacturer.

Equally, artisanal cheese makers are likely to be intimately involved in the curd handling steps, and may contribute their own skin microbiota to the curd. The actual handling of the curd is likely to be determined not only from the recipe for the cheese, but also on the expertise of the cheese maker to adjudge firmness, moisture level, etc., again reinforcing the need for a high level of communication between the cheese-maker and those conducting the challenge trials. Ideally, the cheese-maker would produce the cheese under the supervision of the microbiologist overseeing the challenge trial, whether in the cheese-makers own facility or in the testing laboratory.

4.1.7 Selection of a Laboratory

The results of microbiological testing can be affected by apparently innocuous factors and a high level of discipline is required to achieve reproducible results by following specified methods explicitly. This applies both to the challenge trial methodology (e.g., preparation and administration of inocula, holding conditions, sample times strategy, etc.) and methods for enumeration of the challenge organism, as well as any other organisms of interest. Strict adherence to prescribed procedures is more likely to occur in a laboratory that routinely performance such trials, because they will have the facilities, equipment and expertise to undertake the trials.

It is generally recommended that an accredited laboratory be engaged to undertake the tests because they should have in place systems to assure the reliability of test results, e.g. controls on media quality and performance, monitoring of incubation temperatures etc. Laboratories are usually accredited by independent agencies for specific microbiological analyses.

NACMSF (2010) recommends that a laboratory selected for challenge studies must be able to demonstrate prior experience in conducting challenge studies, with personnel with relevant training and experience, and who will not deviate from the experimental design. In practice, experience in challenge trials may not always be found in accredited (e.g., by a recognized accreditation authority such as IANZ) laboratories and other microbiological facilities in research organizations may be engaged instead. In that case it is important that those organization adopt methods that are validated methods for the microbiological tests to be undertaken, i.e. methods in International or Australian/New Zealand Standards or that have otherwise been shown to be equivalent, or superior, to such methods. Where non-accredited laboratories are engaged, they should adhere to all aspects of quality assurance/control of the methodology as would be employed in accredited laboratories. NACMSF (2010) emphasise that failure to use valid

methods and appropriate controls (i.e., on the performance of the method) may render the results invalid and the study unacceptable, potentially requiring additional time and expense to repeat the study.

4.2 Experimental Design

The specific purpose of the study largely determines the selection of pathogens, strains and inoculum levels, choice of parameters to be tested, duration of the study, types of analysis, etc.

4.2.1 Type of Study

The anticipated or desired fate of pathogens in the product, and the levels of pathogens that might be tolerated at the point of consumption will dictate various aspects of the study design such as whether pathogens need to be enumerated, or whether presence/absence methods will be sufficient, whether high or low concentration of pathogens should be used as inocula, whether the inocula should be prepared so as to minimize the potential lag time, or be in stationary phase, or acid-habituated so as to increase survival in inimical conditions.

The origin of cheese, although probably serendipitous, was as a means to preserve milk. Accordingly, through 'trial and error' it would be expected that, for most cheeses, pathogens in cheese would be inactivated (due to the combination of pH, water activity, organic acids and other inhibitory compounds). However, deviations from 'typical' conditions would have occurred and led to food-borne illnesses and traditional processes were not necessarily safe, particularly compared to expectations of the 20th and 21st century consumers about food safety. Moreover, some pathogens (e.g., *L. monocytogenes*) have very long incubation periods and the association between illness and the food consumed was not readily apparent. With large-scale manufacture of cheeses, and/or improved public health surveillance, evidence for the role of pathogens in cheeses in human illness, became apparent. For more sophisticated cheeses, in which the focus is gastronomic pleasure as opposed to milk preservation, growth could occur prior to completion of fermentation *(see e.g.*, Schoder *et al.*, 2003) and, for some surface ripened cheeses, growth of *L. monocytogenes* has been reported (Ryser and Marth, 1987; Banks, 1994). Pathogens believed to have very low infectious doses (e.g., EHECs) must be eliminated (to below limits of detection) before the product is consumed but for others, such as *S. aureus* or *L. monocytogenes* (in some jurisdictions), low levels may be tolerable. This diversity of 'decision criteria' concerning acceptable cheese-making processes means that cheese-making challenge trials may need to be designed to be able to monitor pathogen growth *and* inactivation, and to enumerate some pathogens or to merely ascertain presence of others, i.e., using presence/absence methods. In yet other cases, if levels of pathogens in raw milk are always below levels of concern, demonstration

that growth is not possible within the cheese at any stage may provide the information required to make a regulatory decision. In that case, the experimental design might employ semi-quantitative methods that demonstrate only that the pathogen is below this tolerable limit without needing to determine the actual level in that cheese.

In other cases, one might be interested in understanding which factors could be manipulated to increase product safety, e.g., would higher temperatures during maturation increase pathogen inactivation. In that case, the experimental design would include conditions that are not representative of the normal (i.e., current) commercial process.

In general, however, challenge trials in cheese will be directed at demonstrating pathogen inactivation to acceptably low levels under conditions representative of the least lethal sets of conditions that might be expected under normal commercial processing[13]. The discussion in subsequent sections will assume that this is the intent of the challenge trials relevant to this report.

4.2.2 Duration of the Study

Various authors recommend that challenge trials should be followed for the full shelf life of the product, and most recommend that a margin of safety be included, i.e., to store and test the product at some time *beyond* the recommended shelf life. (This is to emulate the possibility that consumers might hold product beyond its recommended shelf life). Cheese may be an exception to this general rule, however, because pathogen inactivation is generally expected. Thus, in many cases, unless pathogen levels are at (or reasonably predicted to be at) a tolerable level at the end of the maturation stage (i.e., at the time that the product is released into the market), the product would not be considered acceptable for sale. In other words, the appropriate duration of the study is likely to be the processing time, i.e., to the end of the maturation period[14].

If the rate of pathogen inactivation could be well characterized, the rate of inactivation during the distribution of the product to retail outlets and during consumer handling of the product might be considered as part of the decision regarding the safety of the cheese-making process. To do so, however, knowledge of the storage temperature of the cheese during distribution, warehousing, retail display and consumer handling would be needed, as would knowledge of the rate of inactivation of pathogens of concern at those temperatures so as to be able to make such a

[13] An important exception is the presence and growth of *S. aureus. S. aureus* produces an emetic toxin (*see* Section 3) that is heat stable. While *S. aureus* in foods may die, the toxin, once formed, is not readily eliminated by heating or other microbiocidal factors. Accordingly, absence of viable *S. aureus* does not infer absence of the enterotoxin. Accordingly, a 'before and after' analysis of *S. aureus* will not necessarily give a true indication of the risk to consumers, and testing for the toxin may be needed (*see* Section 4.4.1).

[14] As discussed earlier, in surface-ripened cheeses, the pH may increase later in product life and, accordingly, the challenge trial for such cheeses may need to extend for the full shelf life of the product.

decision. The data needed would *not* normally be collected as part of a challenge trial, other than one specifically designed to assess the effect of post-processing handling on the fate of pathogens, e.g., if the cheese were one that changed in composition during post-processing distribution, sale etc. in a way that would potentially enable regrowth of pathogens.

Some authorities suggest that if temperature abuse is factored into the challenge trial, it is not necessary to include an additional period beyond the product shelf life. Again, for evaluation of a particular cheese-making process, temperature abuse would not be included in the experimental design other than that which could occur during normal processing/maturation of the cheese. Importantly, given that increased temperature could *increase* the rate of inactivation of pathogens, once inimical conditions are achieved, higher than expected temperature during cheese-making does not necessarily translate to "temperature abuse", because it would lead to faster inactivation (in a cheese that did not support growth) and would most likely represents a better result than would be expected under normal (average) conditions of processing. Variability in temperature, and how to include it in the challenge trial, is considered in detail in Section 4.2.3, below.

While the levels and fate of pathogens at the end of the maturation period will usually enable a decision about the safety of the process, if there is potential for the conditions in the cheese to change later in the life of the product (e.g., so as to allow growth of some pathogens), this possibility needs to be included in the design of the challenge trial. This consideration applies particularly to surface-ripened cheese during which proteolysis and consequent de-amination of amino acids, which are a normal part of the maturation of those cheeses, can lead to increased pH and allow pathogen regrowth. If this is possible, consideration should be given to including the entire shelf-life of the cheese in the challenge trial.

Even if the pathogen is reduced to below detectable levels at the end of the maturation period, it must be remembered that no method can ever *prove* the absence of the microbe in product, unless the entire product is tested. Even if data suggests that the pathogen has been eliminated, there is a possibility that viable pathogens may still be present, but not readily cultured, and it must be able to be reasoned that pathogens have been reduced to such a low level, or low *probability* of survival, that the probability of regrowth to harmful levels is tolerably low. For example, if the pathogen is shown to be rapidly eliminated, e.g., well before the end of the maturation period, it may be possible to infer that the probability of survival of any cells of the pathogen is so low that it does not represent a credible public health risk and that the process can be considered to reliably eliminate the pathogen.

Finally, recognizing that artisanal cheese-makers are likely to vary processes subtly from batch to batch to optimize them individually, it will be necessary to consult with the cheese maker to

determine the shortest maturation time and conditions that the cheese-maker would use, or to understand the criteria that the cheese-maker uses to determine when the product is ready for sale.

4.2.3 Storage Conditions: Temperature, Atmosphere, Relative Humidity

As noted earlier, the storage conditions of the cheese during maturation should mimic, as closely as possible, the conditions that are experienced by the product during normal production/handling. For some cheeses temperature will be the main storage factor that varies and requires a decision about the actual temperatures for the fermentation and maturation stages of the challenge trial. The relative humidity of the ripening room/chamber may also need to be emulated. For some cheese, e.g., cheddar styles, the product may be packed in a water-impermeable barrier (wax, plastic) and this will also have to be mimicked if the challenge trial is undertaken away from the normal production facility. If a coating or wrapping is applied, the material used should be obtained from the processor so that gas and moisture diffusion into/out of the challenge trial cheeses are the same as the normal commercial product. If the product is vacuum-packed, the pressure within the pack should also match that of the commercial product.

To determine appropriate times, temperatures and relative humidities for the challenge trial, relative humidity and temperature of maturation need to be monitored during normal commercial production. From this a set of *least* inimical conditions should be determined, remembering that:

i) if conditions are inimical to growth, higher temperatures are more lethal so that conservative conditions for the challenge trial will mimic *lower* temperatures during maturation, and

ii) lower relative humidity will lead to faster desiccation so that if water activity contributes to the prevention of growth using the *higher* levels of relative humidity experienced during normal processing might be expected to be more representative of a 'worst-case scenario'.

Where temperature or relative humidity are considered to be important factors, and their levels are controlled during the challenge trial, they should be measured at regular intervals to ensure that control has been achieved throughout the challenge trial, or at least to characterize the variability in the challenge trial conditions so as to ensure the integrity of the results.

Brining of the formed cheese may also occur. Brine concentration can vary over time due to repeated use. The variation in brine concentration should be characterized, or the variation in salt content in the cheese characterized, so as to be able to mimic the least inimical condition likely to be achieved. In general, lower salt concentrations are likely to lead to more conservative

(i.e., worst-case) estimates of the ability of the cheese process to eliminate pathogens or reduce them to acceptable levels.

Washed rind cheeses (e.g., Munster, Morbier and Chimay) are periodically washed with a brine solution during the maturation process. This process will also have to be characterized (e.g., in terms of frequency, time spent washing with brine, brine concentration and content, etc.) so as to be able to mimic the normal commercial process as closely as possible.

As noted earlier, due to logistic (space, ability to make large batches in the laboratory), cost and technical[15]constraints it may be preferable to make many smaller units of the cheese rather than the size of cheese that is normally produced and marketed. A problem with this is that if the cheese loses moisture during maturation, a higher surface-area: volume ratio is likely to increase the rate of moisture loss. This effect could possibly be ameliorated by controlled relative humidity in the ripening room. Analogously, for surface ripened cheese, the rate of maturation of the entire cheese may depend on the thickness of the cheese and the time it takes for enzymes produced on the surface to migrate into the cheese and cause, e.g., pH increases, production of free fatty acids that might be inhibitory to microbial growth etc. In such cases, care must be taken to ensure that the conditions of storage (mainly relative humidity) are such that the experimental cheese matures at the same rate as the commercial size cheese. Demonstration of this is essential to the integrity of the challenge trial results and may require pre-trial experiments, without pathogens, to ensure that the changes in pH, water activity, total counts etc., occur at the same rate in the experimental cheese as in the commercial product. Smaller, airtight storage vessels, for example can be used to reduce the rate of moisture loss in the cheese by increasing the relative humidity around the cheese.

If post-production storage is included as part of the trial the conditions of packaging of the cheese, and realistic storage temperature etc. need to be determined and reproduced in the trial. Considerations concerning storage temperature were discussed above. If a cheese is distributed without a water-impermeable wrapping (e.g., many surface ripened cheese are distributed in foil wrappers only) and could be stored under conditions of high relative humidity (i.e., that might allow water to be absorbed into the product so that the water activity constraint on microbial growth was lessened) the challenge trial should emulate those high humidity conditions to represent a worst-case situation.

[15] These considerations include that the removal of a sample from a cheese may alter the normal processes of ripening, e.g., by increasing the surface area, removing less permeable 'crusts', changing surface microbiota, etc. In this case it might be preferable for each sample to be an entire cheese. Equally, if the normal commercial cheese size is small, it may not be possible to take sufficient samples from one unit of the cheese over the life of the trial (e.g. some cheeses may not large enough to take multiple, replicate 25 g samples at 7 to 10 sampling times, etc.). In such cases multiple cheeses will again be needed.

Many authorities consider that temperature cycling as may occur during "real" distribution, storage, retail display and home storage, should also be emulated. However, as noted above, for most cheeses pathogen growth will not be possible and the lowest storage temperatures represent the worst-case situation with the possible exception of surface-ripened cheeses in which conditions may become growth permissive for some pathogens. (Note, however, that this is probably only true for *L. monocytogenes* because cheeses are usually held refrigerated and this would prevent growth of all other pathogens considered in this report.)

As a final note, if challenge trials can be conducted in the commercial production facility***, samples inoculated with pathogens or surrogates should be segregated and clearly labelled to prevent inadvertent consumption or release to retail sale.***

4.2.4 Formulation Factors

Unless many challenge trials are able to be undertaken to assess the effects of variability in product characteristics (e.g., pH, water activity, milk composition, etc.) from batch to batch, the aim of a challenge trial is to test each factor relevant to pathogen inactivation or growth at its least stressful level within the range of variability that occurs. The rationale is that, given the cost and time limitations to undertake challenge trials, the challenge trial should aim to characterise the worst case (i.e., least inactivation) that could be expected to occur so that decisions made on the basis of the results are likely to be conservative so as to protect public health. Thus, before starting a challenge trial, it will be necessary to monitor those parameters of the product so as to be able to characterize the worst-case conditions.

In addition to the physico-chemical properties of the cheese it is necessary to consider the likely influence of other organisms in the cheese on the survival and/or growth of pathogens. The presence of competitive microbiota can affect the outcome of a challenge study, particularly one determining the *growth* of the pathogen. However, this is likely to apply to only a small range of cheese types. Moreover, it is expected that raw milk cheeses would be made with starter cultures and that these would dominate the microbiota of the cheese, but it is important that the same starter culture is used and used at levels that are used in the normal process. If the challenge study involves making a smaller batch than is made under the normal commercial conditions, then the amount of starter culture used should be reduced proportionally so that the time to commencement of acidification is as close as possible to that in the commercial product. To place the importance of the starter culture into perspective, Schoder *et al*. (2003) reported that the starter culture used in production of raw milk cheeses had a profound effect on the survival of *L. monocytogenes* in a raw milk cheese, with a mesophilic buttermilk culture delaying acidification and allowing a 60-fold increase in *L. monocytogenes* from the milk within 12 hours.

