FINAL REPORT

B12008 Persistence of *Mycobacterium bovis* and Verocytotoxin-producing *Escherichia coli* (VTEC) in UK-made raw-milk cheeses

Project Manager: Dr P. A. Voysey

Project Teams

1. Work carried out on VTEC

P. A. Voysey, R. A. Green, C. J. Baylis, K. J. Bridgwater and P. Anslow

Microbiology Department
Campden BRI
Chipping Campden
Gloucestershire
GL55 6LD

2. Work carried out on *M. bovis*

R. W. J. Forgrave, J. Donaghy and M. T. Rowe

Food Microbiology Department
Queens University of Belfast (QUB) / Agri-Food and Biosciences Institute (AFBI)
Newforge Lane
Belfast
BT9 5PX

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EXCECUTIVE SUMMARY

WORK ON VTEC

Verocytotoxin-producing *E. coli* (VTEC) is a human pathogen which has occasionally been reported associated with raw milk and the cheese made from it. In the UK though especially, this is a rare event. Elsewhere in the world, a number of serotypes of VTEC including *E. coli* O157:H7 and *E. coli* O26 have been found associated with these foodstuffs. Previously reported work has shown that VTEC strains can survive the various stages of the cheesemaking process and that raw milk and raw milk cheeses are a potential vehicle for VTEC infections.

Aims

The aim of the project was to investigate the behaviour of relevant VTEC serotypes (*E. coli* O157:H7 and *E. coli* O26) through the manufacture of raw milk cheeses as made in the UK. From the information gleaned, authoritative advice can be issued on the risk associated with these hazards should cheeses be found to be contaminated.

Objectives

As far as VTEC work was concerned, the objectives of the project were firstly to carry out a literature review of relevant aspects including: information on VTEC in relation to cheese (outbreaks and methodology); and the microbiology of raw milk and starter cultures. A second objective was to set up a Steering Group of interested parties to advise on the work carried out in the project. The Steering Group would need to meet as required, but at least every 6 months of the project duration.

In order to inform work on the project, it was necessary to determine the range of raw milk cheeses made in the UK, and their characteristics including: pH; salt content; water activity; background microflora; starter cultures used; maturation conditions and duration. It was felt cost-effective to use the write-up of project B12006 (Banks 2006) for this.

The next objective was to secure equipment and devise protocols for the production of cheeses under laboratory conditions. As part of this objective it was necessary to develop a protocol for the inoculation of the Hazard Group 3 bacterium *E. coli* O157:H7 and other VTEC. Once VTECs had been inoculated into the cheeses made, the next objective was to **i**dentify the most appropriate methods to be used for isolation and enumeration of these pathogens. The intention was that once these methods had been identified, they could be used to detect VTEC in uninoculated raw milk cheeses also.

A crucial part of the experimental work was to ensure that the cheese being made in the laboratory had the same characteristics as cheeses being made commercially. To this end, the microbiological content, as well as the pH, salt content and water activity of the cheese used in the inoculation studies were determined.

Once the practical work had been completed, the final objectives of the project were: to produce a final report with conclusions on the survivability of VTEC in different cheese-making processes; to produce peer-reviewed publications on the work; and to hold a dissemination event such that the findings of the project could be discussed by interested parties.

Approaches

A large range of cheese types are made in the UK from pasteurised and unpasteurised milks. It was decided in collaboration with the project Steering Group, that Caerphilly and Camembert type cheeses were the most appropriate for study with VTEC. These are semi-hard and soft cheeses respectively. Caerphilly cheese was chosen because there have been a number of outbreaks of VTEC illness associated with this cheese type (Baylis 2009). It was also decided that the team at QUB could work with this cheese type to look at the behaviour of *M. bovis.* With both teams looking at the behaviour of their respective target microorganisms in the same cheese type, it would be possible for some direct comparisons to be made between the two pathogens being studied.

The first challenge for the project team was to learn how to make cheese as would be done commercially. To this end, a technician from Campden BRI attended a cheesemaking course at Reaseheath College. A 10L vat was purchased and modified so that it could be fitted into a safety cabinet in a containment level 3 microbiology laboratory. (VTEC require handling in a containment level 3 laboratory.) The cheesemaking technique was then modified from a commercial scale to the laboratory scale, but such that cheese with the appropriate attributes (pH, salt, etc.) was produced.

A number of VTEC isolates (O157 and O26) were obtained from various sources. These included isolates from dairy foods and some were outbreak strains. Three of these strains were chosen for inoculation work in the project.

The next challenge was to identify/develop a method which was most suitable for the detection and isolation of VTEC in fatty foods such as milk and cheese. The three chosen VTEC strains were inoculated in turn into milk from which Caerphilly cheeses were made. The milk was purchased from a local farm. The chemical characteristics and microbiological content of the milk and the cheese was measured. The behaviour of the VTEC strains during the cheesemaking process and maturation (for up to 5 months) of the cheeses was monitored. An interpretation was then made on how these findings could be used to

Key findings

Unfortunately, due to technical and project constraints, it was only possible to work on semi-hard (Caerphilly) cheese in this project. It would be useful to look at the behaviour of VTEC in soft (Camembert-type) cheese in the future.

The most suitable method for the detection and isolation of VTEC in fatty foods such as milk and cheese was identified/developed during the project. To this end, for **detection**, the foodstuff (milk/cheese) was initially enriched in modified Tryptone Soya Broth (mTSB) at 41.5°C. Immunomagnetic separation (IMS) was then carried out, and the following plates streaked: ChromID O157 agar [for O157 and O26]; CTSMAC [for O157]; and CT-RMAC [for O26]. For **enumeration**, primary dilutions were carried out in sodium citrate solution and further dilutions in MRD. These were then spread-plated (high levels of VTEC present) and pour-plated (low levels of VTEC present) onto: ChromID O157 agar [for O157 and O26]; and CT-RMAC [for O26].

The chemical characteristics and microbiological content of the milk and the cheese was measured. The behaviour of the VTEC strains during the cheesemaking process and maturation (for up to 5 months) of the cheeses was monitored. During the cheesemaking process itself, counts of VTEC were found to increase. The count for *E. coli* O157 strains 1

and 2 increased by 1.3 log units, whilst the increase for *E. coli* O26 was 3.5 log units. This suggests that low levels of VTEC present in raw milk used to make cheese could increase over the cheesemaking process.

During maturation of the cheese, there was a general decline in VTEC counts. The average time for a 1 log drop in count for the two *E. coli* O157 strains in Caerphilly cheese was 208 days.

The average time for a 1 log drop in count for the *E. coli* O26 strain in Caerphilly cheese 183 days.

These results suggest that should VTEC be present in the milk used to make a semi-hard cheese such as Caerphilly, then the pathogen poses a risk throughout the life of the cheese.

Technical Evaluation/Interpretation

Useful outputs from the project work has been the identification of detection and enumeration methods for VTEC in cheeses

From the evidence gathered from various sources, it appears that the accepted characteristics of Caerphilly cheeses are variable. With the exception of moisture content, which was measured at 47.9-53.6% instead of the literature range of 33.92-46%, the characteristics of the Caerphilly cheeses made for the VTEC work were broadly within the accepted ranges. However, these high moisture levels mean that the 'Caerphilly cheeses' made in the project work were not acceptable as such. Because of the high moisture levels in the cheeses, the findings that: there was an increase in VTEC 'concentration' during cheese manufacture; and there was a slow decline in count over the life of the Caerphilly cheese, have to be questioned. It is simply not possible to say for sure that the results reflect what would happen in industry-made Caerphilly cheese.

What is clear from the work presented, is that VTEC is a potential issue with Caerphilly cheese made from unpasteurised milk.

EXCECUTIVE SUMMARY

SURVIVAL OF *MYCOBACTERIUM BOVIS* IN UK-MADE RAW MILK CHEESES

Mycobacterium bovis is the aetiological agent responsible for tuberculosis (TB) amongst cattle; a broad host range extends not only to other domestic animals and wildlife but also to humans. The link between *M. bovis* infection of cattle and human TB was firmly established by a Royal Commission report in 1911 and the resulting public health arguments eventually formed the basis in 1950 for the introduction of compulsory control measures. This intervention resulted in a gradual decline of bovine TB cases until the early 1980's and included herd screening using the comparative tuberculin skin test, removal and slaughter of test reactor animals and movement restrictions on infected herds. This trend ended with the resurgence in UK cases of bovine TB despite continuing control measures. In most instances the risk posed to humans from *M. bovis* has been limited by routine pasteurisation of milk before sale or in production of milk products. However, coinciding with the heightened prevalence of bovine TB there has been an increased demand from UK consumers for speciality cheeses; often produced using raw milk which has not undergone the pasteurisation process.

The absence of a pasteurisation step removes a major hurdle preventing *M. bovis* contamination and therefore raw milk cheeses may pose a greater risk to the consumer. Furthermore, there is a lack of published data on *M. bovis* inactivation in the raw milk cheese process encompassing both manufacturing and maturation. It was against this background that this investigation sought to characterise the survival kinetics of *M. bovis* during manufacture, ripening and storage of two raw milk cheese types. This data could then form the basis of informed advice to Environmental Health Officers, speciality cheesemakers and consumers, if during routine screening a dairy herd responsible for the milk used in raw milk cheese production generated evidence of *M. bovis* infection.

A project steering group (PSG) was established, from representatives of the UK dairy industry and speciality cheesemakers, providing advice and oversight in the design and execution of the project. Following input from the PSG it was decided to produce typical hard and semi-hard UK cheeses. Cheddar was selected as the most common UK cheese type plus Caerphilly, as it often has a very short maturation time and would overlap with the *E. coli* O157 element of the project. Production protocols for both cheese types had to successfully imitate traditional cheesemaking methods, as outlined by the PSG, whilst satisfying local health and safety regulations. This required that the majority of equipment had to be devised and fabricated, before laboratory scale cheese production could be completed under containment level 3 restrictions. Further input was obtained from veterinary colleagues in the selection and preparation of three *M. bovis* isolates. These were selected as representative of *M. bovis* genotypes responsible for herd breakdowns in the UK and ROI, and included the fully sequenced strain *M. bovis* AF2122/97. Culture preparation methods used, prior to inoculation of cheese, disrupted aggregates of *M. bovis* cells producing a planktonic cell suspension; it was considered that, when isolating *M. bovis* from inoculated cheese samples, this cell suspension would equate better to individual cell survival than an untreated cell suspension in which many more cell aggregates remained.

Established media for the isolation *M. bovis* from clinical samples were recorded in the literature but there were no recognized media for the isolation of *M. bovis* from a cheese matrix. For assessment of *M. bovis* inoculated cheese samples, a panel of media and two

antimicrobial combinations were identified viz. Middlebrook 7H11 and Gallaghers media, and the antimicrobial combinations PANTA and PCAT. Subsequently developed in the course of the project were 7H11++ PANTA and 7H11++ PCAT media enabling, without an initial decontamination stage, enumeration of *M. bovis* from cheese matrices. Statistical analysis confirmed 7H11++ PANTA and 7H11++ PCAT as the best media and in isolation of *M. bovis* from cheese homogenate they outperformed the next best medium, 7H11 PANTA by a minimum of 0.590 log cfu g^{-1}. Preliminary investigation of *M. bovis* selective media also provided evidence of concentration of *M. bovis* from liquid milk into the cheese matrix, and the loss of *M. bovis* in the whey fraction, as calculated from initial cheeses made for media assessment. The average percentage loss of *M. bovis* in whey from Cheddar was 13.2% and 7.4% from Caerphilly.

After fully establishing equipment, media and methods for the production of both Cheddar and Caerphilly cheese, the challenge test phase of the investigation was undertaken. A high-level *M. bovis* inoculum was used $\approx 10^6$ cfu ml^{-1} allowing as many data points as possible during cheese maturation. A total of eighteen Cheddar and Caerphilly cheeses produced had the physicochemical attributes indicative of the respective cheeses, as confirmed by the PSG. DMFit (web edition) was used to generate inactivation curves and *D* values calculated from the maximum rate of inactivation. A 'tailing effect' was observed for inactivation curves produced during twelve month Cheddar maturation, while the rate of decline in *M. bovis* numbers during four months Caerphilly maturation remained constant, producing a linear inactivation curve. Statistical analysis of *D* values was completed using GenStat Release 14.2 (for windows 7), and the mean *D* values, for Cheddar and Caerphilly, calculated as 50.75 and 57.59 days respectively. No other significant difference or interactions were determined between cheese types, *M. bovis* types or media types. Following the challenge test experiments an increase in concentration of *M. bovis* was again observed: calculated for all cheeses there was on average a 4.1-fold increase in the concentration of *M. bovis* from milk to curd. Furthermore, the average percentage loss in the whey fraction was 7.9% when calculated for all cheese and *M. bovis* types. The destination and use of the whey component should therefore be taken into account should a consignment of raw milk cheese be suspected of containing viable *M. bovis.*

Attention should be drawn to the 131.68 day *D* value of Caerphilly G24.130 CA1 on one experimental test run as this value was greater than the *D* values determined for any other Caerphilly cheese test runs. Initially considered anomalous, when viewed with data indicating poor development of titratable acidity (TA), this result may provide evidence of the importance of starter activity and acid development for *M. bovis* inactivation. It should be recognized that, in this particular test run, the trend was consistent over a number of data points and was not a result of skewing over a single datum.

In response to the recommendation of the PSG lower *M. bovis* inoculum cheese was produced. The inoculum levels of *M. bovis* were decreased to approximately 3.0 log cfu g^{-1} as this was deemed more representative of *M. bovis* cell populations likely to be found in naturally contaminated milk. Previous statistical analysis of data from high *M. bovis* inoculum Cheddar and Caerphilly revealed no significant difference or interactions between *M. bovis* types or cheese types. As a result of this and the short time available it was determined that the low inoculum investigation could be undertaken using only one *M. bovis* type. *M. bovis* AF2122/97 was selected as it is a sequenced *M. bovis* strain and is representative of the most common GB spoligotype SB0140 (also known in GB as VLA9). It was hoped that findings for low inoculum *M. bovis* AF2122/97 cheeses would be in

agreement with those produced from the high inoculum investigation and would be valid across all *M. bovis* types.

After completion of challenge tests using only *M. bovis* AF2122/97 inoculum, the mean *D* value of low-inoculum Caerphilly was 21.48 days and 30.49 days for Cheddar. Once more Cheddar cheese inactivation curves exhibited a 'tailing effect' and it may be appropriate to consider the inactivation of *M. bovis* in two phases: initial linear decline followed by the slower decline or 'tailing effect'. No significant difference between cheese types was found but a significant difference was detected between media types.

D values obtained from cheeses incorporating high or low *M. bovis* AF2122/97 inoculum were subjected to ANOVA revealing a significant difference between inoculum level and media type, while a significant interaction was determined between inoculum level and cheese type. Subsequently using Fisher's LSD test no significant difference was found between *D* values of low and high inoculum Cheddar or between *D* values of low inoculum Caerphilly and high inoculum Cheddar. There was however a significant difference between both low inoculum cheese types and high inoculum Caerphilly. The disparity between high and low inoculum Caerphilly may be attributed to adjustments of the Caerphilly protocol, following production of high inoculum Caerphilly and before low inoculum Caerphilly was made. Amendments of procedure may have benefitted *M. bovis* inactivation by enabling improved acid development and increased TA during production of low inoculum Caerphilly.

Following cheesemaking it was immediately apparent that the manufacturing process, in isolation from the ripening phase, was an inadequate control measure for *M. bovis.* It is therefore fortunate that the maturation stage is fundamental to the preparation of Cheddar and Caerphilly and a scenario where cheese could enter the market relying entirely on the manufacturing process for *M. bovis* inactivation is not recommended. However the efficacy of the maturation stage may be dependent on a variety of factors including the cheese type, *M. bovis* type, level of contamination and a potential inactivation role played by cheese microflora. For example, Cheddar and Caerphilly have very different maturation times: raw milk Cheddar can be matured for up to two years, while Caerphilly is usually matured between two and sixteen weeks. Therefore Cheddar, due to a longer possible ripening time, has a greater potential for inactivation of *M. bovis* compared to Caerphilly.

The ripening time required to effect complete inactivation will be dictated by the level of *M. bovis* in the cheese. *M. bovis* contamination level may, itself, be influenced by factors including cheese batch size and herd size. These parameters are important as they can potentially dictate the degree of dilution when *M. bovis* contaminated milk from reactor cows is combined with milk from non-infected animals. Any assessment of maturation time required to obtain a satisfactory level of *M. bovis* inactivation would therefore be assisted by improved knowledge of number and type of *M. bovis* shed in the milk of reactor animals. Finally, TA serves as an indicator of acid development and performance of the starter culture. It was observed that insufficient TA development was associated with poor *M. bovis* inactivation and conversely more rapid TA development achieved improved *M .bovis* inactivation. This suggests that the performance of starter cultures may serve as an indicator of the capacity of the cheese to inactivate *M. bovis* and future studies should consider the potential link between starter culture and raw milk microflora to facilitate the inactivation of *M. bovis* during cheese ripening. This, of course, does not exclude consideration of the possible inclusion of bacteriocin producing starter cultures to increase the lethality of the process for this organism.

LAY PERSON'S SUMMARY

WORK ON VTEC

Verocytotoxin-producing *E. coli* (VTEC) is a human pathogen which has been associated with raw milk and the cheese made from it. This bacterium causes serious infections, particularly in young children and the elderly and these may result from infective doses as low as 10 cells. In 1999 for example, *E. coli* O157:H7 infections were reported as a result of consuming milk from a farm with a faulty pasteurisation unit and from Cotherstone cheese. Although *E. coli* O157:H7 is the best-known serotype of these bacteria other serotypes such as O26 have also been isolated from these foods, including cheese.

This project was initiated to give enforcement officers reliable scientific data and information regarding the behaviour of potential pathogens such as VTEC in raw cow's milk or the cheese made from it.

The first task was to carry out a literature review of information relating to outbreaks associated with VTEC in raw milk and also the microbiological methods adopted to investigate the organism. In order to guide the work on the project a Steering Group of experts and interested parties was formed. The Steering Group met every 6 months throughout the time that the project was in operation. Information on, and characteristics of, raw milk cheeses made in the UK was also gathered at the beginning of the project. This enabled an appreciation of the range of cheeses available to be made.

In order for the behaviour of VTEC to be studied in cheese, it was necessary to make cheese in the laboratory. This is because VTEC is a very hazardous microorganism and it can only be handled under very controlled conditions. Equipment was secured and protocols for cheesemaking developed and verified by professional cheesemakers. Technicians carrying out this work were trained in the art of cheesemaking before the project work could commence. Caerphilly-type cheese was used for work on VTEC as an incident had occurred with this organism in the UK in this type of cheese and it was therefore thought to be of direct relevance to the work being done. A number of cheeses were made in the laboratory conditions and inoculated with VTEC bacteria at the milk stage, such that natural contamination of raw milk was mimicked as closely as possible. The levels of microorganisms used at this stage were quite high, so that the behaviour of the VTEC organisms could be followed as easily as possible.

The VTEC microorganisms used in the work were sourced from a number of places. The bacteria used included strains which had given rise to food poisoning outbreaks as well as isolates from dairy sources.

Appropriate microbiological methods for isolating and enumerating the VTEC bacteria were developed. Important difficulties in working with raw milk cheeses are the nature of the cheese itself (it is difficult to separate VTECs from the cheese curds), and finding VTEC bacteria when there are many more starter culture (lactic acid bacteria) present.

The behaviour of various VTEC strains was studied during the cheesemaking process and also during the maturation process of the cheeses. The characteristics (e.g. fat content, moisture, pH) of the cheeses made had to be checked to show that they were comparable to

commercially made cheeses. To enable this work to be done, the cheeses which had been inoculated with VTEC organisms were irradiated to kill off any microorganisms present.

Key results and conclusions for the work included:

1. Cheeses were successfully made in the laboratory.
2. In order to detect VTEC from milk/cheese, enrichment was first carried out in a microbiological medium called modified Tryptone Soya Broth (mTSB) and this was incubated at 41.5ºC. A process called immunomagnetic separation (IMS) was then carried out and the following media used to isolate the VTEC serotypes:

    a. ChromID O157 agar for O157 and O26;
    b. CTSMAC for O157; and
    c. CT-RMAC for O26

3. To enumerate the VTEC bacteria present in the milk/cheeses, the food was diluted in sodium citrate solution and then spread-plated and pour-plated onto:

    a. ChromID O157 agar for O157 and O26; and
    b. CT-RMAC for O26

4. The chemical characteristics and microbiological content of the milk and the cheese was measured. The behaviour of the VTEC strains during the cheesemaking process and maturation (for up to 5 months) of the cheeses was monitored. During the cheesemaking process itself, counts of VTEC were found to increase. The count for *E. coli* O157 strains 1 and 2 increased by 1.3 log units, whilst the increase for *E. coli* O26 was 3.5 log units. This suggests that low levels of VTEC present in raw milk used to make cheese could increase over the cheesemaking process.

5. During maturation of the cheese, there was a general decline in VTEC counts. The average time for a 1 log drop in count for the two *E. coli* O157 strains in Caerphilly cheese was 208 days.

6. The average time for a 1 log drop in count for the *E. coli* O26 strain in Caerphilly cheese 183 days.

From the evidence gathered from various sources, it appears that the accepted characteristics of Caerphilly cheeses are variable. With the exception of moisture content, which was measured at 47.9-53.6% instead of the literature range of 33.92-46%, the characteristics of the Caerphilly cheeses made for the VTEC work were broadly within the accepted ranges. However, these high moisture levels mean that the 'Caerphilly cheeses' made in the project work were not acceptable as such. Because of the high moisture levels in the cheeses, the findings that there was an increase in VTEC 'concentration' during cheese manufacture and there was a slow decline in count over the life of the Caerphilly cheese, have to be questioned. It is simply not possible to say for sure that the results reflect what would happen in industry-made Caerphilly cheese. What is clear from the work presented, is that VTEC is a potential issue with Caerphilly cheese made from unpasteurised milk.

WORK ON *MYCOBACTERIUM BOVIS*

The bacteria *Mycobacterium bovis* (*M. bovis*) is the cause of the serious cattle disease tuberculosis and can also give rise to a form of tuberculosis in humans. *M. bovis* has been estimated to be responsible for up to 1% of human tuberculosis cases. The wide scale introduction of milk pasteurisation during the 1930's was to prevent milk being a vehicle of transmission of human tuberculosis due to *M. bovis* and milk that has not undergone the pasteurisation process is often referred to as raw milk. Further control measures for *M. bovis* in cattle require routine testing of herds for *M. bovis* using the tuberculin test and slaughter of any test positive animals. Recently, there have been documented cases of human tuberculosis due to *M. bovis*, in the US and the Republic of Ireland, where consumption of raw milk or raw milk products were implicated. This must be seen within a context of an increasing incidence of bovine tuberculosis in the UK and interest in artisan cheeses, some of which are made with raw milk.

The complete cheesemaking process occurs in two distinct phases - the initial manufacturing phase when liquid milk is processed into solid cheese and the maturation or ripening phase when the characteristic taste, texture and aroma of the cheese develops. This study was designed to investigate the survival, if any, of typical UK *M. bovis* types during the manufacture and ripening phase of raw milk cheese and was prompted by a variety of factors: knowledge that tuberculosis is increasing within the UK dairy herd, evidence that consumption of raw milk and raw milk products can be a vehicle of transmission of human tuberculosis, increased interest in artisan cheeses made from raw milk and lack of data on the inactivation kinetics of *M. bovis* during the manufacture and ripening of cheese.

A Project Steering Group (PSG) was instituted, made up of UK artisan cheesemakers and other representatives of the UK dairy industry, to advise on experimental design, interpretation of the results generated and facilitate dissemination of the findings to the industry as a whole. This is, of course, over and above publication in peer reviewed scientific journals. Two UK cheese types were selected for investigation, Cheddar and Caerphilly. Cheddar was chosen as it is a hard cheese and the most commonly produced cheese in the UK. Caerphilly was chosen as a semi-hard cheese which generally receives a shorter ripening period and by inference may pose a greater risk to consumers. In addition three veterinary isolates of *M. bovis* possessing distinctive genetic fingerprints were chosen as representative of *M. bovis* isolated from tuberculosis infected cattle in the UK.

The cheese manufacturing process involves a physical change from liquid milk to solid cheese and critical to this is milk acidification initiated by adding a cheese starter culture to the milk. On this basis, traditional Cheddar and Caerphilly starter cultures were selected. Suitable laboratory cheesemaking equipment had to be devised that would allow the process to be contained and fulfil all health and safety requirements. In addition, existing bacterial growth media for *M. bovis* had been designed principally for veterinary and human clinical samples rather than food. Therefore, another objective of the project was to identify new growth media that would allow detection and enumeration of *M. bovis* from a food.

In the final experimental design it was decided that each of the three *M. bovis* types would be incorporated into a single cheese three times, producing a total of nine cheeses each for Cheddar and Caerphilly cheese types, and thus the overall production was eighteen individual cheeses. For the incorporation of *M. bovis* into each cheese the bacteria would be added to the milk before the starter culture and the cheesemaking process would then proceed as

normal. In the expectation that there may be a rapid decline in numbers during manufacture and maturation a high level of inoculum, approximately 10^6 *M. bovis* bacterial cells per ml of raw milk, was employed to maximise the number of data points obtained during the cheese maturation phase, and hence the accuracy of the subsequent calculated lethality. It should be recognised that lethality is measured in terms of *D* values which in this case is the time taken, under the maturation conditions, for 90% reduction in viable numbers of *M. bovis* and is a unit recognised internationally by microbiologists. A higher *D* value therefore indicates greater resistance of the organism under the pertaining conditions.

Statistical analysis of *D* values was completed and resulted in calculated average *D* values of 50.75 and 57.59 days respectively for test Cheddar and Caerphilly cheeses. The cheeses were all tested chemically and the parameters were within the limits for their commercial counterparts and this was verified by the PSG. It should be noted that there was on average a 4.1 fold concentration of *M. bovis* from liquid milk to solid cheese, probably because of the affinity of the lipid rich *M. bovis* cell wall for the cream fraction. There was also an average percentage loss of the pathogen in the drained whey fraction of 7.9%. This is considered important as it calls into question disposal of whey should a reactor animal be detected whose milk is destined for raw milk cheese production: in particular, the final location of the suspect whey and its manner of disposal. Also during this stage of the investigation there was some evidence that poor performance of the Caerphilly starter, manifested as reduced acidification of milk, was associated with poor *M. bovis* inactivation during the cheese ripening phase.

Following consideration of the results generated, the PSG suggested that the concentration of pathogen inoculum should be reduced as this would better simulate the likely conditions encountered in the event of *M. bovis* contamination of raw milk cheese. The inoculum level of *M. bovis* used for cheesemaking was subsequently lowered to approximately 100 to 1000 times less than the inoculum level used previously and only one *M. bovis* type was used, due to the limited time available. In addition, adjustments were made to the preparation of Caerphilly starter culture to improve acid development during Caerphilly manufacture; preparation of Cheddar starter culture remained unaltered. After cheese preparation and subsequent statistical analysis of sampling data the calculated *D* values for low inoculum cheeses were Cheddar 30.49 days and Caerphilly 21.48 days. Statistical analysis of low inoculum *D* values revealed the 21.48 day *D* value was significantly lower than *D* values of the same *M. bovis* type in high inoculum Caerphilly cheese. It is believed that this disparity may have been due to adjustments made to the Caerphilly manufacturing process to improve starter activity and acid development. This may represent further evidence of an association between the performance of cheese starter culture and eventual inactivation of *M. bovis*. However, it cannot be discounted that reducing *M. bovis* numbers from high to low inoculum level plays some role in the reduced *D* value observed in low inoculum Caerphilly.

It was clear, from microbiological analyses of both cheese types, that the manufacturing process alone (excluding maturation) would be insufficiently hostile to ensure lethality of the organism. It should be noted that there was a decline in viable numbers of *M. bovis* during maturation, albeit after an initial concentration upon transition from milk to curd, and therefore the length of the maturation stage is likely to have an impact on the contamination level of the organism. This must be seen in the context of cheese type and that Caerphilly has traditionally a much shorter maturation time than Cheddar. The efficacy of maturation phase in facilitating *M. bovis* inactivation will be dictated by a variety of other factors including *M. bovis* type, initial level of contamination and a potential inactivation role played by advantageous bacteria constituent in the raw milk and cheese starter culture. Furthermore

any initial level of *M. bovis* contamination will be dictated by other variables, including the herd size and cheese batch size as *M. bovis* contaminated milk from reactor cows is combined with milk from non-infected animals. Overall, despite the manufacturing process providing insufficient inactivation of *M. bovis*, the cheese maturation phase in combination with the constituent properties of the cheese appears to be critical for *M. bovis* inactivation.

In respect of further work on this topic, it is recognised that only three types of *M. bovis* and two types of cheese were investigated. This perhaps does not provide sufficient evidence to extrapolate the results to other *M. bovis* types, and in particular milk from other species. In addition, inclusion of advantageous bacteria in starter cultures producing antibacterial bacteriocins may increase the lethality of the manufacturing and maturation processes. These issues are worthy of further investigation.

The project met all the objectives set at the outset, along with additional targets set as a result of initial findings. The results and conclusions were considered of sufficient import that they were presented at a meeting of the UK Advisory Committee on the Microbiological Safety of Food in order to provide an evidence base to inform the Food Standards Agency, local authorities and hence Environmental Health Officers. It is anticipated that, although already subjected to internal audit by relevant referees appointed by the FSA, the results will be submitted to peer reviewed journals to ensure wider dissemination.

PROJECT OBJECTIVES

The objectives of the project were agreed as follows:

1. **Carry out a literature review of key aspects relating to the project**
   These aspects are to include: information on VTEC in relation to cheese (outbreaks and methodology); information on *M. bovis* in relation to cheese (outbreaks and methodology); the microbiology of raw milk and starter cultures; and the potential for use of predictive models (especially for *M. bovis* in relation to cheeses).

2. **Steering Group**
   A Steering Group of interested parties to be set up to advise on the work carried out in the project. The Steering Group to meet as required, but it is anticipated that this will be every 6 months.

3. **Information on UK raw-milk cheeses**
   Determine the range of such cheeses made in the UK, and their characteristics including: pH; salt content; water activity; background microflora; starter cultures used; maturation conditions and duration. Report write-up of project B12006 to be used for this.

4. **Cheese production (*M. bovis*)**
   Devise protocols for the production of cheeses under laboratory conditions. This objective to include a protocol for the inoculation of the Hazard Group 3 bacterium *M. bovis*.

5. **Cheese production (VTEC)**
   Devise protocols for the production of cheeses under laboratory conditions. (These will be the same as in 04). This objective will include a protocol for the inoculation of the Hazard Group 3 bacterium *E. coli* O157:H7 and other VTEC.

6. **Methodology (*M. bovis)***
   Identify the most appropriate methods to be used for isolation and enumeration of *M. bovis.*

7. **Methodology (VTEC)**
   Identify the most appropriate methods to be used for isolation and enumeration of *E. coli* O157:H7 and other VTEC.

8. **Challenge test work (*M. bovis)***
   Using spiked raw cow's milk quantify the number of *M. bovis* during each stage of cheese production and maturation. This practical work to be repeated to reduce variability and allow modelling of the data.

9. **Challenge test work (VTEC)**
   Using spiked raw cow's milk quantify the number of *E. coli* O157:H7 and other VTEC during each stage of cheese production and maturation. This practical work to be repeated to reduce variability.

10. Analysis of cheese

The microbiological content, as well as the pH, salt content and water activity of the cheese used in the inoculation studies will be determined. It is envisaged that the cheese will be irradiated to allow the chemical analyses to be undertaken; alternatively duplicate, uninoculated cheese to be produced and measured.

11. Final Report

A final report will be written with conclusions on the survivability of VTEC and *M. bovis* in different cheese-making processes.

12. Produce peer-reviewed publications

It is envisaged that articles will be produced during the project on:

- Applicability of methodology of isolation and enumeration of both organisms in high-fat foods
- Conclusions relating to the survivability of both bacteria in cheese-making.

13. Dissemination event

A seminar event is envisaged at the end of the project to disseminate findings to interested parties.

INTRODUCTION TO THE PROJECT

Research has shown that there is a potential for *Mycobacterium bovis* to survive in cheeses made from unpasteurised milk. A great deal is known about the survival of Verocytotoxin-producing *E. coli* (VTEC) O157:H7 in some foods, however not in fermented dairy foods such as cheese, where starter cultures and naturally occurring microorganisms may compete with this pathogen.

The project described here set out to investigate the persistence of *M. bovis* and relevant VTEC serotypes such as O157:H7 through the manufacture of raw milk cheeses as made in the UK. Benchmark cheeses were identified for use in the project, and these were inoculated with these key pathogens.

The intended outcome from the project was to enable the safety of raw milk cheeses with respect to these two microorganisms to be assessed.

PROJECT TEAM

The project team consisted of experts in the areas of *M. bovis*, VTEC and cheese. In addition to Campden BRI and The Queens University Belfast, the Project Partnership comprised:

Dr Ed Komorowski (Dairy UK Ltd.);
Dr Chris Edwards / Tony Breeze (Reaseheath College);
Dr Ken Burgess / Intisar Khan (Dairy Crest Ltd.); and
Dr Mike Johnston (Dairy Council for Northern Ireland).

The project partnership was supported by a Steering Group which comprised:

Randolph Hodgson (Neal's Yard Dairy)*
James Montgomery (Montgomery's Dairy)*
Jemima Cordle (Westcombe Dairy)*
Dr Paul Neaves (Williams & Neaves – The Food Microbiologists)*
Diana Brydson (First Milk)
Mervyn McCaughey (Five Mile Town Dairy)
Paul Segynowycz (Joseph Heler Cheese)
Katherine Spencer (Cheddar Gorge Cheese Co.)

*Also representing the Specialist Cheesemakers Association.

The Project Partners met six times and the Steering Group met three times during the project to guide and advise Campden BRI and The Queens University Belfast.

ACKNOWLEDGEMENTS

The project team acknowledge funding for the work in this project from the Food Standards Agency (FSA).

We are indebted to individuals and organisations making up the project partnership and steering group for their invaluable help in carrying out the project work. Help provided by the Specialist Cheesemakers Association in relation to cheese production is gratefully acknowledged.

The team at Campden BRI would like to acknowledge the assistance through provision of VTEC cultures from:

- Estelle Loukiadis: ENVL UMAP, Ecole Nationale Vétérinaire de Lyon, Unité de Microbiologie Alimentaire et Prévisionnelle, France.
- Tom Cheasty and Geraldine Smith: HPA, Health Protection Agency Centre for Infections, Colindale, London, UK.

The team at QUB / AFBI would like to acknowledge the assistance from Robin Skuce and Carl McCormick (Veterinary Sciences, AFBI) in selection and preparation of *M. bovis* cultures, and also Anne Fisher (Food microbiology, AFBI) for assistance with the experimental work and Dr Alan Gordon (Biometrics, AFBI) who performed the statistical analysis of their results.

PROJECT SEMINAR

A successful seminar was held towards the end of the project (8th March 2011) at Campden BRI. At this event, findings from the project were discussed as well as associated subjects. Fifty five delegates from industry attended the seminar. Posters were shown illustrating work carried out on the project as well as work carried out by other workers working in this area. More details of the seminar can be found at:
http://www.campden.co.uk/training/events/cheese-safety-overview.htm

LAYOUT OF THIS PROJECT REPORT

The work carried out on this project was carried out at 2 centres: Campden BRI where work on VTEC was done; and QUB where work on *M. bovis* was done. Although work on the 2 microorganisms shared the theme of persistence in UK raw milk cheeses, it makes sense to consider the work separately. To this end, this final report is divided into 2 parts. In the first, work carried out on VTEC is described and considered. In the second, work carried out on *M. bovis* is treated in the same way.

PART 1 WORK CARRIED OUT AT CAMPDEN BRI ON VTEC

CONTENTS

1. INTRODUCTION

The scientific problem

Verocytotoxin-producing *E. coli* (VTEC) O157:H7 is a human pathogen which has been associated with raw milk and the cheese made from it. This bacterium causes serious infections, particularly in young children and the elderly, and these may result from infective doses as low as 10 cells. In 1999, for example, *E. coli* O157:H7 infections were reported as a result of consuming milk from a farm with a faulty pasteurisation unit, and from Cotherstone cheese. Although *E. coli* O157:H7 is the best known serotype of these bacteria, other serotypes such as O26 have also been isolated from these foods including cheese. Quinto & Cepeda (1997) report organisms from serotypes O5, O2 and O51 from cheese made from raw milk. As with *M. bovis*, a pasteurisation process would eliminate these bacteria

Enforcement officers currently have no reliable scientific data and information regarding the survivability of potential pathogens such as VTEC, in raw cow's milk or the cheese made from it. The work carried out in this project, which is described here, aimed to address this concern.

State of the art in this area

There is some information and data available on the prevalence of *E. coli* O157:H7 in raw milk and raw milk cheeses (see for example Coia *et al*. 2001), but this information needs bringing together in a precise and managed way, which was an aim of this project.

Another area where information and data are lacking, is the methodology used to isolate and enumerate VTEC, especially non-O157 VTEC. Again this was investigated in this project.

Anticipated outcomes

For this study, it was originally envisaged that different types of cheeses made from UK unpasteurised milk would be taken to represent 'cheese' - for example, a hard and a soft cheese. These products would differ in their characteristics with respect to the environment they present to VTEC or any other microorganism found to be present. Information on values for characteristics such as salt and pH were derived from the expertise of the project team, but measurements were also made. Due to constraints, only one representative cheese was in effect studied in the project.

Work in the project set out to develop and evaluate methods for detecting, isolating and enumerating VTEC O157:H7 from milk and cheeses. The ability to detect and isolate non-O157 VTEC (e.g. O26, etc.) from cheeses was also investigated during the study.

Cheeses were manufactured in the project from milk inoculated with VTEC bacteria. The microflora and characteristics of the cheeses were monitored throughout the life of the cheeses, such that the impact on the growth and survivability of VTEC was evaluated. This could enable the safety of raw milk cheeses to be assessed more precisely.

2. INFORMATION ON UK RAW MILK CHEESES

Contents

Figure 2.1 An example of change in Titratable Acidity (TA) and pH during the stages of Cheddar Cheese manufacture.

It is intended that this section of the report should be read in conjunction with the findings of Dr J G Banks presented in his report entitled 'Risk Assessment of *L. monocytogenes* in UK retailed cheese'. Dr Banks' report was written as the culmination of a Food Standards Agency-funded study B12006. The report can be obtained from the FSA website at:
http://www.food.gov.uk/science/research/foodborneillness/microriskresearch/b12programme/B12projlist/b12006/

1. Introduction

This section will outline which raw milk cheeses are produced in the UK, and discusses their characteristics such as pH, water activity / salt and moisture content, background microflora, details of starter cultures used in cheese manufacture and maturation conditions. Only cheeses made from cows' milk will be considered. Information gained from this part of the study, will be used to identify suitable benchmark cheeses for use in the project.

The focus of this project is on cheeses made from raw milk. The pasteurisation process was 'introduced' to the dairy industry in the UK principally to inactivate any vegetative pathogens such as *Mycobacterium tuberculosis, Coxiella burneti, Rickettsia* and *Staphylococcus aureus* in milk. The process will also kill other vegetative pathogens and spoilage bacteria.

Despite the apparent advantages of using pasteurised milk, there is still a demand for raw milk and the products made from it. As far as making cheese from raw milk is concerned, more complex and individually characteristic flavours can be produced. The use of starter cultures with pasteurised milk restricts (through suppression of the natural flora and production of lactic acid), the development of these flavours.

Despite the perceived food safety risks associated with unpasteurised dairy products, these goods have a good safety record. For example, in England and Wales (1995-6), cheese (including unpasteurised) caused only 1.6% of the number of general food-poisoning outbreaks recorded (Williams & Neaves 1999). Data presented by ACMSF (ACM/1008 (2010)), shows that between the years of 1992 to 2009, 20/2521 (0.8%) of foodborne infectious intestinal disease outbreaks were linked to raw drinking milk and raw cream. The ACMSF however recommend that pasteurisation is an important control measure in reducing the risks from consumption of raw milk.

Mycobacterium bovis is a member of the *M. tuberculosis* complex of bacteria with the capability of causing disease in humans. Wild badgers may act as a reservoir of infection and can spread the disease to cattle that are susceptible to subsequent lung disease caused by *M. bovis* (Krebs *et al*. 1998, Rowe and Donaghy 2008).

Because of milk pasteurisation and tuberculin screening of herds, the current risk posed by *M. bovis* to human health in the UK is considered negligible. However, if the hurdle of pasteurisation is removed, the risk is likely to increase. This is one of the conclusions arrived at by Lake *et al*. (2002) when they carried out a risk profile of *M. bovis* in New Zealand milk. It is recognised that the incidence of tuberculosis disease in UK cattle caused by *M. bovis* is increasing, with a reported 1% of cattle herds in the South West of England being affected annually (HPA 2003). Despite this, a risk assessment carried out by the ACMSF in September 2011 entitled 'The possible health risks to consumers associated with *M. bovis* and unpasteurised milk and milk products" (see

http://www.food.gov.uk/multimedia/pdfs/committee/acm1047a.pdf), concluded that the risk to human health from *M. bovis* in unpasteurised cows' milk and milk products is very low.

Transmission of *M. bovis* can occur between animals and from animals to humans and *vice versa*. As with *M. tuberculosis*, transmission is most common through an aerosol route, but also occurs through ingestion of contaminated milk and meat (Kovalyov 1989). There are a number of similarities between *M. bovis* and *M. tuberculosis*, including the way they develop disease. Some implied opinions can be drawn from these similarities, and further parallels can be drawn from our knowledge of *M. paratuberculosis*. This has increased substantially when there were reports that this bacterium could survive standard pilot plant pasteurisation protocols (Gao *et al.* 2002).

Another human pathogen which has been associated with raw milk and the cheese made from it is Vero cytotoxin-producing *E. coli* (VTEC) O157:H7. However, it is recognised that in the UK and other countries surveyed, the incidence of this and other VTECs is very low (de Louvois and Rampling 1998, Baylis 2009).

E. coli (VTEC) O157:H7 causes serious infections, particularly in young children and the elderly, and these may result from infective doses as low as 10 cells (Baylis 2009).

There have been a number of reports in the UK of VTEC illness associated with unpasteurised milk and cheese. For example, between 1992 and 1996 three *E. coli* O157 outbreaks were reported - one was associated with unpasteurised milk, the other two were associated with pasteurised milk where the pasteurisation process had failed (Baylis 2009).

As far as cheese made from unpasteurised milk is concerned, in one case a 12 year old boy in Somerset, England developed HUS following infection by *E. coli* O157, after eating cheese made with unpasteurised milk. This case involved consumption of Wedmore, a Caerphilly-type cheese containing chives produced by a small company with limited national distribution. A second case involved cheese made from unpasteurised milk (Cotherstone cheese), where two people were infected with *E. coli* O157 in North Yorkshire, England (Baylis 2009).

Three outbreaks of *E. coli* O157 infection in Scotland, between 1994 and 1999, involved consumption of cheese made from unpasteurised milk (Reid 2001). In all three outbreaks the hygiene standards of the premises involved appeared to be satisfactory. However, the raw milk at two premises was being stored at temperatures that facilitated growth of the bacteria. Furthermore at one premises no starter culture was being used and in another the maturation step was found to be too short to enable sufficient reduction in pH and subsequent decrease in the bacterial population in the product. Poor traceability and lack of adequate labelling of contaminated products were also highlighted in two of the outbreak investigations.

As with *M. bovis*, a pasteurisation process would eliminate these bacteria.

Some speciality cheeses are produced from milk which has not received a full pasteurisation process, these are discussed below.

2. Cheese Types

There is little general agreement as far as classifying cheese is concerned. The most widely accepted basis for classification is the moisture content (Scott 1986; Farkye and Vedamuthu 2002). The general 'rule-of thumb' is that the higher the moisture, the softer the cheese. A general classification is illustrated in Table 2.1.

Table 2.1 Classification of cheese in respect of composition (Banks 2006)

Cheese type	Water in fat-free substance (%)	Fat in dry matter (%)	Class
Extra hard	<51	>60	High fat cheese
Hard	49-55	>45<60	Whole milk cheese
Half fat	53-63	>25<45	Half fat cheese
Semi-soft	61-68	>10<25	Low fat cheese
Soft	>61	>10	Skim milk cheese

As far as this document and study is concerned, the categories of cheeses used are: 'hard'; 'semi-hard'; 'soft'; and 'blue-veined'. Other categories such as 'fresh', 'acid coagulated' or 'whey' cheese will be mentioned but not discussed in great detail.

The most popular varieties of hard cheeses in the U.K. are Cheddar and Cheshire. Caerphilly and Lancashire are the most popular of the semi-hard cheeses, with Cottage cheese of the soft cheeses.

2.1 Hard Cheese

The majority of Cheddar cheese sold in the U.K. is made from highly mechanised processes (Banks, 2006). Bulk raw milk has a typical lactic acid content of 0.16% before heat treatment. The raw milk is collected into refrigerated silos, and maintained between 4^{o}C and 7^{o}C (typically 3°C). However, for on-farm cheesemaking, the milk may be delivered from the milking parlour to the dairy without cooling and is used immediately. Pasteurisation is legally defined as at least 72^{o}C for 15 seconds on plate heat exchangers and at least 63°C for 30 minutes in batch pasteurisation.

The manufacture of a representative hard cheese (Cheddar) is described on pages 58-61 in Banks (2006).

Hard cheeses and other cheeses made from raw milk are often considered to have a much more intense, complex and individually distinctive flavour than the same cheese made from pasteurised milk. This has been ascribed to more proteolysis and lipolysis by the raw milk microflora in the cheese. Non-starter lactic acid bacteria, enterococci, propionic acid bacteria, enterococci and yeasts have not been destroyed by pasteurisation of the milk and may therefore grow to higher cell numbers in hard cheeses made from raw milk than in the same cheeses made from pasteurised milk (Beuvier *et al.* 1997).

During the ripening of cheese, glycolysis, lipolysis and proteolysis occur which leads to flavour formation in the cheese. Glycolysis (conversion of lactose to lactic acid) leads to the acidic taste associated with cheese. This is carried out by starter culture organisms. Other

compounds such as diacetate, acetate and acetaldehyde are also produced which are important in flavour formation.

Lipolysis results in hydrolysis of the milk fat and the production of glycerol and free fatty acids, many of which have characteristic flavours. There are several potential sources of lipase in cheese, including: milk; starter culture, pregastric esterase in rennet; and lactic acid bacteria present in the milk.

The sources of proteinases in cheese include the milk itself, lactic acid bacteria including starter culture and other microorganisms found in milk which could include coryneforms, micrococci, staphylococci, yeasts and moulds (Robinson 2002).

The implication is that the indigenous microflora have an important role in development of the flavour of the cheese.

Staphylococcus aureus is the commonest cause of mastitis in dairy cows. Both it and other pathogens (e.g. *E. coli*, *L. monocytogenes*, etc.) can be present in the raw milk, and can grow to high numbers during cheese manufacture and ripening (Cogan and Beresford 2002). The rate and extent of growth will be dependent on how rapid acid production is and the cooking temperatures used; for example, the high cooking temperature (54°C) and the length of time the curd is held at this temperature (~60 minutes) in Swiss and Italian cheeses will inactivate most, if not all, pathogens that might be present. Generally, it is only semi-hard and soft cheeses – particularly those in which the pH of the surface increases during ripening – that cause problems (Cogan and Beresford 2002). Ryser and Marth (2007) however, did report die-off of *Listeria monocytogenes* in Cheddar cheese during ripening. It is clear though that although significant amounts of raw milk cheeses are produced, especially on mainland Europe, they are relatively few cases of food poisoning associated with raw milk cheeses.

To prevent the growth of pathogens in cheese, Cogan and Beresford (2002) suggest:

- The milk should be produced following Good Agricultural and Good Hygiene Practices and be held at 4°C until cheese manufacture starts,
- The total count of the milk should be <20,000 cfu/ml,
- An active starter, good hygiene, attention to detail and a HACCP system should be in operation.

Further information on safe production of cheese can be found in EC Regulation 853/2004 'Laying down specific hygiene rules for food of animal origin' (*http://www.food.gov.uk/multimedia/pdfs/h2ojregulation.pdf*) and 'The Specialist Cheesemakers Code of Practice' (2011).

2.2 Semi-Hard Cheese

Semi-hard cheeses are made up of many varieties. The majority are produced from pasteurised milk, with the addition of starter cultures. The curd may be scalded and is usually pressed. Curd is scalded at a variety of temperatures: Leicester to typically 35°C; Wensleydale and Cheshire typically to 32-34°C. The maximum scald temperature is determined by the recipe; if the scald is too high then the starter bacteria may be destroyed. Higher levels of acidity are obtained over a shorter make-time compared to Cheddar.

The manufacture of UK cheeses representative of this type: Cheshire; Derby; Leicester; Gloucester; Caerphilly; and Lancashire, are described in Banks (2006), pages 62-64.

2.3 Soft Cheese

This is the largest family of cheeses. Soft cheeses can be grouped into four categories:

- **unripened** (e.g. Cottage Cheese), for which curd is formed by acidification of milk to or near the isoelectric pH of casein (pH 4.6);
- **surface mould-ripened** (e.g. Camembert), where the mould *Penicillium camemberti* is used to ripen the cheese;
- **bacterial surface "smear"-ripened** (e.g. Limburger), where the cheese is ripened using the bacterium *Brevibacterium linens;* and
- **pickled** (e.g. Feta), where the cheese is stored in 6-10% brine before being sold.

Many of these cheeses are produced from raw milk, and maturation can be based on the natural lactic flora, which is selected for from the processing conditions and environment.

The production of a range of soft cheeses (Mozzarella, Gorgonzola and Ricotta are described by Banks (2006) on pages 67-93.

2.4 Blue-Veined Cheese

Some of the cheeses in the semi-hard cheese group are internal mould ripened. An example of an internal mould ripened semi-hard cheese is Stilton. Internal mould ripened cheeses are of great interest when considered as substrates for growth of pathogenic bacteria because of the growth of a mould, *Penicillium roqueforti* which probably has an inhibitory effect on some pathogens (Laporte *et al.* 1992).

The manufacture of this group of cheeses is described by Banks (2006) on pages 66-67.

3. UK Produced Raw Milk Cheeses

There are very few types of raw, fresh cheese in the UK retail market (Banks 2006). But many speciality farmhouse cheeses, made from un-pasteurised milk are available and considered high cuisine foods. Un-pasteurised milk enables the naturally occurring microbial population to generate any specific flavour attributes to the cheese.

3.1 Raw milk cheeses produced and on sale in the U.K.

Tables 2.2 to 2.4 list cheeses produced from un-pasteurised milk and sold in the U.K. (Information taken from The Specialist Cheesemakers Association (see http://www.specialistcheesemakers.co.uk/index.htm for more details) and the "British Cheese Directory" (Harbutt 2002, and Harbutt 2008).

Table 2.2 Some raw milk <u>hard</u> cheeses produced and on sale in the U.K.

CHEESE NAME	CHEESE TYPE
Appleby's Cheshire	Cheshire
Appleby's Double Gloucester	The Gloucesters
Appleby's Smoked Cheshire	Cheshire
Ashmore Farmhouse Cheese	Cheddar type
Berwick Edge	Gouda variation
Birdwood Dunlop	Territorial
Birdwood Forester	Hard
Birdoswald	Ayshire (Dunlop)
Caerfai cheddar	Farmhouse cheddar type
Carrow	Clothboard cheddar type 2% mustard seed
Caws Aberaeron + flavours	Flavour added
Caws Cenarth with Garlic & Herbs	Cream taste; lemon/garlic
Chapman's Leicestershire	Unpasteurised traditional Red Leicester
Cheddar	Cheddar: Traditional Farmhouse
Cheddar with Garlic & Herbs	Flavour added
Cheddar with Chives	Flavour added
Cheddar with Port	Flavour added
Cheshire	Cheshire
Clachnaben	Crumbly cheese
Crofton	Hard
Cuddy`s Cave	Hard
Cumberland Oak Smoked	Flavour added
Cumberland Farmhouse	Hard
Cumberland Herb	Garlic
Daylesford Organic Cheddar	Cheddar
Doddington	Cheddar variation
Duckett`s Caerphilly	Caerphilly
Double Glocester	Double Gloucester
Double Gloucester	Double Gloucester (pasteurised & un-pasteurised)
Double Gloucester	The Gloucesters
Double Gloucester	The Gloucesters
Double Worcester	Worcester
Dunlop	Organic
Dunsyre White	Hard
Drumkain	Hard
Elgar Mature	Mature Cheddar
Farmhouse Cheddar	Cheddar
Goodwood	Flavour added
Harefield	Hard
Herefordshire Oak Smoked	Little Hereford cheese Smoked
Herefordshire Sage	Little Hereford + fresh sage

CHEESE NAME	CHEESE TYPE
Isle of Mull	Hard
Isle of Mull Flavel	Flavour added
Isle of Mull Cheddar	Cheddar
John Bourne`s Organic Cheshire	Cheshire
Keen's unpasteurised Extra Mature Cheddar	A complex Cheddar
Keen's unpasteurised Traditional Cheddar	Traditional Cheddar
Kelsae	Hard
Lincolnshire Poacher	Matured Cheddar (15-20 months
Leicestershire Tiger	Unpasteurised Red Leicester + chillies
Lightwood Smoked	Flavour added
Little Hereford	Original Herefordshire recipe
Llanboidy with Laverbread	Young subtle flavoured cheese
Llangloffan Cheshire	Cheshire
Llangloffan Farmhouse	Hard
Llangloffan Granston	Hard
Llangloffan Red with Chives & Garlic	Flavour added
Llangloffan Organic	Hard
Loch Arthur Farmhouse	Traditional cheddar
Loch Arthur Cheddar	Cheddar
Loch Arthur with various Herbs	Flavour added
Loddiswell Cheese	Hard
Mullachag	Hard
Menallack	Hard full fat cheese matured 2 months
Menallack Chives & Garlic	Flavour added
Menallack Farmhouse	Unpasteurised; mould ripened
Menallack Vintage	Unpasteurised : matured
Merlins Sandwich	Flavour added
Monkland	Hard
Monkland with Chives & Garlic	Flavour added
Montgomery`s Jersey Shield	Hard
Montgomery`s Traditional	Cheddar
Montgomery`s Smoked Cheddar	Flavour added
Mrs Kirkham's Traditional Lancashire	Unpasteurised cow's milk cheese
Norfolk Dapple	Clothboard cheddar type
Noson Lawen	Flavour added
Old Barford	-
Old Plawhatch Cheese	Hard
Olde Gloster	Medium Double Gloucester
Red Leicester	Red Leicester
Rumbolds Farmhouse	Hard
Remarkable Valley	Hard
Scrumpy Sussex	Flavour added
Severn Sisters	Reduced fat
Severn Vale	Medium cheddar style

CHEESE NAME	CHEESE TYPE
Single Glocester	Delicate/ creamy
Single Gloucester	Single Gloucester. Pasteurised & unpasteurised milk
Single Gloucesters	The Gloucesters
Smoked Birdwood Dunlop	Flavour added
Smoked Lincolnshire Poacher	Flavour added
Smoked Teifi	Flavour added
St Aubyns	Hard
St Florence	Hard
St Laudus	Rodded; matured 3 months
St Thomas	Organic artisan Cheshire cheese
Staffordshire Organic	Organic artisan cheddar style
Staffordshire Oak Smoked	Flavour added
Staffordshire Organic with Wild Garlic	Flavour added
Stichill Hard	Hard
Strata Florida + Flavours	Flavour added
Sweet Milk	Hard
Teifi	Artisan
Teifi Farmhouse	Hard
Teifi with Flavours added	Flavour added
Teme Valley	Hard
Times Past Black Waxed Truckle	Cheddar
Times Past Cheshire	Cheshire
Times Past Double Gloucester	The Gloucesters
Times Past Draycott Red	Flavour added
Times Past Cheddar	Cheddar
Times Past Red Leicester	Red Leicester
Times Past Red Leicester with onions	Flavour added
Tobermory Truckle	Hard
Traditional Cheddar	Matured traditional cheddar
Traditional Cheddar	Traditional matured cheddar
Traditional Farmhouse Cheddar	Traditional cheddar
Traditional Cheddar	Cheddar
Traditional Double Gloucester	The Gloucesters
Traditional Single Gloucester	The Gloucesters
Traditional Lancashire	Lancashire
Twineham Grange	Hard
Westcombe Raw Milk Cheddar	Cheddar
Westcombe Red	Hard
Westerly Plain	Hard
Westmorland Original Mature	Lancashire
Westmorland Wonders	Lancashire with garlic/ spices
Whitmore	Hard
Wrekin Gold	Grainy texture
Wrekin White	Firm aromatic cheese

Table 2.3 Some raw milk semi-hard cheeses produced and on sale in the U.K.