4.2.5 Location of Challenge Study

As discussed above, the challenge study would ideally be undertaken in the premises of the cheese-maker, using the same equipment, milk, starter cultures, maturing rooms etc. as are normally used, but this is unlikely to be possible except in the case of the largest and most well-resourced cheese producers. The main problem is the deliberate introduction of pathogens into a food manufacturing operation and particularly cheese-making, in which there is no well-validated critical control point or corresponding critical limits. Thus, challenge trials of raw-milk cheese processes are likely to be conducted in research facilities, either in a pilot plant or in a food microbiology laboratory. Alternatively, if it is considered impossible to reliably replicate the cheese-making process in a pilot plant or research laboratory, consideration should be given to using non-pathogenic surrogate organisms for the challenge trial as discussed in Section 4.1.4[16].

For reasons outlined above, great attention must be given to ensuring that the cheese-making process reproduces the commercial process as closely as possible. To maximize the likelihood that the challenge trial does mimic the commercial process as closely as possible, it is desirable that the cheese-maker makes the cheese in the laboratory, or other facility, under the supervision of a food microbiologist experienced in challenge trials. There should be discussion with the cheese-maker prior to the making of cheese/challenge trial to ensure that all equipment and resources needed to make the product, in as normal a way as possible, are available in the laboratory where the challenge trial will be undertaken. Similarly, as mentioned in Section 4.1.6, ideally milk from the same source as the normal cheese-making process will be used in the challenge trial. If this involves transport over a longer time than would normally occur, particular attention should be given to the conditions of transport of the milk to the testing laboratory so that the microbial composition of the raw milk is equivalent to that which would normally be used by the cheese-maker.

Refrigerated transport/storage of the milk will almost certainly be required but transporting the milk at a lower temperature, to compensate for (potentially) increased transport time should be considered. While it is impossible to generalize equivalent time-temperature conditions for all the organisms likely to be present in the milk, it is probable that most contaminants in the milk are mesophiles and that their growth during transport of the milk would be prevented by refrigeration to 4 or 5°C. Spoilage organisms are likely to be psychrotrophs and might be expected to have T_{min} values (Ratkowsky *et al.*, 1982) in the range ~ -5 to -10°C. Table 4 indicates temperatures required when the time between milking and cheese-making is varied so as to

[16] Irrespective of the location of the trial it should be remembered that the cheese produced, and the whey remaining after curd formation, may be heavily contaminated with pathogens. Disinfection of the contaminated material is needed prior to disposal of the whey or of the contaminated cheese remaining at the completion of the challenge trial.

produce equivalent microbial growth as that at 4°C, assuming that most organisms able to grow in the milk under refrigeration are psychrotrophs with an average T_{min} of -7.5°C. The values in Table 4 can be used as a guide to temperatures required for transport for times other than those that are normal between the milking and the commencement of cheese-making.

Whatever the location of the trial, product lots used for the challenge study should be representative of the normal production with the exception of the manipulation of acidity, moisture, salt etc., that are *most permissive* to pathogen growth or survival as discussed in Section 4.2.4 above.

Table 4. Indicative temperatures required to compensate for different times between milking and commencement of cheese-making for the purposes of challenge trials.

Time between milking and commencement of cheese making *relative to the normal arrangements*	Indicative temperature (°C) required to achieve equivalent microbial growth of psychrotrophs (assuming that the milk is normally transported/held at 4°C)
0.5	8.8
0.75	5.8
1	4.0
1.2	3.0
1.5	1.9
2	0.6
3*	-0.9
5*	-2.4
10*	-3.9

- the required temperature for these relative times is below the freezing point of milk which could alter the microbiota free of the milk (*see text for more details*)

4.2.6 Sampling times and Sample Numbers

The number of samples required for the challenge trial can dictate the volume of product/number of units of product that need to be produced, and challenged, to generate reliable results from the trial. As such, the number of samples should be determined prior to the start of the challenge trial so that sufficient product is available. Additionally it is usual to include additional samples in case some samples are mishandled and cannot be used with confidence. Additional samples are also required as controls, e.g., uninoculated controls to ensure that the

introduction of the challenge organisms does not alter the physico-chemical properties, or development of normal microbiota, of the cheese.

In principle, only two samples are required[17]: one that enumerates the introduced pathogens in the cheese at the commencement of the trial and another at the end of the trial to determine whether the pathogens initially present have been eliminated, or reduced to acceptable levels or prevented from increasing to unacceptable levels. NACMSF (2010) warns, however, that determining whether a product supports growth of a pathogen or not is rarely as simple as comparing final and initial counts. There are numerous potential sources of variability in cheese-making processes, including the reliability of the sampling process and microbiological enumeration methods. Scotter *et al.* (2001) determined that the variability in the results of the ISO method for enumeration of *L. monocytogenes* in foods ranged from 0.17 to 0.45 logCFU.g^{-1}, depending on the food product and level of contamination. For this reason a greater number of samples will be required when variability is high, i.e., to increase confidence that the results obtained are representative and that differences in counts are significant given the attendant variability.

Samples number will be determined by the number of replicates required at each sampling time and the number of sample times. Sampling times will vary according to the aim of the challenge trial. If the aim is merely to assess the microbiological safety of the process, only two sampling times (with sufficient replicates) are required, as suggested above[17]. However, in many cases the challenge trial will also seek to gain information about which steps in the overall cheese-making process are most critical to the overall efficacy of the process. Moreover, taking samples at different times throughout the process can be considered as a form of replication as well, because the numbers of introduced pathogens in successive samples are not independent, but are related to one another by virtue of the number originally introduced to the cheese and the effects of the processes on that number.

Various commentators suggest that a minimum of five samples should be tested during a challenge trial. In cheese processing a number of distinct steps that might be expected to differentially affect microbial levels in the product can be identified. These include:

i) Warming of the milk prior to addition of the starter culture. In this step pathogen growth could be anticipated.

ii) Fermentation. In this step growth of pathogens could also occur, but might be expected to slow as the pH decreases/organic acid levels increase.

[17] See also footnote 13 which explains the special case of *S. aureus* because it produces a toxin that can persist in food even when no viable cells of *S. aureus* remain.

iii) Curd formation. In this step, concentration of the pathogens into the curd can be expected and must be borne in mind when planning microbiological analyses and consequent calculations and interpretations of pathogen numbers (*n.b*. at this point units of pathogen concentration will change from '*per* mL' to '*per* g.').

iv) Heating of the curd. In some cheeses the curd is heated to aid syneresis, leading to a harder style of cheese. While the temperature used is usually relatively mild, in combination with other hurdles some inactivation of pathogen may occur, or could lead to increased growth rate if conditions allow growth of the target pathogen.

v) Maturation. During maturation it is expected that conditions would be inimical to pathogen growth and are likely to cause inactivation of pathogens. As noted earlier, some surface ripened cheeses might allow growth of pathogens later in the maturation process.

It is desirable to enumerate pathogens at the completion of each of the above stages to determine their relative contribution to the overall safety of the product, and thereby to identify cheese-processing steps that are most critical to the microbiological safety of the product. This knowledge will be important for the development of food safety management programs (e.g., Risk Management Programme or Food Safety Programme) specific to the production of particular styles of cheese. Following from the above discussion it should be apparent that there is no need to take and process samples at regular time intervals. Rather, the aim should be to characterize the effects of distinct process steps. In this regard, it may be desirable to not only determine the net change in pathogen levels during each step, but also to determine the kinetics (rate of change) of microbial changes during that step. Quantification of the rates of change can facilitate extrapolation of changes and prediction of the effects of increased or reduced time or conditions for different process steps. This is likely to be most relevant to the cheese maturation steps, for which there is greater scope for varying duration, e.g., to achieve greater levels of inactivation.

As noted above, while single samples are, in principle, adequate, the numerous sources of variability in microbial levels, e.g., due to the variability in the cheese, the distribution of organisms in the cheese and methods employed, it is preferable to process multiple samples at each sampling time. Processing of replicates of an individual samples (e.g., duplicate plates prepared from a single dilution series) are relatively unimportant, as they only provide information about the reproducibility of the techniques used and operator processing the sample. More valuable information can be obtained by enumerating pathogens in replicate distinct samples (whether material drawn from a single cheese, or samples taken from different cheeses produced in the same batch) and yet more valuable information could be obtained by processing analogous samples from distinct *batches* of the cheese. The latter samples would characterize batch-to-batch variability. Samples from replicate cheeses from a single batch characterize the

variability due to heterogeneity in the cheese, e.g., regions in a mould-ripened cheese that develop different physicochemical properties from other parts of the same cheese (i.e., because they are, or are not, directly in contact with the mould). If samples at each sampling time are drawn from the same cheese, or samples are drawn from individual cheeses produced in the same batch, duplicate or triplicate samples are generally recommended. Duplicate samples are the minimum which allow a mean and standard deviation to be calculated, thereby providing a minimal estimate of the extent of variability. If the duplicates produce widely disparate results, the possibility of an anomalous result (i.e., an "outlier") can possibly be identified by comparison with the results of samples analysed at the previous and subsequent sampling time. However, if the variability is large in all samples, the reliability of this approach can be limited. Several commentators recommend that triplicate samples be processed at each time point, so as to increase the chance that if an anomalous result eventuates it will be better able to be recognized as anomalous by comparison with the other two replicates of that sample at that time. If the heterogeneity is random, the part of the cheese sampled should also be selected randomly to maximize the chance that the results will be representative of the whole cheese. In other cases in which the heterogeneity is a characteristic of the cheese, e.g., surface-ripened cheese in which the surface might be expected to be significantly different from the interior of the cheese, it would be more appropriate to systematically sample those different parts to determine whether the fate of pathogens differs significantly in each region.

If a minimalist approach to the challenge trial is adopted, (i.e., that microbial levels are enumerated only at the beginning and end of the trial) it is usual to test a greater number of replicates (e.g., 5 to 10) at both the beginning and end of the cheese-making process to better characterize the potential variability in the fate of target microorganisms during production. The decision to adopt such an approach would also depend on the purpose of the challenge trial, but the information gained by taking samples at multiple time points during the process will generally be more informative than that obtained from a larger number of replicates at only two time points.

It is also desirable to enumerate the organisms in the inoculum to be used for the challenge trial as well as enumerating the pathogens in the food after the inoculation process. Enumerating the inoculum is useful for determining the number of serial dilutions required to enumerate the pathogen in the product. Additionally, in some cases there may be instantaneous loss of challenge organism viability upon introduction to the food and this will be useful additional information to understand the microbial ecology of the cheese and the process. This is possibly more likely to happen in cheese challenge trials that are designed to evaluate the consequences of post-processing contamination.

NACMSF (2010) also recommend that the number of samples and replicates should be increased in situations of higher variability or uncertainty but also note that "sampling schemes for food microbiology experiments are often dictated by common practice, not solely based on statistical design." As explained in earlier sections, the many potential sources of variability in cheese making mean that it is more practical to characterize those sources and design a 'worst-case' scenario so that only one (replicated) challenge is needed rather than attempting to characterize the influence on process safety of those individual sources of variability. There are potential problems with this approach, however, which are discussed in Section 4.6.2. Statistical considerations concerning the number of samples have been alluded to earlier, and will be considered in greater detail in Section 4.5.

Inoculating individual packages may be more appropriate for studies considering the effect of post-processing contamination or when production cannot be readily replicated in the laboratory. Inoculation methods that result in highly variable inoculum levels will need more samples to be taken at each time to establish statistical validity.

4.3 Inoculation

4.3.1 Inoculum Preparation

As with all aspects of the challenge trial, the objective is to characterize the fate of pathogens in the product/process under conditions that are as close as possible to the usual commercial situation but, due to resource limitations, to characterize a worst-case situation for the process that would lead to risk management decisions that are conservative, i.e., that encompass a wide margin of safety to compensate for the lack of complete information. This also applies to the condition of the challenge organisms used in the challenge study. Thus, depending on the nature of the challenge trial the challenge organisms are prepared in a way that miminises their lag time upon addition to the food or food ingredients, or maximizes their resistance (and survival) in conditions that are inimical to growth (*see* footnote 9, p. 21). In the case of cheese challenge trials both situations can arise and careful thought needs to be given to the preparation of the microbial cultures to be used for the challenge trial.

Two types of challenge trial involving cheese can be envisaged. The first is to test the ability of the overall cheese-making process to eliminate pathogens or to control their numbers to acceptable levels. In this situation the pathogens would be added directly to the milk used for cheese making and the challenge organisms should be prepared so as to be able to grow as soon as the

temperature of the milk allows it[18]. This requires growing the challenge organisms in milk at the lowest temperature possible that still allows the pathogen to grow. The aim is to condition the cells for growth at low temperatures in milk. For *S. aureus, E. coli* and *Salmonella*, 10°C is close to the lowest temperatures that enable their growth. For *L. monocytogenes*, growth in milk at 5°C is possible, albeit slow and a slightly higher temperature might be selected for practical reasons (e.g., to lessen the time required to produce the challenge organism inoculum).

The second type of challenge study envisaged would be to assess post-processing contamination and would involve inoculation of microorganisms onto cheese at some later point at which contamination could occur, e.g., after curd cutting and moulding, during ripening, etc. Depending on the cheese and stage, the cheese will most likely be less favourable to pathogen growth (e.g., due to lower water activity, lower pH and higher organic acid levels) than raw milk and the inoculum should be prepared in a way that maximizes its resistance to relevant stresses. This could involve growth at a relevant temperature in an acidified medium with added salt, and/or organic acids to mimic the conditions in the cheese, or growth to stationary phase. If growth is not possible under the conditions in the cheese growth to stationary phase in a medium that is as close as possible to the conditions in the cheese but that still allows growth should be adopted to maximize the induction of resistance mechanisms (*see* footnote 9, p. 21).

Challenge organisms should be maintained in a way that preserves their characteristics so that they do not change between successive trials. Stock cultures should be prepared with enough aliquots to undertake the complete series of trials planned. This is to minimize sub-culturing and thereby to avoid genetic changes that affect the phenotypic properties of an organism. International Guidelines for laboratories (ISO/IEC 17025, 2005; AOAC, 2006) indicate that there should not be more than five passages from the primary reference culture material. For some organisms even this number of sub-cultures may alter phenotypic properties, particularly organisms with plasmids that are readily shed. Similarly, organisms are frequently stored frozen (either -20°C or -80°C) and each freeze-thaw cycle kills some proportion of the population. As such repeated freeze-thaw will reduce the population of the stock culture and may also select for a sub-set of cells that are best able to resist the effects of freezing. For challenge trials specifically involving spore-forming organisms, and for trials in which the normal mode of contamination of the product during commercial processing and handling arises from spores, the spores can be prepared beforehand and stored frozen. While in some challenge trials spores are often heat shocked prior to inoculation, this is relevant to challenge trials involving cheeses because thermal inactivation steps are not included in most cheese-making processes. However, if a thermal inactivation step does occur as part of normal processing, a heat shock step to mimic this could

[18] i.e., based on the idea that no other inhibitor of growth in the raw milk will change during the processing in a way that would *lessen* the inhibition of growth of the challenge organisms.

be appropriate. Alternatively the spores might be introduced at a point prior to the heating step in the cheese-making process being evaluated.

For either inactivation or growth studies, adaptation of cells should attempt to mimic the likely physiological state of the organism at the time it contaminates the food. Thus, inocula used for challenge trials should also be prepared under defined and reproducible conditions otherwise results can vary between trials. As discussed earlier, the physiology of bacteria will alter as a function of the growth environment, including nutrients in the culture medium[19], and the stage of the population growth curve (e.g., stationary *vs.* exponential phase). For inoculation into raw milk, the obvious growth medium would mimic milk as closely as possible but must be sterile. UHT milk, or a medium of near neutral pH, containing lactose, casein etc. would be appropriate. For inoculation onto cheese, a culture adapted to the water activity, acidity (including specific organic acids) of that cheese will be more appropriate. As discussed earlier, the temperature of incubation of the inoculum should also be chosen to match the conditions to which the organism will be introduced, which will depend on the mode of contamination (discussed further in Section 4.3.2).