CHEESE NAME	CHEESE TYPE
Alderwood	Wash rind
Ashmore Tillingbourne	Semi-soft
Birdwood Dunlop	Territorial
Brecon Blue	Blue
Brecon Blue	Blue
Caerfai caerffili	Organic caerffili cheese
Caerfai Leek & Garlic Caerffili	Organic Caerffili
Caerffili with Spring	Caerphilly with flavour added
Caerffili with Green Onion	Caerphilly with flavour added
Farmhouse Herb & Garlic	Flavour added
Caws Cenarth Caerffili	Caerffili; cream taste ; hint of lemon (award)
Caws Cenarth Oak Smoked	Caerphilly: smoked
Caws Nantybwla	Caerphilly
Caerphilly	Traditional Caerphilly Pasteurised & unpasteurised
Celtic Promise	Washed rind
Coulommier	Salty
Crabtree	Smooth & creamy
Crannog	Small waxed cheeses (herbs also)
Criffel	Rind washed
Crofton	Naturally rinded
Cuddys Cave	Creamy Dale variation
Doddington Blue	Creamy soft blue
Dorset Blue Vinny	Blue
Dunsyre Blue	Blue
Exmoor Jersey Blue	Blue
Glen Moray Carola	Semi soft
Granston Blue	Blue
Gloucester Woad	Blue
Gorwydd Caerphilly	Traditional Mature Caerphilly Unpasteurised
Harold Lukins Lactic Cheese	Semi-soft
Kebbuck	Washed curd
Lancashire	Lancashire
Landsker	Blue
Lightwood Smoked	Oak smoked cheddar
Maisie`s Kebbuck	Traditional cheese of the Upper Clyde Valley
Monkland	Brine bathed cheese
Monkland with Garlic & Chives	Monkland +garlic & chives
Morven	Waxed cheese
Nantybwla with Garlic	Flavour added
Nantybwla with Laverbread	Flavour added
Nantybwla with Leek & Garlic	Flavour added

CHEESE NAME	CHEESE TYPE
Norfolk Tawny	Washed in Norfolk Strong dark ale
Partridges Blue	Blue
Penyston	Semi-soft
Saval	Washed rind
Sharpham Rustic	Monet texture
Sharpham Rustic Chive & Garlic	Savory notes + chives & garlic
Sir Benfro	Semi soft
Smoked Cuddys	Oak smoked
Smoked Caerphilly	Caerphilly. Flavour added
Somerset Blue	Blue
Teifi Caerphilly	Caerphilly
Times Past Caerphilly	Caerphilly
The Original	Mild cheese
Wedmore	Caerphilly and chives Pasteurised & unpasteurised
Willett Blue	Blue

Table 2.4 Some raw milk soft cheeses produced and on sale in the U.K.

CHEESE NAME	CHEESE TYPE
Bath Brie	Soft
Bewcastle	Soft curd
Brierley	Soft White
Caws Cenarth	New cheese
Caws Cenarth Perl Wen	Combination of Caerphilly & Brie
Crannog	Fresh
Crannog with Chives	Fresh
Crannog with Green Peppercorns	Fresh
Crannog with Herbs	Fresh
Cream Cheese	Lactic curd cheese
Finn	White rind
Fromage Frais	Fresh
Gorgeous Spread	Fresh
Gwyn Bach	Fresh
Herbie Cheese	Fresh
Kelston Park	Ripened
Lightwood Chaser	Triple cream Vignotte-style
Little Aston	Fresh cheese with band of golden cheese curd
May Cheese	Fresh
Ruddle Court Traditional Fresh Cow Cheese	Vegetarian Cheese
Sharpham	
Sharpham Elmhirst	Triple cream soft cheese
Stichill Garlic & Chives Log	Fresh
Stichill Soft	Fresh
Stichill Soft Mint & Rosemary	Fresh
St. Nicholas	Ripened fresh soft cheese; natural moulds
Tunworth	Ayreshire cows milk
Waterloo	Soft White
Wealden Round	Fresh with herbs & garlic
Werston	Ayreshire cows milk + tarragon/ chives/ salt
Westerly Cumin	Flavour added
Wingreen	Soft mould ripened Camenbert- style
Wummle	Soft mould ripened

4. Cheesemaking

The control of cheesemaking plays a large part in determining the growth, survival or death of bacterial pathogens. Varieties of cheese differ in their composition and the technology used to make them. This in turn, has an impact on their risk from foodborne disease. Factors such as the rate of acid development, titratable acidity of the fresh curd, salt concentration, pH changes during maturation and maturation time all have an impact.

Williams and Neaves have summarised the attributes of different cheeses in Table 2.5.

Table 2.5 Summary of attributes of some cheeses (Williams and Neaves).

Cheese type	Rate of acidification	Hard / Soft cheese	Maturation
1. Cheddar	Rapid	Hard	Long ('mature', 18+ months) or Short ('mild', a few weeks)
2. Caerphilly	Rapid	Hard	Short (within a week of production or 'matured' for up to 6 months)
3. Camembert	Slow	Soft	Short
4. Gouda	Slow	Hard	Long
5. Ashby Blue	Slow	Semi-soft	Short

The rapid (4 h) acidification in cheeses such as Caerphilly can be viewed as a barrier to the growth of pathogens in the cheese. However, the maturation of this cheese can be quite short, therefore if a pathogen such as *Salmonella* is able to survive the cheesemaking process, it may still give rise to illness.

5. Characteristics of Cheeses

In addition to the impact of factors involved in the cheesemaking process, the attributes of the cheeses themselves need to be taken into account when considering risk factors for cheeses.

5.1. pH and Titratable Acidity

The measurement of acidity in cheeses and the ingredients used to make them is discussed by Banks on pages 23-28 of his report (Banks 2006). He gives typical pH values for different cheeses in Table 11 on pages 104-105.

The biological, biochemical and physical changes which take place in milk from the cow to when the cheese produced is fully ripe, are often controlled or influenced by the pH (hydrogen ion concentration) of the liquid or curd. The acid-base equilibrium of the milk or whey is a useful indication of the processing of the cheese. It is important that comparison of pH values between processes in cheese manufacture is done at the same temperature.

Cheesemakers are more familiar with 'titratable acidity' than pH. The advantage of using pH is that the reading is instantaneous. However, the probe used can easily become 'poisoned' by proteins, fats and salts becoming adsorbed onto the surface of the electrodes used. There is no correlation between pH values and acidity measurements (Scott 1986).

Traditionally phenolphthalein has been used as an indicator in the titration of milk. The rate of acid production in the milk/curd is measured by the change in acidity over unit time. The rate of acid development at each stage of the cheesemaking process influences the subsequent stages. Some variation of acidities at stages of cheese processing may be due to:

- the quality of the milk as growth medium for bacteria
- the quality and activity of the starter in the cheese milk
- the milk ripening time
- the temperature of the milk
- the size of curds at cutting
- the temperature regimes employed
- elapsed time and drainage time
- quantity of salt and method of salting
- pressing times and temperatures.

The cheese making process is one of continuing activity of bacteria or enzymes so that measurement of this activity by acid production must have reference to time.

An example of the variation of pH and titratable acidity during the manufacture of cheddar cheese is given in Figure 2.1

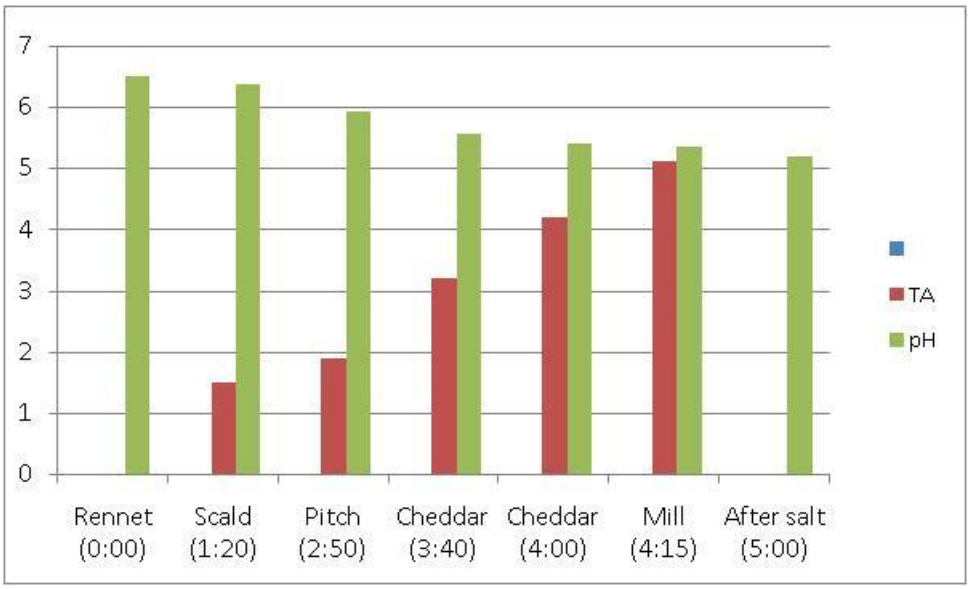


Figure 2.1 **An example of change in Titratable Acidity (TA) and pH during the stages of Cheddar Cheese manufacture.** TA is expressed as '% equivalent to lactic acid'. TA and pH are both represented on the 'y axis'. (Data courtesy of J. Montgomery).

5.2 Salt content

The use of salt in cheese making is discussed by Banks on pages 30-31 and 40-43 of his report (Banks 2006).

He states that it is the concentration of salt dissolved in the cheese moisture which determines the inhibitory effect of the salt. Salt can be applied by immersion in brine, or by dry application whereby salt is mixed with fresh curds or applied to the outside of the cheese. Salting tends to inhibit acid production by the cheese starter cultures. Therefore the salt must not be added before the acidity has fully developed.

Even distribution of salt in a large dry-salted cheese may take 3 to 4 days, but in smaller soft cheese the salt is well distributed in 16 hours. Until the salt is completely distributed in the curd, the pH of the curd may be unstable (Scott 1986).

The percentage of salt in cheese varies significantly from 0.25% for cottage cheese to 7% for some blue cheeses.

5.3 Water activity

The amount of water available for microbial growth (water activity) is linked in cheeses to the moisture content and the amount of salt present. Examples of water activity values for different cheeses are given in Table 10 (pages 102-103) of Banks' report (Banks 2006).

5.4 Moisture content

The total amount of water present in cheeses (moisture content) can be used to categorise cheeses, as described in section 2.

5.5 Background microbiological flora

The microbiology of raw milk is a diverse subject and is not covered here in great detail. Comprehensive accounts such as Bramley and McKinnon (1990) and Chambers (2002) are useful sources of detailed information.

There are 3 basic sources of microbial contamination of milk:

(i) from within the udder (in the teat canal);
(ii) from the exterior of the teats and udder; and
(iii) from the milk handling and storage equipment.

The main groups of mesophilic, aerobic microorganisms and their respective genera and species that comprise the microflora commonly found in raw UK-produced milk are given in Table 2.6.

Table 2.6 Types of aerobic mesophilic microorganisms in fresh milk (adapted from Chambers 2002)

Microorganism group	Example genera
Micrococci (30-99% incidence)	*Micrococcus* *Staphylococcus*
Streptococci (0-50% incidence)	*Enterococcus*
Gram positive rods (non-spore forming) (<10% incidence)	*Microbacterium*
Spore-formers (<10% incidence)	*Bacillus*
Gram negative rods (<10% incidence)	*Pseudomonas*
Miscellaneous Groups (<10% incidence)	*Streptomyces*, Yeasts, Moulds

The pasteurisation of milk will inactivate most of the vegetative bacteria listed above, however their presence may need to be considered in the manufacture of raw milk cheese.

5.6 Starter cultures used in cheese manufacture

Production of most types of cheese depends on the fermentation of the lactose by lactic acid bacteria to form mainly lactic acid. The low pH of fresh cheese curd (5.0-5.2) helps to suppress the growth of spoilage and pathogenic bacteria. At one time, the fermentation of lactose in milk for cheese making depended on natural contamination of the milk with lactic acid bacteria from the environment. When large volumes of cheese are being produced, a reliable fermentation is needed. This can best be achieved by use of a starter culture of known content.

The traditional microflora of starter cultures used in cheese manufacture includes members of the genera: *Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Lactobacillus, Bifidobacterium, Enterococcus, Propionibacterium* and *Brevibacterium.* A comprehensive account of starter cultures and their use in cheese manufacture is given by Tamime (2002).

Examples of starter organisms are given in Table 2.7.

Table 2.7 Lactic acid bacteria employed as starter cultures (adapted from Chapman and Sharpe 1990)

Bacteria	Examples of Usage
Mesophilic starters:	
Streptococcus lactis subspecies *cremoris*	Hard-pressed cheese (Cheddar)
	Semi-hard cheese (Lancashire)
Streptococcus lactis subspecies *lactis*	Blue-veined cheese (Stilton)
Leuconostoc	Surface mould-ripened cheese (Brie)
Streptococcus lactis biovar *diacetylactis*	Cottage Cheese
Thermophilic starters:	
Streptococcus thermophilus with *Lactobacillus delbrueckii* sub-sp. *lactis* subspecies *lactis*	Hard cheese (Emmenthal)
Mixed starters:	
Streptococcus lactis subspecies *lactis* with *Streptococcus thermophilus*	Italian pasta filata (Mozzarella)

5.7 Maturation conditions and duration

Cheese ripening is a very complex and slow biochemical process, and is therefore relatively expensive. Therefore incentives to reduce costs where possible encourage shorter maturation times (Fox and Tobin 1999). Semi-hard cheeses are ripened during a storage period ranging from 4 to 5 weeks up to 3 months.

The changes in composition of hard and soft cheeses during manufacture and maturation are represented in Tables 2.8 and 2.9.

Table 2.8 Compositional Profile of Hard Cheese Production (Williams & Neaves 2007)

Parameter	Milk Intake	In vat	Into mould	Next day	Maturation	
					Start	End
Time into cheese making	0h	0.5h	5h	20h	24h	6-24m
Temperature (°C)	4	32	22-25	22	8-10	10-12
Aqueous salt (%)	0.1	0.1	4.5	4.5	4.5	5.5
pH	6.8	6.8	5.0	5.0	5.0	5.2

Table 2.9 Compositional Profile of Soft Cheese Production (Williams & Neaves 2007)

Parameter	Milk Intake	In vat	Into mould	Next day	Maturation	
					Start	End
Time into cheese making	0h	0.5h	5h	20h	24h	21-28d
Temperature (°C)	4	30	22-25	22	10-15	10-15
Aqueous salt (%)	0.1	0.1	0.1	0.1	2.5	2.5
pH	6.8	6.8	6.7	4.8	4.8	7.5

Table 2.10 Summary of cheese characteristics

CHARACTERISTIC (Typical)	CHEESE TYPE		
	HARD (e.g. Cheddar)	**SEMI-SOFT** (e.g. Caerphilly)	**SOFT** (e.g. Cottage)
pH	5.0 - 5.2 *	4.6- 5.4	5.1-6.1*
NaCl %	1.75- 1.95 *	1.0- 2.0	0.25-1.0*
Water activity (a_w)	0.920 – 0.968* (ave 0.955)	0.980- 0.986* (ave 0.982)	0.983- 0.990* (ave 0.987)
Moisture %	20- 42*	44-55*	>55*
Background flora			
Starter cultures	*Lactobacillus cremoris* [@]	Defined lactic acid bacteria **or** indigenous microflora [@]	Thermophilic or mesophilic lactic acid bacteria [@]
Maturation conditions	60+ days at 5-12^{o}C (raw milk)*	Quick ripened* (10-14 days and 8-14°C))	High care under hygienic conditions

* Banks (2006).
[@] Robinson (2000)

6. Microbiological risks associated with cheeses

The microbiological safety of cheeses have been widely considered and reviewed (see for example Johnson *et al*. 1990).

The safety record for cheese is generally good considering the amount of this commodity consumed world-wide. Having said this, there has been a number of food poisoning episodes associated with cheese. The most important microorganisms in this regard are *L. monocytogenes*, *Salmonella* spp. and *E. coli* (VTEC) (Fernandes, 2009). Cheeses made from raw milk are particularly at risk since they may become contaminated by pathogens initially present in the milk. Pathogens could also enter during processing if hygiene and process controls are inadequate. According to Fox (Fox *et al*. 2000), 28% (9) of the 32 food poisoning outbreaks associated with cheese in the period 1970-2000 involved cheese made from raw milk. Several organisms were involved, but *Salmonella* spp., *Staphylococcus*

aureus and *Listeria monocytogenes* were the most common. Various enterotoxigenic *E. coli* including O157 were also involved.

The characteristics of individual cheeses will influence the potential presence and survival of pathogens. Process and storage temperature; acid production by starter cultures; and the addition of salt are all important in this regard. The majority of hard and semi-hard cheeses (Cheddar, Caerphilly and Red Leicester) appear to present little risk. For soft cheese the risk is considered low for Mozzarella, Ricotta, Quark and Cream Cheese due to processing and packaging (Banks, 2006). However, possible risk does exist for Blue-veined, surface film-ripened and mould ripened cheeses, due to production processes and controlling factors (i.e. neutralisation of acidity due to metabolites of mould / surface bacterial growth in later ripening stages (Banks 2006)).

Growth of some pathogens, such as *E. coli* and *Staph aureus* may occur during curd production if the organisms are present in the raw milk, but the extent of growth will be dependent on the organism and the rate of acid production and cooking (scalding) temperature. Generally, semi hard and soft cheeses are more likely to have problems because of their relatively high moisture content (Cogan and Beresford 2002).

Hargrove *et al*. (1969) studied the die-off of *Salmonella* in Cheddar cheese. At pH 5.03 and 5.23, the normal pH of Cheddar, they died off quickly, whereas at pH 5.7, they did not die off at all. In some soft cheeses there is an increase in pH during ripening (e.g. mould-ripened), and growth of some pathogens such as *Listeria monocytogenes* can occur (Fox *et al*. 2000).

A number of cases of food poisoning associated with soft cheeses are described by Farkye and Vedamuthu (2002). The organisms involved include *Listeria monocytogenes*, *Salmonella*, and *Staphylococcus aureus.* There have also been cases associated with *Escherichia coli* O157:H7 (in French Brie and Camembert (D'Aoust 1989)), which has been found to exhibit unusual heat and acid tolerances

- ***Listeria* spp.**

An outbreak of listeriosis in California in 1985 involved 142 cases and resulted in 48 deaths. The outbreak was associated with Mexican-style cheese. The processing environment and equipment were found to be contaminated and the proper pasteurisation of the milk questioned (Fernandes 2009). Further outbreaks were reported in the years following this incident, e.g. Vacherin Mont d'Or soft cheese in Switzerland; and Brie de Meaux raw milk cheese in France (1995).

Surface-ripened cheeses are especially vulnerable to recontamination and growth of *Listeria monocytogenes.* As ripening proceeds, the development of mould on the surface raises the pH from ~5.0 to 6.0-7.0. This combined with the high moisture content and temperature of the ripening rooms (~10°C) creates conditions in which rapid growth of *L. monocytogenes* is possible. Counts of 10^7 cfu/g have been found at the surface of Camembert after 56 days (Ryser and Marth 1987). The same process may occur during the ripening of blue-veined cheeses.

Although growth of *Listeria* is less likely to occur in cheeses where there is no rise in pH during ripening, the pathogen may survive for long periods. For example, viable cells were found in Cheddar cheese stored for 434 days (Ryser and Marth 1987), and raw-milk soft or

semi-hard cheese that had undergone aging for 60 days was implicated in an outbreak in Canada in 2002 (Norton and Braden 2007).

In order to prevent issues with *L. monocytogenes* in cheese manufacture it is essential that Good Hygienic Practices are adhered to, and that good quality milk is used to make the cheese. Vulnerable consumers such as the pregnant and elderly in the UK are advised not to eat surface-ripened cheeses.

- ***Escherichia coli***

Enteropathogenic *E. coli* (EPEC) is a cause of gastroenteritis in humans, but its growth is inhibited during cheese manufacture by acidity and pH. If the starter culture fails however, EPEC can cause issues. In the U.S.A. in 1971, EPEC caused 387 cases of illness associated with imported Camembert (Marrier *et al.* 1973).

The enterohaemorrhagic strain of *E. coli* (EHEC), known as *E. coli* O157:H7 is considered to be a potentially high-risk pathogen in cheese, because of its ability to tolerate low pH values for long periods and its association with unpasteurised milk. Several outbreaks have occurred with this bacterium in cheese, including one associated with soft cheese made from unpasteurised milk in France, and a small outbreak in the NE of England in 1999 associated with Cotherstone cheese (Anon 1999), which was also made from unpasteurised milk. Control of raw milk quality and checks on the health of dairy cattle are essential for the production of unpasteurised soft cheeses.

- ***Salmonella* spp.**

Salmonellae can be isolated in milk from infected animals, but as for *E. coli* do not survive pasteurisation. In cheese made from raw milk, *Salmonella* can survive the cheese making process. This pathogen can also gain entry to cheese through post-pasteurisation contamination. If acid production during manufacture is slow, salmonellae may grow during cheese making (Hargrove *et al.* 1969), and have been known to survive for more than 60 days in some cheeses (D'Aoust *et al.* 1985).

There have been a number of large outbreaks of salmonellosis associated with Cheddar cheese (Fernandes 2009). These illustrate the ability of salmonellae to survive for long periods in cheese at refrigeration temperatures (D'Aoust *et al.* 1985). Other cheese types including Mozzarella have been linked to outbreaks of salmonellosis.

- ***Staphylococcus aureus***

Low numbers of *S. aureus* are relatively common in raw milk. This may be due to contamination from the udder surface or shedding of the organism into milk by cows with sub-clinical mastitis. The hazard posed by *S. aureus* (production of a heat-stable toxin) can be controlled by storage of milk at low temperatures together with pasteurisation and adequate post-process hygiene to prevent recontamination. There have only been a few outbreaks over recent years, these have been associated with slow acid production by starters, or faulty process control.

- **Other pathogens**

Outbreaks of infection with other foodborne pathogens have rarely been associated with cheese. Cases associated with *Clostridium botulinum, Shigella* spp. and *Brucella* spp. have been reported, but none associated with *Campylobacter, C. perfringens* or *Bacillus cereus* (Teuber 2000).

- **Toxins**

Mycotoxins could be present in cheese either as a result of using contaminated milk (derived from animal feed), or as a consequence of mould growth on the cheese during ripening and storage.

The biogenic amines histamine and tyramine may be found in cheese. They are produced as a result of the decarboxylation of the amino acids histidine and tyrosine which is carried out by Lactic Acid Bacteria and members of the Enterobacteriaceae during cheese ripening. These biogenic amines can lead to a series of symptoms including: increased blood pressure (tyramine); flushing, headaches, nausea (histamine). Cases of both tyramine-induced hypertensive crisis and histamine poisoning have been reported associated with cheese (Teuber 2000).

7. Conclusions

A wide variety of cheeses are made in the UK using raw cow's milk. A number of microbial pathogens have the potential to give rise to problems for consumers of cheese.

3. RAW MILK AND RAW MILK CHEESES AS VEHICLES FOR INFECTION BY VEROCYTOTOXIN-PRODUCING ESCHERICHIA COLI

ABSTRACT

Raw milk and raw milk cheeses can be a source of foodborne pathogens, including Verocytotoxin (Shiga toxin)-producing *Escherichia coli* (VTEC/STEC). Outbreaks of VTEC O157: H7 infections have been attributed to the consumption of raw milk and associated dairy products. Although the general prevalence of VTEC O157 in raw milk and raw milk cheeses is low, it can be higher for non-O157 VTEC. The clinical significance of many of these VTEC is unclear, although some are associated with disease. Studies show that *E. coli* O157 strains can survive the various stages of the cheese making process and that raw milk and raw milk cheeses remain a potential vehicle for VTEC infections.

INTRODUCTION

Many of the common enteric pathogens such as *Salmonella*, *Escherichia coli* O157: H7 and *Campylobacter* are carried in the intestinal tract of ruminants, including domestic animals used in milk production, e.g. cows, sheep and goats. Preventing faecal material contaminating the milk is an important step in reducing the prevalence of pathogens entering raw milk. Effective cleaning procedures, including removing faecal material from udders prior to milking, can reduce the risk, although heat-treatment of raw milk is the most important process used to eliminate the risk from viable vegetative pathogenic bacteria and provide a safe product.

Raw milk can be a significant source of foodborne pathogens, and there have been numerous foodborne outbreaks associated with direct consumption of raw milk, milk that has been inadequately heat-treated, or milk that has been re-contaminated after heat-treatment. The presence of pathogens in milk, is likely to arise from contamination by faecal material during the milking process. Good hygiene, including the removal of faecal material from udders and ensuring a clean environment, is therefore important. Contaminated milking parlour equipment and floors can facilitate the spread of these pathogens to the udders; subsequently milking equipment including teat cups, pipelines, filters and bulk storage vessels can become colonised (O'Loughlin and Upton 2001). Failure to implement effective cleaning and disinfection procedures can result in contamination of subsequent batches of product.

Besides the risks associated with consuming raw milk there are concerns over the safety of cheeses made from raw milk. Although cheese can be made safely with raw milk, there have been foodborne outbreaks linked to raw milk cheeses caused by *Salmonella*, *Campylobacter*, *Staphylococcus aureus* and *E. coli* O157: H7 (Zottola and Smith 1991; De Buyser *et al.* 2001). Nevertheless, raw milk remains popular for cheese making because of enhanced organoleptic properties, notably the flavour it imparts to the final product. In Europe, cheese manufacture is controlled by the Food Hygiene Regulations (European Commission 2004a, b) and cheeses are generally considered to be one of the safest foods consumed. However, dairy products including cheese, can occasionally contain pathogenic bacteria. The most dangerous of the vegetative pathogens associated with raw milk and raw milk cheeses are the Verocytotoxin-producing *E. coli* (VTEC), especially VTEC O157: H7.

Verocytotoxin-producing *E. coli*

Escherichia coli is a common Gram-negative, non-spore forming bacterium belonging to the family Enterobacteriaceae. It is found in the gastrointestinal tract of man and other animals, although it can be found in water, soil and food, often as a result of faecal contamination. Because of this association, *E. coli* is used as an indicator of potential faecal contamination of foods and water (Baylis and Petitt 1997). Most *E. coli* strains are harmless commensal organisms, however pathogenic strains have evolved which are responsible for distinct types of clinical disease in man.

There are two recognised groups of pathogenic *E. coli*. Extraintestinal pathogenic *E. coli* (ExPEC) represent *E. coli* associated with urinary tract infections and newborn meningitis, whilst the intestinal pathogenic *E. coli* (IPEC) are responsible for a range of diarrhoeal diseases. Within the IPEC there are currently six distinct groups of *E. coli* that are associated with foodborne disease: Verocytotoxin-producing *E. coli* (VTEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC) and the diffusely adherent *E. coli* (DAEC). These diarrhoeagenic *E. coli*, the associated virulence mechanisms and their role in disease are described elsewhere (Sussman 1997; Nataro and Kaper 1998; Donnenberg 2002; Baylis *et al.* 2006).