In challenge trials a specific number, or density, of challenge organisms in the product at the commencement of the trial is usually sought (see Section 4.3.3.). Thus, it is necessary to have knowledge of the density of the inoculum prior to inoculation onto the product. The densities of cells in inocula can generally be approximated by measurement of optical density (OD) of broth cultures, it is preferable to enumerate viable cells (or spores) in the inoculum *prior* to inoculation to the milk/cheese in addition to the enumeration of the levels achieved in the product immediately after inoculation. This data can help to resolve, e.g., if levels in the product are unexpectedly low whether that is due to failure of the inoculum/low viability, whether the milk/cheese is more inimical to microbial growth than expected and has caused immediate inactivation of the inoculum, if the milk/cheese in some ways reduces recovery of the inoculum, etc. Conversely, if the milk to be used for the challenge trial is naturally contaminated this would be revealed by such having both sets of enumeration data.

If low inocula densities are used, the reliability of any single determination of concentration may be low if (petri) plates derived from the lowest possible dilutions result in only a few colonies[20].

---

[19] Bacteria will tend to produce enzymes that are needed to catabolise nutrients available in their environment, and for synthesis of those needed compounds are not. Accordingly, the composition of the medium used to culture the inoculum could affect its responses when introduced into raw milk or cheese.

[20] The reliability of the enumeration of any sample can be estimated from a Poisson distribution. As a rule of thumb, the standard error of the count is approximated by the square root of the number of items, e.g. colonies, counted from the sample. Thus, a count of one has a confidence intervals of ± 100%, a count of 10 has confidence intervals of approximately ±30%, a count of 100 has confidence intervals of ±10%.

As such, a greater number of replicates may be desirable to gain greater confidence in the determination of the initial contamination level.

Where 'cocktails' of strains are used as inocula, the levels of the individual strains in the combined inoculum should be approximately equal. If broth cultures are used to generate the inocula a simple way to achieve this is to measure the optical density (OD) of the cultures to determine the relative numbers of cells in each, and to combine volumes of each inoculum strain so as to achieve approximately equal numbers in the pooled inoculum. Alternatively, the inoculum broths can be diluted with fresh broth to achieve equal optical densities, and then equal volumes (of appropriate dilutions - *see* Section 4.3.2, below) combined to produce a single, 'master', inoculum.

4.3.2 Mode(s) of Inoculation

It is impossible to produce sterile milk (Leedom, 2006) and milk taken aseptically from a cow's udder contains microorganisms, though typically less than 10^2 – 10^3 CFU.ml^{-1} (Adams and Moss, 2000). Equally, during the course of milking, organisms can contaminate the milk from i) soil, ii) dirty equipment, (e.g., through shipment and unclean lines), and iii) organisms on the outside of the teat, or on the hide etc., or in the teat canal.

Cheese-making is a complex process with many steps and possible variations around those steps. Equally there are many stages at which contamination could occur. While the least controllable of these is probably contamination of the milk from the cow (e.g., due to sub-clinical mastitis), followed by other sources near the udder during milking, contamination is known to be possible at later stages as well. The microbial ecology of cheese is "dynamic" and can change at many stages in the life of the cheese. Thus, even if contamination were reliably eliminated by the fermentation, recontamination and growth could occur if the product characteristics changed at later stages. Thus consideration needs to be given, when designing the challenge trial, to the potential sources of pathogens into, or onto, the cheese and the manner in which they arise, so as to accurately mimic the natural process(es) of contamination.

Key considerations concerning the mode of inoculation are to maintain the intrinsic or extrinsic properties of the foods and to simulate contamination that could realistically occur in the commercial production of the product.

To achieve the first objective, the medium suspending the inoculum should not contribute nutrients, or inhibitory compounds, that would alter the microbial ecology of the food. To achieve this aim, inoculum volumes are usually very small in relation to the amount of food to which they are added. Thus, the suspending medium usually consists only of water and salt to make the

medium isotonic to the cells being inoculated (e.g., a 0.85% saline solution). If the product is acidic, or alkaline, the pH might also be adjusted.

To these ends, after growth in a suitable medium under appropriate conditions the inoculum is often 'washed'. Washing of cell suspensions involving centrifugation to separate the cells from the growth medium, removal of the growth medium, and re-suspension of the cells in, for example, a basal salt medium. This process is often repeated to minimize carry-over of nutrients or metabolites from the growth culture.

Simulating post-processing contamination

For most challenge trials relevant to the safety of raw-milk cheese processes, it will be appropriate to inoculate the raw milk to be used for cheese making. Post processing contamination can also occur, but is not a hazard unique to raw milk cheese. It is possible however, that cheeses derived from raw milk will offer different potential for microbial growth or inactivation compared to pasteurized milk cheeses and it may be of interest or relevance to inoculate formed raw-milk cheese

Several methods of inoculation of formed cheese have been used. The aim is to inoculate the challenge strain(s) as evenly as possible over the surface of the product. These include the use of very soft paintbrushes (to avoid altering, or to minimize alteration to, the surface characteristics of the cheese). Sterile velvet pads can also be used to gently transfer inoculum to the surface of the product, but it is perhaps more difficult to control the inoculum levels. Other methods include use of an atomizer, or other sprayer that emits a gentle stream, to spray the suspension of organisms onto the product surface. If using atomizers, or other sprayers, extreme caution is needed to contain the spread of the spray. Accordingly, spray application needs to be done in an environment that contains the spray and which can be decontaminated afterwards. Typically, a biological hood, with controlled airflow, HEPA filters on the effluent air, and that can be disinfected after use would be needed.

Whatever the mode of application time should be allowed, post-inoculation, for the medium suspending the inoculum to dry off, or be absorbed fully into the product.

It may be necessary to simulate post-processing contamination using product that has already been packaged. In some case products can be inoculated through the packaging material. Again, the aim is to minimize changes to the ecology of the product, e.g., due to changes in availability of oxygen or concentration or other gases that may influence the microbial ecology of the product. Thus, if inoculating through packaging, care must be taken to maintain the gaseous atmosphere in the product. This is often done by placing a small, self-adhesive rubber septum on the packaging,

and injecting through the rubber. This strategy minimizes the egress or ingress of gases through the hole. For additional confidence the septum can be covered/sealed with other impervious material after the inoculation process.

Adding inocula via a *syringe* through packaging can make it very difficult to inoculate evenly over all surface of the product and, alternatively, products may have to be un-packed, inoculated using methods described above, and then repackaged in the same packaging material and, if appropriate, with the same gas composition in the packaging. In this context, a wax coating on a cheese is a packaging method as it aims to minimize oxygen levels on the surface of the cheese to prevent growth of surface molds. The temperature of the molten wax should be considered, as it may be lethal to the inoculum.

Similarly, when taking samples from material that has been packaged, the same levels of care are needed either not to disrupt the environment/microbial ecology of the cheese by the sampling, or to restore it after the sample has been taken.

Simulating contamination of milk

As noted above, it would be expected that for most challenge trials relevant to the safety of raw-milk cheese processes, it will be appropriate to inoculate the challenge organisms into the raw milk to be used for cheese making.

Inoculation of milk will be a much simpler process than inoculation of formed cheese but the same concerns about carryover of materials from the suspending medium are relevant. To minimise this carryover the methods described above can be used. Additionally, once the inoculum is prepared, if dilution is needed, the dilutions can be performed in a sub-sample of the milk itself which will dilute the suspending medium. Another alternative that has been employed in challenge studies is to use a lyophilised (freeze-dried) culture added directly to the milk. While a lyophilized preparation has a small volume, it still contains the contents of the suspending medium but in a concentrated form due to the freeze-drying process. As such, the medium in which the lyophilized cells are suspended should also be considered and controlled so as to minimise the contamination of the raw milk with foreign compounds.

After inoculation, it will still be necessary to mix the inoculum through the milk. Stirring of the milk and curd is a normal part of cheese making. Accordingly, it would be expected that the required mixing should occur as a natural part of the cheese-making process. However, if a process does not include mixing, some other method of mixing the inoculum through the cheese will be required. Given that the inoculum contains pathogens, this should not be done with an exposed hand or arm as is traditionally done by some artisanal cheese makers

4.3.3 Inoculum Size

As discussed in Section 4.32, it is not possible to obtain sterile milk from a milking animal. Milk taken directly from the udder typically contains hundreds to thousands of bacteria per millilitre, principally derived from the teat canal. Numerous studies (Adesiyun, 1994; van Schalk *et al*., 2002; Berry *et al*., 2008; D'Amico *et al*., 2008; Elmoslemany *et al*., 2009, Rysanek *et al*., 2009a, 2009b; D'Amico and Donnelly, 2010) indicate that typical levels in "bulk tank milk" (i.e., milk that is pooled from all animals on a farm, or from multiple farms) are tens of thousands of cells of bacteria per milliliter. The sources of these additional bacteria can be from mastitic cows, from contaminated teats and dirty milking equipment etc, and/or from faecal contamination of milk. Levels of 50,000 to 100,000 cells per ml are considered 'normal' in various jurisdictions and consistent with raw milk of acceptable quality. Higher levels are considered indicative of poor hygiene in the dairy plant or mastitis in the herd.

In the case of contamination due to mastitis, published evidence reviewed by Elmoslemany (2009) suggests that it is reasonable to assume that pathogenic bacteria will often dominate the total bacterial count for that milk. Given this and the above data, it is reasonable to assume that, in cases where bacterial levels are high, they may be high due to the presence of high levels of pathogens. Of the organisms that cause mastitis in cows, however, only a relatively small number can cause human food-borne illness. *S. aureus* is a common cause of mastitis and *E. coli,* and *L. monocytogenes* can also cause mastitis. Importantly, mastitis - particularly sub-clinical mastitis – is not unusual among herds.

Potential pathogen loads in raw milk are relevant to the design of challenge trials for cheese. This is because the overall safety of the cheese making process depends on the ability of the process to reduce pathogen loads to acceptable levels prior to release of the cheese for sale. Conversely, if only low levels of pathogens are ever present, using an unrealistically high inoculum could overwhelm the mechanisms in the product that would normally limit, or inactivate, pathogens. Similarly, if an unrealistically high level were used in the challenge study it could underestimate the risk if extensive growth were possible but was limited by the onset of stationary phase, i.e., the true growth potential could be underestimated. As such, knowledge of realistic levels of pathogens is needed to design challenge trials.

Unfortunately, while there is much data concerning prevalence of mastitis among herds, there is relatively little *quantitative* information on resulting levels of pathogens. Equally, while there is a much data on the prevalence of pathogens in raw milk, there is very little data on pathogen levels in raw milk. Accordingly, an unequivocal recommendation on pathogen levels of relevance for challenge studies in raw milk cheeses is not possible. This section reviews information that can

be used to inform both the design of challenge trials for raw milk cheeses and for determination of levels of inactivation that are required for realistic levels of pathogens of relevance in raw milk used in cheese production.

Effect of Mastitis on Bacterial Loads in Raw Milk

Mastitis is an inflammation of one or several mammary quarters[21] often due to a bacterial infection. If there are signs of infection (sore, swollen or inflamed teats, or other signs of systemic illness in the animal) the mastitis is described as 'clinical mastitis'. 'Sub-clinical' mastitis describes the situation when there are no overt signs of illness but there is evidence of increased microbial levels with evidence of the presence of a pathogen or potential pathogen, and/or increased somatic cell count (SCC).

SCC levels are correlated, but not simply, with levels of mastitis pathogens in milk from the infected udder (Figure 3): SCC levels of several hundred thousand or more are usually associated with infection. By international agreement SCC counts in excess of 100,000 ml^{-1} in quarter foremilk are considered indicative of infection while in composite milk (e.g., bulk tank milk) an SCC of >50,000 ml^{-1} may be considered indicative of mastitis in the herd contributing to the milk. The relationship between SCC and bacterial count in the milk varies with the pathogen causing the infection (Fenton *et al*., 1995). For example, *S. aureus*, while a major cause of mastitis,

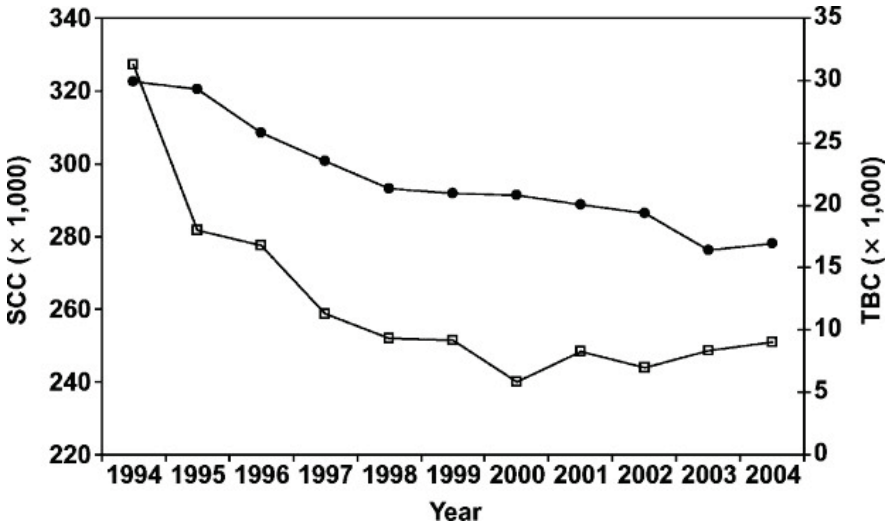

Figure 3. Annual trend in geometric mean somatic cell count and total bacterial counts. While not directly paired results, the trends suggest that the relationship between SCC and bacterial loads is variable (Reproduced from Berry *et al.*, 2006). Similar data can be found in Elmoslemany *et al*. (2009).

[21] Milk animals typically have four mammaries. Each is referred to as a "quarter". With forequarters and hindquarters also distinguished.

is rarely present at very high levels and is usually associated with sub-clinical mastitis. In the context of mastitis "major" pathogens are those that can cause 'clinical' mastitis and include *S. aureus*[22] and coliforms, including *E. coli* (Djabri *et al.*, 2002)."Minor" pathogens describe other organisms that usually cause only sub-clinical infections. *L. monocytogenes* is included in this group. Mastitis-causing organisms are also classified according to their potential for spread from animal to animal. Thus, "contagious" mastitis organisms are those that can be spread from udder to udder. Environmental mastitis, however, arises from contamination of the udder from contact with manure, soil, bedding etc. and is usually associated with clinical symptoms but of shorter duration. When mastitis occurs, it is often limited to one quarter only.

The literature on mastitis is mainly concerned with use of the SCC as an indicator of infection but infection is usually only assessed qualitatively (i.e., the presence of the pathogen in the milk) rather than enumeration of bacterial levels in the milk. Sub-clinical infections are usually characterized by bacterial (pathogen) contamination levels < 10^5 cfu.ml^{-1} in the milk from the infected udder. These pathogens will be diluted in bulk milk, i.e. when contaminated milk is combined with non-contaminated milk.

Clinical mastitis is usually evident in the animal (see clinical signs described above) but also in the milk. Milk quantity from an overtly infected udder declines, the milk may have obvious clots or other changes in appearance etc (Harmon, 1994), and such milk is unlikely to enter the bulk milk if the milker is alert. Levels of pathogens in clinical mastitis can be very high, e.g., 10^8 cfu.mL^{-1} for *E. coli* but currently there are no recognized guidelines regarding bacterial levels that indicate a 'true' infection (Torres *et al*., 2009).

Given that sub-clinical mastitis is more likely to be a source of elevated pathogen levels in raw bulk milk used for cheese making than clinical mastitis, levels of pathogens observed in sub-clinical mastitis were sought. As noted above, such data is scarce in the published literature, presumably a reflection of the use of SCC as an indicator of infection, rather than specific pathogen levels. For this reason, data from bulk tank milk is also included in the following section. As noted, mastitis is usually only present in one quarter at a time and milk from the sub-clinically infected udder will be diluted with uncontaminated milk by 10 to 100-fold or even greater depending on the herd size and frequency of sub-clinical mastitis in other animals in the herd.

---

[22] While *S. aureus* can cause peracute, acute, chronic, and subclinical mastitis, the chronic subclinical form is the predominant form (Harmon, 1994).