The Verocytotoxin-producing *E. coli* (VTEC), which are also commonly referred to as Shiga-toxin producing *E. coli* (STEC), comprise over 400 serotypes of *E. coli* (Scheutz and Strockbine 2005). The common feature which defines this group is their ability to produce a distinct range of toxins termed Verocytotoxins (VT) which show potent cytotoxicity against Vero cells (Konowalchuk *et al.* 1977). Owing to the similarity in structure and biological activity of these toxins and the toxin produced by *Shigella dysenteriae* type 1 (O'Brien and Holmes 1987), they are also termed Shiga toxins (Stx). Two antigenically distinct types of VT (VT1 and VT2) were initially identified (Scotland *et al*. 1985), although there are now at least 3 sub-types of VT1 (VT1, VT1c and VT1d) and six sub-types of VT2 (VT2a, VT2c, VT2d, VT2e, VT2f, and VT2g) recognised (EFSA 2007). The genes for these toxins (*vtx*/*stx*) are generally encoded by prophages of the lambda (λ) family and VTEC strains may produce either VT1 or VT2 alone or together. The most common combination of toxin types associated with severe human disease are VT2 and VT2c (Friedrich *et al.* 2002; Persson *et al.* 2007). In contrast, VT1c and other toxin types, (e.g. VT2e, VT2f and VT2g), are commonly associated with milder disease or are rarely associated with human disease (Friedrich *et al.* 2003; Beutin *et al.* 2007).

Clinical manifestations of infection with VTEC range from asymptomatic carriage through mild diarrhoea to life threatening conditions such as haemorrhagic colitis (HC) and the severe complications of haemolytic uraemic syndrome (HUS) and thrombotic throbocytopenic purpura (TTP), which can result in kidney damage, renal failure and even death (Tarr *et al.* 2005). Some VTEC, notably those belonging to serotype O157: H7, are associated with severe disease. Besides toxin production, some VTEC have the ability to cause attaching and effacing (A/E) lesions on epithelial cells using similar mechanisms to those found in EPEC. The process of A/E is mediated by a pathogenicity island termed the locus of enterocyte effacement (LEE), which is an important cluster of genes in the bacterial chromosome involved in this process. The LEE encodes a type III secretion system and the *eaeA* gene, which codes for the outer membrane protein intimin, which mediates binding of the bacterium to the host cell surface. Strains carrying *eaeA* are often associated with more

severe forms of disease. Additional virulence factors can be carried on large plasmids found in some VTEC. In VTEC O157, a large (*ca* 92 kb) plasmid termed pO157 carries the gene *ehxA* which encodes for enterohaemolysin, *katP* for catalase-peroxidase and *espP* which codes for an extracellular serine protease (Burland *et al.* 1998). These and other genes have all been associated with pathogenicity, although their exact role in disease is not yet fully understood. Some VTEC strains lacking *eae* and *ehxA* genes have, on occasion, been shown to cause human illness, so these genes may not necessarily be essential for a VTEC to cause disease (Neill 1997).

Not all VTEC are associated with severe human disease, however, and there are many VTEC serotypes that have been isolated from animals and foods which have rarely or never been associated with cases of human infection. Therefore, the clinical significance of these VTEC serotypes or the potential risk they pose has not yet been fully elucidated. Some studies have shown differences between VTEC from dairy products, including cheese, and those commonly isolated from humans. In a study by Pradel *et al*. (2008), VTEC strains from dairy food were predominantly carrying *vtx1* and few were harbouring the *eae*, *espP* and *katP* which are commonly associated with disease-causing strains isolated from patients.

Dairy animals as a source of VTEC

Cattle have long been identified as a major reservoir for VTEC, including VTEC O157: H7 (Chapman *et al.* 1993a; Hancock *et al.* 1994). Cattle are known to shed a range of VTEC, including VTEC O157: H7, and others associated with human disease. In a study of 514 VTEC isolates from diarrheic and healthy cattle in Spain, 20% carried *vtx1*, 54% carried *vtx2* and 26% carried both toxin genes (Blanco *et al.* 2004). In addition, enterohaemolysin and intimin genes were detected in 63% and 29% of the isolates, respectively. Overall, 85% of the cattle isolates were identified as those associated with human disease and 54% were serotypes associated with human HUS.

Although raw meat and undercooked meat products have been commonly associated with outbreaks of VTEC O157: H7 infection, raw milk and dairy products have also been implicated in cases of human infection, including HUS. The prevalence of VTEC in dairy cattle and their products has been reviewed by Hussein and Sakuma (2005). In this review the prevalence of VTEC in faeces of dairy cattle was reported to be between 0.2 and 48.8% and 0.4 to 74.0% for *E. coli* O157 and non-O157 VTEC, respectively. VTEC serotypes isolated from dairy cattle and associated with disease include O2: H29, O22: H8, O26: H11, O103: H2/H$^-$, O111: H8/H$^-$, O113: H21, O145: H$^-$ (Hussein and Sakuma 2005).

Besides cattle, other domestic animals used to produce milk, such as goats and sheep, also harbour these bacteria and shed them in their faeces. Compared to cattle, higher prevalence of *E. coli* O157 and other VTEC in sheep and goats has been reported (Beutin *et al.* 1993; Sidjabat-Tambunan and Bensink 1997; Fagan *et al.* 1999). In Germany, reports indicate prevalence rates for non-O157 VTEC in sheep, goats and cattle of 67%, 56% and 21%, respectively (Beutin *et al.* 1993). Similar results were found during another German study, which reported VTEC prevalence rates of 32%, 75% and 18%, respectively in sheep, goats and cows (Zschock *et al.* 2000).

In addition to the high prevalence of VTEC in sheep and goats, these animals appear to harbour a broad range on VTEC serotypes, including VTEC associated with human infection. In a study by Rey *et al*. (2003), comparison of 253 VTEC strains isolated from sheep in Spain

revealed 43% to be carrying *stx* (*vtx*) 1 genes, 4% carried *vtx* 2 and 53% carried both toxin genes. In addition, the virulence associated genes *ehxA* and *eae* were present in 47% and 4% of strains, respectively. The majority of these VTEC strains belonged to 6 serogroups (O6, O91, O117, O128, O146 and O166) and 71% belonged to 9 serotypes (O6: H10, O91: H^-, O117: H^-, O128: H2, O128: H^-, O146: H21, O166: H28, O76: H19 and O157: H7). Rey *et al.* (2003) also reported the discovery of ten new VTEC O: H serotypes, not previously described.

In dairy herds, faecal contamination of udders poses a risk of pathogens entering the raw milk. Preventing faecal material from contaminating the milk is therefore an important step in reducing the prevalence of VTEC and other pathogens in raw milk. The implementation of effective cleaning procedures to remove faecal material from udders prior to milking can reduce the risk but not eliminate it entirely. The risk of transferring pathogens, including VTEC, to milk can be further reduced by hygiene precautions during milking.

Good animal husbandry on the farm reduces udder infections, which can allow pathogenic bacteria to be introduced into the milk. Bovine mastitis can be caused by *E. coli* (Jones 1990) but there is little if any published evidence that mastitis can be caused by *E. coli* O157. Turutoglu and Madul (2002) reported isolating *E. coli* from 50 (13%) of 382 mastitic milk samples in the Burdur province of Turkey, but none of these were identified as belonging to serogroup O157. There is also little published information to indicate that VTEC strains are responsible for mastitis, although they could potentially can gain access from the udder to the milk in mastitic cows (Stephan and Kühn 1999). Both VTEC and other *E. coli* come from the same faecal origin and they can readily be isolated from the cow's environment so there is no reason to assume that VTEC cannot enter milk by the same routes used by other enteric bacteria.

Pasteurisation is an effective treatment for removing vegetative pathogens including VTEC from milk. Without this step there is always the risk of pathogenic bacteria being present in the milk. Raw milk and dairy products produced using unpasteurised milk, such as cheeses, have been responsible for outbreaks of foodborne illness (Djuretic *et al.* 1997). With the increased popularity of products such as cheeses made from unpasteurised milk, strict hygiene and good quality and process control during their manufacture are essential.

Outbreaks associated with raw milk and associated cheese

Raw Milk

Raw milk consumption is considered potentially hazardous and has been associated with several types of infection including brucellosis, cryptosporidiosis, campylobacteriosis, *E. coli* O157 infection, listeriosis, salmonellosis, yersiniosis, tuburculosis and staphylococcal enterotoxin poisoning (Potter *et al.* 1984). Despite the apparent dangers, consumption of raw milk does still occur. In England and Wales the most common vehicle of infection associated with milkborne outbreaks between 1992 and 2000 was unpasteurised milk (52%) (Gillespie *et al.* 2003). The second most common vehicle reported by Gillespie *et al.* (2003) was milk sold as pasteurised (37%) with most of the outbreaks (67%) being linked to farms. The most common pathogens detected in these outbreaks were *Salmonella* (37%), VTEC O157 (33%) and *Campylobacter* (26%).

Direct consumption of raw milk is much less common than consumption of pasteurised milk, although it is still permitted in some countries. In the UK, the Animal Health Dairy Hygiene Inspectorate (formerly the Dairy Hygiene Inspectorate) has estimated consumption of raw cows' drinking milk to be of the order of 0.01% of cows' milk consumption (FSA 2005). In Scotland, the sale of raw milk has been banned since 1983 following a number of milk related outbreaks and more recently the sale of raw milk and cream from any species was banned. In England and Wales, the sale of raw milk is still permitted, but only directly to the consumer by registered production holdings such as at the farm gate or in a farmhouse catering operation, or through milk roundsmen. Although there are no known sales in Northern Ireland, similar controls to those in England and Wales currently apply. In England and Wales the sale of raw milk from sheep, goats or buffaloes is not subject to the same restrictions imposed on raw cows' drinking milk, which has tight marketing controls, although other requirements and restrictions are imposed on these.

In the UK and USA there have been several outbreaks associated with milk and dairy products. In the USA between 1973 and 1992, raw milk accounted for 46 outbreaks reported to the Centers for Disease Control (CDC) involving *Campylobacter* (57%), *Salmonella* (26%), *Staphylococci* (2%) and *E. coli* O157: H7 (2%) and 13% which could not be linked to a particular agent (Headrick *et al.* 1998). In the UK, 20 general foodborne outbreaks associated with consumption of milk and dairy products were reported to the PHLS Communicable Disease Surveillance Centre between 1992 and 1996 (Djuretic *et al.* 1997). The pathogens involved were *Salmonella* (55%), *Campylobacter* (25%), VTEC O157 (15%) and *Cryptosporidium parvum* (5%). Milk was implicated in 16 outbreaks, of which 10 were associated with unpasteurised milk. Interestingly, of the three *E. coli* O157 outbreaks reported, only one was caused by unpasteurised milk. The other two outbreaks were caused by pasteurised milk, where post pasteurisation contamination and failure in pasteurisation respectively, were identified as key factors. In 1994, an outbreak caused by VTEC O104: H21 in Montana, which resulted in 4 cases of HC, was also attributed to post pasteurisation contamination of milk (Centers for Disease Control 1995).

Two cases of HUS associated with consumption of raw milk provided early evidence that raw milk can be a vehicle for the transmission of *E. coli* O157 to humans (Martin *et al.* 1986). In Sheffield, UK, *E. coli* O157 was linked to untreated milk (Chapman *et al.* 1993b). Since then there have been several documented cases or outbreaks of *E. coli* O157 infection caused by consumption of raw milk. These include the consumption of raw goats' milk in Austria in 2001 (Allerberger *et al.* 2001) and British Columbia in 2001 (McIntyre *et al.* 2002) and raw cows milk in the USA in 2005 (Denny *et al.* 2008) and in 2006 (Centers for Disease Control 2008). In Austria, unpasteurised cows' milk has been associated with 2 cases of HUS caused by VTEC O26 (Allerberger *et al.* 2003). In the event of an *E. coli* O157 outbreak associated with raw milk, failure to restrict the distribution of contaminated product and prevent further consumption can lead to prolonged outbreaks that are intermittent and unpredictable (Keene *et al.* 1997).

Besides direct consumption of raw milk, defective pasteurisation and post process contamination can both yield milk containing pathogens, leading to outbreaks of infection. One example of this was the Red House Dairy outbreak in West Lothian, Scotland, in 1994, which resulted in over 100 people becoming infected by *E. coli* O157: H7 phage type (PT) 2 VT2 (Upton and Coia 1994). This outbreak resulted in 9 children developing HUS and one elderly woman developed TTP. This remains the largest reported milkborne outbreak of *E. coli* O157 infection and at the time it was the first involving a heat-treated milk supply. Since

then there have been others; for example in North Cumbria, UK, an outbreak involving 60 cases of infection from *E. coli* O157 PT 21/28, VT2 and VT2c in 1999 was caused by defective pasteurisation (CDSC 1999b, d, c).

Cheese

Several outbreaks involving pathogenic *E. coli* have been caused by consumption of contaminated cheese. In the USA, imported French cheese was associated with outbreaks involving pathogenic *E. coli* in soft ripened Camembert cheese in 1971 (Marrier *et al.* 1973), and ETEC in semi-soft cheese (Brie) in 1983 (MacDonald *et al.* 1985). The outbreak in 1971 involved an enteroinvasive *E. coli* O124 and was reported to be the first adequately documented occurrence of enteropathogenic *E. coli* foodborne disease in the USA. The French authorities later identified a malfunctioning filtration system used to filter river water used in cleaning at the site of the cheesemaker and the organism was also isolated from a curdling tank and from ripening cheese.

Infections caused by VTEC, especially strains belonging to serotype O157: H7, can be potentially life threatening, especially if the disease progresses to HUS. In France a cluster of 4 cases of HUS in children, caused by *E. coli* O157 VT2, was traced to consumption of cheese made with unpasteurised mixed cows' and goats' milk (Deschênes *et al.* 1996). In Somerset England, a 12 year old boy developed HUS following infection by *E. coli* O157 PT 2, VT2 after eating cheese made with unpasteurised milk (CDSC 1998). This case involved consumption of Wedmore, a Caerphilly-type cheese containing chives produced by a small Company with a national distribution (Anon 1998). In 1999, a cheese made with unpasteurised milk (Cotherstone cheese) was responsible for 2 cases of infection by *E. coli* O157 PT 21/28, VT 2 in North Yorkshire, UK (CDSC 1999b, a).

Despite the retail sale of raw cows' milk being prohibited in Scotland since 1983, there have been several documented outbreaks of *E. coli* O157 infection associated with cheese. Three outbreaks of *E. coli* O157 infection in Scotland, between 1994 and 1999, involved consumption of cheese made from unpasteurised milk (Reid 2001). In all three outbreaks the hygiene standards of the premises involved appeared to be satisfactory. However, the raw milk at two premises was being stored at temperatures that facilitated growth of bacteria. Furthermore, at one premise no starter culture was being used and in another the maturation step was found to be too short to enable sufficient reduction in pH and subsequent decrease in the bacterial population in the product. Poor traceability and a lack of adequate labelling of contaminated products were also highlighted in two of the outbreak investigations.

In 1998, an outbreak involving 55 laboratory confirmed cases of *E. coli* O157: H7 infection in Wisconsin, USA was linked to consumption of fresh cheese curds which had inadvertently been produced using vats previously used to produce cheese made from unpasteurised (raw) milk (Centers for Disease Control and Prevention 2000). In Alberta, Canada, unpasteurised gouda cheese was responsible for an outbreak of 13 cases of VTEC O157: H7 infection in 2002 (Honish *et al.* 2005) and in 2003 another outbreak involving contaminated cheese was responsible for at least 10 cases, including HUS in one child patient (Anon 2003). In 2004, two cases of HUS caused by VTEC O157 in France were traced to consumption of unpasteurised goats cheese made by an independent producer (Espié *et al.* 2006).

Prevalence of VTEC in raw milk and dairy products

Although contaminated raw milk and cheese made from unpasteurised milk have been responsible for outbreaks of VTEC O157 infection, published studies indicate a low prevalence or a failure to detect this pathogen in these products (Table 3.1).

Table 3.1 Prevalence of VTEC O157 in raw milk and unpasteurised dairy products

Country	Type of products tested	No. positive/ No. tested (%)	Reference
Australia	Raw milk[§]	0/147 (0)	Desmarchelier *et al.* (1998)
Belgium	Soft/semi-soft (cow, ewe & goat milk) cheeses	0/153 (0)	Vivegnis *et al.* (1999)
Egypt	Raw cows milk	3/50 (6.0)	Abdul-Raouf *et al.* (1996)
France	Raw milk[§], Unpasteurised cheeses	0/205 (0) 0/180 (0)	Fach *et al.* (2001)
Germany	Raw milk[§]	1/273 (0.4)	Klie *et al.* (1997)
Italy	Raw cows milk	0/227 (0)	Colombo *et al.* (1998)
Italy	Raw milk[§]	0/100 (0)	Massa *et al.* (1999)
Italy	Raw goats milk for cheese making	1/60 (1.7)	Foschino *et al*. (2002)
Italy	Various dairy products (Italian cheeses and butter)	0/1313 (0)	Conedera *et al.* (2004)
The Netherlands	Raw cows milk from bulk tanks	0/1011 (0)	Heuvelink *et al.* (1998)
Peru	Soft cheese	8/102 (7.8)	Mora *et al.* (2007)
Portugal	Raw milk cheeses	0/70 (0)	Almeida *et al*. (2007)
Spain	Raw Ewes' milk	3/84 (3.6)	Caro *et al*. (2006)
Spain	Raw milk soft cheese	1/221 (0.4)	Quinto and Cepeda (1997)
Switzerland	Raw (cows, sheep's, goat's milk) milk cheeses- soft, semi hard, hard cheese)	0/796 (0)	Stephan *et al*. (2008)
UK	Raw cow's milk	3/1097 (0.3)	de Louvois and Rampling (1998)
UK (Scotland)	Raw cow's milk	0/500 (0)	Coia *et al*. (2001)
UK (Scotland)	Raw milk cheeses (Scottish & imported)	0/739 (0)	Coia *et al*. (2001)
USA	Raw milk[§]	11/115 (9.6)	Padhye and Doyle (1991)
USA	Raw cows milk	0/603 (0)	Hancock *et al.* (1994)
USA	Raw milk[§] Soft & semi-soft cheeses	0/42 (0) 0/65 (0)	Ansay and Kaspar (1997)
USA	Raw milk (cows, goat, sheep) for cheese making)	1/133 (0.75)* goats milk	D'Amico *et al*. (2008)

However, as with prevalence studies for cattle and meats, the methodology used and the ability to recover these pathogens will influence the results. Although standard methods exist for detecting *E. coli* O157 and methods have been developed to detect non-O157 VTEC,

many published studies have employed different approaches for the detection of these bacteria in milk and cheese samples. Despite this, and irrespective of the methods used, past surveys and published studies report the incidence of VTEC O157 in these types of samples as consistently low. The findings of one study suggest that most raw milk intended for cheese making is of high microbiological quality with a low incidence of pathogens (D'Amico *et al.* 2008). Occasionally there are reports of higher isolation rates and there is evidence that the incidence can be higher on case-farms linked to outbreaks (Wells *et al.* 1991).

In Spain a study of VTEC in ovine and caprine milk and other dairy products showed the presence of VTEC in bulk tanks, fresh cheese curds and cheese, and one bulk tank was positive for VTEC O157 (Rey *et al*. 2006). In a survey of the prevalence of *E. coli* O157 in the UK dairy industry, this pathogen was not detected in 1146 samples comprising dairy products (raw milk, imported soft cheese), environmental samples and cattle faeces (Neaves *et al.* 1994). A study of 1097 samples of unpasteurised milk purchased from 242 retail outlets in England and Wales between May 1996 and July 1997 revealed the presence of *E. coli* O157 in 3 samples, and unacceptable aerobic plate counts or coliform counts in a fifth of samples not containing pathogens (de Louvois and Rampling 1998). This would tend to suggest that there is no correlation between high levels of indicator organisms, such as coliforms and the presence of *E. coli* O157. Unfortunately, the numbers of *E. coli* O157 found in contaminated samples is often not reported, but where this is information has been obtained it would appear to be very low. For example, Foschino *et al.* (2002) reported the level of *E. coli* O157 in the one positive sample of goats' milk was to be 1.5 cells/mL. In a study by D'Amico *et al*. (2008) the only a positive sample was obtained after enrichment and it was reported that the level of *E. coli* O157 contamination in the sample was <1 CFU/mL. Interestingly, confocal scanning laser microscopy (Auty *et al.* 2005) has indicated that *E. coli* O157 can occur as single cells or in small clumps of about 10 cells, usually within the protein matrix of the cheese. Therefore some care should be taken in interpreting low colony forming unit counts, as each CFU could comprise a greater number of individual cells.

Diversity of serotypes and virulence factors

The presence of VTEC in cheese is undesirable because of their potential to cause disease. However, the problem is complicated because many VTEC serotypes have not been associated with human disease whereas others, notably O157: H7, O26: H11, O111: H^-, O145: H^-, O103: H2 and O121: H19, are known to cause disease in man, including HUS (EFSA 2007). The concept of seropathotype (Karmali *et al.* 2003) provides a useful method of assessing disease potential by dividing VTEC into categories (A to E) based on their relative prevalence and their association with outbreaks, severe disease, serotype and presence of OI-122 (a pathogenicity island linked to virulence, although its exact role has yet to be elucidated).

Studies to date indicate that VTEC serotypes carrying *eae* and toxin subtypes commonly associated with those responsible for human disease can be isolated from raw milk and raw milk cheese (Hussein and Sakuma 2005; Rey *et al.* 2006; Stephan *et al.* 2008). However, Pradel *et al.* (2008) showed that most VTEC isolated from dairy products in France were different to those isolated from patients and that the dairy VTEC strains predominantly carried *vtx1* with a minority carrying *eae*, *esp*A and/or *kat*P. In the study by Caro and García-Armesto (2007), *vtx1* was the only toxin gene carried by the 3 strains of VTEC isolated from Spanish raw ewes' milk cheese and none of these strains possessed the *eaeA* or *ehxA* genes.

The diversity of VTEC serogroups/serotypes isolated from raw milk and cheeses is shown in Table 3.2 and Table 3.3, respectively.

Table 3.2 Prevalence of VTEC in milk

Product	Country	No. positive/ No. tested (%)	Serotypes/serogroup	Reference
Raw milk	Canada	15/1720 (0.9)	No VTEC O157 isolated. Serotypes isolated: O?: H-, O121: H7, O163: H19, O136: H16, O26: H11, O136: H12, O91: H21, O?: H8, O1: H20, O113: H21, O142: H38, O?: H21	Steele *et al.* (1997)
Raw milk	France	44 (21.5)* 14/205 (7.0)**	No VTEC O157 isolated. Serogroups isolated: O3 O91, O110, (*vtx1* only). O6, O117, O77 (vtx2 only). O76/O22 (both *vtx1* & *vtx2*)	Fach *et al.* (2001)
Raw milk cheeses	France	136/1039 (13.1)	No VTEC O157 isolated. Serotypes isolated: O6: H10, O76: H19, O109: H25, O174: H8 (*vtx1* only).O22: H8, O109: H25 (*vtx2* only).O?: H8, O174: H8*, O76: H19 O6: H1, O175: H16, O6: H10, O162: H10 (both *vtx1* & *vtx2*). *Only *eae* positive strain	Vernozy-Rozand *et al.* (2005)
Raw bulk tank milk (goat's and ewe's milk)	Switzerland	55/344 (16.3) Goat's milk 8/63 (12.7) ewe's milk	No VTEC O157 isolated. Serotypes isolated O5:H$^-$, O76:H19 (all *vtx2c*), O91: H?, O113: H4, O113: H4, O174 (OX3): H8 (all *vtx2d*) ONT: H19 (*vtx2)* Only ONT: H19 was eae positive. All EHEC-*hyl* positive except O87: H16 and O174 (OX3): H8	Muehlherr *et al*. (2003)

Table 3.3 Prevalence of VTEC in cheese

Product	Country	No. positive/ No. tested (%)	Serotypes/serogroups and virulence factors identified	Reference
Cheeses (NS)	France	60/603 (9.9)	No VTEC O157 isolated. Serotypes and serogroups isolated: OX3: H2, O91: H21 (both *vtx1* & *vtx2*), O113, O8, O15, O103 (toxin type not stated). All strains *eae* negative	Pradel *et al.* (2000)
Unpasteurised cheeses	France	55/180 (30.5)* 16/180 (9.0)**	No VTEC O157 isolated. Serogroups isolated: O5, O76, O110, O117, O136 (*vtx1* only). O6, O77, O91, O113 (*vtx2* only). O79, (both *vtx1* & *vtx2*). Only O5 strains *eae* positive	Fach *et al.*(2001)
Pasteurised cheeses	France	4/45 (8.9)* 0/45 (0)**	N/A- no isolates obtained	Fach *et al.* (2001)
Unpasteurised bulk tank milk (sheep and goat's milk)	Spain	39/360 (10.8)	O157: H7 serotype (*vtx2* and *ehxA*) positive was isolated from one (0.3%) bulk tank sample. Other serotypes identified: O27: H18*, O76: H19*, O91: H28* (*vtx1*). ONT: H7, ONT: H9, O157: H7*, O45: H38 (*vtx2*). ONT: H21 (both *vtx1* & *vtx2*). Only O157: H7 serotype showed *eae* virulence gene. **ehxA* positive	Rey *et al.* (2006)
Fresh (sheep and goat's milk) cheese curd	Spain	4/103 (3.9)	No VTEC O157 isolated. No isolates obtained for characterisation	Rey *et al.* (2006)
Cheeses (sheep and goat's milk)	Spain	2/39 (5.1)	No VTEC O157 isolated. No isolates obtained for characterisation	Rey *et al.* (2006)
Raw ewe's milk cheese	Spain	3/83 (2.4)	ONT, O14 (2 isolates) All *vtx1*, (*eaeA* and *ehxA* negative)	Caro & García-Armesto (2007)
Raw milk cheeses (goats, sheep and cows milk)	Switzerland	5/52 (9.6) Soft cheese 34/744 (4.6) Semi-hard and hard cheese	No VTEC O157 isolated. Serotypes isolated: O2:H27, O15:H16, O22:H8, O91:H21, O109:H16, O113:H4, O174:H21 1 strain Or: H45 was *vtx1* positive. All other strains carried *vtx2* or *vtx2* variants) 2 strains of O91:H21 and 1 strain Or: HNT were EHEC *hyl* positive	Stephan *et al*. (2008)

* *vtx* (*stx*) positive by PCR ELISA; ** positive by Vero cell assay; [§] Origin not specified (assumed to be cows' milk); (NS) not specified

It is evident from the results of various studies that a diverse range of VTEC serotypes carrying several virulence associated genes can be encountered in these products. The prevalence of VTEC O157 is generally very low (Table 3.1), whereas non-O157 VTEC can be encountered more frequently (Table 3.2 and Table 3.3). Our understanding of VTEC in cheese, is however, currently restricted to a limited number of studies and only where it has been possible to obtain and further characterise isolates. Isolates from cheese samples that are PCR positive for the presence of *vtx* genes, can be difficult to obtain. Vivegnis *et al.* (1999) reported obtaining isolates from only 5 (29%) of 17 PCR (*vtx*) positive samples and Vernozy-Rozand (2005) recovered strains from 18 (13%) of 136 *vtx*-positive samples. Greater isolation success was reported by Stephan *et al.* (2008) who recovered VTEC from 16 (41%) of the 39 *vtx*-positive samples with the aid of a colony dot-blot hybridization procedure.

Survival of VTEC during cheese manufacture

Although the reported prevalence of VTEC in raw milk is low, it is known that raw milk intended for cheese making does occasionally contain pathogenic bacteria, including *E. coli* O157 (Table 3.1) and other VTEC serotypes (Table 3.2 and Table 3.3). The manufacture of cheese involves a series of different stages, including curd formation, drainage, setting, ripening and storage. The exact process will vary depending on the type of cheese and there are additional processes such as brining which are associated with the manufacture of some cheeses. Owing to the wide diversity of cheeses made throughout the world, it is therefore difficult to completely understand how pathogens such as VTEC will behave during the cheese making process and their survival during storage and maturation. Furthermore, some strains of *E. coli* O157: H7 appear to be more acid resistant, being able to withstand pH values as low as 3.0 (Jordan *et al.* 1999), and there have been outbreaks associated with low pH foods including fermented meats (Getty *et al.* 2000; MacDonald *et al.* 2004) and yogurt (Morgan *et al.* 1993). Consequently, there is a potential risk that some VTEC strains, and especially strains of *E. coli* O157: H7, could survive the low pH associated with the cheese manufacturing process.