Schoder *et al*. (2003) reported on a case of ovine mastitis with *L. monocytogenes* in a flock of 130 sheep and in which shedding persisted for approximately three months. A mean count of 50, 000 cells *L. monocytogenes* per milliliter was observed, but with numbers ranging from 90 to 300,000 cfu.ml^{-1}. The bulk milk from the herd was contaminated at a level of ~6,000 cfu.ml^{-1}, reflecting the dilution of contaminated milk with uncontaminated milk in the bulk tank. Bourry *et al*. (1995) presented data for bovine mastitis due to *L. monocytogenes* and reported that levels in naturally contaminated cows were low, in the range 720 – 2000 cfu.ml^{-1}. They also noted that sonication of the affected milk increased the count, suggesting that the cells of *L. monocytogenes* were present as clumps. In one case, however, 60,000 cfu *L. monocytogenes* ml^{-1}was found. Similarly, Wagner *et al.* (2000) reported *L. monocytogenes* at 40,000 cfu.ml^{-1} in milk from one quarter of a sub-clinically infected cow during high lactation.

S. aureus is the commonest causes of mastitis in cattle (Harmon, 1994) and, as a major pathogen, is frequently observed at sub-clinical levels. In bulk tank milks Rysanek *et al*. (2009a, b) observed mean (± S.D.) *S. aureus* levels of 2.53 ± 0.32 $\log_{10}$cfu.ml^{-1}in 12% of samples and a mean contamination level of 2.0 ± 0.95 $\log_{10}$cfu.ml^{-1} (generic) *E. coli* in 7% of samples. Thus, for the upper one per cent of cases, contamination levels of 1,000s to 100s of thousand of cells per milliltre could be expected. Similar levels were reported for other mammary pathogens. Typical frequencies were 10 – 20 %, and typical levels were 2.5 to 3 $\log_{10}$cfu.ml^{-1}.

Do Cormo *et al.* (2002) found levels of *S. aureus* in raw milk of 24,000 cfu.ml^{-1}. Torres *et al*. (2009) recovered levels of 100 to 20,000 cfu.ml^{-1} of *S. aureus* from individual cows, and up to 10,000 cfu.ml^{-1} coliforms. Schoder *et al.*(2010) found that 11.3% of samples from farm bulk tanks had *S. aureus* levels > 2000 cfu.ml^{-1}. Heidinger *et al*. (2009) presented distributions of *S. aureus* levels in raw milk as part of a risk assessment of consumption of raw milk. The *S. aureus* levels were derived from bulk milk data from dairies in California as part of routine monitoring. Of 51,963 samples analysed, 25.3% contained measurable levels of *S. aureus.* A limitation of the data set was that the techniques used meant that levels in excess of 40,000 cfu.ml^{-1} could not be enumerated and were reported only as >40,000 cfu.ml^{-1}. Notably, however, samples in excess of this level were reported, and found in *bulk* tank milk. El-Zubeir *et al*. (2008) found 15.3% of samples of raw milk contained *S. aureus* in Western Cape, South Africa, and 14.3% contained *E. coli.* Adesiyun (1994) found *S. aureus* in 94.3 % (of 478) samples of raw milk in Trinidad sampled at collection centres. Mean levels were in the range 100, 000 to 1,000,000 cfu.ml^{-1}but the author concluded that the quality of raw milk in Trinidad was poor.

D'Amico *et al.* (2008) specifically examined bulk tank milk from farms in the state of Vermont, USA that produced artisanal cheeses. Five farms produced cheese from cow's milk, four from goat's milk and two from sheep's milk. Total bacterial counts ranged from <1 cfu.ml^{-1}to

9,300,000 cfu.ml^{-1}. Most results were in the range 1000 – 10,000 cfu.ml^{-1} but 3.4% of samples were in excess of 100,000 cfu.ml^{-1}. Counts were not obviously different as a function of species of animal, but goat samples yielded the highest counts. In the same survey, *S. aureus* levels were determined. *S. aureus* was detected in 33.3% of samples at levels > 1 cfu.ml^{-1} and the most frequently contaminated milk was from sheep, but 20 – 30% of goats milk and cows milk samples were contaminated. Levels of *S. aureus* ranged up to 20,000 cfu.ml^{-1}, but the mean level was 250 cfu.ml^{-1}. In the same survey, other pathogens were detected in 3% of samples, reinforcing the status of *S. aureus* as a major mastitis pathogen. In a "follow up" study several years later (D'Amico and Donnelly, 2010) total bacterial counts were not found to differ significantly between animal milk source and 7% of cow milk samples exceeded 10^5cfu.ml^{-1}, but no sheep or goat milk samples exceeded this level. Between 30 and 50% of samples from all animals types were positive for *S. aureus* but levels were significantly lower is sheep milk samples than in cow or goat milk samples. Coliform counts were as high as 70,000 cfu.ml^{-1} in cow's milk and 11,000 in goat milk, but less than 200 cfu.ml^{-1} in sheep milk.

Le Loir *et al.* (2003) also studied *S. aureus* in cows with mastitis and found that 15 – 80 % of strains isolated were able to produce staphylococcal enterotoxins, i.e., it can be assumed that if *S. aureus* are present they are probably toxigenic. In this regard, in recent years many new staphylococcal enterotoxins have been recognized (Chiang *et al.,* 2008) and it is probably that many strains not identified as enterotoxin producers in the earlier literature, when fewer enterotoxins were able to be distinguished and detected, would now be regarded as enterotoxin positive.

O'Mahoney and Austin (1991) reported that in Irish dairies, 27% of bulk tank milk samples had counts for mastitis pathogens in excess of 10,000 cfu.ml^{-1} and that 11% of samples had counts of mastitis pathogens in excess of 20, 000 cfu.ml^{-1}.

Schoder *et al.* (2001) noted that 49.1% of bulk tank milks from sheep and goat dairies in Austria exceeded the acceptable total bacterial count level of 10^5 cfu.ml^{-1}. Also, foodborne pathogens were isolated from 6.3 % (10/160) of the bulk tank milk samples.

In relation to *Mycobacterium*, Spahr and Schaforth (2001) state that cows in the latter stages of infection with *M. avium* subsp. *paratuberculosis* shed viable cells into their milk, albeit at low levels, e.g., 2 – 8 cfu per 50 millilitres. They also noted that faecal material from clinically infected cows may contain much higher levels of pathogens and suggested that fecal contamination of raw milk may provide a much larger contribution of pathogenic contaminants.

Faecal Contamination

Some potential pathogens (e.g. *Mycobacterium* spp., *Salmonella*, *E. coli, L. monocytogenes*) are excreted in the faeces of cows. Data describing concentrations of pathogens in faeces could be used to estimate potential pathogen levels derived from faecal contamination in raw milk. In general, data for pathogen concentrations in cattle faeces are scarce, particularly for *Salmonella*, for which culture methods are usually qualitative rather than quantitative.

Duffy *et al.* (2010) reported that the mean concentration of *Salmonella* in faeces of sheep was 1.43 log(MPN).g^{-1}. Fegan *et al.* (2004) reported levels up to 2,800 MPN.g^{-1} in bovine faeces, while Fegan *et al.* (2005) reported somewhat lower levels in a subsequent report. They noted however, that the highest level observed was 11,00 MPN *Salmonella* per gram in a rumen sample. Rhoades *et al.* (2009) reviewed available data for *L. monocytogenes*, EHEC and *Salmonella* in cattle faeces, but presented no quantitative data for *Salmonella*, or *L. monocytogenes.*

Relatively more quantitative data are available for enterohaemorrhagic *E. coli* levels. Rhoades *et al.* (2009) reported that levels ranged up to $10^6 g^{-1}$ faeces in Scottish studies and up to $10^7.g^{-1}$ faeces in a USA study. Wells *et al.* (2009) reported levels in cattle faeces in USA and, as in the Scottish studies, observed that levels were usually low (i.e. ≤100 cfu.g^{-1}) but that a few percent of EHEC positive animals excreted levels up to 10^6cfu.g^{-1} faeces. They also observed that for animals with diets supplemented with wet distiller grains the distribution of EHEC levels excreted was "shifted" to approximately 10-fold higher levels, i.e., a few percent of these animals excreted levels up to 10^7cfu.g^{-1} faeces. Fegan *et al*. (2009) reported mean levels of *E. coli* O157:H7 in cattle faeces of 2.37 $\log_{10}$MPN per gram but also observed that one animal had a level of 5.66 $\log_{10}$MPN.g^{-1}. In Trinidad, Adesiyun isolated *E. coli* from 20% of raw milk from raw milk collection centres. The mean counts ranged from 6.6 x 10^2 (± 1.1 x 10^2) to 4.0 x 10^5 (± 4.5 x 10^5) cfu.ml^{-1}.

Myocobacterium bovis levels of up to 10^9cfu.g^{-1} faeces in later stages of disease have also been reported (Spahr and Schaforth, 2001).

The data, while very limited, can be used to generate a 'rough' estimate of potential levels for these pathogens due to contamination from faeces. A typical milk volume for cows per milking is 10 L. Assuming a worst-case scenario, if tens grams[23] of faeces were to contaminate that volume of milk, and contained 10^7 of a pathogen per gram of faeces, the final concentration in the milk would be 10^4 per millilitre of milk before that milk is combined with the milk from other animals, and which might be expected to reduce the concentration further. For small animals (goats and sheep) the faeces are more coherent (i.e., less sloppy) and such gross contamination is less likely.

[23] It seems likely that more than 10g of faeces would be readily detected, and even 10g represents an unlikely, extreme, scenario.

Conversely the milk volume will be less and consequences of faecal contamination would be somewhat higher. Finally, in a mechanised dairy, filters on the milking apparatus would further limit the potential contamination. In the case of hand milking, such contamination would, presumably, be readily detected and prevented or the milk discarded.

Other considerations

A number of studies have identified that there are many factors that can affect the bacterial load in bulk milk, including diary hygiene, teat washing (D'Amico and Donnelly, 2010), whether cows are fed on pasture or in confinement, and whether cows are fed grain from distilleries. Such information may be taken into account when assessing the adequacy of the inactivation level achieved by the cheese process that is the subject of a challenge trial.

Recommendation

From the above discussion, and adopting a conservative approach, pathogens levels in milk used for cheese-making could be as high as 10^4 to 10^5 cfu.ml^{-1}. This level also is suitable for pragmatic reasons in that, as inactivation of pathogens is desired in the cheese-making process, it is more appropriate to use relatively high levels so that the magnitude of inactivation of pathogens that is achieved can be measured with greater accuracy, i.e., up to 4 to 5 decimal reductions can be monitored using standard cultural enumeration methods.

Accordingly, an inoculum concentration of 10^4 to 10^5 cfu.ml^{-1} in raw milk to be used making the cheese to be challenged is recommended. This level in the milk makes allowance for the concentration effect of curd formation. This level can be applied for all pathogens considered.

Some commentators (e.g., IFTS&T, 2003a; Notermans *et al*., 1993) recommend levels of 10^6 to 10^7 cfu.ml^{-1} to be used when validating a process lethality step. For cheese, however, a higher level is not desirable because it is expected that concentration of the pathogen will occur during curd formation. Additionally, under improper conditions, pathogen growth could occur during the early stage of the fermentation. Accordingly, the level of inoculation has to allow for potential growth of the challenge organism (in addition to the expected concentration into the curd). Thus, the inoculum level needs to be such that the initial concentration in the milk is well below the stationary phase level of the challenge organism, typically 10^8 to 10^9 cfu.ml^{-1} in raw milk. Also, higher pathogen levels in bulk milk would be unusual, from the discussion presented above.

The levels recommended are consistent with suggested levels given by the various authorities and experts, cited earlier, whose recommendations form the basis of this report.

Where it is desirable to use lower inoculum levels, experimental procedures can be modified to increase the lower detection limit, e.g., by taking multiple samples when pathogen levels are

expected to be low, or by using MPN methods rather than plate count methods. Practical advice when using low inoculum levels is given by NACMSF (2010).

4.4 Sampling and Analytical Methods

4.4.1 Analytical Methods

Enumeration of challenge organisms in cheese, rather than evaluation of the "absence" of the challenge organism, is needed to evaluate the safety of the cheese-making process. Enumeration should use methods that permit the accurate and reproducible recovery of the challenge micro-organisms. Methods that are described in national or international standards are developed to satisfy these requirements. Some methods for detection and/or enumeration of pathogenic organisms in foods differ for particular types of foods and it is also important that the methods adopted are accredited and appropriate for microbiological analysis of cheese, because conditions that are inimical to the growth of most bacteria will usually prevail, or because high fat levels may interfere with recovery of challenge organisms, etc.

Due to the inimical conditions, the challenge organisms introduced to the cheese are likely to become damaged over time and may require 'resuscitation' before they are subject to selective media for enrichment or enumeration. Ideally, methods used would enable direct enumeration of the pathogens from the food, or suspensions of it, e.g., by reliably forming colonies on agar plates from each cell of the challenge organism in the aliquot plated out, and that any colonies present unambiguously indicate the presence of a cell of the challenge organism in the sample. In practice, few microbiological media achieve this goal. For example, selective media usually include compounds that inhibit the growth of organisms that are *not* the challenge species. Those selective agents, however, can also cause stress to the challenge organisms, particularly if those cells have been injured by the conditions in the cheese, and can prevent them their growth and from forming visible colonies, potentially leading to incorrect results. Accordingly, "resuscitation" procedures that enable the target cells to repair their injuries before being exposed to selective agents in selective/differential media are often required to ensure recovery of all cells. In addition, few microbiological media are 100% selective for the organism of interest and even when typical and distinctive colonies are produced additional test procedures may be required to confirm that the typical colonies did arise from surviving cells of the challenge organism.

Baird-Parker Agar is highly selective and diagnostic for *Staphylococcus aureus*, while PALCAM is selective for *L. monocytogenes.* PALCAM is specified in EN ISO 11290-2 (1998) and subordinate national standards (e.g., AS/NZS 1766.2.16.1:1998, or Australian Standard 5104.24.4 – 2009) for direct enumeration of *L. monocytogenes*, after a short resuscitation period of one hour at 20°C

and subsequent incubation at 37°C for 24 – 48 hours. Baird-Parker Agar is generally used without a resuscitation step for many types of foods. *Escherichia coli* O157 can be enumerated directly on CT-SMAC (Cefixime Tellurite Sorbitol MacConkey) agar. Maher *et al*. (2001) used this method for a challenge trial involving *E. coli* O157 in raw milk cheese. They also used a resuscitation technique which involving pour plating of samples onto a non-selective agar (in this case Brain Heart Infusion Agar), leaving the samples to recover from injury by incubation for 3 hours at 37°C, before pouring CT-SMAC over the plate as a selective medium for enumeration. This general resuscitation method, i.e., plating onto non-selective media with a short incubation for resuscitation of injured cells before pouring selective agar over the plates and completing the incubation, is also recommended by NACMSF (2010).

Conversely, a potential problem will be to identify accredited quantitative methods for some of the pathogens of interest, e.g., *Salmonella*. While Salmonella determination is usually applied qualitatively (i.e., "are Salmonella present in a specified quantity of the food?"), for the purposes of challenge testing, quantitative methods are preferable because they are more informative. Qualitative methods can be made quantitative by applying them as most probable number (MPN) methods to achieve resuscitation and enrichment. In practice, however, this will be very time and labour intensive because each MPN tube requires, in principle, that the full Salmonella isolation/identification methodology is followed which can take up to five days of culture and sub-culture and confirmation tests

In development of rapid methods for Salmonella enumeration, based on the MPN approach, a common approach to reduce the workload and time required for results is to replace post-enrichment stages that have traditionally relied on biochemical tests for phenotypic characteristics with genetic detection methods, such as polymerase chain reaction (PCR) or specific antibodies (e.g., immuno-magnetic separation, ELISA methods) to unambiguously identify target cells. However, any non-standard or non-accredited method, or any deviation/refinement of a standard method should be supported with compelling evidence that the performance of the method is equivalent to the standard, accredited, method.