In raw milk cheese operations there are different factors and complex interactions that occur during production. During the ripening process the indigenous microflora of the raw milk can out-compete the pathogens. These bacteria, together with the presence of starter culture and low pH, are important factors for controlling pathogens in cheese. Whilst the effects of intrinsic factors such as pH on the growth and survival of microorganisms are well known, less is understood about the interactions that can occur between the specific pathogens such as *E. coli* O157 and the starter culture organisms or natural microflora found in cheese. Commercial cheese manufacturers generally use defined starter cultures for cheese production. In contrast, producers of artisan cheeses or small-scale cheese makers may rely upon the natural microflora from the milk or starter cultures that have not been clearly defined or even properly identified. Rash and Kosikowski (1981) studied the influence of lactic acid starter bacteria on EPEC survival during the manufacture of Camembert cheese. They showed that successful elimination of the pathogen could be achieved and that using a combination of *Streptococcus cremoris* and *Lactobacillus helveticus* or *L. bulgaricus* as starter cultures, more effective than using a single species or *S. cremoris* and *S. diacetylactis* in combination. During the production of a typical

Brazilian fresh cheese (Minas cheese), the addition of a type O lactic culture to the milk has been shown to improve reduction of *E. coli* O157 (Saad *et al.* 2001).

Concern over the safety of traditional raw milk cheeses eaten in some countries has prompted research into the survival of *E. coli* O157 in these products. Soft Hispanic cheese has become popular in the USA. These typically contain no starter culture, have a pH value of 6.6, a moisture content of 60% and low brine (1.61%) and have been shown to support the growth of *E. coli* O157 if the storage temperature is ≥ 10°C (Kasrazadeh and Genigeorgis 1995). The manufacturing process of Paneer, which is a popular cheese in India, has been shown to allow the growth and survival of *E. coli* O157: H7 (Wahi *et al.* 2006) and this pathogen has also been shown to survive during the manufacture and storage of other cheeses (Tsegaye and Ashenafi 2005; Lekkas *et al.* 2006). In Camembert and Feta cheeses, *E. coli* O157 has been shown to survive the manufacturing process and persist for 65 and 75 days' storage, respectively (Ramsaran *et al.* 1998). One of the most popular cheeses consumed is Cheddar cheese. This cheese can be made with unpasteurised or pasteurised milk, although the latter is more common. In a study by Reitsma and Henning (1996), *E. coli* O157: H7 was reported to survive the Cheddar cheese manufacturing process and could be recovered after 60 days' ripening. When the cheese was made using milk containing 10^3 CFU/mL *E. coli* O157, a 2-log reduction was demonstrated after 60 days' ripening, but the pathogen could still be detected after 158 days. When the milk contained 1 CFU/mL of this pathogen, the 60 days' ripening period yielded a reduction to 1 or <1 CFU/g in the cheese, but it could not be detected at 158 days. A later study by Schlesser *et al* (2006) confirmed that the 60 day ageing period is inadequate to eliminate *E. coli* O157: H7 during ripening of Cheddar cheese. However, it is worthy to note that 2 of the strains used by Schlesser *et al.* were chosen for their tolerance of acidic conditions, being strains associated with outbreaks linked to cider and fresh apple juice.

Understanding the behaviour of pathogens during cheese manufacture is difficult to determine and it is inevitable that much of our information has been derived from artificially contaminated cheese and cheese produced in the laboratory using artificially contaminated milk. Using a laboratory-scale smear ripened cheese produced from raw milk, Maher *et al.* (2001) showed that *E. coli* O157: H7, at an initial level of 1.52 log CFU/mL in the milk, was able to grow by 1.3 log cycles during the cheese manufacture stage. A dramatic increase was observed, following the heating of the curd from 32 to 37°C and the pathogen subsequently survived 70 days during the ripening process and could be detected by enrichment after 90 days. A laboratory procedure reported by Leuschner and Boughtflower (2002) for producing soft cheese artificially contaminated with low levels of pathogens, also showed that *E. coli* O157 can survive the cheese manufacture process, including curd formation, drainage, setting and ripening. Other studies have shown that *E. coli* O157 can survive for up to 21 days in pasteurised and unpasteurised whey (Marek *et al.* 2004) and in cheese brine for several weeks under typical brining conditions (Ingham *et al.* 2000).

Methods for the detection and isolation of VTEC in cheese

In the past there has been less information available on the prevalence of non-O157 VTEC in raw milk and cheese, although with the more widespread adoption of

molecular methods and the availability of immunomagnetic beads for O26, O111, O103 and O145, this information has started to emerge. Methods for the detection and confirmation of non-O157 VTEC are still developing and improving. However, most tests for these bacteria are currently being performed by Government agencies and specialist laboratories in hospitals or those involved with public health protection and research. In contrast, there are a number of methods available for the detection and confirmation of VTEC O157, including commercial media and test kits (Baylis and Limburn 2006). Some methods have also become published standards which have been widely adopted and are used for regulatory testing of foods. Examples include the International Organisation for Standardization (ISO) method for the detection of *E. coli* O157 (Anon 2001) which has been validated for use with milk (Scotter *et al.* 2000), and the US Food and Drug Administration Bacteriological Analytical Manual (BAM) method (Feng and Weagant 2002).

One of the difficulties of comparing results from different studies is the diversity of methods that have been used and the potential impact that this could have on the recovery and isolation of VTEC from milk and cheese products. Although there are several media that have been developed and used successfully for the isolation of *E. coli* O157 in foods some of these media may not necessarily be suitable for use with dairy products. Compared with other foods, dairy products, especially cheese, can present several practical difficulties to the microbiologist trying to isolate VTEC. Interference by competitor organisms can be particularly problematic and selective agars such as SMAC plates can be difficult to read because of heavy growth by competing bacteria (Hammack *et al.* 1997; Vernozy-Rozand *et al.* 2005). Cheese can contain particularly high numbers of competing bacteria so it is important that these are suppressed or inhibited by the enrichment and plating media to enable successful growth and isolation of the target organism. The population of background microflora in cheese has been reported to be 106 CFU/g or above (Drysdale *et al.* 2004). Increasing the incubation temperature of the enrichment medium to *ca* 42°C for samples containing high numbers of competing bacteria has been shown to improve isolation of *E. coli* O157 from meats and dairy samples (Baylis *et al.* 2001; Drysdale *et al.* 2004).

Method specificity is also improved by using the immunomagnetic separation (IMS) technique. This has become an integral part of many *E. coli* O157 detection methods, including EN ISO 16654. The technique uses paramagnetic beads coated with antibodies to specifically capture the target organism, which can then be separated from other organisms and food debris in the enriched sample using a magnetic field. After gently washing the beads, the target organism can be concentrated into a smaller volume and the beads are typically spread over the surface of a suitable selective medium where cells can grow and develop into typical colonies. Although beads were originally available only for serogroup O157, others are now commercially available for the major VTEC serogroups (O157, O26, O111, O103 and O145) previously identified as being commonly associated with human diseases by the World Health Organisation Scientific Working Group (1999). However, the fat content of dairy products can adversely affect the IMS technique by preventing the capture of immunomagnetic beads by the magnetic field (Baylis *et al.* 2001). In a study by Vernozy-Rozand *et al*. (1997), detection of *E. coli* O157 in replicate raw milk cheese samples artificially contaminated at levels of 8 and 20 CFU/25g yielded fewer positives by traditional enrichment and IMS procedure compared to detection

by immunoassay (VIDAS *E. coli* O157; bioMérieux). The authors attributed the fewer positive results by IMS to be due to the fatty matrix interfering with the settling of bead particles.

The ability to grow low numbers of cells to detectable levels is a critical step in the isolation of VTEC from foods. The choice of enrichment medium and the type and concentration of selective agents must be chosen with care, especially if the method is intended to detect and isolate a wide range of VTEC. Growth of some VTEC, including strains of *E. coli* O157, can be poor in some enrichment media, including those developed for the detection of serogroup O157 (Baylis 2008). Antibiotics and other selective agents are often used to reduce interference from background microflora, although some VTEC can also be sensitive to these compounds (Hussein and Bollinger 2008). The enrichment medium stipulated in EN ISO 16654 is modified Tryptone soya broth supplemented with 20 mg/l novobiocin. For the detection of *E. coli* O157 in cheese and other dairy products using the VIDAS system, the addition of 2.25 g/L acriflavin to the enrichment medium mTSB has been used (Cohen and Kerdahi 1996; Vernozy-Rozand *et al.* 1997). Ogden *et al.* (2001) found that buffered peptone water supplemented with 8 mg/L vancomycin (BPW + V) incubated at 42°C, followed by IMS and plating beads onto SMAC and a chromogenic medium (Rainbow or CHROMagar), gave optimum recovery of *E. coli* O157 from spiked samples including cheese. Further work by this group confirmed that enrichment in BPW or BPW + V at 42°C gave the best recovery of VTEC O157, O26 and O111 from foods including cheese (Drysdale *et al.* 2004).

Alternative methods have become popular because of their potential to reduce the overall test time and their greater specificity compared to conventional culture methods. This is highlighted by two published studies where *E. coli* O157 in cheese (Vernozy-Rozand *et al.* 1997) and milk (Foschino *et al.* 2002) was not detected following enrichment and plating on CT SMAC agar. In both studies the same sample tested by an immunological method (VIDAS; bioMérieux) yielded a positive result and the bacteria in the sample was subsequently isolated and confirmed. Detecting all VTEC in a food normally involves confirming the presence of *vtx* genes or VT in an enriched sample. Immunological methods, e.g. ELISA, are now available to detect VT, and the polymerase chain reaction (PCR) technique is most commonly used to detect *vtx* gene sequences. Inhibitors or competing microflora in the food sample can, however, sometimes prevent the growth of the target organisms, which then fail to reach detectable levels after enrichment. Furthermore, components in the food can inhibit certain techniques such as the PCR. In cheese, fats and proteinases have been reported to adversely affect PCR reactions (Wernars *et al.* 1991; Rossen *et al.* 1992; H.A. Powell 1994; McKillip *et al.* 2000). Some success in removing inhibitors found in cheese has been achieved by utilising proprietary DNA extraction kits (Fratamico *et al.* 2000). Centrifugation is a step commonly incorporated into many DNA extraction protocols including commercial kits to aid removal of inhibitory compounds. Buoyancy centrifugation is another approach which has been used to separate bacteria from food and which can help to remove potential PCR inhibitors. This technique has been used to enable successful PCR detection of *E. coli* O157 in a range of foods including milk, blue cheese and Camembert cheese (Lindqvist 1997).

After enrichment, one of the most common media used for the isolation of *E. coli* O157 is CT SMAC, a medium that was originally developed for clinical specimens. In a study by Hammack *et al.* (1997), hemorrhagic coli (HC) agar incubated at 43°C was reported to recover significantly more unstressed *E. coli* O157 from Brie cheese than a variety of SMAC formulations. To estimate the number of *E. coli* O157 in samples generally involves direct plating onto a selective diagnostic medium. Whilst CT SMAC remains a common medium for the isolation of *E. coli* O157 from food enrichments, this medium is often too inhibitory and therefore unsuitable for direct plating of samples containing stressed cells (McCarthy *et al.* 1998). In cheese VTEC cells may be sub-lethally injured by the presence of lactic acid and the low pH of the product. Under these circumstances, using a chromogenic medium (O157:H7 ID medium; bioMérieux) supplemented with 0.005 mg/L cefixime and 2.5 mg/L potassium tellurite and a pour plate method was shown to give better recovery and higher counts of *E. coli* O157 in cheese during ripening compared with CT SMAC (Jordan and Maher 2006). For the isolation of non-O157 VTEC there are few truly diagnostic media available. In the case of O26 strains, Hiramatsu *et al*.(2002) demonstrated that rhamnose MacConkey agar (RMAC) with and without cefixime (50 µg/mL) and potassium tellurite (2.5 mg/L) provided good diagnostic and selective properties. For other VTEC serotypes, detection often involves primary isolation on a suitable *E. coli* -specific chromogenic medium such as TBX, followed by confirmation of *vtx* genes in a selection of typical colonies. However, some chromogenic media have been developed to assist with the isolation of non-O157 VTEC (Bettelheim 1998) or specific serogroups, e.g. O111, O26, O103 and O145 (Hara-Kudo *et al.* 2002; Possé *et al.* 2008).

CONCLUSIONS

Direct consumption of raw milk remains a high risk and there is a large amount of documented evidence highlighting the different types of pathogens that can be transmitted by this product and outbreaks that have been traced to this source. Compared with other bacterial pathogens, *E. coli* O157: H7 and other VTEC can cause severe disease and even death. The manufacture of cheese made with raw milk is commonplace in some countries and has increased in popularity in others. Studies show that despite the low incidence of *E. coli* O157 in these products, occasionally this pathogen can be isolated, although other VTEC serotypes are generally present at a higher frequency. The serotypes/serogroups isolated from these products can be quite diverse and often include VTEC that are less frequently associated with human disease or which do not carry virulence associated genes such as *eae*, which is often found in VTEC isolated from patients. Therefore, the clinical significance of these VTEC is not fully understood. These results show the complexity of the current situation, especially when it comes to risk assessment of these products. Studies with artificially contaminated cheese confirm that *E. coli* O157 is capable of surviving the cheese manufacture process and subsequent ripening and storage periods. More research is needed to fully appreciate the survival and prevalence of VTEC in cheese made with raw milk. Strict hygiene is essential during the production of these products. In the UK there have been few if any reported outbreaks associated with a milk product since 2003. Although the hazards still exist, this would suggest that the dairy industry now has better control over the manufacturing process and hygiene practices have been maintained or improved.

4. EXPERIMENTAL PLAN

The practical side of the project had two distinct areas of investigation.

The aim of the first of these was to look at the behaviour of relevant VTEC strains in milk and cheese (as opposed to other environments / foods). It was necessary to ensure that the methods used in the practical work were able to isolate VTEC strains from dairy substrates in the presence of competing microflora from the raw milk.

For this first phase, the work was broken down under the following headings and is described in Section 5 of this report:

1. Growth of VTEC O157 and O26 strains in pure culture
2. Growth and colony morphology of strains of competitor organisms and cheese micro flora on selective media after enrichment
3. Evaluation of enumeration methods and immunomagnetic separation procedure for VTEC O157 and O26 in cheese

Once the isolates for use in the experimental work had been acquired, and methodology had been developed for detecting the behaviour of them in milk and cheese, work on making cheese and carrying out challenge testing could begin.

This second phase of the experimental work can be broken down under the following headings:

3. Equipment and ingredients for making cheese
4. Method for cheese making
5. Cheesemaking results
6. VTEC persistence in cheese results

These are discussed in more detail in Section 6 of this report.

5. METHODOLOGY FOR ISOLATING AND DETECTING VTEC IN CHEESE

5.1 Introduction

A number of methods exist for the isolation and detection of VTECs in foodstuffs. However, it was envisaged at the start of this project, that some work would be needed to evaluate (and possibly develop) the best methods for detecting and enumerating *E. coli* O157:H7 and other VTECs in association with fatty foods such as milk and cheese.

Some work from the literature was consulted to help to clarify which serotypes of VTEC should be covered in the project. There is evidence of both O157:H7 and O26 being associated with cheeses.

Methodologies have been developed by Herman, Heyndrickx and De Zutter in Belgium (FEDRA project CP/58 – 2003-2005) for use in isolating VTEC (including O26, O111, O103, O145 as well as O157) from a range of foods. In addition ISO Working Group (WG6) is developing a standard method for VTEC in foods and feeds. Standard protocols as well as those from specialist papers and the method proposed by WG6 was consulted to enable the most appropriate method to be identified.

This work has been broken down into 3 main areas of study:

1. Growth of VTEC O157 and O26 strains in pure culture
2. Growth and colony morphology of strains of competitor organisms and cheese micro flora on selective media after enrichment
3. Evaluation of enumeration methods and immunomagnetic separation procedure for VTEC O157 and O26 in cheese.

Study 1 Growth of VTEC O157 and O26 strains in pure culture

1.1 Objectives:

- Determine the best enrichment medium or media for detection of VTEC O157 and O26 strains
- Confirm enrichment temperature (37°C or 41.5°C)
- Establish any differences in growth between the strains (especially the cheese outbreak strains compared with other VTEC)
- Select most appropriate isolation medium/media (detection/enumeration)

1.2 Approach

The growth of each strain of *E. coli* O157 and O26 was established using pure cultures and the Bioscreen C growth analyser (ThermoFisher Scientific, UK).

1.3 Media

Six broths were studied:

- Tryptone Soya Broth (TSB, Oxoid CM129); non-selective control
- modified Tryptone Soya Broth (mTSB, Lab M Lab165)
- mTSB supplemented with 20 mg/l Novobiocin (Lab M X150) (mTSB+N)
- mTSB supplemented with 12 mg/l Acriflavine (Sigma A8126) (mTSB+A)
- Buffered Peptone Water (BPW, Oxoid CM509)
- BPW supplemented with 8 mg/l Vancomycin (Sigma V2002) (BPW+V)

1.4 Strains

Details of the 31 *E. coli* strains used in this project are given in Table 5.1. Nineteen were O157 strains and 12 were O26 strains.

All cultures were maintained on storage beads (Protect, Technical Service Consultants Ltd.) at -75°C in the Campden BRI Culture Collection. A duplicate set of cultures was maintained on storage beads at -20°C in the Containment Level 3 (CL3) laboratory. Prior to use, strains were subcultured onto Nutrient Agar (NA, Oxoid CM3) incubated overnight at 37°C.

Table 5.1 Strains of *Escherichia coli* O157 and O26 used in this project

No	Campden code	Serotype	Source	Origin	*E. coli* pathotype	Toxin (VT) type
1	9890	O157:H-	Clinical	VIDRL	VTEC	VT2
2	12628	O157:H-	Steak	HPA	VTEC	VT1, VT2
3	13558	O157:H-	Clinical	RKI	VTEC	VT1, VT2c
4	13551	O157:H7	Environmental (waste water)	RKI	VTEC	VT2, VT2c
5	13555	O157:H7	Clinical	RKI	VTEC	VT1, VT2
6	13559	O157:H7	Clinical	RKI	VTEC	VT2c
7	14260	O157:H7	Cheese	Grampian General Hospital	VTEC	VT2
8	14261	O157:H7	Bovine (sporadic)	Grampian General Hospital	VTEC	VT2
9	14262	O157:H7	Bovine (sporadic)	Grampian General Hospital	VTEC	VT2
10	4495	O157:H7	Animal	PHL Sheffield	VTEC	VT1, VT2
11	9892	O157:H7	Clinical	VIDRL	VTEC	VT1
12	12506	O157:H-	Clinical	PHL Preston	VTEC	VT2c
13	12507	O157:H7	Clinical	PHL Preston	VTEC	VT1, VT2
14	16413	O157:H7	Unpasteurised cheese (UK outbreak 1997)	HPA	VTEC	VT1, VT2
15	16414	O157:H7	Cheese	HPA	VTEC	VT2
16	12626	O157:H7	Raw milk	HPA	VTEC	VT2
17	16039	O157:H7	Radish sprouts	Birmingham University	ND	VT negative
18	16040	O157:H7	Ground Beef	Birmingham University	ND	VT negative
19	16244	O157:H7	NCTC 12900	NCTC	ND	VT negative
20	12848	O26 :H11	Clinical	VIDRL	VTEC	VT1
21	9896	O26:H-	Clinical	VIDRL	VTEC	VT1
22	12842	O26:H-	Clinical	VIDRL	VTEC	VT1
23	9889	O26:H11	Clinical	VIDRL	VTEC	VT1
24	3488	O26:H11	Clinical	HPA	VTEC	VT1
25	3490	O26:H11	Clinical	HPA	VTEC	VT1
26	12512	O26:H11	Clinical	PHL Preston	VTEC	VT1
27	12514	O26:H11	Clinical	PHL Preston	VTEC	VT1
28	16484	O26:H11	Raw milk Camembert cheese (French outbreak 2005)	ENVL UMAP	EPEC	VT negative
29	16485	O26:H11	Raw milk Cantel cheese	ENVL UMAP	VTEC	VT1
30	16486	O26:H11	Sheep milk	ENVL UMAP	VTEC	VT1
31	16487	O26:H11	Fresh raw milk cheese before refinement	ENVL UMAP	VTEC	VT1

Key:
VIDRL, Victoria Infectious Disease Reference Laboratory, Melbourne, Australia.
HPA, Health Protection Agency Centre for Infections, Colindale, London, UK.
RKI, Robert Koch Institute, Berlin, Germany.
Grampian General Hospital, *E. coli* Reference Laboratory, Aberdeen, Scotland.
PHL Sheffield, Regional Public Health Laboratory, Sheffield, UK.
PHL Preston, Regional Public Health Laboratory, Preston, UK.
ENVL UMAP, Ecole Nationale Vétérinaire de Lyon, Unité de Microbiologie Alimentaire et Prévisionnelle, France.
NCTC, National Collection of Type Cultures, Colindale, London, UK.
VTEC, Verocytotoxin-producing *E. coli*
EPEC, enteropathogenic *E. coli*
VT, Verocytotoxin
ND, Not Determined

1.5 Temperatures

Two incubation temperatures were studied 37°C and 41.5°C.

1.6 Procedure

Each strain was cultured in Nutrient Broth (NB, Oxoid CM1) incubated overnight at 37°C. Cultures were then serially diluted in Maximum Recovery Diluent (MRD, Lab M LAB103). To confirm inoculum levels, 0.1 ml aliquots of appropriate dilutions were spread in duplicate onto pre-poured plates of Nutrient Agar (NA, Oxoid CM3). Plates were incubated at 37°C for 24 h.

An aliquot (50µl of 10^{-4} dilution) of each culture was added to each 10ml of test medium separately and after gentle mixing 400µl of medium was removed and dispensed into the well of a Bioscreen honeycomb plate. Three replicates per strain and medium combination was set up for each temperature. Duplicate Bioscreen plates were set up with one plate incubated at 37°C in one Bioscreen instrument and the other plate incubated at 41.5°C in a second Bioscreen instrument. Both Bioscreen instruments were under the control of the Biolink software. Plates were shaken (with low intensity) for 5 s prior to every reading. The optical density (O.D.) of each well was measured using the wide band filter (420-580 nm) at 12 minute intervals over a 26 h period.

A total of 31 strains were tested in 6 media over 2 temperatures [in triplicate], i.e. over 1000 curves were produced. The following attributes were assessed:

- Growth / no growth;
- Time to detection (lag);
- Mean generation time; and
- Specific growth rate.

1.7 Expected results

The purpose of this study was to establish the best potential medium/media for use in subsequent studies. If appropriate more than one medium will be taken forward to later studies which will involve enrichment of VTEC O157 and O26 in cheese samples.

The study will help confirm the choice of strains for the project (2 strains of VTEC O157 and 1 strain of VTEC O26) and how the growth and behaviour of the cheese outbreak strains compares with other VTEC strains from these serogroups.

1.8 Actual results

Cultures of O157 VTEC associated with dairy foodstuffs were received from HPA and elsewhere. A panel of 31 VTEC strains (19 O157 and 12 O26 – see Table 1) and other *E. coli* were selected for growth studies. A panel of O26 strains isolated from cheese or raw milk in France were supplied by the National E. coli Reference Laboratory, Lyon for use in this study.

A typical trace from the Bioscreen from this work is given in Figure 5.1.

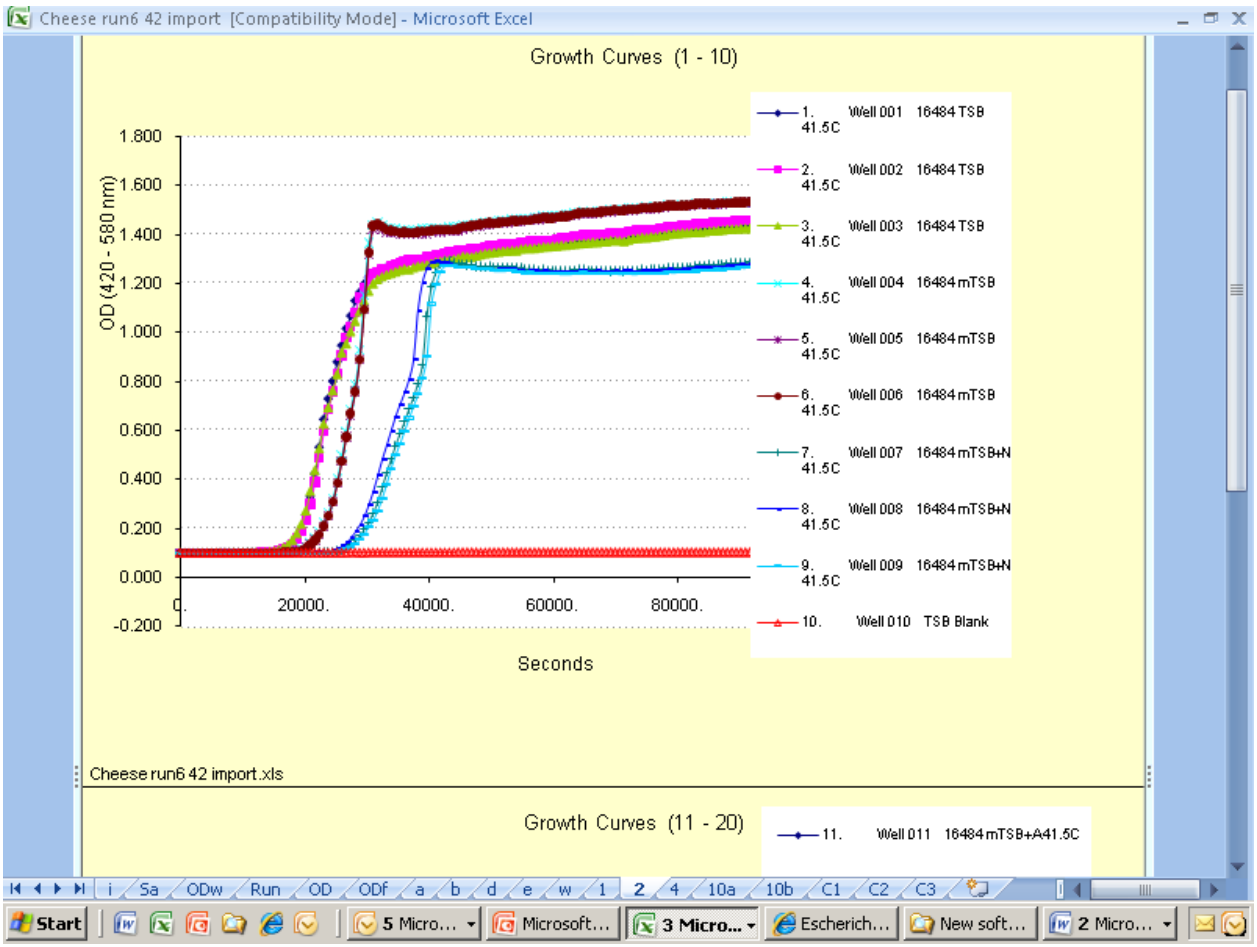


Figure 5.1 Bioscreen trace showing growth of VTEC O26 strain 16484 in different broths.

Initial studies compared growth of the VTEC strains in pure culture in different enrichment media at 37°C and 41.5°C. Some of the outputs of this work are summarised in Table 5.2.

Table 5.2 Minimum generation times (hours) for VTEC strains in various media at 37°C and 41.5°C

Strain	Origin	TSB	mTSB	mTSB+N	mTSB+A	BPW	BPW+V
16484 O26:H11 i) 37°C	French outbreak	36.8	36.0	NG	NG	53.5	49.4
ii) 41.5°C		31.9	37.6	40.3	NG	44.6	46.5
16485 O26:H11 i) 37°C	Cantel cheese	35.3	42.7	58.8	NG	51.4	50.8
ii) 41.5°C		33.6	34.6	57.6	NG	45.3	45.5
16486 O26:H11 i) 37°C	Sheep milk	33.4	34.6	NG	NG	48.4	48.1
ii) 41.5°C		35.2	31.1	NG	NG	50.3	44.8
16487 O26:H11 i) 37°C	Raw milk cheese	38.0	39.6	71.4	NG	53.5	53.9
ii) 41.5°C		39.4	36.4	61.6	NG	46.8	54.5
16488 O26:H11 i) 37°C	Clinical	31.5	20.1	NG	NG	53.9	53.1
ii) 41.5°C		36.9	25.6	50.7	NG	50.8	50.3
16040 O157:H7 i) 37°C	EDL933	28.7	42.6	57.3	NG	44.8	46.9
ii) 41.5°C		41.2	38.4	48.2	NG	45.4	43.9
14260 O157:H7 i) 37°C	Cheese	25.8	42.5	71.1	NG	53.2	49.9
ii) 41.5°C		46.4	39.5	52.0	NG	50.7	53.1

NB, NG = no growth

The VTEC strains were analysed for growth or no growth at the set temperatures and media. Results were analysed by drafting a 'dot plot of $\log_{10}$ (minimum Generation Time (mins.)) by medium' (see Figure 5.2).