Indirect methods that rely on identification and quantification of mRNA transcripts as quantitative markers of cell viability (e.g., RT-PCR) may need careful interpretation. Conventional wisdom argues that mRNA transcripts are very short-lived in cells and that, as such, their presence is indicative of viable cells. mRNA in cells is short lived is because it is actively degraded by nucleases synthesized by viable cells. Any treatment that inactivates nucleases, however, can effectively prolong the longevity of mRNA and confound the interpretation of results (Zhang *et al.*, 2010).

Alternative methods with better performance than standard methods for specific food/pathogen combinations are periodically presented in the refereed scientific literature. Provided that strong documented evidence of the accuracy of these methods and their equivalence or superiority to standard, or other accredited, methods is available (or even preferable) their use may be acceptable for challenge trials.

Pathogen enumeration or toxin quantification

In the case of toxigenic organisms (e.g., *S. aureus, C. botulinum*) it may be thought relevant to determine toxin levels rather than to enumerate the organisms themselves. However, toxins only arise from viable cells and their metabolism, and toxins are usually only detectable when the organisms have reached high levels, typically at least tens of thousands of cells. Botulin is only produced by growing cells and *C. botulinum* levels of10^5 CFU.g^{-1} or ml of food, or higher, typically are required to generate detectable toxin levels (Lund and Notermans, 1993; Elliott and Schaffner, 2001).Some reports have suggested that toxin production can occur in the absence of growth (Hyttia *et al*., 1999; C. Moir, *pers. comm*.. 2007). While Hyytia *et al*. (1999) suggested that this may be due to growth in one part of the product being balanced by inactivation in other parts of the test product, no experimental evidence was presented to support this hypothesis. As noted earlier (Section 4.3.3), levels of *S. aureus* of up to 10^5 CFU.g^{-1} or ml of food are rarely associated with foodborne illness, and this accords with approaches taken by a number of regulatory authorities including NZFSA (K. Shaw, *pers. comm*.. 2011). Also, in the case of *S. aureus* a very high proportion of strains are toxigenic but not all toxins are readily quantifiable. For this reason enumeration of *S. aureus* levels, rather than determination of toxin levels, is often considered sufficient (NACMSF, 2010). Moreover, as the methods for enumeration of *S. aureus or C. botulinum* will detect the organism at lower levels that that at which toxin can be detected, enumeration of the pathogen will provide more information about the ecology, and attendant risk, from toxigenic pathogens in cheese making, than will be obtained by enumeration of the toxins alone. Nonetheless, testing for toxins in at least some samples, at some sample times, will provide additional information for the evaluation of the risk from toxigenic pathogens potentially present in cheese. This is particularly important for processes in which conditions might kill pathogenic cells but leave toxins produced by those cells unaffected (*see also* footnote 13).

4.4.2 Sampling

Section 4.2.6 discussed aspects of the sampling plan including number of samples and considerations when multiple samples are take from a single cheese. Replicate samples are needed to establish sampling variability and, where systematic variation can be expected between, for example, the rind and core of a cheese, those sites should be sampled separately at each sampling time as well.

Dividing a large batch into discrete portions for testing at each sampling time reduces the risk of contamination (and alteration of product properties due to repeated sampling), but there is a limit because the size and shape of the cheese will affect the ripening process. Thus, when multiple samples are taken from a single cheese it will be necessary to minimize changes to the microbial ecology of the product as a result of that sampling or to have a sufficiently large cheese so that samples can be taken from sites unlikely to have been affected (e.g. due to drying, oxygenation etc., due to exposing new surfaces) by previous sampling. In this regard a cork borer can be useful for taking samples in a way that minimizes the changes to the bulk of the cheese.

As noted in Section 4.2.6 the size of the cheese made can be an important part of the challenge trial experimental design. In some cases it may be appropriate and preferable to make many small cheeses to facilitate sampling/testing and minimize changes due to sampling compared to the size of cheese normally produced. Alternatively, it may be decided that a batch of full size cheeses is made and that sufficient cheeses are made so that each cheese represents one sample and that sufficient cheeses are made to include replication at each time point in the challenge trial. In other cases, aliquots of one, or a few, cheeses may be able to be used as samples.

Even if worst-case type experimental designs are employed, replicates are essential to reveal the extent of variability in the cheese making process, attributes of the milk, etc. or method reliability. In this regard, 'technical' replicates and 'biological' replicates can be distinguished. Technical replicates are identical samples taken to reveal method repeatability, i.e., they evaluate variations in results that are due only to the methodology. Biological replicates are samples drawn from replicates of the process, e.g. multiple sites within a cheeses, multiple distinct cheeses, cheeses from multiple different batches of production that evaluate the variability in outcomes due to variations in the process or variations between the process conducted at different times.

4.4.3 Physico-chemical Properties and "Background" Microbiota

As suggested above, because of the large number of variables that can affect the survival of pathogens in cheese, a longer-term aim is develop a quantitative understanding of the factors relevant to cheese-making that determine the survival of pathogens. As such, in addition to testing the levels of the challenge organisms over time during the cheese process, it is useful to measure physico-chemical properties of the cheese (e.g. pH, organic acid levels, a_w, etc.), which would logically correspond to "Operator Defined Process Measures") and levels of other microorganisms in the cheese so as to be able to determine whether correlations exist between these factors and the survival of the challenge organisms. This data will also be needed to establish that the challenge trial is also adequately representative of the normal commercial

process. Monitoring the physico-chemical properties of the cheese over time will be particularly important for cheeses for which it is known that ripening can result in changes in product composition (e.g., pH rise in surface-mold ripened cheeses) that might allow pathogen regrowth.

Enumeration of the background microbiota may be achieved by total counts, or by counts for specific organisms that may be known to dominate the product. In addition, it is useful to monitor physicochemical factors and the background microbiota in cheeses that were not inoculated with the challenge organisms (i.e., uninoculated controls) to determine whether the presence of high numbers of challenge organisms affects the normal processes of cheese development and characteristics of the cheese, so as to ensure that the cheese-making process in the challenge trial really is representative of the normal process.

Given this long-term desire to be able top predict the safety of cheeses without recourse to challenge studies, it is preferable to enumerate target cells and to plot these as a function of time during the cheese making process so as to be able to relate changes in the target pathogen levels to physicochemical conditions, background microbiota and storage conditions. The desire is to use the data to understand the microbial ecology of the product so predictions can be made about the fate of specific pathogens from measurements of time and the physicochemical parameters of the product and to minimize the need for challenge trials (*see also* Sections 1.2.3, 2.1.3 and 4.1.1).

4.5 Variability/Statistical Aspects and Data Interpretation

As discussed in Section 4.2.6, determining the fate of a pathogen in a food/food process is rarely as simple as comparing final and initial counts, and the interpretation of inconsistent or highly variable results is an important and complicated issue that requires both microbiological and statistical expertise. As noted in Section 4.2.6, the significance of differences in microbiological counts depends on the variability in the enumerations. Sources of variation include variation in the process itself, in the quality/attributes of the milk, in the strains of pathogens that actually contaminate the milk, and methodological/operator inconsistencies. The combined magnitude of these sources of variation will dictate the variability in counts and, hence, the significance of differences in pathogen counts at the beginning and end of the challenge trial.

Replication and statistical methods can be used to determine whether differences in counts at specific sampling points indicate true growth or inactivation or whether they are simply due to sampling and measurement errors. Equally, microbiological expertise can assist in assessing whether spikes or depressions in data over the time course of a process represent real microbiological phenomena or are due to methodological/sampling variability (e.g., due to

difference in microbiology within a single product, between units of the same batch or differences between batches of product).

Powell (2009) presented a comprehensive statistical analysis of *L. monocytogenes* growth challenge study designs, emphasizing that different challenge protocols to evaluate a single criterion can produce vastly different levels of confidence that growth (as defined by the criterion) did, or did not, occur in the food. Importantly, Powell (2009) questions whether any of the existing challenge study designs can provide a high level of assurance that growth of *L. monocytogenes* of 1 logCFU.g^{-1} has occurred, or not.

The statistical issues raised by Powell (2009) are reasonably well known and discussed in the statistical literature (D. Ratkowsky, *pers. comm.*, 2011) and are the reason that worst-case experimental designs for challenge studies have usually been recommended by commentators such as NACMSF (2010) and IFTS&T (2003a), i.e., that the number of trials need to characterize the variability in the process can not be practically achieved. NACMSF (2010) opined that "one of the limitations of these studies is the balance between statistical validity and practicality" and that " because of these constraints, variability is generally addressed through the use of worst-case scenarios, which should provide conservative results".

When adopting a worst-case experimental design approach, knowledge of the manufacturing or production variability is needed to determine the appropriate test parameters for the challenge study. As noted in previous sections, variability within lots should be determined by measuring formulation factors such as pH, a_w, etc. The greater the variability the more samples of product need to be evaluated for the challenge trial, i.e., the more measurements need to be made to determine the upper and lower control limits, etc. The acceptability limits of inactivation, or growth, specified for interpretation of a challenge study defines the limit of acceptability for that product/process from then on, but the result is only valid for the most extreme conditions tested. If some aspect of the process changes to a less stringent condition, a new challenge trial will be needed.

Whilst replication of each sample is important, as noted earlier evaluating changes in challenge organism levels over time and relating these changes to the physico-chemical properties of the cheese and the storage conditions enables far more insightful interpretation of results. In this case, where some understanding of the relationship between different sampling times is available, mathematical "regression" of the data can add statistical power to the interpretation of the results. The ideal is to use the data to understand the microbial ecology of the product – i.e., so that predictions of the microbiological consequences of those changes can be made, rather than having to do more challenge studies for each change. In this regard IFT (2003a) commented that "combining the quantitative inoculum data for each time point with data on the background

microbiota and the relevant physicochemical parameters gives a powerful and broad representation of the microbiological stability of the formulation under evaluation."

NACMSF (2010) provides a useful summary of the potential pitfalls in interpretation of challenge study results. For reasons outlined above, they stated that *universally acceptable rules for interpreting test results are not available*. This highlights the need for the expertise of a food microbiologist as well as rigorous statistical analysis to correctly interpret challenge trial results as indicating the safety, or otherwise, of a cheese making process involving raw milk.

4.5.1 "Compounding Conservatism"

A limitation of the worst-case scenario approach, however, is that it can produce a result that is overly conservative. That is, challenge trials that use the combination of worst-case conditions for each factor, represent a situation that is highly unlikely to occur in reality. This consequence was discussed by Cassin *et al.* (1996) who coined the term "compounding conservatism" to describe it, pointing out that the decisions based on the outcome of worst-case scenarios could lead to overly stringent and impractical food safety regulations. If sufficient data were available a less stringent value for each variable could be used in setting the conditions for the challenge trial, e.g., 95th percentile values for each relevant variable. However, in practice, this level of detail about the distributions of values for each variable affecting cheese safety is unlikely to be available and, accordingly, the challenge trial will necessarily devolve to using the most extreme recorded values for each variable in the process. It should also be noted that the most extreme *recorded* value also might not be the most extreme value that could occur. As such, the challenge trial may not fully represent the worst-case but rather approximate a worst-case situation.

4.6 Communicating the Results

The challenge trials protocols described in this report will be undertaken to make decisions about the safety of raw milk cheeses and it is anticipated that the decision about the safety of the process will be made by people other than those that supervised and performed the challenge trial. Accordingly the results of the challenge trial, and sufficient information to understand their significance for process safety, will have to be documented.

Throughout this report factors potentially affecting the fate of pathogenic organisms in the cheese, and the extent of their variability, have been emphasized. Accordingly all of these factors, e.g., sources of milk, the time of times and temperatures of all process steps, curd handling operations, starter cultures used, final product characteristics (e.g., pH water activity, moisture

content etc.) in the normal process, as well as magnitude of variations in these parameters, must be reported.

Similarly, the same details about the conduct of the challenge trials must be presented so as to be able to evaluate how closely the challenge trial mimics the normal, commercial process, and the extent to which it represents a worst-case for the overall process, in addition to specific details of the challenge trial, e.g., strains of pathogens used and their mode of preparation (stationary phase, exponential phase, washed cells, etc) and level and mode of inoculation. The numbers of replicates, modes of sampling, methods used for analysis, both microbiological and physico-chemical, should also be documented or specific reference given to readily available sources. There should be a description of the rationale of the trial, particularly if there are deviations from the commercial process. Such deviations would be almost inevitable due to practical compromises that have to be made because the challenge trial will usually not be able to be conducted in the normal processing area/facility.

Results of the trial including:

- levels of pathogens
- level of background microbiota
- physico-chemical attributes

as a function of time and related to specific process steps, should be presented clearly and succinctly, stating time of sampling, site sample taken from (if from a single cheese), as well as results (including average value and estimate of variability, e.g., standard deviation). Use of tables and graphs usually aids communication of results, particularly changes in measured parameters over the time course of processing.

To aid interpretation of the integrity of the trial there should also be a commentary discussing the degree to which the trial is a reliable representation of the process, identifying any possible shortcomings, problems with the trial and presenting other pertinent information that would influence the interpretation of the results of the trial.

5 Recommendations

The following presents a succinct summary of recommendations of this report for the conduct of challenge trials of raw milk cheeses in New Zealand.

General

The most fundamental requirement of a challenge trial of a food/food process is that it accurately mimics the conditions that the challenge organisms would experience in the actual food during all stages of processing. A second fundamental requirement is that the variability in the process is also characterized so that the *range* of process efficacy can be determined and appropriate decisions made. Accordingly the conditions employed by the cheese maker that could affect the fate of pathogen during processing should be thoroughly measured. For pragmatic reasons a worst-case approach is suggested for the challenge trial design, based on these measurement, to minimize the number of trials needed to co characterize the variability in process conditions so as to develop conservative interpretations of the efficacy of the process.

Pathogens of Interest

The pathogens of that are most commonly associated with raw milk cheese outbreaks and that are most relevant in New Zealand are:

Pathogenic *E. coli*

L. monocytogenes

Salmonella

S. aureus

Strains to be used

A cocktail of 3 to 5 strains of each pathogen should be used. Cocktails should include well-characterised strains, reference strains, strains known to have been involved in outbreaks from cheese, and strains with high resistance to the inimical conditions encountered during cheese making. A list of suggested strains is for each species is given in Section 4.1.5

Inocula should be introduced into the raw milk at levels of $\sim 10^4$ to 10^5 cfu.ml^{-1} representative of actual high levels of contamination. Inocula should be pre-cultured in milk to avoid carry-over of

non-milk suspending media, and in exponential growth phase to reflect the 'worst-case' approach adopted.

For evaluation of post-processing contamination, different methods are required and are described in Section 4.3.2

Location and Conduct of Challenge Trial

Given the desire to use frankly pathogenic strains, rather than avirulent surrogates, challenge trials will have to be conducted away from the processing plant. Given this, the design of the challenge trial must seek to identify and reproduce as closely as possible the normal methods of manufacture. Ideally, the cheese-maker would produce the cheese under the supervision of the microbiologist overseeing the challenge trial.

Officially endorsed microbiological methods should be used, or methods that have been validated (for cheese testing) against such standard methods.

Replicate samples should be tested at each sampling time. Samples should be taken from each distinct stage in the cheese making process, e.g., raw milk, milk after addition of the inoculum, during curd formation etc. To fully evaluate variance trials should be replicated, i.e. different batches of cheese should be prepared.

Caution must be exercised that the removal of a sample does not change the microbial ecology of the cheese so as to affect the fate of pathogens remaining in the cheese and that may be sampled subsequently. Alternatively, multiple cheeses, each representing a separate sampling time, may be preferable.

The amount of cheese made must be sufficient to enable analysis of the expected number of samples needed to evaluate the process.

Analysis and Interpretation of Data.

Universally acceptable rules for interpreting challenge trial results are not available. This highlights the need for the expertise of a food microbiologist as well as rigorous statistical analysis to correctly interpret challenge trial results as indicating the safety, or otherwise, of a cheese making process involving raw milk. The microbiologist and statistician should be involved in the design of the trial as well as analysis of results.

Documentation

Clear communication of the conduct and results of the trial will be required to enable an independent assessor to determine whether the process investigated in the challenge trial can be considered to be "safe" for a public health. This will involve description of the commercial process and its variability, rational of the challenge trial, detailed description of the conduct of the challenge trial, results of the trial and presentation of other information and discussion relevant to interpretation of the results that would enable a food safety manager to be able to determine, with confidence, the safety of the process.