One strain (012512) of O26:H11 failed to grow in mTSB and BPW+V. Also, mTSB+N and mTSB+A performed poorly across all factors.

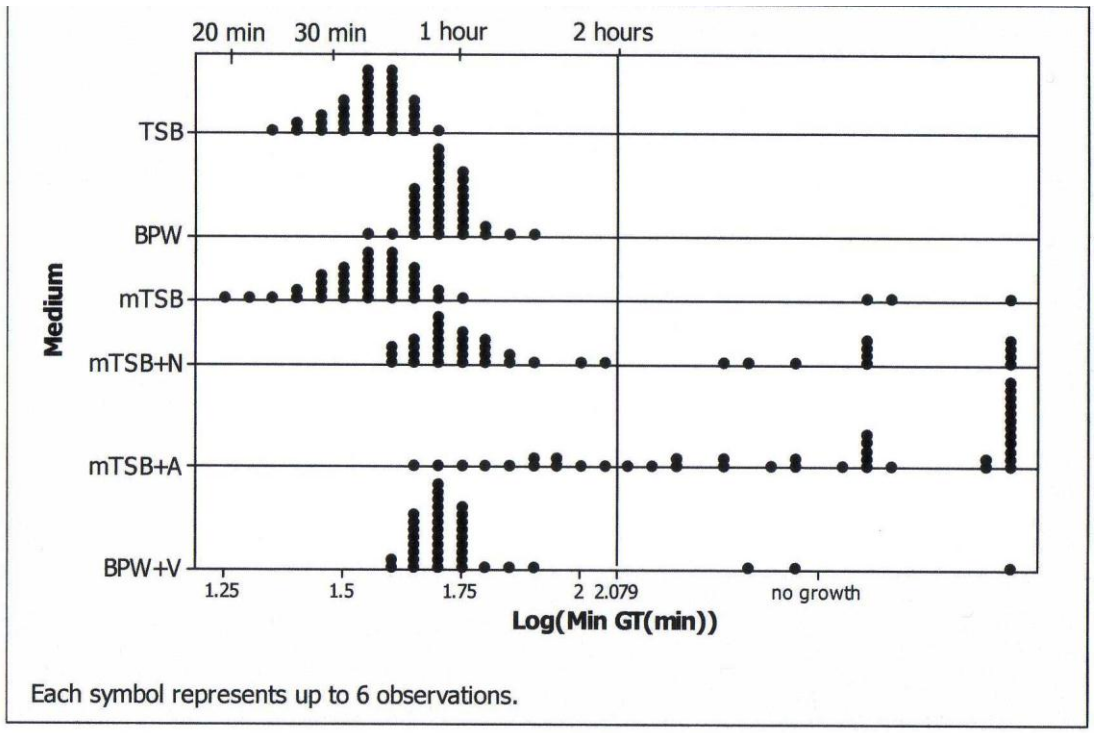


Figure 5.2 Dot plot of $\log_{10}$ (minimum Generation Time (mins.)) by medium

The impact of growth media and growth temperatures were also analysed statistically. A summary of findings is shown in Figure 5.3.

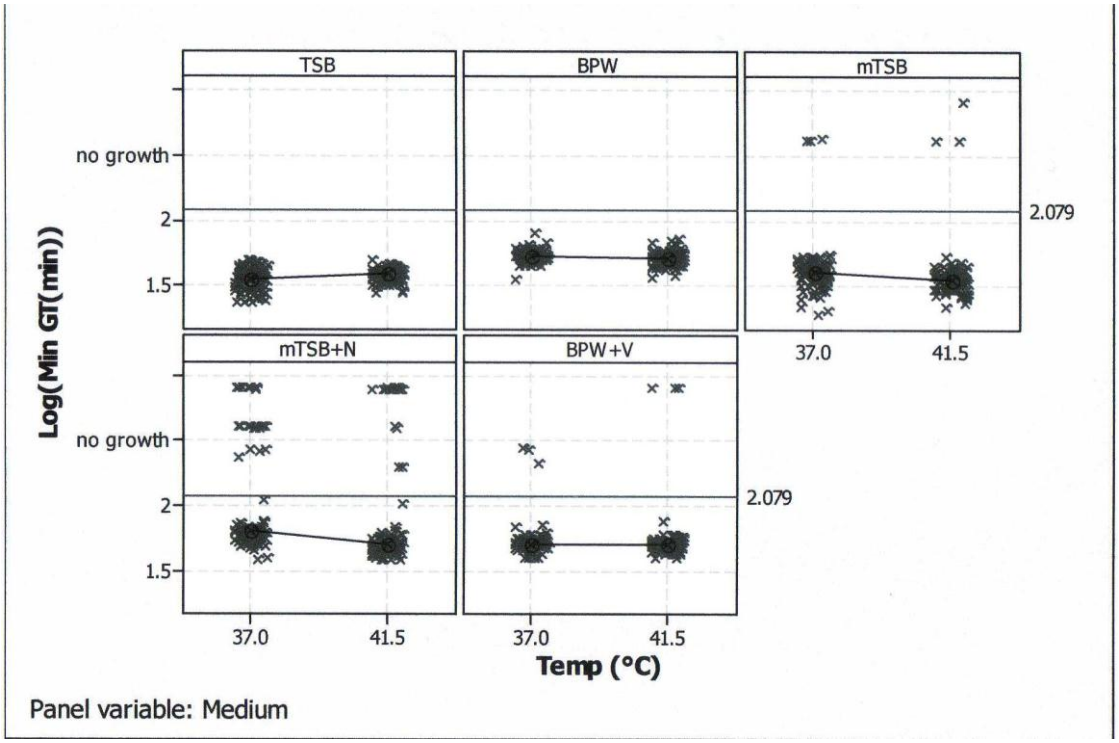


Figure 5.3 Incubation medians of log_{10}(minimum Generation Time (mins.)) by medium

As a result of this work, it was noted that:

- Differences between different temperatures for a given medium were small;
- A substantial proportion of results for mTSB+N showed no growth
- The ANOVA analysis indicated highly significant evidence of effects of medium ($p<0.001$), and of the Medium: Temperature interaction ($p<0.001$), but only weak evidence ($p=0.081$) of an effect of Temperature.

More growth study results are given in Table 5.3.

Table 5.3(a) Growth (+) / no growth (-) for VTEC strains using the Bioscreen technique.

Strain	Serotype	TSB	mTSB	mTSB+n	mTSB+A	BPW	BPW + V
		Growth Curves at 37°C/41.5°C					
16413	O157:H7*	+/+	+/+	+/+	-/-	+/+	+/+
16414	O157:H7*	+/+	+/+	+/+	(weak)	+/+	+/+
12626	O157:H7$	+/+	+/+	+/+	+/+	+/+	+/+
12848	O26:H11	+/+	+/+	-/weak	-/-	+/+	+/+
9896	O26:H-	+/+	+/+	(weak)	-/-	+/+	+/+
12628	O157:H-	+/+	+/+	+/+	-/-	+/+	+/+
13558	O157:H-	+/+	+/+	+/+	(weak)	+/+	+/+
13551	O157:H7	+/+	+/+	+/+	+/-	+/+	+/+
12842	O26:H-	+/+	+/+	-/-	-/-	+/+	+/+
9889	O26:H11	+/+	+/+	-/-	-/-	+/+	+/+

NB, *HPA Cheese outbreak strains
$ Raw milk strain

Table 5.3(b) Growth (+) / no growth (-) for VTEC strains using the Bioscreen technique.

Strain	Serotype	TSB	mTSB	mTSB+n	mTSB+A	BPW	BPW + V
		Growth Curves at 37°C/41.5°C					
13555	O157:H7	+/+	+/+	+/+	+/+	+/+	+/+
13559	O157:H7	+/+	+/+	+/+	-/-	+/+	+/+
14260	O157:H7	+/+	+/+	+/+	+/-	+/+	+/+
3488	O26:H11	+/+	+/+	-/weak	-/-	+/+	+/+
3490	O26:H11	+/+	+/+	-/weak	-/-	+/+	+/+
14261	O157:H7	+/+	+/+	+/+	+/-	+/+	+/+
14262	O157:H7	+/+	+/+	+/+	+/-	+/+	+/+
4495	O157:H7	+/+	+/+	+/+	weak/-	+/+	+/+
12512	O26:H11	+/-	+/+	-/-	-/-	+/+	-/-
12514	O26:H11	+/+	+/+	-/-	-/-	+/+	+/+

The conclusion from this work was that 41.5°C is the most suitable temperature to use and that modified Tryptone Soy Broth (mTSB) the most suitable medium. This is because the combination of mTSB incubated at 41.5°C supported the growth of more strains of VTEC.

Some VTEC strains were found to be sensitive to selective agents used in media (acriflavin and novobiocin). Some strains were less/more sensitive at different temperatures.

Study 2 Growth and colony morphology of strains of competitor organisms and cheese micro flora on selective media after enrichment.

2.1 Objectives

- To confirm which combinations of enrichment broth and plating media can support the growth of competitor bacteria.
- Assess inhibition of competitors and starter culture microorganisms.
- To assess the impact of natural microflora from cheese and raw milk on the enrichment and isolation procedure

2.2 Approaches

This study was used to identify potential interference problems caused by natural micro flora present in the cheese and raw milk and competitor organisms commonly found in cheese.

2.3 Samples

a) Cheeses:

- Caerphilly
- Camembert

Cheese from more than one source/manufacturer was used (e.g. supermarkets as well as delicatessen and farm shop cheeses). Full details of the cheese (including composition if known) were recorded.

2.4 Enrichment media

The following media were used:

- modified Tryptone Soy Broth (mTSB)
- Buffered Peptone Water (BPW)

2.5 Temperatures

Both 37 ±1°C and 41.5 ±1°C were used in this study.

2.6 Isolation media

- Sorbitol MacConkey Agar (SMAC)
- SMAC supplemented with Cefixime (0.05 mg/l) and Potassium Tellurite (2.5 mg/l) (CT-SMAC)
- Rhamnose MacConkey Agar (RMAC)
- RMAC supplemented with Cefixime (0.05 mg/l) and Potassium Tellurite (2.5 mg/l)
- Typtone bile X-glucuronide agar (TBX)
- TBX supplemented with 8mg/l Vancomycin (TBX+V)
- ChromID O157:H7 ID agar (bioMerieux)

2.7 Brands

In our experience, different brands of the same media have been known to give rise to slightly different results. To look at this different brands of certain media were assessed (e.g. TBX (Oxoid, Merck, Lab M, Neogen Acumedia).

The media investigated are given in Table 5.4

Table 5.4 Media used in this study

Agar	Code	Supplement
TSA Oxoid	TSA	None
SMAC Oxoid	SO	None
SMAC Lab M	SL	None
CT-SMAC Oxoid	CTO	1 vial (Oxoid SR0172E) rehydrated with 2ml SDW per 500ml agar.
CT-SMAC Lab M	CTL	1 vial (Lab M X161) rehydrated with 5ml SDW per 500ml agar.
RMAC Lab M	R	None
CT-RMAC Lab M	CTR	1 vial (Lab M X161) rehydrated with 5ml SDW. To 100ml agar, add 1ml from rehydrated vial. To 200ml agar, add 2ml from rehydrated vial.
TBX Oxoid	TO	None
TBX Merck	TM	None
TBX Acumedia	TA	None
TBX Oxoid + 8mg/L vancomycin	TVO	To 500ml agar, add 0.5ml of 8mg/ml (0.8%) vancomycin solution.
TBX Merck + 8mg/L vancomycin	TVM	To 500ml agar, add 0.5ml of 8mg/ml (0.8%) vancomycin solution.
TBX Acumedia + 8mg/L vancomycin	TVA	To 500ml agar, add 0.5ml of 8mg/ml (0.8%) vancomycin solution.
ChromID	ID	None

2.8 Procedure

Suitable portions of cheese (e.g. 25 g) were homogenised in 9 x enrichment broths (e.g. 225 ml) and incubated at 37 ±1°C and 41.5 ±1°C. After 18-24h incubation, each enriched sample was streaked onto the isolation media listed above. The plates were incubated at 37 ±1°C. Visible growth was recorded with the colony morphology evident on each plate. The identity of any interfering background flora was used to establish suitable antibiotics and concentrations that could be used.

2.9 Expected Results

This study set out to confirm which combination enrichment and isolation medium allows the growth of a range of competitor organisms. It will also confirm which strains can grow on the isolation media and the colony morphology of these strains compared with the target organisms (VTEC O157 and O26).

2.10 Actual results

Some work was done to look at the behaviour of competitor organisms isolated from Caerphilly and Camembert cheeses in association with combinations of enrichment broth and plating media.

Figure 5.4 gives a breakdown of types of growth on plates using each of the media listed for isolates from (i) Caerphilly and (ii) Camembert cheeses. In each case the isolates were directly streaked onto the agars concerned and incubated appropriately before growth was assessed.

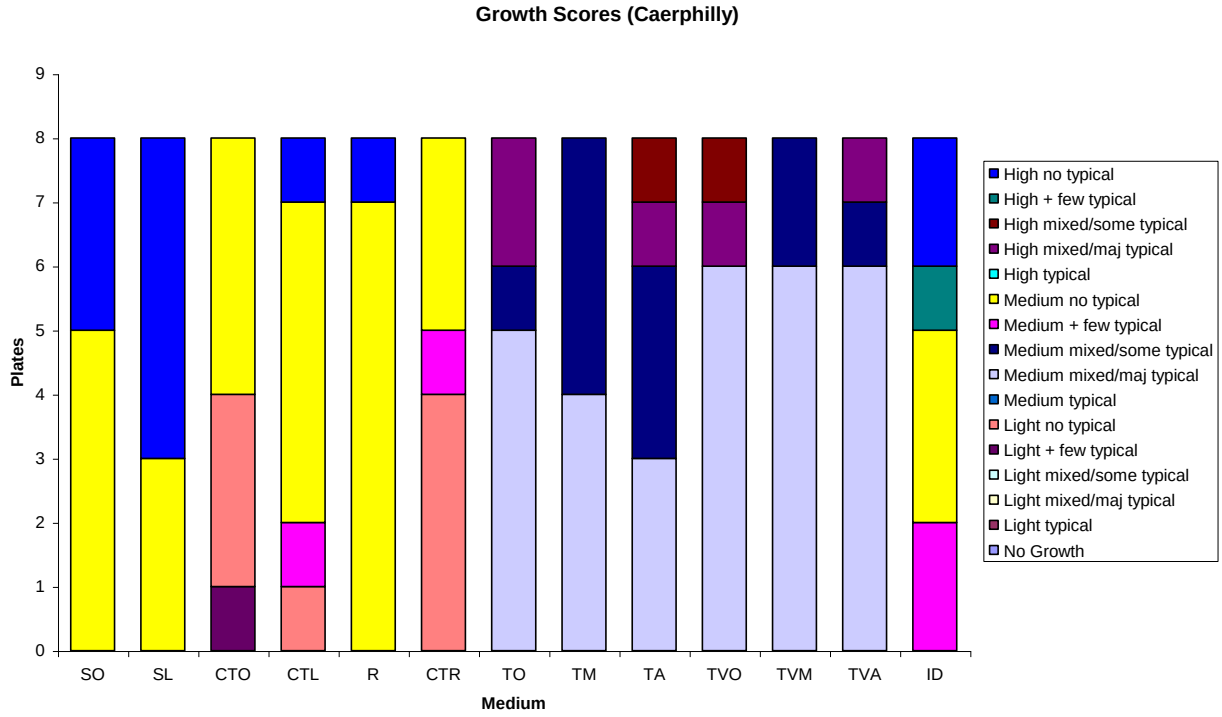


Figure 5.4(a) Types of growth on plates using each of the media listed for isolates from Caerphilly cheese.

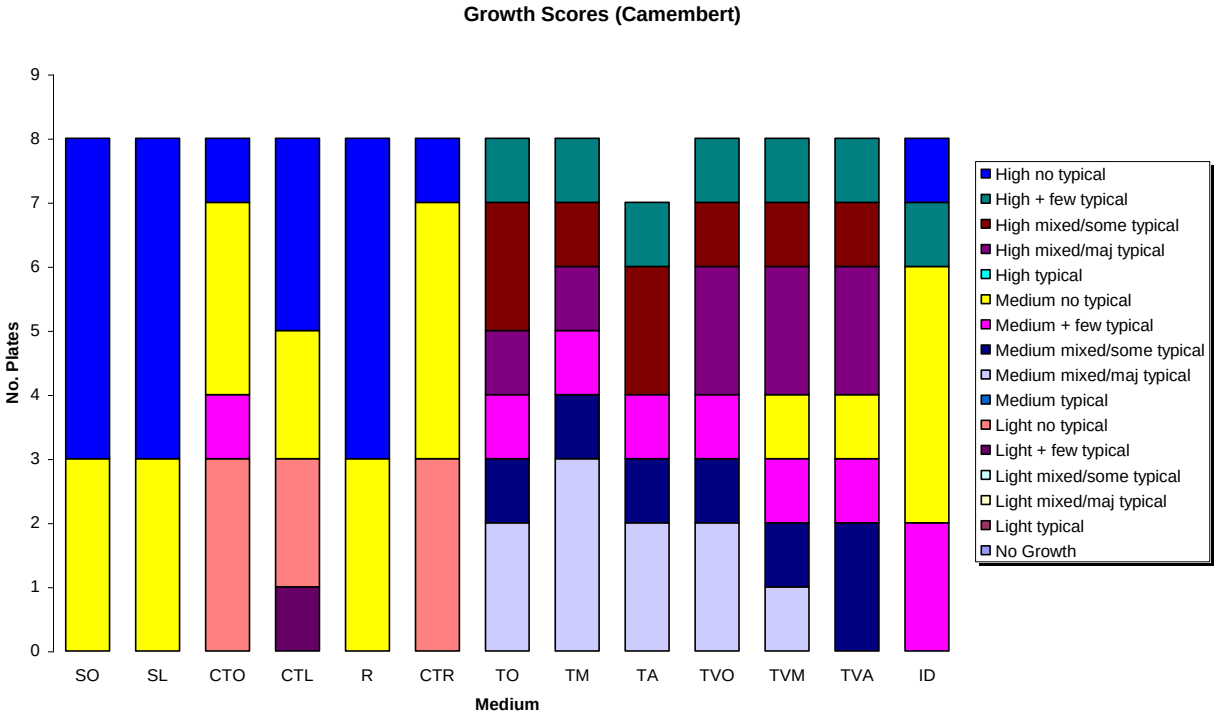


Figure 5.4(b) Types of growth on plates using each of the media listed for isolates from Camembert cheese.

Figure 5.5 gives the extent of growth seen (blank = no growth, 1 = little growth, 5 = lots of growth).

Medium	SO	SL	CTO	CTL	R	CTR	TO	TM	TA	TVO	TVM	TVA	ID
NG	0	0	0	0	0	0	0	0	0	0	0	0	0
LA													
LB													
LC													
LD				1									
LE			3	2		3							
MA													
MB							2	3	2	2	1		
MC							1	1	1	1	1	2	
MD			1				1	1	1	1	1	1	2
ME	3	3	3	2	3	4					1	1	4
HA													
HB							1	1		2	2	2	
HC							2	1	2	1	1	1	
HD							1	1	1	1	1	1	1
HE	5	5	1	3	5	1							1
	8	8	8	8	8	8	8	8	7	8	8	8	8

Figure 5.5(a) The extent of growth seen (blank = no growth, 1 = little growth, 5 = lots of growth) for cheese isolates on different media.

Caerphilly													
Medium	SO	SL	CTO	CTL	R	CTR	TO	TM	TA	TVO	TVM	TVA	ID
NG													
LA													
LB													
LC													
LD			1										
LE			3	1		4							
MA													
MB							5	4	3	6	6	6	
MC							1	4	3		2	1	
MD				1		1							2
ME	5	3	4	5	7	3							3
HA													
HB							2		1	1		1	
HC									1	1			
HD													1
HE	3	5		1	1								2
	8	8	8	8	8	8	8	8	8	8	8	8	8

Figure 5.5(b) The extent of growth (blank = no growth, 1 = little growth, 5 = lots of growth) for cheese isolates on different media.

Some studies were carried out with Caerphilly and Camembert cheese starter cultures and isolates. Growth of the cocktail of strains appears to be inhibited by a combination of growth temperature and time without the need for additional antibiotics. Bile salts in some media have additional inhibitory effect on the starter culture strains.

2.11 Conclusions

The objectives set out for Study 2 were achieved. Starter culture and raw milk competitor microorganisms were found to grow on the range of media tested to varying degrees. The media found to be most useful for detection and enumeration of the target microorganisms in this study (VTEC), namely:

- ChromID 157 agar (detection of VTEC O157 and VTEC O26)
- CTSMAC (detection of VTEC O157)
- CTRMAC (detection of VTEC O26)

and

- ChromID 157 agar (enumeration of VTEC O157 and VTEC O26)
- CTRMAC (enumeration of VTEC O26)

did not appear to be any less selective for these competing microorganisms than the other media tested. Thus it was concluded that it would be possible to detect the target VTEC bacteria using these media without undue hindrance from other bacteria that are likely to be present in cheeses made from raw milk.

Study 3 Evaluation of enumeration methods and immunomagnetic separation procedure for VTEC O157 and O26 in cheese.

3.1 Objectives

- Determine the specificity of the Immunomagnetic Separation (IMS) procedure used in conjunction with the chosen combination of enrichment and isolation media.
- Establish if there are any interference problems with fat (cheese/milk).

3.2 Approaches

A critical part of this project is the recovery and quantification of VTEC O157 and O26 throughout different stages in the cheese manufacturing process. Accurate results are therefore dependent on the enumeration method. Two, possibly 3 approaches can be used. **Direct plating** can be used when expected levels are >10 cfu/g and **most probable number** (MPN) is suitable for when numbers have declined to below 50 cfu/g (or it can be used for higher levels if required). **Quantitative real Time PCR** is a third option, but this is more complex and would require additional time and resources to develop unless a published method is available.

In this part of the work, a direct comparison of MPN and direct plating will be performed to establish any differences. The best plating medium for these techniques will also be determined.

The Immunomagnetic separation (IMS) technique provides great specificity and sensitivity of detection and isolation of the target organism. Paramagnetic beads (Dynabeads) for O157 and O26 antigen will be used in this study.

This study will evaluate the recovery from cheese samples which can interfere with the IMS technique because of interference from fat.

3.3 Enumeration of *E. coli* O157 and other VTEC Summary of methods

Different approaches exist for the enumeration of *E. coli* O157 from foods although quantification remains less common than detection for this pathogen.

The main approaches available are direct plating and most probable number (MPN). The main issue with enumeration methods is method specificity and the limit of detection/sensitivity of the method. Plating methods normally involve testing the initial suspension which is usually a 1:10 dilution of a solid sample and subsequent dilutions. A 1ml aliquot is normally dispensed into a 90mm diameter Petri dish. Therefore, the sensitivity of plating methods using a 1ml inoclulum of a 1:10 homogenate is ≥10 CFU/g. By comparison MPN techniques have a low limit of 0.3 MPN/g but the range can be restricted unless more dilutions/tubes are used and results tend to show greater variability. To improve the limit of detection of direct plating methods the alternative is to dispense 10ml of the 1:10 homogenate into a large (140mm diameter) Petri dish. This gives a sensitivity of ≥1 CFU/g.

3.4 Approaches to be considered

During the cheese making/maturation process high (ca 10^6 cfu/g) levels of VTEC O157/O26 in the initial cheese would be expected to decrease over a period of time. This decline is not yet known but the number to be recovered/enumerated will also dictate the method being used:

Numbers of target organism (CFU/g)	**Suitable method**	**Comments**
10^6 down to 10^2	0.1ml spread plates 1ml pour plates	
10^2 down to 10	1ml pour plate 10ml pour plate	
1-10	10ml pour plate	Include Detection

3.5 Published methods

Table 5.5 Summary details of published methods for detecting VTEC in cheese

Matrix	Sample prep	Plating method	Incubation	Reference
cheese	10g sample diluted 1:10 in 2% trisodium citrate, homogenised 4min	**Pour plate:** 10ml homogenate in 140mm Petri dish + 40-50ml medium (CT SMAC and CT-O157:H7 ID agar) 0.05 (cef) and 2.5 (K_2TeO_3) mg/L	41°C 16 to 20h	Jordan and Maher 2006
Cheese (survival)	10g sample diluted 1:10 in 2% trisodium citrate,	**Pour plate:** 10ml homogenate in 140mm Petri dish + 40-50ml medium (CT SMAC. For cheese non selective resuscitation step: **pour plate** BHIA 3h at 37°C followed by overlay with CT-SMAC	41°C 16h	Maher *et al*. 2001
Cheese (survival	Poorly described	**Spread plate** 2ml of 1:10 dilution across 6 plates (LOD 5 CFU/g) For E. coli O157: TBX spread plate	37°C 24h	Leuschner and Boughtflower 2002
Cheese (survival)	10g sample + pre-warmed 0.1% peptone water homogenised 1min	Deep-plated **(pour plate?)** in SMAC+MUG volume not specified	37°C 24h	Spano *et al*. 2003
Cheese whey (survival)	1ml whey into 10ml TSB	**Spread plate** on SMAC+MUG volume not specified	37°C 24h	Marek *et al*. 2004
Cheese brine survival	Poorly described	Duplicate **spread plates** SMAC	35°C 24h	Ingham *et al*. 2000
Dairy products (survival)	Serial doilution in 0.1% peptone water	**spread plates** SMAC	37°C 24h	Tsegaye and Ashenafi 2005
Cheese	25g sample + 225ml 0.1% BPW homogenised 60s	Serial dilution in BPW, duplicate **spread plates** on SMAC and Fluorocult O157:H7 agar	37°C 48h	Leekas *et al*. 2006

Matrix	Sample prep	Plating method	Incubation	Reference
Cheese (survival)	10g sample + quarter strength ringers solution.	**Spread plate** SMAC + BCIG	37°C 24h	Ercolini *et al*. 2005
Cheese survival	Sample (25g) + 225ml 0.1% peptone water homogenised (time?) + serial dilution	1ml Petrifilm EC count plate + HEC kit (no longer available)		
Cheese survival	10g + 90ml tryptone salt solution homogenised 3 min + serial dilution	**Spread plates** on CT-SMAC and O157:H7 ID agar	37°C 24h	Vernozy-Rozand *et al*. 2005
Milk	10g + 90ml 0.1% peptone solution	**Spread plates** on SMAC	37°C 48h	Issa and Ryser 2000
Foods inc cheese	10g sample diluted 1:10 in 2% trisodium citrate,	**Spread plates** on O157:H7 ID agar	37°C 18h	Auty *et al*. 2005
Cheese survival	10g + 90ml MRD homogenised for 1min. Diluted in MRD	1ml **pour plate** CT SMAC. Injury protocol: layer 1: 20ml SMAC layer 2: 7ml BHIA+ 0.6% YE then 1ml sample followed by 7ml BHIA+YE? (no difference found between media)	37°C 24h	De Lamo-Castellvi *et al*. 2006
Cheese survival	Cheese + 20ml 0.1% peptone water homogenised 2min	0.1ml **spread plates** SMAC	37°C 24-48h	Kasrazadeh *et al*. 1995
Cheese survival	25g cheese 1:10 with pre-warmed (42°C) tryptose broth + 2% trisodium citrate, homogenised 3 min	0.1ml **spread plates** on SMAC	37°C 24-48h	Govaris *et al*. 2002
Chocolate	25g + BPW+SM+BG	0.1ml and 0.5ml **spread plates** O157:H7 ID agar	37°C 18-24h	Baylis *et al*. 2004

3.6 Method variables

There were a number of variables that needed to be worked through so that decisions could be made on which of the available options could be used in the practical investigation. These were:

- Which enrichment medium to use (mTSB or BPW);
- Stomacher mixing times (1 or 2min);
- Pour or spread plates (10ml must be poured 1ml could be done as 2 x 0.5 ml spread or 1ml pour)

NB Spread plates are more convenient and remove the danger of additional stress induced by thermal injury (warm molten media).