This page left blank deliberately

6. References

AAM (American Academy of Microbiology). (2008). *Mycobacterium avium paratuberculosis*: Infrequent Human Pathogen or Public Health Threat? Accessed 1 January 2011 at http://academy.asm.org/index.php/colloquia-reports/-food-microbiology/192-mycobacterium-avium-paratuberculosis-infrequent-human-pathogen-or-public-health-threat-august-2008c.

Adams, M.R. and Moss, M.O. (2008). *Food Microbiology 3rd Edn*. RSC Publishing, Cambridge, UK. 463 pp.

Adesiyun, A.A. (1994). Bacteriological quality and associated public-health risk of pre-processed bovine-milk in Trinidad. *International Journal of Food Microbiology,* **21**:253-261.

Altekruse, S.F., Timbo, B.B., Mowbray, J.C., Bean, N.H. and Potter, M.E. (1998). Cheese-associated outbreaks of human illness in the United States, 1973 to 1992: Sanitary manufacturing practices protect consumers. *Journal of Food Protection,* 61:1405-1407.

Ammon, A. (1997). Surveillance of enterohaemorrhagic *E. coli* (EHEC) infections and haemolytic uraemic syndrome (HUS) in Europe. *Euro Surveillance*, **2**(12):pii=133. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=133.

Angelidis, A.S., Boutsiouki, P. and Papageorgiou, D.K. (2010). Loss of viability of *Listeria monocytogenes* in contaminated processed cheese during storage at 4, 12 and 22°C. *Food Microbiology*, **27**:809-818.

Animal Products Act (2009). Animal Products Act (Raw Milk Cheese Specifications) Notice 2009. Downloaded 28 Feb 2011, from http://www.foodsafety.govt.nz/elibrary/industry/Animal_Products-Sets_Requirements.pdf

AOAC (2006). *Guidelines for laboratories performing microbiological and chemical analyses of food and pharmaceuticals: an aid to interpretation of ISO/IEC 17025:2005.*AOAC International, Gaithersburg, USA.

Aureli, P., Di Cunto, M., Maffei, A., De Chiara, G., Franciosa, G., Accorinti, L., Gambardella, A.M. and Greco, D. (2000). An outbreak in Italy of botulism associated with a dessert made with Mascarpone cream cheese. *European Journal of Epidemiology*, **16**:913-918.

Baker, M.G., Lopez., L.D., Cannon, M.C., de Lisle, G.W. and Collins, D.M. (2006). Continuing *Mycobacterium bovis* transmission from animals to humans in New Zealand. *Epidemiology and Infection*, **134**:1068-1073.

Banks, W. (1994). Microbiology of milk. *Milk Industry (Tech.Process. Supp.),***96**:18-20.

Bayliss, C.L. (2009). Raw milk and raw milk cheeses as vehicles for infection by verocytotoxin-producing *Escherichia coli. International Journal of Dairy Technology*, **62**:293-307.

Bemrah, N., Sanaa, M., Cassin, M.H., Griffiths, M.W. and Cerf, O. (1998). Quantitative risk assessment of human listeriosis from consumption of soft cheese made from raw milk. *Preventative Veterinary Medicine*; **37**: 129-145.

Berry, D.P., O'Brien, B., O'Callaghan, E.J., Sullivan, K.O. and Meaney, W.J. (2006). Temporal trends in bulk tank milk somatic counts and total bacterial count in Irish dairy herds during the past decade. *Journal of Dairy Science*, **89**:4083-4093.

Boor, K.J. (2005). Origin of the 60-day minimum holding period requirement for United States cheeses made from sub- or unpasteurized milk. *Food Protection Trends*, **25**:767-770.

Bourry, A., Poutrel, B. and Rocourt, J. (1995). Bovine mastitis caused by *Listeria monocytogenes:* characteristics of natural and experimental infections. *Journal of Veterinary Microbiology,***43**:125-132.

Bowman, J.P., Chang, K.J.L., Pinfold, T. and Ross, T. (2010). Transcriptomic and phenotypic responses of *Listeria monocytogenes* strains possessing different growth efficiencies under acidic conditions. *Applied and Environmental Microbiology*, **76**: 4836-4850.

Buazzi, M.M., Johnson, M.E. and Marth, E.H (1992). Fate of *Listeria monocytogenes* during the manufacture of mozzarella cheese. *Journal of Food Protection*, **55**:80-83.

Buchanan, R.L., Golden, M.H. and J.G. Phillips (1997). Expanded models for the non-thermal inactivation of *Listeria monocytogenes. Journal of Applied Microbiology*, **82**:567-577.

Campbell, D.A., Cowden, J.M., Morris, G., Reilly, W.J. and O'Brien, S.J. (1996). All milk products should be pasteurized (Letter t[24]o the Editor). *British Medical Journal*, **312**:1099.

Cassin, M.H,.Paoli, G.M., McColl, R.S. and Lammerding, A.M. (1996). A comment on Hazard assessment of *Listeria monocytogenes* in the processing of bovine milk.(Journal of Food Protection, 57: 689–697, 1994).*Journal of Food Protection***59**: 341–343.

CDC (Centers for Disease Control and Prevention). (2008). Outbreak of multi drug-resistant *Salmonella enterica* serotype Newport infections associated with consumption of unpasteurized Mexican-style aged cheese – Illinois, March 2006 – April 2007. *Morbidity and Mortality Weekly Report*, **57**:432-435.

CDC (Centers for Disease Control and Prevention). (2009). *Campylobacter jejuni* infection associated with unpasteurized milk and cheese-Kansas, 2007. *Morbidity and Mortality Weekly Report*, **57:**1377-1379.

CDC (Centers for Disease Control and Prevention). (2010). Accessed 10 January 2011 at http://www.cdc.gov/ecoli/2010/cheese0157/.

Chiang, Y.C., Liao, W.W., Fan, C.M., Pai, W.Y., Chiou, C.S. and Tsen, H.Y. (2008). PCR detection of Staphylococcal enterotoxins (SEs) N, O, P, Q, R, U, Y and survey of SE types in *Staphylococcus aureus* isolates from food-poisoning cases in Taiwan. *International Journal of Food Microbiology,***121**:66-73.

Contreras, A., Sanchez, A., Corrales, J.C., Sierra, D., Gonzalo, C. and Paape, M.J. (2005). Mastitis in small ruminants. Pp. 67 – 74 in *Mastitis in Dairy Production: Current Knowledge and Future Solutions* Ed. H. Hogeveen, Wageningen Academic Publishers, Wageningen, NL.

Cornu, M., Billoir, E., Bergis, H., Beaufort, A. and Zuliani, V. (2010). Modelling microbial competition in food: Application to the behaviour of *Listeria monocytogenes* and lactic acid flora in pork meat products. *International Journal of Food Microbiology* (in press, doi:10.1016/j.fm.2010.08.007).

Cree, L.M. and Reid, M.H. (2009). Development of the UK food samples surveillance system. *Public Health,* **123**:89–94.

Czaplicki, A. (2007). "Pure Milk Is Better Than Purified Milk": Pasteurization and Milk Purity in Chicago, 1908-1916. *Social Science History*, **31**:411-433.

D'Amico, D.J. and Donnelly, C.W. (2010). Microbiological quality of raw milk used for small-scale artisanal cheese production in Vermont: Effect of farm characteristics and practices. *Journal of Dairy Science*, **93**:134-147.

D'Amico, D.J., Druart, M.J. and Donnelly, C.W. (2008a). 60-day aging requirement does not ensure safety of surface-mold-ripened soft cheeses manufactured from raw or pasteurized milk when *Listeria monocytogenes* is introduced as a post processing contaminant. *Journal of Food Protection*, **71**: 1563-1571.

D'Amico, D.J., Druart, M.J. and Donnelly, C.W. (2010). *Escherichia coli* behaviour of O157:H7 during the manufacture and aging of Gouda and stirred-curd cheddar cheese manufactured from raw milk. *Journal of Food Protection*, **73**: 2217-2224.

D'Amico, D.J., Groves, E. and Donnelly, C.W. (2008b). Low incidence of foodborne pathogens of concern in raw milk utilized for farmstead cheese production. *Journal of Food Protection*, **71**:1580-1589.

Das, D., Baker, M., Venugopal, K. and McAllister, S. (2006). Why the tuberculosis incidence rate is not falling in New Zealand. *Journal of the New Zealand Medical Association*, **119** (1243), 11 pp. accessed 6 May 2011 from http://www.nzma.org.nz/journal/119-1243/2248/content.pdf

De Buyser, M-L., Dufour, B., Maire, M. and Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology,***67**:1-17.

Djabri, B., Bareille, N., Beadueau, F. and Seegers, H. (2002). Quarter milk somatic cell counts in infected dairy cows: a meta-analysis. *Veterinary Research*, **33**:335-357.

Donaghy, J.A., Totton, N.L. and Rowe, M.T. (2004). Persistence of *Mycobacterium paratuberculosis* during manufacture and ripening of cheddar cheese. *Applied and Environmental Microbiology*,**70**:4899- 4905.

Donnelly, C.W. (2004). Growth and survival of microbial pathogens in cheese. Pp. 541 – 559 in *Cheese: chemistry, physics, and microbiology* Ed. P.F. Fox, P.L.H. McSweeney, T.M. Cogan and T. P. Guinee. Elsevier/Academic Press, Amsterdam.

Duffy, L.L., Grau, F. H. and Vanderlinde, P. B. (2000). Acid resistance of enterohaemorrhagic and generic *Escherichia coli* associated with foodborne disease and meat. *International Journal of Food Microbiology*, **60**:83-89.

Duffy, L.L., Small A. and Fegan, N. (2010). Concentration and prevalence of *Escherichia coli* O157 and *Salmonella* serotypes in sheep during slaughter at two Australian abattoirs. *Australian Veterinary Journal,* **88**:399-404.

Durmaz, H. (2009). Fate of *Listeria monocytogenes* in carra cheese during manufacture and ripening. *Journal of Food Safety*, **29**:253-260.

EC (European Commission) (2002). Trends and sources of zoonotic agents in animals, feeding stuffs, food and man in the European Union and Norway in 2002. Accessed on sss22 January 20100 at http://ec.europa.eu/food/food/biosafety/salmonella/zoonoses_reps_2002_en.htm

EFSA (European Food Safety Authority) (2007). Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union,* **L 322**, 7 December 2007: 12-29.

EFSA (European Food Safety Authority). (2007). Request for updating the former SCVPH opinion on *Listeria monocytogenes* risk related to ready-to-eat foods and scientific advice on different levels of *Listeria monocytogenes* in ready-to-eat foods and the related risk for human illness. *The EFSA Journal*, **599**:1-42

Eglozos, S., Huang, B.X., Dykes, G.A., Fegan, N., Bell, K. and Studdard, E. (2008). A survey of microbiological quality of frozen unpasteurized goats' milk in Queensland, Australia. *Australian Journal of Dairy Technology*, **63**:79-81.

El-Zubeir, I.E.M., Gabriechise, V. and Johnson, Q. (2008). Comparison of chemical composition of raw and pasteurized milk of the Western Cape, South Africa. *International Journal of Dairy Science*, **3**:137 – 143.

Elliott, P.H. and Schaffner, D.W. (2001). Germination, growth, and toxin production of nonproteolytic *Clostridium botulinum* as affected by multiple barriers. *Journal of Food Science*, **66**:575-579.

Elmoslemany, A.M. (2009). The association between bulk tank milk analysis for raw milk quality and on-farm management practices. PhD Thesis, University of Prince Edward Island, Canada. 201 pp.

eMedicine (2010). Brucellosis. Accessed on 19 December 2010 at http://emedicine.medscape.com/article/213430-overview.

EpiCentre (Institute of Veterinary, Animal and Biomedical Sciences, Massey University). (2008). A systematic review of the human disease evidence associated with the consumption of raw milk and raw milk cheeses. A report prepared for the New Zealand Food Safety Authority. Accessed 15 December 2010 from http://www.nzfsa.govt.nz/science/research-projects/final-report-rawmilk.pdf

EU-SCSVMPH (2003). Opinion of the Scientific Committee on Veterinary Measures relating to Public Health On Salmonellae in Foodstuffs. Access 12 January 2011 at http://ec.europa.eu/food/fs/sc/scv/out66_en.pdf

FAO/WHO (Food and Agriculture Organization and World Health Organization of the United Nations). (2002). Risk assessment of *Salmonella* in eggs and broiler chickens. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00100 Rome, Italy or

http://www.fao.org/DOCREP/005/Y4392E/y4392e00.htm

FAO/WHO (Food and Agriculture Organization of the United Nations and World Health Organization). (2004). Risk assessment of *Listeria monocytogenes* in ready-to-eat foods. TECHNICAL REPORT. Microbiological risk assessment series 5. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00100 Rome, Italy. or http://www.fao.org/es/esn/food/risk_mra_listeria_report_en.stm.

Fegan, N., Higgs, G., Duffy, L. and Barlow, R.S. (2009). The effects of transport and lairage on counts of *Escherichia coli* O157 in the feces and on the hides of individual cattle. *Foodborne Pathogens and Infectious Disease*, **6**:1113-1120.

Fegan, N., Vanderlinde, P., Higgs, G., Desmarchelier, P. (2004). Quantification and prevalence of *Salmonella* in beef cattle presenting at slaughter. *Journal of Applied Microbiology*, **97**, 892–898.

Fenlon, D.R., Logues, D.N., Gunn, J. and Wilson, J. (1995). A study of mastitis bacteria and herd-management practices to identify their relationship to high somatic cell counts in bulk tank milk. *British Veterinary Journal*, **151**:17-25.

Fox, P.F., Guinee, T.O., Cogan, T.M. and McSweeney, P.L.H. (2000). *Fundamentals of Cheese Science*, Aspen Publishers, N.Y. 608 pp.

Friedly, E.C., Crandall, P.G., Ricke, S., O'Bryan, C.A., Martin, E.M. and Boyd LM. (2008). Identification of *Listeria innocua* surrogates for *Listeria monocytogenes* in hamburger patties. *Journal of Food Science,***73** :M174-8.

FSANZ (Food Standards Australia New Zealand: Risk Assessment Microbiology Section) (2009). Microbiological Risk Assessment of Raw Milk Cheese. Accessed 15 December 2010 from http://www.foodstandards.gov.au/_srcfiles/P1007%20PPPS%20for%20raw%20milk%201AR%20SD3%20Cheese%20Risk%20Assessment.pdf. 296 pp.

FSANZ (Food Standards Australia New Zealand: Risk Assessment) (2004). Association between Johne's Disease and and Crohn's Diease. A microbiological review. Technical report Series Np. 35. Accessed 1 January 2011 from : http://www.foodstandards.gov.au/_srcfiles/edit_Report_JD%20and%20CD-%20Final%20Dec%202004.pdf

FSNet (2006). Accessed 21 January 2011 at http://archives.foodsafety.ksu.edu/fsnet/2006/6-2006/fsnet_june_23.htm

Glass, K.A., Kaufman, K.M. and Johnson, E.A. (1998). Survival of bacterial pathogens in pasteurized process cheese slices stored at 30°C. *Journal of Food Protection*, **61**:290-294.

Gould, G., Franken, P., Hammer, P., Mackey, B. and Shanahan, F. (2005). *Mycobacterium avium* subsp. *paratuberculosis* (MAP) *and the Food Chain*.ILSI Europe Report Series. Report prepared under the responsibility of the ILSI Europe Emerging Pathogen Task Force. (*n.b.,* also published in *Food Protection Trends*, **25**:268-297).

Hages, E. (2010). Non-thermal inactivation of pathogens in raw milk cheeses. PhD Thesis, University of Tasmania. 208 pp.

Hamann, J. (2005). Diagnosis of Mastitis and Indicators of Milk Quality.pp. 82-90.in *Mastitis in Dairy Production: Current Knowledge and Future Solutions* Ed. H. Hogeveen, Wageningen Academic Publishers, Wageningen, NL.

Harmon, R.J. (1994). Physiology of mastitis and factor affecting somatic cell counts. *Journal of Dairy Science*, **77**:2103-2112.

Heidinger, J.C., Winter, C.K. and Cullor, J.S. (2009). Quantitative microbial risk assessment for *Staphylococcus aureus* and *Staphylococcus* enterotoxin A in raw milk. *Journal of Food Protection*, **72**:1641-1653.

Hill, A. and Warriner, K. (2010). Cheese Safety 101: Part 2: I have seen the enemy and he speaks Latin. Ontario Cheese Society: Accessed on 19 December 2010 at: http://www.ontariocheese.org/cheese_safety/cheese_safety_2.php

Howard, P. (2006). Mastitis pathogens present in bulk tank milk from seven dairy herds in the Waikato region, New Zealand. *New Zealand Veterinary Journal,* **54**:41-43.

http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=39883

Hyytiä, E., Heilm, S., Mokkila. M., Kinnunun, A. and Korkeala, H. (1999). Predicted and observed growth and toxigenesis by *Clostridium botulinum* type E in vacuum-packaged fishery product challenge tests. *International Journal of Food Microbiology*, **47**:161-169.

ICMSF (International Commission for Microbiological Specification of Foods) (2005). *Microbial Ecology of Food Commodities* 2nd Edn. Kluyver Academic, N.Y., 736 pp.

ICMSF (International Commission on Microbiological Specifications for Foods) (*in press*). Chapter 2. Validation of Control Measures*in Microorganisms in Foods 8. Use of Data for Assessing Process Control and Product Acceptance* Kluwer Academic/Plenum Publishers, New York, USA.

ICMSF (International Commission on Microbiological Specifications for Foods) (1996). *Microorganisms in Foods 5.Characteristics or Microbial Pathogens.* Blackie Academic and Professional, London.

IFST (Institute of Food Science and Technology) (1998). *Food Safety and Cheese. Institute of Food Science and Technology Position Statement. Cited in Fox et al. (2000) q.v.*

IFTS&T (Institute of Food Technologists Scientific and Technical Panel). (2003a). Chapter VI Microbiological Challenge Testing. pp. 46 – 50 in: *Comprehensive Reviews in Food Science and Food Safety*, **2** (supplement): 46 – 50.

IFTS&T (Institute of Food Technologists Scientific and Technical Panel) (2003b). Chapter VIII: Framework Developed to Determine Whether Foods Need Time/Temperature Control for Safety. Pp. 55-62 in 'Evaluation and Definition of Potentially Hazardous Foods'. Comprehensive *Reviews in Food Science and Food Safety*, **2(supp)**:3-109.

Irlinger, F. and Mounier, J. (2009). Microbial interactions in cheese: implications for cheese quality and safety. *Current Opinion in Biotechnology*, **20**:142-148.

ISO/IEC 17025 (2005). General requirements for the competence of testing and calibration laboratories. Available from:

Johnson, E.A., Nelson, J.H. and Johnson, M. (1990). Microbiological safety of cheese made from heat-treated milk. III. Technology, discussion, recommendations, bibliography. *Journal of Food Protection*, **53**:610-623.

Kagkli, D.M, Iliopoulos, V., Stergiou, V., Lazaridou, A. and Nychas, G.J. (2009) Differential *Listeria monocytogenes* strain survival and growth in Katiki, a traditional Greek soft cheese, at different storage temperatures. *Applied and Environmental Microbiology*, **75**:3621-3626.

Kasrazadeh, M. and Genigeorgis, C. (1994). Potential growth and control of *Salmonella* in hispanic type soft cheese. *International Journal of Food Microbiology*, **22**:127-140.

Kérouanton, A., Hennekinne, J.A., Letertre, C., Petit, L., Chesnau, O., Brisabois, A. and de Buyser, M.L. (2007). Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *International Journal of Food Microbiology*, **115**:369-375.

Kocharunchitt, C., King, T., Gobius, K., Bowman, J. and Ross, T. (*submitted*). Integrated transcriptomic and proteomic analysis of the physiological response of *Escherichia coli* O157:H7 Sakai to steady-state conditions of cold and water activity stress. *Submitted to Molecular and Cellular Proteomics, March, 2011.*

Kongo, J.M., Gomes, A.P. and Malcarai, F.X. (2008). Monitoring and identification of bacteria associated with safety concerns in the manufacture of Sao Jorge, a Portugese traditional cheese from raw cow's milk. *Journal of Food Protection*, **71**:986-992.

Lake, R., Hudson, A., Cressey, P. and Gilbert, S. (2005). *Risk Profile: Listeria monocytogenes in soft cheeses.* Institute of Environment al Science and Research Limited. Christchurch. Downloaded 2 February 2011 from: http://www.foodsafety.govt.nz/elibrary/industry/Risk_Profile_Listeria_Monocytogenes_Soft-Science_Research.pdf

Lake, R., Gilbert, S., Lok Wong, T. and Cressey, P. (2009). Risk profile: *Mycobacterium bovis* in milk. Institute of Environmental Science & Research Limited, Christchurch, New Zealand.

Leedom, (2006). Milk of nonhuman origin and infectious diseases in humans. *Clinical Infectious Diseases*, **43**:610 –615.

Linton, R.H. and Harper, N. (2008). Survival and growth of foodborne microorganisms in processed and individually wrapped cheese slices. *Journal of Environmental Health*, **70**:51-54

Little, C.L., Rhoades, J.R., Sagoo, S.K., Harris, J., Greenwood, M., Mithani, V., Grant, K. and McLauchlin, J. (2008). Microbiological quality of retail cheeses made from raw, thermized or pasteurized milk in the UK. *Food Microbiology*, **25**:304-312.

Liu, S.W., Puri, V.M. and Demirci, A. (2009). Evaluation of *Listeria innocua* as a suitable indicator for replacing *Listeria monocytogenes* during ripening of Camembert cheese. *International Journal of Food Science & Technology*, **44**:29-35.

Lund, B. M. and S.H.W. Notermans. (1993). Potential hazards from REPFEDS. pp. 279 - 304 in A.H.W. Hauschild and K.L. Dodds (Eds). *Clostridium botulinum*: Ecology and Control in Foods. Marcel Dekker, Inc., NY. 412 pp.

MAFNZ (Ministry of Agriculture and Forestry New Zealand) (2010). "Brucellosis". Accessed on 19 December, 2010 at: http://www.biosecurity.govt.nz/pests/brucellosis

Maher, M.M., Jordan, K.N., Upton, M.E. and Coffey, A. (2001). Growth and survival of *E. coli* O157:H7 during the manufacture and ripening of a smear ripened produced from raw milk. *Journal of Applied Microbiology,***90**:201-207.

Marler, W.D. (2010a). The raw milk beat goes on: a look at the literature and the 60-day raw milk cheese again rule. Part 1. Accessed 10 December, at http://www.marlerblog.com/uploads/image/cheese%20piece%20Final%20updated%2012_18_10.pdf

Marler, W.D. (2010b). Comparing the food safety record of pasteurized and raw milk products. Part 3. Accessed 10 December, at http://www.marlerblog.com/uploads/image/2010%20ALL%20dairy%20through%20NOV%2013.pdf

McClelland, M., Sanderson, K.E., Spieth, J., *et al.* & Wilson, R.K. (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature*, **413**:852-856.

McQuestin, O.J., Shadbolt, C.T. and Ross, T. (2009). Quantification of the relative effects of temperature, pH, and water activity on inactivation of *Escherichia coli* in fermented meat by meta-analysis. *Applied and Environmental Microbiology*, **75**:6963 -6972.

Mejlholm, O. and Dalgaard, P. (2007). Modeling and predicting the growth boundary of *Listeria monocytogenes* in lightly preserved seafood. *Journal of Food Protection*, **70**:70-84.

Mellefont, L.A. and Ross, T. (2007). Effect of potassium lactate and potassium lactate-sodium diacetate blend on *Listeria monocytogenes* growth in modified atmosphere packaged sliced ham. *Journal of Food Protection*, **70**:2297-2305.

Mellefont, L.A., McMeekin, T.A. and Ross, T. (2008). Effect of relative inoculum concentration on *Listeria monocytogenes* growth in co-culture. *International Journal of Food Microbiology,* **121**:157-168.

Moraes, P.M., Vicosa, G.N., Yamazi, A.K., Orttolani, M.B.T. and Nero, L.A. (2009). Foodborne pathogens and microbiological characteristics of raw milk soft cheese produced and on Retail sale in Brazil. *Foodborne Pathogens and Disease*, **6**:245-249.

Mossel, D. A. A., Corry, J. E. L., Struijk, C. B., and Baird, R. M. (Editors, 1995). *Essentials of the Microbiology of Foods: A Textbook for Advanced Studies*, John Wiley & Sons, New York. 699 pp.

Muehlherr, J.E., Zweifel, C., Corti, S., Blanco, J.E. and Stephan, R. (2003). Microbiological quality of raw goat's milk and ewe's bulk-tank milk in Switzerland. *Journal of Dairy Science*, **86**:3849-3856.

Murdough, P.A., Deitz, K.E. and Pankey, J.W. (1996). Effects of freezing on the viability of nine pathogens from quarters with subclinical mastitis. *Journal of Dairy Science*, **79**:334-336.

MWLC (Manaaki Whenua Landcare Research). (2011). Possums and TB. Accessed 6 May 2011 from http://www.landcareresearch.co.nz/publications/infosheets/possums/possums_tb.pdf

NACMSF (National Advisory Committee on Microbiological Criteria for Foods). (2010). Parameters for Determining Inoculated Pack/Challenge Study Protocols. *Journal of Food Protection*, **173**:140 – 202.

Notermans, S., in't Veld, P., Wijztes, T. and Mead, G.C. (1993). A user's guide to microbial challenge testing for ensuring the safety and stability of food products. *Food Microbiology*, **10**:145-157.

NZMOH (New Zealand Ministry of Health) (2010). Public Health Surveillance: annual Surveillance Summary. Accessed 24 December 2010 at: http://surv.esr.cri.nz/surveillance/annual_surveillance.php

O'Brien, M., Hunt, K., McSweeney, S. and Jordan, K. (2009). Occurrence of foodborne pathogens in Irish Farmhouse cheese. *Food Microbiology*, **26**:910-914.

O'Mahony, P.A. and Austin, F.H. (1991). A study of on-farm factors affecting bacteriological quality of bulk farm milk. *Irish Journal of Agricultural Research*, **30**:113-119.

Orotlani, M.B., Yamazi, A.K., Moraes, P.M., Vicosa, G.N. and Nero, L.A. (2010). Microbiological quality and safety of raw milk and soft cheese and the detection of autochthonous lactic acid bacteria with antagonistic activity against *Listeria monocytogenes, Salmonella* spp., and *Staphylococcus aureus. Foodborne Pathogens and Disease*, **7**:175-180.

Ostyn A., De Buyser, M.L., Guillier, F., Groul, Félix, B., Salah, S., Delmas, G. and Hennekinne, J.A. (2010). First evidence of a food poisoning outbreak due to staphylococcal enterotoxin type E, France, 2009. *Euro Surveillance,* **15**(13):pii=19528. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19528

Park, S.F. (2002). The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *International Journal of Food Microbiology*, **74**:17-188.

Pereira, C.I., Graca, J.A., Ogando, N.S., Gomes, A.M.P. and Malcata, F.X. (2009). Bacterial dynamics in model cheese systems, aiming at safety and quality of Portugese-style traditional ewe's cheese. *Journal of Food Protection*, **72**:2243-2251.

Potter, M.E., Kaufmann, A.F., Blake, P.A. and Feldmann, R.A. (1984). Unpasteurized milk: the hazards of a health fetish. *JAMA*, **252**: 2048 – 2052.

Rampling, A. (1996). Raw milk cheese and Salmonella (Editorial). *British Medical Journal*, **312**: 67.

Ratkowsky, D.A., Olley, J., McMeekin, T.A. and Ball, A. (1982). Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology*, **149**: 1-5.

Reitsma, C. and Henning, D. (1996). Survival of enterohaemorrhagic *Escherichai coli* O157:H7 during the manufacture of cheddar cheese. *Journal of Food Protection*, **59:**460-464.

Rhoades, J.R., Duffy, G. and Koutsoumanis, K. (2009). Prevalence and concentration of verocytotoxigenic *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes* in the beef production chain: A review. *Food Microbiology,* **26**:357-376.

Rosengren, A., Fabricius, A., Sylven, S. and Lindvist, R. (2010). Occurrence of foodborne pathogens and characterization of *Staphylococcus aureus* in cheese produced on farm-dairies. *International Journal of Food Microbiology*, **144**:263-269.

Ross, T. and Dalgaard, P. (2004). Secondary Models. pp. 63 – 150 in *Modelling Microbial Responses in Foods*. Ed. R. McKellar and X. Lu. CRC Press, Boca Raton, USA.

Ross, T., McQuestin, O.J. and Vanderlinde, P.B. (2004). Predictive model for the inactivation of *E. coli* in uncooked comminuted fermented meat products (FINAL REPORT of Project PRMS.021a AUGUST, 2004). Meat and Livestock Australia, Sydney, pp. 109.

Ross, T., Ratkowsky, D.A., Mellefont, L.A. and McMeekin, T.A. (2003). Modelling the effects of temperature, water activity, pH and lactic acid concentration on the growth rate of *Escherichia coli. International Journal of Food Microbiology*, **82**:33– 43.

Rowe, M.T. and Donaghy, J. (2008). *Mycobacterium bovis:* the importance of milk and dairy products as a cause of human tuberculosis in the UK. A review of taxonomy and culture methods, with particular reference to artisanal cheeses. *International Journal of Dairy Technology*, 61: 317-326.

Rüegg, M and Blanc, B. (1977). Beziehungen zwischen Wasseraktivität, Wasser-Sorptionsvermögen und Zusammensetzung von Käse.*Milchwissenschaft/Milk Science International*, **32**:193-201.

Rysanek, D., Zouharova, M. and Babak., V. (2009a). Monitoring major mastitis pathogens at the population level on examination of bulk tank milk samples. *Journal of Dairy Research*, **76**:117-123.

Rysanek, D., Zouharova, M. and Babak., V. (2009b). Major mammary pathogens as contributors to total bacterial counts in raw milk. *Acta Veterinaria Brno*, **78**: 455 – 461.

Ryser, E.T. (1997). Incidence and behaviour of *Listeria monocytogenes* in cheese and other fermented dairy products. Pp. 411 – 503 in *Listeria, Listeriosis and Food Safety 2nd Edition, Revised and Expanded.* E.T. Ryser and E.H. Marth (editors). Marcel Dekker, N.Y.

Ryser, E.T. and Marth, E.H. (1987). Fate of *Listeria monocytogenes* during manufacture and ripening of Camembert cheese. *Journal of Food Protection*, **50**:372 378.

Salter, M., Ross, T. and McMeekin, T.A. (1998). Applicability of models for non-pathogenic *Escherichia coli* for predicting the growth of pathogenic *Escherichia coli. Journal of Applied Microbiology*, **85**: 357 - 384.

Sandroua, D.K. and Arvanitoyannisa, I. S. (2000). Application of Hazard Analysis Critical Control Point (HACCP) System to the Cheese-Making Industry: A Review. *Food Reviews International,* **16**: 3, 327 — 368.

Schlesser, J.E., Gerdes, R., Ravishankar, S., Madsen, K., Mowbray, J. and Teo, A.Y.L. (2006). Survival of a five-strain cocktail of *Escherichia coli* O157:H7 during the 60-day aging period of cheddar cheese made from unpasteurized milk. *Journal of Food Protection*, **69**:990-998.

Schoder, D., Winter, P., Kareem, A., Baumgartner, W. and Wagner, M. (2003). A case of sporadic ovine mastitis caused by *Listeria monocytogenes* and its effect on contamination of raw milk and raw-milk cheeses produced from it. *Journal of Dairy Research*, **70**: 395-401.

Schoder, D., Zangana, A and Wagner, M. (2010). Sheep and goat raw milk consumption: a hygienic matter of concern? *Archiv fur Lebensmittelhygiene*, **61**:229-234.