3.6.1 Agar Media:

- CT SMAC
    - SOR negative O157:H7 are colourless on this medium,
    - other *E. coli*/Enterobacteriaceae (SOR positive) are red.
- RMAC or CT RMAC
    - Rhamnose (RHA) negative VTEC O26 appear colourless on this medium,
    - others (RHA positive) are red.
- ChromID O157:H7 agar
    - VTEC O157 appear green on this medium,
    - other *E. coli* (GUD-positive) are blue.

3.6.2 Enumeration

Ideally more than one medium should be used. For the purposes of our work, 2 were used:

- CT SMAC (O157) and CT RMAC (O26)
- CT RMAC (O26) and ChromID O157 (O157 + O26)*

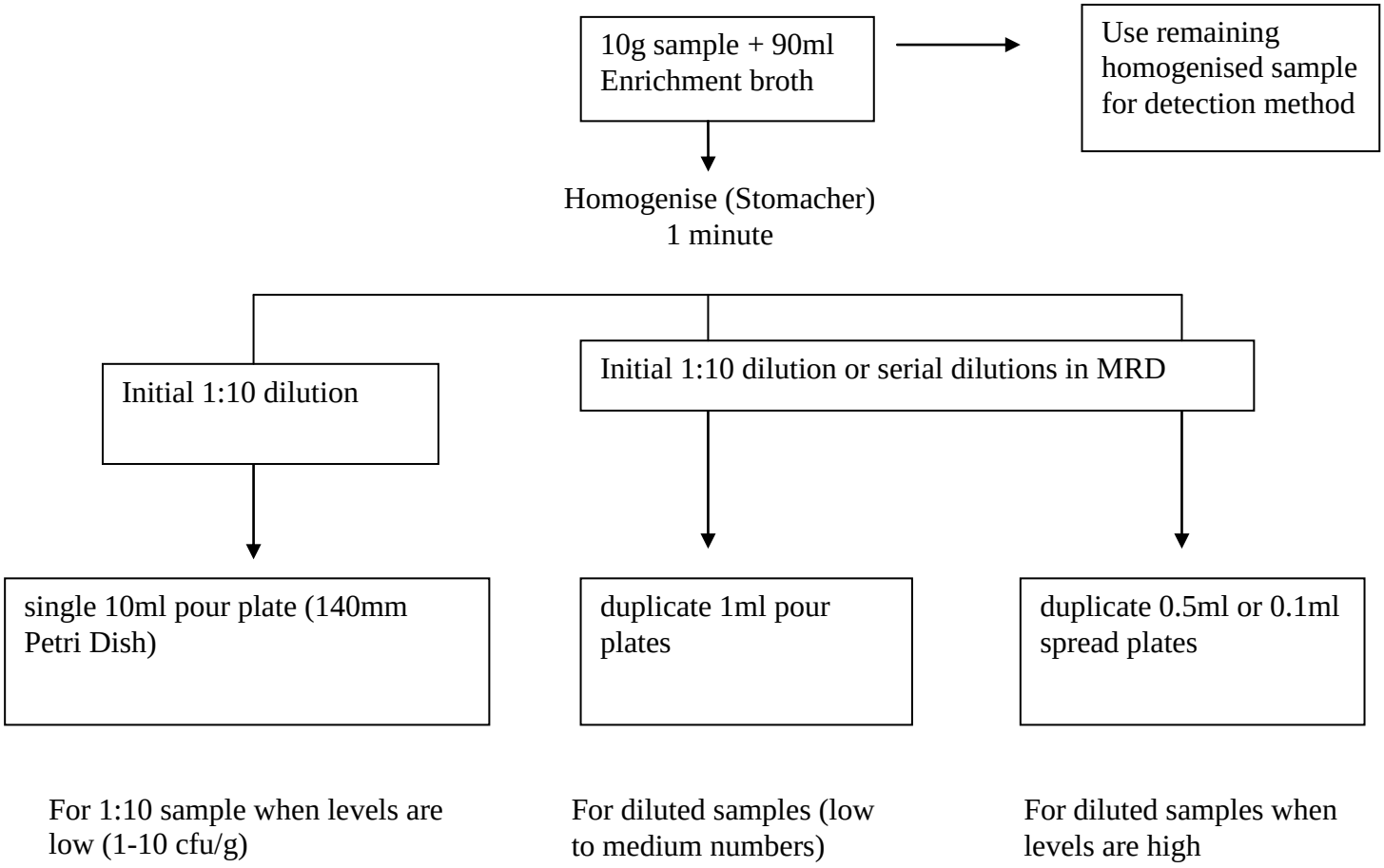
*The ability to enumerate VTEC O26 accurately with ChromID agar will depend on the presence of naturally occurring *E. coli* as these will be phenotypically the same as the VTEC O26 in terms of colony morphology (i.e. blue GUD positive colonies).

If ChromID O157 agar provides a VTEC O157 count there is no need for CT SMAC as well. What is uncertain is the ability for ChromID to provide the VTEC O26 count without interference from other *E. coli*.

We need to establish if the French cheese strains are sensitive to cefixime 0.05mg/l and K_2TeO_3 2.5 mg/L in RMAC and if it is needed in ChromID agar. If the selected VTEC O26 strain (the one we intend to use in the cheese) is sensitive to CT we need to see how much interference we get from cheese without CT present.

A Caerphilly cheese produced with VTEC O157 and O26 at Campden BRI was used to compare the media and morphologies for the O26 and O157 strains (added as a cocktail).

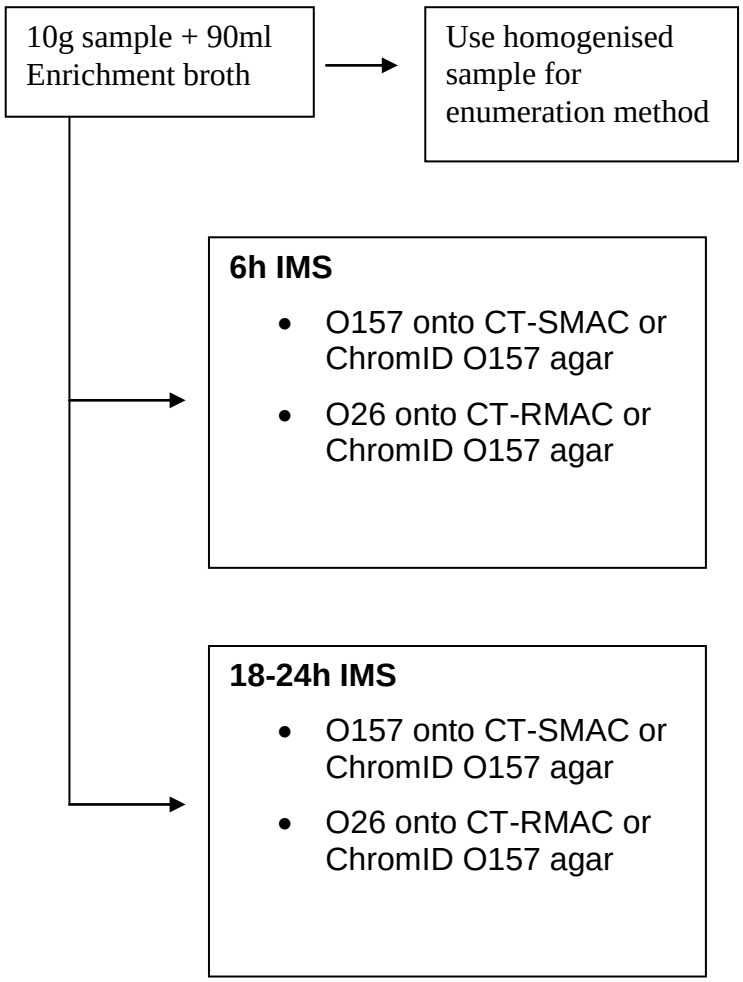
Summary of enumeration method



3.6.3 Detection
1st medium of choice: CT-SMAC is for VTEC O157.
2nd medium of choice: ChromID O157 (for VTEC O157 and O26).

Incubation of plates will be at 37°C for 24h

Summary of detection method



3.7 Cheeses

Only Caerphilly cheese was used in this work as there was insufficient resource to look at Camembert cheese.

3.7.1 Procedure

Work carried out on cheese is illustrated in Figure 5.6. The cheese used in this work was Caerphilly cheese made at Campden BRI so that its constituents and properties were known.

Caerphilly cheese made at Campden BRI which had been inoculated with *E. coli* O157 was also tested with Immuno-Magnetic Separation (IMS) detection and enumeration procedures. In this work, 10g cheese samples were mixed with 90 ml broths. The broths used were:

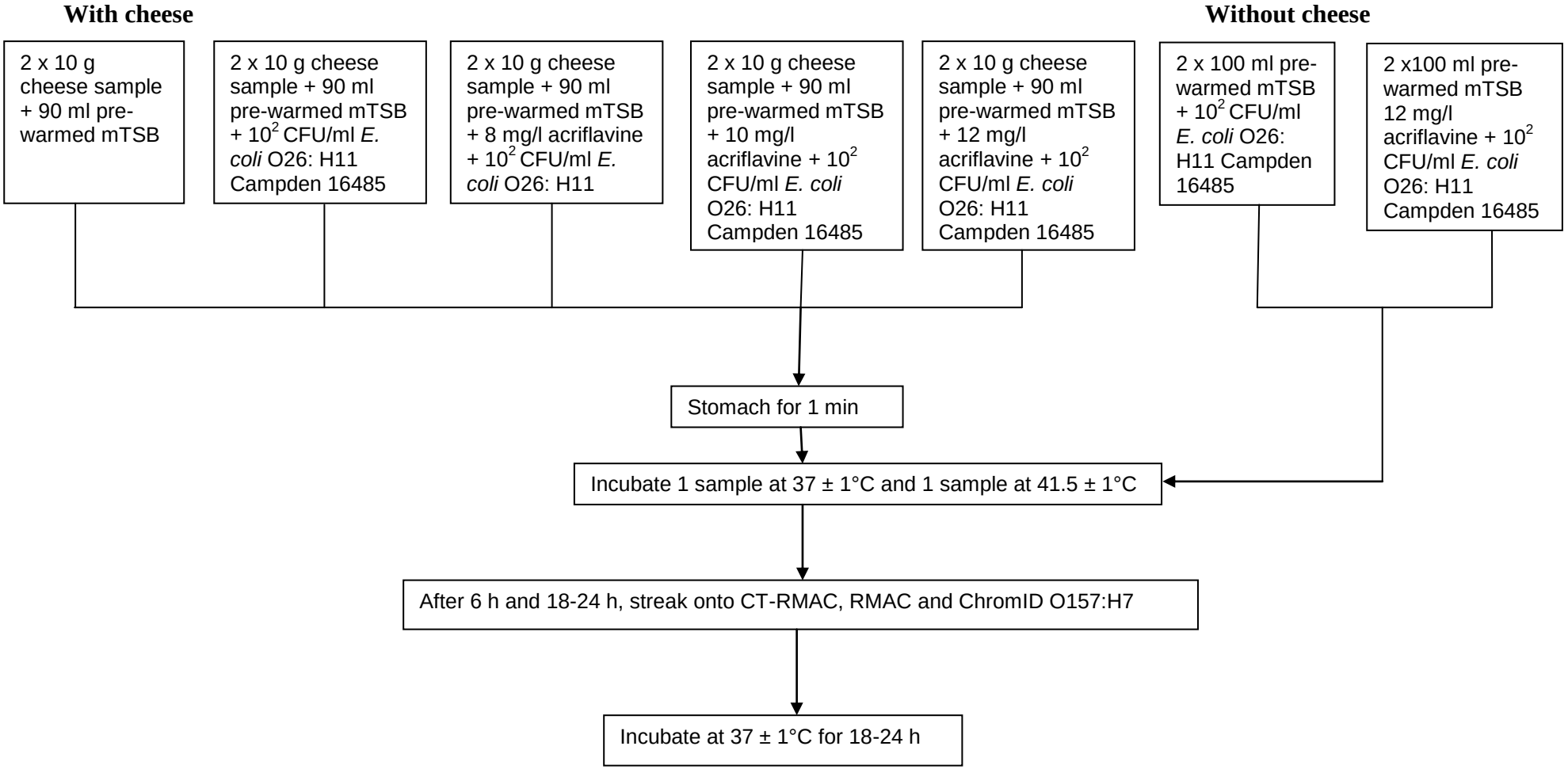
- pre-warmed modified Tryptone Soya Broth (mTSB); and
- pre-warmed mTSB with 12 mg/l arcriflvine

These were incubated at 37 and 41.5°C, and IMS with O157 beads was carried out after 6h and 18-24h incubation. The broths were then streaked onto CT-SMAC and ChromID agars.

Prior to incubation, the broth of one mTSB sample was plated out:
plate 1 x 10ml into 140 mm Petri dish pour plate and 2 x 1ml pour plate from initial 1 in 10 dilution plus 2 x 0.1/0.5 spread plate for all dilutions.

ChromID agar was used for plating, which was incubated at 37 ± 1°C for 18-24h. (RMAC agar was used for the same procedure to look for VTEC O26.

Figure 5.6 Representation of work carried out using cheese to determine the best methods for detection and enumeration of VTEC O157 and O26.



3.7.2 Expected results

This study was designed to show how direct plating for the quantification of VTEC O157 and VTEC O26 compare.

The intention of the study was also to confirm the ability of the IMS technique in combination with the chosen enrichment and isolation medium to recover VTEC O157 and VTEC O26 strains from cheese.

3.7.3 Actual results

A comparison of detection methods (enrichment and plating media) was carried out. This included use of the Immuno-Magnetic Separation (IMS) technique after 6h and 24h.

A variety of isolation media for the enumeration of VTEC O157 and VTEC O26 from cheese was tested. In fact a direct plating enumeration technique, rather than a MPN method was used in preference in this study. (A review of the literature showed that the direct plating technique was a common approach.

For high populations 0.1ml spread plates were used and when the population was expected to have fallen to lower numbers (< 50 cfu/g) 1ml pour plates using 140mm petri dishes were used.

The following detection and enumeration methods were identified as being most suitable to detect VTEC in cheeses.

(i) Detection

a) Enrichment in mTSB at 41.5°C
b) Immuno-magnetic separation (IMS) at 6h and/or 24h
c) Streak onto:
    a. ChromID O157 agar (VTEC O157 and VTEC O26)
    b. CTSMAC (VTEC O157)
    c. CT-RMAC (VTEC O26)

(ii) Enumeration

a) Dilute in primary enrichment (mTSB) with sodium citrate.
b) Further dilutions if required are made in MRD.
c) Spread plates (0.1ml) are to be used when levels are high.
d) Pour plates are used when levels fall:
    - 1ml in 90mm Petri dishes and/or
    - 10ml in 140mm Petri dishes.
e) ChromID O157 agar (VTEC O157/O26).
f) CT RMAC (VTEC O26).

Results showed that some strains, but especially the VTEC O26 strains (including an outbreak strain) were inhibited in media supplemented with acriflavin, even at levels below the recommended concentrations (12 or 10 mg/l). The inhibition was greater when bile salts

were present in the medium. Subsequent studies have shown that this inhibition is negated by the presence of cheese.

6. CAERPHILLY CHEESE PRODUCTION PROTOCOL AND EXPERIMENTAL RESULTS

CONTENTS

8. Discussion of results

1. Introduction

The ultimate objective of this project was to determine if strains of VTEC were able to persist in cheese made from contaminated raw milk. In order to investigate this, a cheese production system was developed in the laboratory to replicate commercial production systems. Once the cheesemaking technique had been developed and established, strains of VTEC were added to raw milk, and this was then made into cheese. The cheese was packaged and held under conditions representative of those it would experience throughout its shelf life if it was made commercially. Samples of the cheese were taken over its life and the number of persisting VTEC bacteria enumerated.

2. Equipment and Ingredients

2.1 Cheese vat

An Armfield FT20MkII Cheese Vat (Armfield Limited, Hampshire) was purchased to make the cheese on a laboratory scale. The vat had a 10L capacity and the unit had integral: pH and temperature probes and displays; vat agitator and speed control; and water heater and temperature sensor. However, because VTEC are classified as 'containment level 3 (CL3)' bacteria, they have to be handled under more stringent conditions than most bacteria. Consequently the cheesemaking unit had to fit inside a safety cabinet, which in turn was sited inside a CL3 laboratory. Some modifications had to be carried out on the vat (Figure 6.1), so that it had the correct dimensions to fit inside the safety cabinet, but still be representative of an industrial unit.

Figure 6.1 Laboratory cheese vat inside a safety cabinet

2.2 Unpasteurised milk

The milk used for cheesemaking was obtained from a local farm. It was collected the day before cheesemaking so that initial tests could be performed. These initial tests were:

- 2.2.1 'Delvotest' (DSM, Delft, The Netherland) – to ensure that the milk was free of antibiotics.
- 2.2.2 Chemistry – acidity; fat; moisture; pH; salt.
- 2.2.3 Microbiology – Aerobic Plate Count (APC); coagulase positive *Staphylococcus aureus*; coliforms; Enterobacteriaceae; *E. coli* O157. (NB, with hindsight it would have been useful to have carried out a 'non-starter culture lactic acid bacteria count' in addition to these tests. It was not realized that this count was important at the time of testing.)

2.3 Rennet

The rennet used to coagulate the milk protein was 'Naturen Plus 175', 165 IMCU/ml, Chr. Hansen's, Denmark.

2.4 Starter Culture

The starter culture used was 'MT36' supplied by A. J. & R. G. Barbers, Somerset, England.

2.5 Other

Disposable containers for 20L liquid
Funnel for transfer into above containers
15ml Sterile Distilled Water (SDW)
10ml pipettes and bulb
Thermometer
Pasteur pipettes
Small beaker
Salt (sufficient to add to achieve approximately a 2% level in the final cheese)
Burette and indicator solution
30ml tubes for measuring Titratable Acidity (TA)
Water bottle to clean pH probe
Standing solution for probe
Calibration solutions for probe
2x palette knives
2x spatulas
cheese knife
2x slotted spoon
Disposable muslin squares
Large beaker of disinfectant

3. Method

Following consultation with the Steering Panel associated with the project, which included members of the Specialist Cheesemakers Association (SCA), it was decided to investigate the persistence of VTEC in **Caerphilly** Cheese. Caerphilly is a semi-hard cheese, with a relatively short maturation. It has also been implicated in illness from VTEC in the UK (Anon. 1998).

In order to learn how to make Caerphilly Cheese, a cheesemaking course was attended. This was run by Chris Ashby (AB Cheesemaking) at Reaseheath College, Cheshire.

The method used in this project was adapted from that used at Reaseheath, but was monitored by SCA.

3.1 Starter

The starter was stored frozen until required. At this point, an aliquot of starter culture was thawed by removing it from the freezer during the morning of the day prior to cheesemaking, and incubated at 22^{o}C overnight.

The method followed for cheesemaking is outlined in the subsequent steps:

3.2 Milk

- Fill the bath used to heat/cool the vat with water.
- Add 10L of unpasteurised milk to the cheese vat. Measure the starting pH and temperature of the milk.
- Switch on the stirrer of the vat, and the water bath heater (set to 33^{o}C). *This will enable 32^{o}C to be achieved in the milk in the vat. This will take up to 45 minutes.*
- When the temperature of the milk reaches 32^{o}C, measure and record the pH of the starter culture (4.30-4.50)*, measure the pH of the milk (6.68) and then add the starter to the milk. **This is 0h0m**.

3.3 Rennet

- Whilst maintaining the temperature of the milk at 32^{o}C, allow it to 'ripen' for one hour and measure the pH (6.55) at the end of ripening.
- Pipette 2.0 ml stock rennet into 20mL SDW. Add this diluted rennet to the milk and stop and remove the stirrer after 3 mins.
- After 15 mins look for 'flocculation' by drop-wise adding the milk to warm water using a Pasteur pipette. Flocculated milk will drop to the bottom and grains/granules will be visible. The time taken from rennet to flocculation is χ. Record the acidity and temperature at flocculation.
- The time from this point to 'cutting' should be around 2χ (*more likely to be 45 mins*.). Check for a clean break (curd should pull away from the sides of the vat and have a bouncy nature when touched. Insert a thermometer at a 45^{o} angle if required - the fracture surface should be smooth). Record the pH when this is achieved (6.5).

3.4 Cutting

- Cut the curd with the cheese knife. The sharp edge is the cutting edge - this reduces tearing.
- Change the orientation of the knife to obtain small cubes of curd - roughly 1cm^{3} in size. Try to keep them regular to unsure that the whey is lost from each at a similar rate.
- Leave the curds for 10 mins under whey. Replace the stirrer and stir for 10 mins without heating.
- Record the pH/acidity.

3.5 Scald

- Increase the temperature of the water bath, so that the curds achieve 34^{o}C in 10 mins.
- Maintain the temperature at 34^{o}C for a further 20-50 mins while stirring gently, during which time the curd will tighten up. Measure the pH (6.20). *The particles should be firm and bounce. Ensure that the curd gets no hotter than 34.4^{o}C.*
- Switch off the pump when the water bath achieves 35/36^{o}C.

3.6 Pitch

- Remove the stirrer and leave the curds to pitch for 10 mins. This is slightly longer than before as we have a small volume and the whey will drain relatively quickly from the vat. The time can be shortened if the acidity is high. Record the pH of the curd (6.00).
- Push the curds 1/3 of the way up the vat and start to drain the whey by switching on the pump. Take at least 10 mins to slowly drain the whey.
- Squeeze the whey from a lump and measure its acidity and pH and that of the curd.

3.7 Drain

- Make a channel in the curd along the middle of the vat. Record the pH (5.90) and acidity.
- Continue to drain the whey.

3.8 Curd Handling

- After 5 minutes cut the curd into large (up to 9") blocks and turn over carefully.
- Record the acidity and temperature every 5 mins.
- Cut and pile the curd every 5 mins until the pH reaches less than 6.
- Up to 20 mins may be required for this

3.9 Mill

- Cut the curd into 2.5cm^{3} pieces.
- Add 24g of salt in two portions, mixing well each time.
- Allow the curd to 'mellow' for 15 mins and stir twice during this time.
- Record the pH/acidity and temperature of the curd at the end of milling.

3.10 Fill Mould

- Line the mould with cheese cloth.
- Fill with curds, push the lid down onto the cheese cloth and top up the mould. All the curd should fit into the mould.

3.11 Press

- Put the press into a tray and bag to trap the whey.
- Press the cheese for 16 hours at 22-25^{o}C. This was done using a press where a metal plate was pressed against the cheese using a

screw-threaded jack. The pressure was kept applied to the cheese, however it was not measured.

(According to SCA, for a professional cheesemaker, a pressure of 0.3kg per cm^2 is required. (Or 2cwt for 2h and 5-10cwt overnight for a 25cm diameter cheese).)

3.12 Pack and store

- Melt wax in a pan on a hot plate.
- Use a paint brush to apply the molten wax in even layers on all surfaces of the cheese.
- Store bagged and in a tray at 10-12^{o}C.

*Numbers in brackets indicate target pHs suggested by the SCA.

4. Cheesemaking Results

A template for Caerphilly cheese manufacture is given in Table 6.1. The pH and Titratable Acidity values at each processing stage are as advised by the Specialist Cheesemakers Association (SCA) or AB Cheesemaking.

Table 6.1 Template for Caerphilly cheese manufacture as advised by the SCA / A B Cheesemaking.

PROCESS	TIME	pH	TITRATABLE ACIDITY (TA)
DATE:			
FARM PROVIDING MILK:			
AGE OF OLDEST MILK (Hrs):			
PASTEURISED / UNPASTEURISED:			
LITRES: 10			
pH/TA INCOMING MILK:		6.77	0.16
TEMP INCOMING MILK (◦C): 6			
pH STARTER		4.32	
AMOUNT STARTER (L): 0.14			
START REAL TIME			
START MILK TEMP (◦C): 21			
START TIME	0h0m		
START MILK pH		6.68	
QUANTITY RENNET (mL): 2			
RENNETING TEMP: 32◦C			
RENNET TIME	1h0m		
pH AT RENNETING		6.55	0.18
CUT TIME	1h45m		
pH AT CUTTING		6.5	0.1-0.12
STIR START TIME	2h0m		
HEAT ON TIME	2h10m		
SCALD TEMP (◦C): 34			0.14
TIME OF PITCH	2h40m		
pH AT PITCH		6.2	0.16
TIME OF WHEY OFF	2h50m		
CURD pH AT WHEY OFF		6	0.19
TIME BEFORE SALTING	3h20m		
pH BEFORE SALTING		5.9	
AMOUNT OF SALT (Kg): 0.024			>0.3
TIME OUT OF PRESS	22h0m		
pH OUT OF PRESS		4.8	
Weight of cheese produced (Kg): ~1.2			
DAIRY TEMP MAX:			
DAIRY TEMP:			

At Campden BRI, following advice from SCA, pH rather than Titratable Acidity (TA) was measured throughout the cheese making process. TA was measured at Queens University Belfast (following training with AB Cheesmaking).

4.1 Uninoculated Cheeses

In an attempt to perfect the cheesemaking technique, a series of 13 Caerphilly Cheeses were made without being inoculated with VTEC strains. The pH values achieved at each stage in the production of example cheeses is given in Table 6.2. These values are represented graphically in Figure 6.2.

Table 6.2 pH values achieved for example Caerphilly cheeses not inoculated with VTEC.

Cheese-making Stage	Target pH	pH values for cheeses '2' and '6'	
		'2'	'6'
Milk	6.77	6.68	6.29
Starter	4.35	NR	5.12
Start Milk	6.68	6.46	NR
Renneting	6.55	6.44	5.95
Cutting	6.5	6.46	6.29
Pitch	6.2	6.7	5.8
Curd at Whey off	6	5.89	5.8
Before Salting	5.9	6.66	6.27
Out of Press	4.8	5.02	5.12

NR, not recorded

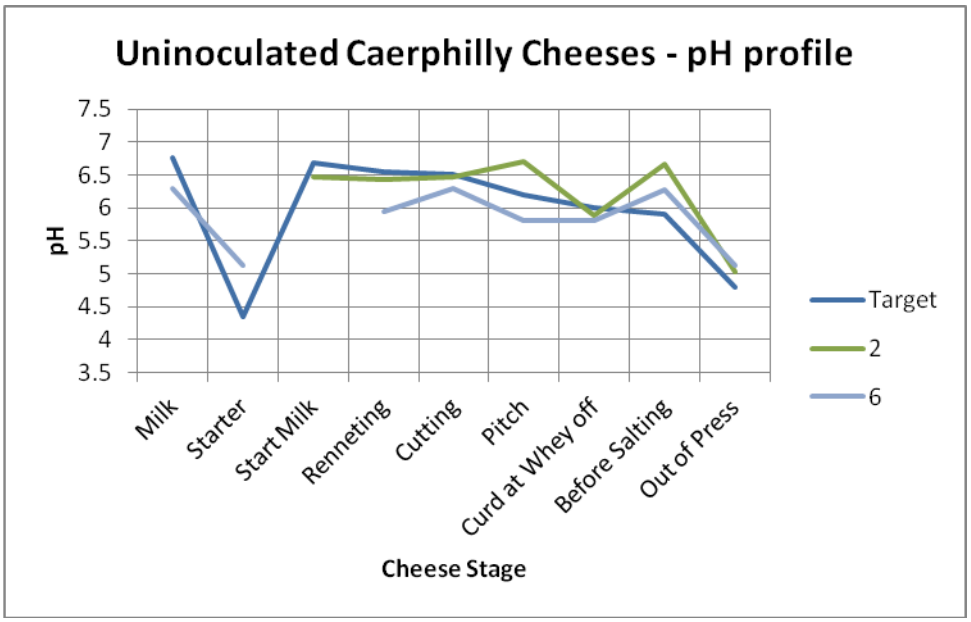


Figure 6.2 pH values achieved for example Caerphilly cheeses not inoculated with VTEC

As can be seen, the values obtained for the cheeses, (with the exception of an apparent small increase in pH prior to salting, which cannot easiily be explained) were in line with target levels anticipated.

These cheeses and the batches of milk from which they were made, were analysed for their chemical characteristics. The values obtained (plus those from some other batches) are given in Table 6.3.