Schvartzman, M.S., Maffre, A., Tenenhaus-Aziza, M., Sanaa, M., Butler, F. and Jordan, K. (2010). Modelling the fate of *Listeria monocytogenes* during manufacture and ripening of smeared cheese made with pasteurised or raw milk. *International Journal of Food Microbiology*, doi:10.1016/j.ijfoodmicro.2010.11.032

Scotter, S.L., Langton, S., Lombard, B., Lahellec, C., Schulten, S., Nagelkerke, N., in't vedl, P.H., and Rollier, P. (2001). Enumeration of *Listeria monocytogenes* in foods. *International Journal of Food Microbiology*, **70**:121-129.

Shabala, L., Lee, S.H., Cannesson, P., and Ross, T. (2008). Acid and NaCl limits to growth of *Listeria monocytogenes* and influence of sequence of inimical acid and NaCl levels of inactivation kinetics. *Journal of Food Protection,* **71**:1169-1177.

Sommers, C.H., Geveke, D.J and Fan, X. (2008). Inactivation of *Listeria innocua* on frankfurters that contain potassium lactate and sodium diacetate by flash pasteurization. *Journal of Food Science,* **73**:M72–M74.

Sommers, C.H., Geveke, D.J. Pulsfus, S. and Lemmenes. B. (2009). Inactivation of *Listeria innocua* on frankfurters by ultraviolet light and flash pasteurization. *Journal of Food Science*, **74**:M138–M141.

Spahr, U. and Schaforth, K. (2001). Fate of *Mycobacterium avium* subsp *paratuberculosis* in Swiss hard and semihard cheese manufactured from raw milk. *Applied and Environmental Microbiology*, **67**:4199-4205.

Sung, N. and Collins, M.T. (2000). Effect of three factors in cheese production (pH, salt, and heat) on *Mycobacteroium avium* subsp. *paratuberculosis* viability. *Applied and Environmental Microbiology*, **66**:1334-1339.

Tanaka, N. (1982). Challenge of pasteurized process cheese spreads with *Clostridium botulinum* using in-process and post-process inoculation. *Journal of Food Protection*, 45:1044-1050.

Tanaka, N., Traisman, E., Plantinga, P., Finn, L., Flom, W., Meske, L. and Guggisberg, J. (1986). Evaluation of factors involved in antibotulinal properties of pasteurized process cheese spreads. *Journal of Food Protection*, **49**:526-531.

Tienungoon, S., Ratkowsky, D.A., McMeekin, T.A. and Ross, T. (2000). Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl and lactic acid. *Applied and Environmental Microbiology*. **66**:4979-4987.

Torres, A., Rajala-Schultz, P.J. and DeGraves, F.J. (2009). Diagnosis of intramammary infections at dry-off based on sampling strategy, epidemiology of pathogens, and agreement beyond chance. *Journal of Veterinary Diagnostic Investigations,* **21**: 427-436.

van Duynhoven, Y.T.H.P., Isken, L.D., Borgen, K., Besselse, M, Soethoudt, K. , Haitsma, O., Mulder, B. Notermans, D.W., De Jonge, R., Kock, P., Van Pelt, W., Stenvers, O. and Jan Steenbergen, V. (2008). A prolonged outbreak of *Salmonella Typhimurium* infection related to an uncommon vehicle: hard cheese made from raw milk. *Epidemiology and Infection* (*Cambridge),* **37**:1548-1557.

Van Schalk, G., Lotem, M. and Schukken, Y.H. (2002). Trends in somatic cell counts, bacterial counts, and antibiotic residue violations in New York State during 1999-2000.*Journal of Dairy Science*, **85**: 782-789.

Vestergaard, E.M. (2001). Building product confidence with challenge studies. *Dairy, Food and Environmental Sanitarian*, **21**:206-209.

Wagner, M., Podstatzky-Lichtenstein, L., Lehner, A., Asperger, H., Baumgartner, W and Frandl, E. (2000). Prolonged excretion of *L. monocytogenes* in a sub-clinical case of mastitis.*Milchwissenschaft-Milk Science International*, **55**:3-6.

Weisbacker, A. (2007). A legal history of raw milk in the United States. *Journal of Environmental Health*, **69**:62-63.

Wells, J.E., Shackleford, S.D., Berry, E.D., Kalchananand, N., Guerni, M.N., Varel, V.H., Arthur, T.M., Boilevic, J.M., Freetly, H.C., Wheeler, T.L., Ferrell, C.L. and Koohmaraie, M. (2009). Prevalence and level of Escherichia coli O157:H7 in feces and on hides of feedlot steers fed diets with or without wet distillers grains with solubles. *Journal of Food Protection*, **72**:1624-1633.

West, H.G. (2008). Food fears and raw-milk cheese. *Appetite*, **51**:25-29.

Zhang, D.L., Ross, T. and Bowman, J.P. (2010). Physiological aspects of *Listeria monocytogenes* during inactivation accelerated by mild temperatures and otherwise non-growth permissive acidic and hyperosmotic conditions. *International Journal of Food Microbiology,* **141**:177–185.

7. Appendices

Appendix 1: Cheese Categorisation

Over 1,000 different types of cheese can be distinguished. The names of cheeses derive from:

- place of origin
- type (animal) of milk
- shape
- duration of ripening
- method of ripening
- microbes involved.

By international agreement, however, only 35 distinct varieties are recognised.

A useful categorisation of types of cheese is according to their hardness:

- ***soft and/or not ripened, "fresh"***(e.g. Cottage, Queso Blanco, Quarg, Mozzarella). These styles do not rely on rennet ofr curd formation - they are simply acidified
- ***brined*** (e.g. Feta, Domiati)
- ***mould ripened***(e.g. Brie, Camembert, Roquefort, Gorgonzola, Danish Blue)
- ***bacterial surface ripened*** (e.g. smear cheeses, Brick, Limburger, Mont d'Or, Munster)
- ***semi-hard*** (e.g. Caerphilly, Wensleydale, Colby, Edam)
- ***hard/semi-hard***: open textured 'eyeholes' (e.g. Havarti, Emmental, Gruyere, Tilsiter)
- ***hard***: close texture (e.g. cheddars)
- ***extra-hard*** (e.g., Reggiano, Parmesan/Grana, Romano)

In general, the harder the cheese the longer is has been left to mature.

Other characteristics, such as the presence of eye-holes (due to gas formation by bacteria) are also used to differentiate types of cheese.

FSANZ (2009; Appendix 3) provides a good discussion of various schemes for classification of cheeses.

Dairy Australia provides a very comprehensive and illustrated 'on-line' guide to cheese nomenclature and styles and can be accessed at:

http://www.dairyaustralia.com.au/Products-and-Recipes/Dairy-Products/Cheese/Types-of-Cheese.aspx

Appendix 2: Example Protocols

This appendix contains examples of design of a challenge protocol for two cheeses to illustrate some of the considerations in the experimental design.

Example 1. Gouda, 10 kg wheel

Gouda is a semi-hard cheese. It is produced in a large wheel shape from cow's milk. It is covered in a wax during maturation.

According to Table 3, physico-chemical parameters of the cheese (pH 5.1, a_w 0.953) may allow growth of *L. monocytogenes*, *Salmonella*, *E. coli* and *S. aureus*, as well as other foodborne bacterial pathogens. As such, the trial should consider the fate of each of these organisms.

In cheese-making, approximately 10L of milk produces 1 kilogram of cheese. Accordingly, for a 10kg wheel of gouda, 100 L of raw milk is needed. Milk should be obtained from the normal source used by the cheese-maker.

For the trials, each cheese is large enough to enable multiple samples to be taken, but two replicate cheeses per organism will be required *per challenge organism* to assess within batch variation, and two separate batches should be assessed per organism to assess between batch variation. Uninoculated controls (i.e. not containing pathogens) will also be needed. Accordingly, to test all pathogens of interest, twenty 10kg wheels of gouda will be needed - four for each pathogen tested (two trials involving two replicates) and two uninoculated control trials involving two replicates each.

Inocula should be prepared in suitable broth at temperatures as described in Section 4.3.1. For each batch, approximately 2 x 10^8 exponential phase cells of each of the challenge strains are required for each cheese to be made. (The number of cells is based on the volume of milk to be used in the manufacture of the cheese, and inoculating to achieve 10^5 pathogen cfu.mL^{-1} in the milk based on a five strain mixture). 2 x 10^8 cells can be obtained from ~20 mLs of a 10^7 cfu.mL^{-1} culture, or equivalent combination of volume and cell density, but remembering that at levels greater than ~5 x 10^7 cfu.mL^{-1} the culture will be beginning to enter stationary phase.

The exponential phase culture can be centrifuged to remove the growth medium (decanted or aspirated off) and the pellet resuspended in a small volume (e.g., 5 - 10 mL) of sterile milk. The five cultures resuspended in milk are combined and allowed to incubate at ~10°C for ~ 1 hour to reestablish growth. This culture is added to the milk as soon after milking as possible. The milk should be handled normally after that, and cheese produced in exactly the same manner as the normal commercial process.

Samples should be taken and tested for the level of the specific pathogen added to the milk as well as temperature, pH, water activity and total aerobic (microbial) count ("TVC") at each of the following points in the process:

i) Upon inoculation into the milk

ii) After warming but before addition of starter culture and rennet

iii) After addition of starter culture and rennet

iv) After curd has formed and whey discarded but before curd handling

v) After washing and brining of the curd

vi) From the soft curd after formation of the wheels but before waxing

At each of the above times duplicate samples should be analysed for each of the tests described.

vii) At equal intervals during the maturation, at least three duplicate samples should be taken and the tests described above undertaken. Alternatively, at least five unreplicated samples at equal intervals should be taken and the tests described above undertaken.

Additionally, pathogen levels in the inoculums hold be determined prior to inoculation of the raw milk, and the same suite of tests undertaken on the raw milk before introduction of the challenge strains.

For each set of tests, approximately 30 - 50g of material are needed from each replicate to complete the tests. For *Salmonella*, unless performed by MPN, 25g are needed. A further 10g are needed for the TVC and approximately 5 - 10 g each for pH and water activity determinations. If Salmonella is enumerated by MPN ~35g are needed, and a further 20g for TVC and physico-

chemical tests. For other pathogens that can be directly plated for enumeration (and possibly including and 'on-plate' resuscitation step) a 10 g sample only is required for pathogen enumeration, in addition to the amounts needed for the other tests.

Unless specifically assessing spatial differences in microbiology or physico-chemical properties of the cheese (not expected to be required in this style of cheese because it is not surfaced ripened), the sample taken could be homogenized by aseptically mincing it into pieces 1 – 2 mm in diameter/side length and mixing these small pieces, prior to preparation for microbiological, pH or water activity testing. This will minimize differences due to micro-environments in the sample and when attempting to correlate physico-chemical properties to microbiological outcomes.

For this type of cheese, multiple samples can be taken from the same cheese (e.g. using a cork borer) but subsequent samples need to be taken at a site far enough away from previous sampling sites so as to be unaffected by removal of the previous sample. Also, the sampling site should be resealed with wax. All sampling sites should be randomly selected.

Example 2. Washed rind cheese (300g); bacterial surface ripened

Bacterial surface-ripened cheeses include Limberger, Tilsit, and brick. They are also called 'smear' or 'red smear' cheeses because of the development of red-orange colour on their surfaces during ripening primarily due to the growth of the bacterium, *Brevibacterium linens*. The ripening process involves microbes that catabolise amino acids to produce alkaline metabolites, such as ammonia. This, in turn, leads to de-acidification of the cheese, which is most pronounced near the surface where these proteolyic organisms are, potentially allowing the growth of pathogens that were initially inhibited by the acidity.

According to Table 3, representative physico-chemical parameters of these cheeses (pH 5.3, a_w 0.98) may allow growth of *L. monocytogenes*, *Salmonella*, *E. coli* and *S. aureus*, as well as other foodborne bacterial pathogens. As such, the trial should consider the fate of each of these organisms.

The relatively small size of individual cheeses of this style, require multiple cheeses will to be made for the challenge trial. Also, with this style of cheese, significant differences in the physico-chemical properties of the cheese are expected in the core compared to the rind, with pH expected to be higher nearer to the rind (i.e. the site of proteolytic activity and deamination processes that lead to production of ammonium ions) As such, it would be expected that one cheese would yield only one full set of samples for analysis, i.e., for pH, water activity and microbial counts both at the core and near the surface/rind. At least two inoculated samples will be needed for each sampling time, as well as two uninoculated controls. The samples taken from the liquid milk (i.e., prior to curd formation) for analysis will reduce the volume by a relatively

insignificant amount and should not need to be considered in determining the amount of milk needed to produce enough samples of cheese. Other sample times include:

- after curd formation
- after salting/moulding
- after application of the surface-ripening culture
- three time points during the maturation period

requiring a minimum of six samples, in duplicate. Thus, a minimum of twelve 300g cheeses *per pathogen* are required for the challenge study for *both* the inoculated and uninoculated products. The trial should include two 'biological' replicates, i.e., the trial should be done twice to estimate between-batch variability. Typically 10L of milk are required to produce 1 kg of cheese. Accordingly, ~75L of milk per trial will be required to produce enough inoculated and uninoculated samples. Milk should be obtained from the normal source used by the cheese-maker.

Inocula should be prepared in suitable broth at temperatures as described in Section 4.3.1. For each batch, approximately 1.5 x 10^8 exponential phase cells of each of the challenge strains are required for each batch of cheese to be made. (The number of cells is based on the volume of milk to be used in the manufacture of the cheese, and inoculating to achieve 10^5 pathogen cfu.mL^{-1} in the milk based on a five strain mixture). 1.5 x 10^8 cells can be obtained from ~15 mLs of a 10^7 cfu.mL^{-1} culture, or equivalent combination of volume and cell density, but remembering that at concentrations greater than ~5 x 10^7 cfu.mL^{-1} the culture will be entering stationary phase.

The exponential phase culture can be centrifuged to remove the growth medium (decanted or aspirated off) and the pellet resuspended in a small volume (e.g., 5 - 10 mL) of sterile milk. The five cultures (i.e., five different strains) resuspended in milk are combined and allowed to incubate at ~10°C for ~ 1 hour to reestablish growth. This culture is added to the milk as soon after milking as possible. The inoculated and uninoculated milks should be handled normally after that, and cheese produced in exactly the same manner as the normal commercial process.

Samples should be taken and tested for the level of the specific pathogen added to the milk as well as temperature, pH, water activity and total aerobic (microbial) count ("TVC") at each of the following points in the process:

i) upon inoculation into the milk

ii) after warming but before addition of starter culture and rennet

iii) after addition of starter culture and rennet

iv) after curd has formed and whey discarded but before curd handling

v) after moulding and brining of the curd

vi) after application of the surface ripening culture/washing

vii) at (at least) three equal intervals during the maturation, duplicate samples (at least) should be taken and the tests described above undertaken. Alternatively, at least five unreplicated samples at equal intervals should be taken during maturation and the tests described above undertaken.

Additionally, pathogen levels in the inoculum should be determined prior to inoculation of the raw milk, and the same suite of tests undertaken on the raw milk before introduction of the challenge strains.

For each set of tests, approximately 30 - 50g of material are needed from each replicate to complete the tests. For *Salmonella*, unless performed by MPN, 25g are needed. A further 10g are needed for the TVC and approximately 5 - 10 g each for pH and water activity determinations. If Salmonella is enumerated by MPN ~35g are needed, and a further 20g for TVC and physico-chemical tests. For other pathogens that can be directly plated for enumeration (and possibly including an 'on-plate' resuscitation step) a 10 g sample only is required for pathogen enumeration, in addition to the amounts needed for the other tests.

For this style of cheese spatial differences in microbiology or physico-chemical properties of the cheese are specifically tested by taking samples from the core and near the surface/rind. Samples (30 – 50 g) from each of those sites will generally be quite soft and so could be homogenised in a stomacher prior to testing, for microbiological, pH or water activity testing. This will minimize differences due to micro-environments in the sample and when attempting to correlate physico-chemical properties to microbiological outcomes in samples taken near the surface and from the core of the cheeses.