Table 6.3 Chemical and microbiological characteristics of example uninoculated Caerphilly cheeses and the milk from which they were made.

Chemical / Microbiological Value	Milk / Cheese batch			
	'2'	**'3'**	**'5'**	**'6'**
MILK				
Acidity (Lactic) (g/100g)		0.2		0.74
Fat (Weibull-Stoldt) (g/100g)		4.7		10.6
Moisture (Microwave) (g/100g)		86.9		83
pH		6.73		
Salt (Volhard)		0.2		0.2
Delvotest (antibiotics)	negative	negative	negative	negative
APC (per g)			8400	2520
Coagulase +ve staphylococci (per g)			<10	
Coliforms (per g)			80	
Enterobacteriaceae (per g)				
E. coli O157 (per 25g)			ND	
CHEESE				
Acidity (Lactic) (g/100g)	0.73	0.82	1.66	0.57
Fat (Weibull-Stoldt) (g/100g)	26	21.5	26.7	20.7
Moisture (Microwave) (g/100g)	48.4	49.2	47.9	50
pH	5.02	5.16	4.88	5.12
Salt (Volhard)	1.4	1.6	0.8	1

NB, blank box indicates a not recorded value

Comments

The characteristics obtained are quite close to those anticipated. The only exceptions were:

a) the salt level which should have been 2% in the cheeses. This level was not reached, although cheese '3' had a final level of 1.6%. The protocol for adding salt as directed by AB Cheesemaking and the SCA was followed carefully. The only explanations for why 2% was not achieved are:
   - that insufficient salt was added; or
   - that the salt was not evenly distributed throughout the cheese and some was lost in the whey that was squeezed out in pressing.

b) moisture, which should have been approximately 43%. Insufficient whey appears to have been squeezed out during pressing, and coating the cheese in wax has retained this moisture throughout the life of the cheese.

4.2 Inoculated cheeses

A total of 4 inoculated Caerphilly cheeses were made (inoculated with VTEC O26 and one of the O157 strains). The pH values obtained at each stage in the manufacture of these cheeses is given in Table 6.4. These values are represented graphically in Figure 6.3.

Table 6.4 pH values achieved for Caerphilly cheeses inoculated with VTEC.

Cheese-making Stage	Target pH	pH values for inoculated cheeses			
		'10' / 'A'	'11' / 'B'	'12' / 'C'	'13' / 'D'
Milk	6.77	6.82	6.82	6.71	6.8
Starter	4.35	NR	4.64	4.54	4.61
Start Milk	6.68	6.59	6.67	6.17	6.72
Renneting	6.55	6.59	6.61	6.51	6.62
Cutting	6.5	6.63	6.62	6.5	6.6
Pitch	6.2	6.55	6.64	6.56	6.62
Curd at Whey off	6	6.65	NR	NR	6.68
Before Salting	5.9	NR	NR	NR	NR
Out of Press	4.8	5.39	6.02	6.1	6.13

NR, not recorded

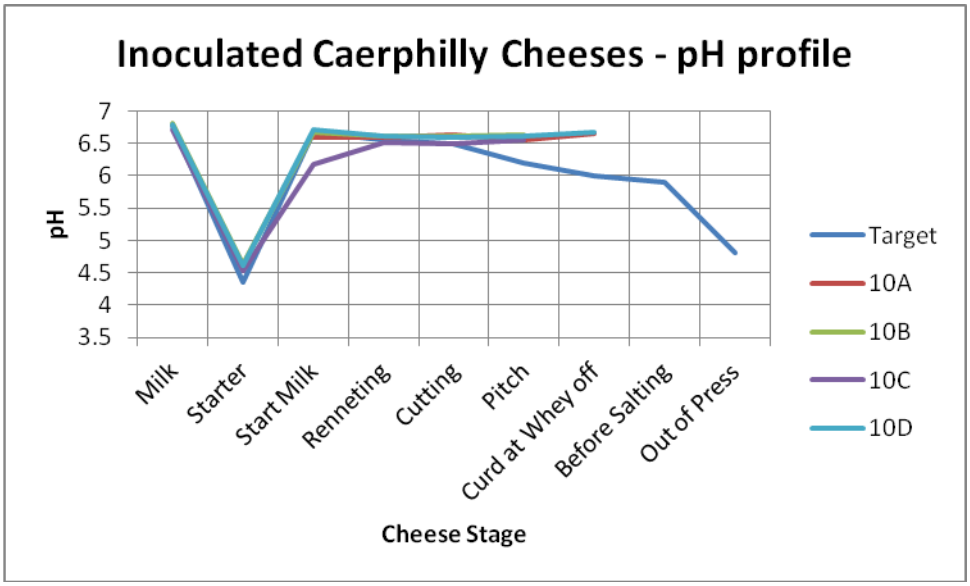


Figure 6.3 pH values achieved for example Caerphilly cheeses not inoculated with VTEC

The Titratable Acidity (TA) values for these cheeses during manufacture are given in Table 6.5. The values are represented graphically in Figure 6.4.

Table 6.5 Titratable Acidity (TA) values achieved for Caerphilly cheeses inoculated with VTEC.

Cheese-making Stage	Target TA	pH values for inoculated cheeses			
		'10' / 'A'	'11' / 'B'	'12' / 'C'	'13' / 'D'
Milk	0.16	0.14	0.14	0.16	0.14
Starter			0.63	0.75	0.76
Start Milk					
Renneting	0.18	0.16	0.13	0.16	0.18
Cutting	0.11	0.09	0.1	0.1	0.12
Scald	0.14			0.11	0.12
Pitching	0.16	0.07	0.1	0.11	0.09
Curd handling	0.19				
Into mould	0.3				

NB, blank box signifies value not recorded.

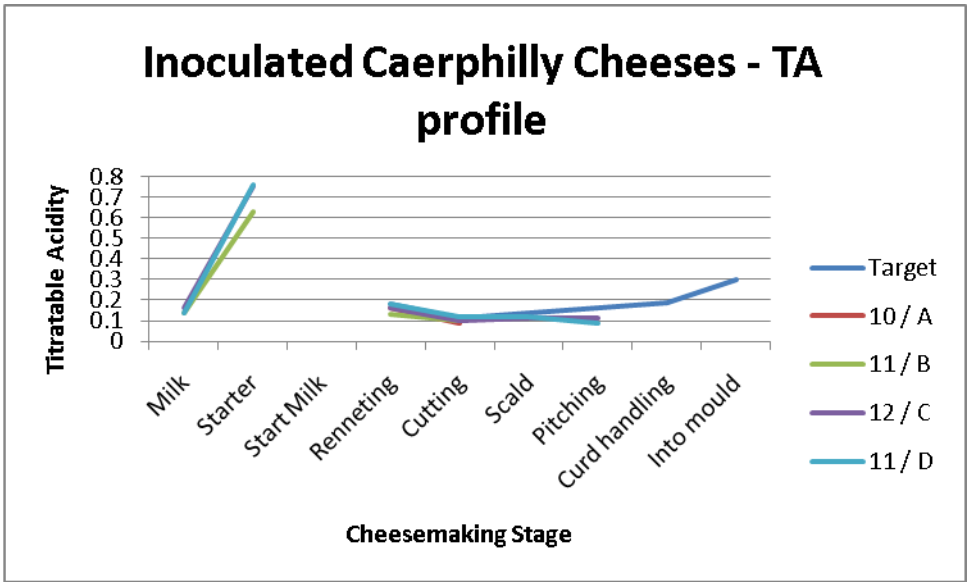


Figure 6.4 Titratable Acidity (TA) values achieved for example Caerphilly cheeses not inoculated with VTEC

These cheeses (at beginning and end of life) and the batches of milk from which they were made, were analysed for their chemical characteristics. The values obtained are given in Table 6.6.

Table 6.6 Chemical and microbiological characteristics of inoculated Caerphilly cheeses and the milk from which they were made.

Chemical / Microbiological Value	Milk / Cheese Batch							
	'10' / 'A'		**'11' / 'B'**		**'12' / 'C'**		**'13' / 'D'**	
	Start (T0)	End (21 weeks)	Start (T0)	End (19 weeks)	Start (T0)	End (17 weeks)	Start (T0)	End (15 weeks)
MILK								
Acidity (Lactic) (g/100g)	0.23		0.15		0.13		0.36	
Fat (Weibull-Stoldt) (g/100g)	5.1		3.6		3.7		3.6	
Moisture (Microwave) (g/100g)	86.3		88		85.2		88.3	
pH	6.76		6.77		6.59		6.73	
Salt (Volhard) (g/100g)	0.2		0.2		0.2		0.1	
Delvotest (antibiotics)	negative		negative		negative		negative	
APC (per g)	1300		2600		1100		8100	
Coagulase +ve staphylococci (per g)	<10		<10		<10		<10	
Coliforms (per g)	20		30		100		40	
Enterobacteriaceae (per g)	25		20		85		10	
E. coli O157 (per 25g)	ND		ND		ND		ND	
Inoculum level: O157 (log)	4.65		4.57		2.76		2.76	
Inoculum level: O26 (log)	4.6		4.63		2.7		1.79	
CHEESE								
Acidity (Lactic) (g/100g)	0.48	0.92	0.15	0.6	0.15	1.02	0.13	1.06
Fat (Weibull-Stoldt) (g/100g)	23.6	24	21.4	21.6	23.1	23.4	22.8	21.4
Moisture (Microwave) (g/100g)	49.8	47.9	50.5	51.3	50	47.9	49.6	53.6
pH	5.39	6.57	6.02	6.95	6.1	5.85	6.13	5.76
Salt (Volhard) (g/100g)	0.8	0.9	0.8	1.1	1.1	1	0.8	1

Comments

As was seen in the uninoculated cheeses, the salt levels were lower and the moisture levels higher than was anticipated in the inoculated cheeses. In addition, the pH profiles (and TA profiles) were not as acidic as was anticipated.

The reasoning for low salt and high moisture levels have been discussed in 4.1. Bearing in mind the fact that the pH profiles for the uninoculated cheeses were acceptable, the most logical explanation for a lack of acidification with the inoculated cheeses is that the presence of the VTECs interfered with the starter cultures and/or the acidification process in the cheeses.

5. Sampling of cheeses inoculated with VTEC strains

Each of the cheeses inoculated with VTEC strains was sampled throughout its life (normally 3-4 months (12-16 weeks)), and tested for numbers of persisting cells. The method used to sample the cheeses in this study consisted of the following steps:

a) Use a sterile knife to make a 'doorway' in the wax surrounding the cheese.
b) Use a cork borer to extract the samples of cheese required for microbiological testing and pH.
c) Carry out testing for VTECs.
d) Measure the pH of the cheese.
e) Plug the hole made by the cork borer and re-seal the wax using a knife warmed in molten wax.
f) Return the cheese to the incubator at 10-12°C.

6. VTEC persistence in cheese - results

Results for the behaviour of the VTEC strains in the four inoculated Caerphilly Cheeses are given in the following four Tables and Graphs.

6.1 Caerphilly Cheese A / 10

Table 6.6.1 Counts of VTEC in Caerphilly Cheese A / 10 over cheese production and storage at 10-12◦C for 21 weeks (5 months)

Caerphilly Cheese A						
Strains and inoculum levels:						
Organism	Campden code	CFU/ml of milk	Log_{10} CFU/ml of milk			
E. coli O157:H7	16244	4.50E+04	4.65			
E. coli O26:H11	16485	4.00E+04	4.6			
			E. coli O157 count on ChromID O157:H7		*E. coli* O26 count on CT-RMAC	
Sample code	Test date	pH reading	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
A milk	27/04/2010		<5	<0.7	<5	<0.7
A whey	27/04/2010		2.0E+03	3.30	7.8E+05	5.89
A curd	28/04/2010		1.3E+06	6.12	1.1E+08	8.04
A1	28/04/2010	5.39	4.1E+07	7.61	6.0E+08	8.77
A2	05/05/2010	5.18	3.5E+07	7.54	1.9E+08	8.28
A3	12/05/2010	5.00	2.6E+07	7.42	2.2E+08	8.35
A4	18/05/2010	4.98	2.5E+07	7.40	2.2E+08	8.34
A5	26/05/2010	5.02	1.2E+07	7.06	2.3E+08	8.36
A6	09/06/2010	5.45	1.7E+06	6.23	2.1E+08	8.33
A7	23/06/2010	5.06	1.3E+05	5.10	1.2E+08	8.07
A8	07/07/2010	5.62	1.8E+06	6.26	1.4E+08	8.15
A9	17/08/2010	5.98	6.5E+04	4.81	7.0E+07	7.85
A10	22/09/2010	6.84	1.0E+04	4.00	9.1E+06	6.96

The data from Table 6.6.1 is represented graphically in Figure 6.6.1.

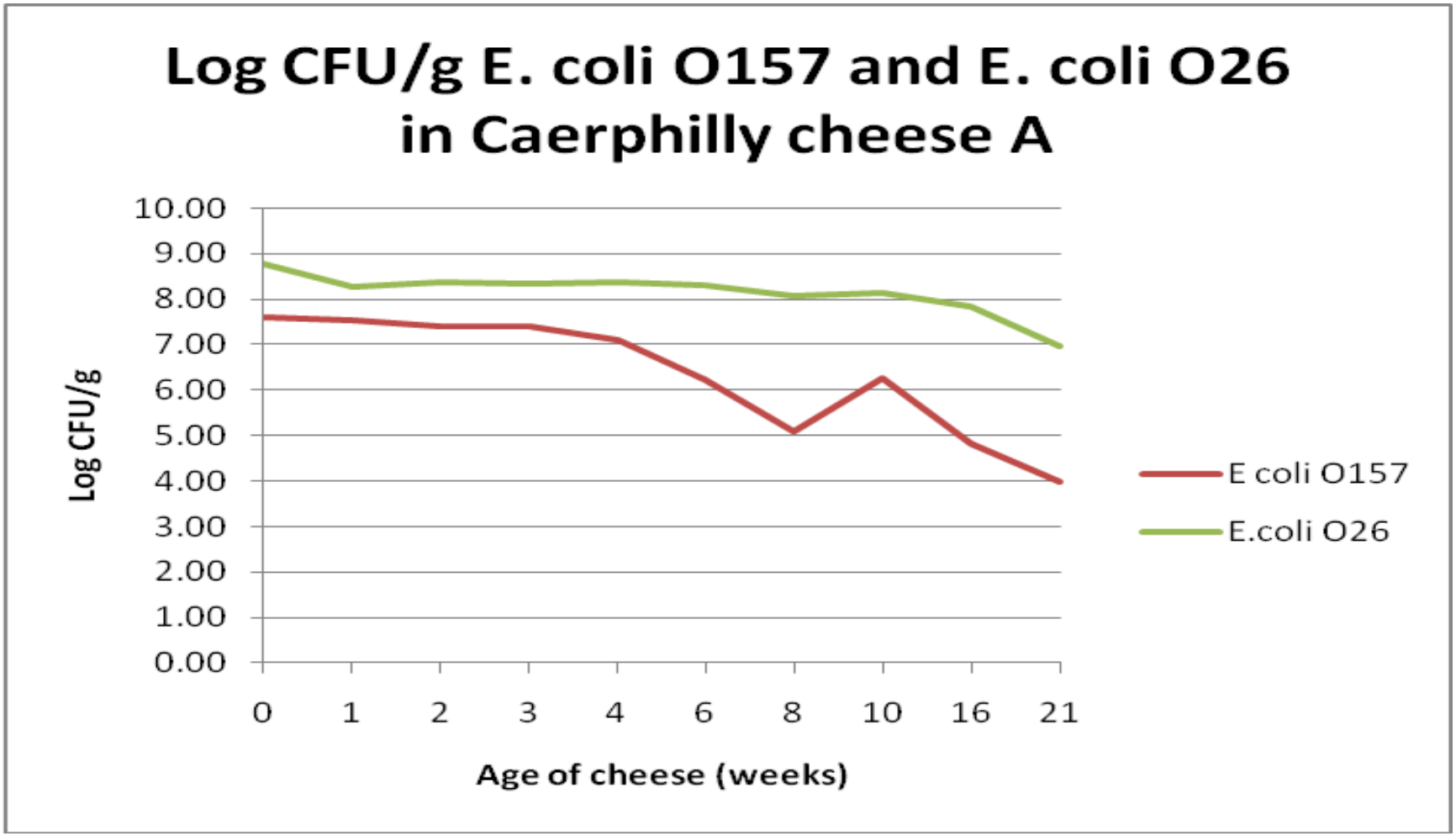


Figure 6.6.1 Persistence of VTEC microorganisms in Caerphilly Cheese A / 10 over its life at 10-12˚C

6.2 Caerphilly Cheese B / 11

Table 6.6.2 Counts of VTEC in Caerphilly Cheese B / 11over cheese production and storage at 10-12◦C for 19 weeks (5 months)

Caerphilly Cheese B						
Date made:	11/05/2010					
Strains and inoculum levels:						
Organism	Campden code	CFU/ml of milk	Log_{10} CFU/ml of milk			
E. coli O157:H7	16414	3.8E+04	4.57			
E. coli O26:H11	16485	4.3E+04	4.63			
			E. coli O157 count on ChromID O157:H7		*E. coli* O26 count on CT-RMAC	
Sample code	Test date	pH reading	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
B milk	11/05/2010		<5	<0.7	<5	<0.7
B whey	11/05/2010		1.2E+04	4.08	4.0E+05	5.60
B curd	11/05/2010		1.1E+06	6.04	1.1E+08	8.03
B1	12/05/2010	6.08	5.2E+06	6.72	1.2E+08	8.08
B2	18/05/2010	5.87	4.3E+06	6.64	1.3E+08	8.10
B3	26/05/2010	5.26	7.2E+06	6.86	1.3E+08	8.12
B4	09/06/2010	4.95	2.9E+06	6.46	2.9E+08	8.46
B5	23/06/2010	4.85	9.0E+05	5.95	2.3E+08	8.36
B6	07/07/2010	4.84	8.5E+05	5.93	1.6E+08	8.21
B7	17/08/2010	5.33	7.0E+04	4.85	2.8E+07	7.44
B8	22/09/2010	6.27	1.0E+08	8.00	2.5E+06	6.39

The data from Table 6.6.2 is represented graphically in Figure 6.6.2.

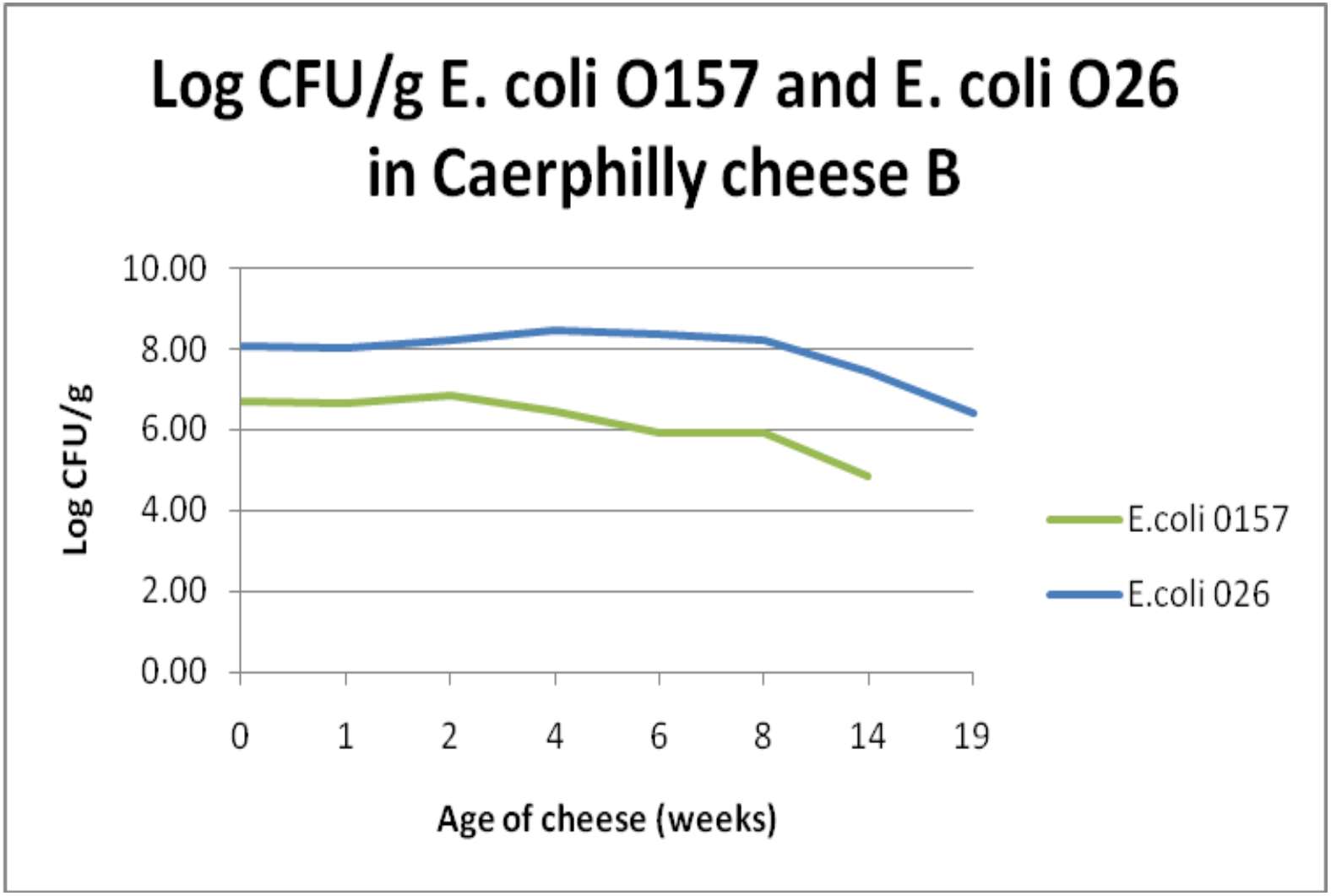


Figure 6.6.2 Persistence of VTEC microorganisms in Caerphilly Cheese B / 11 over its life at 10-12°C

6.3 Caerphilly Cheese C / 12

Table 6.6.3 Counts of VTEC in Caerphilly Cheese C / 12 over cheese production and storage at 10-12°C for 17 weeks (3 months)

Caerphilly Cheese C						
Date made:	25/05/2010					
Strains and inoculum levels:						
Organism	Campden code	CFU/ml of milk	Log_{10} CFU/ml of milk			
E. coli O157:H7	16244	5.8E+02	2.76			
E. coli O26:H11	16485	5.0E+02	2.70			
			E. coli O157 count on ChromID O157:H7		*E. coli* O26 count on CT-RMAC	
Sample code	Test date	pH reading	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
C milk	25/05/2010		<5	<0.7	<5	<0.7
C whey	25/05/2010		2.0E+02	2.30	1.8E+04	4.26
C curd	25/05/2010		9.5E+03	3.98	2.3E+06	6.36
C1	26/05/2010	6.27	9.5E+05	5.98	1.1E+08	8.04
C2	09/06/2010	5.10	8.5E+05	5.93	3.8E+08	8.58
C3	23/06/2010	4.88	9.0E+05	5.95	1.9E+08	8.28
C4	07/07/2010	4.82	1.5E+05	5.18	1.2E+08	8.09
C5	17/08/2010	5.01	3.5E+04	4.54	4.6E+07	7.67
C6	22/09/2010	5.22	6.1E+05	5.78	2.3E+07	7.37

The data from Table 6.6.3 is represented graphically in Figure 6.6.3.

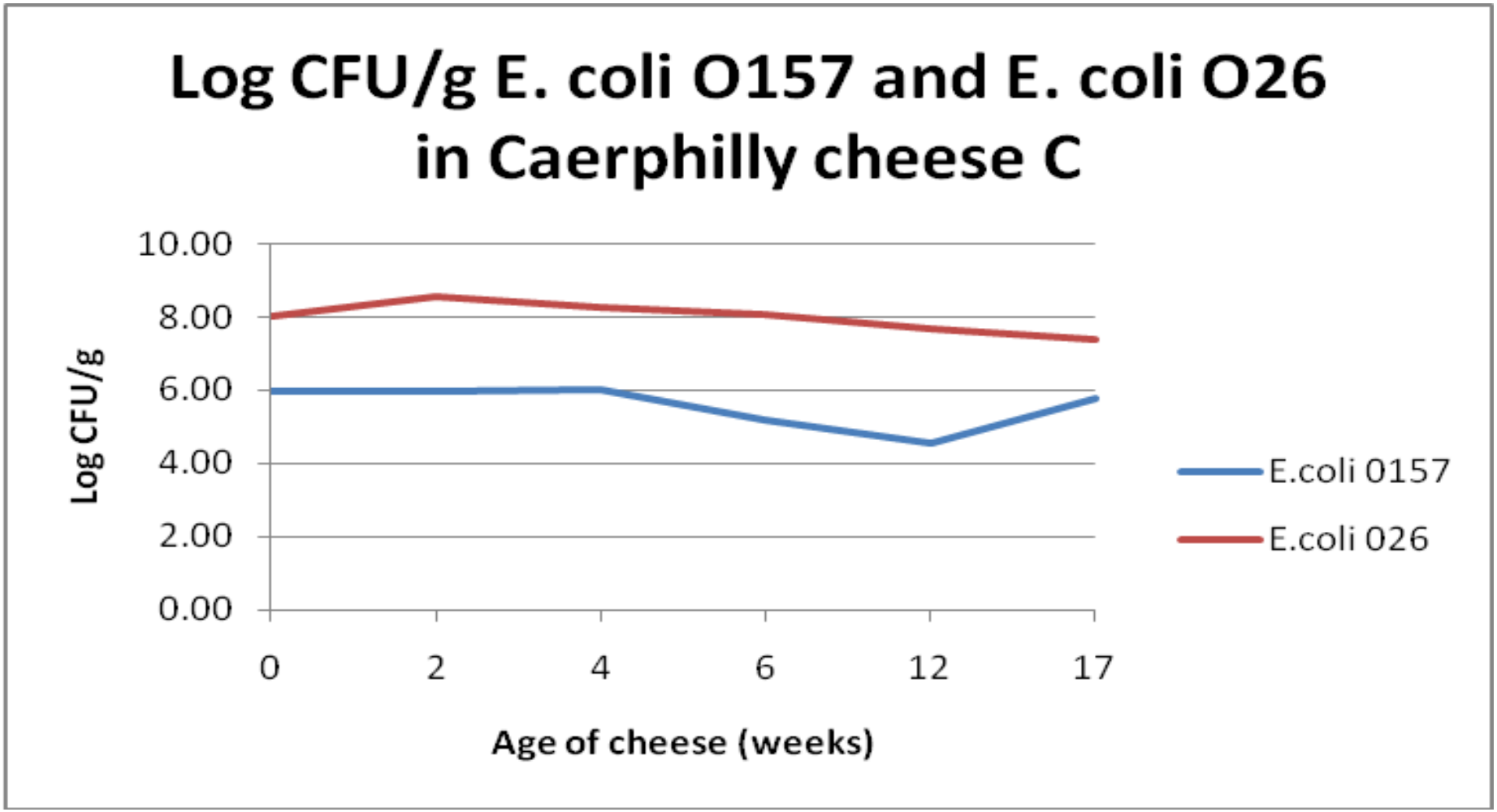


Figure 6.6.3 Persistence of VTEC microorganisms in Caerphilly Cheese C / 12 over its life at 10-12°C

6.4 Caerphilly Cheese D / 13

Table 6.6.4 Counts of VTEC in Caerphilly Cheese D / 13 over cheese production and storage at 10-12°C for 17 weeks (3 months)

Caerphilly Cheese D						
Date made:	08/06/2010					
Strains and inoculum levels:						
Organism	Campden code	CFU/ml of milk	Log_{10} CFU/ml of milk			
E. coli O157:H7	16414	5.8E+02	2.76			
E. coli O26:H11	16485	6.0E+01	1.79			
			E. coli O157 count on ChromID O157:H7		*E. coli* O26 count on CT-RMAC	
Sample code	Test date	pH reading	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
D milk	08/06/2010		<5	<0.7	<5	<0.7
D whey	08/06/2010		<50	<1.7	3.5E+02	2.54
D curd	08/06/2010		7.5E+03	3.88	1.5E+05	5.18
D1	09/06/2010	6.45	3.5E+05	5.54	7.7E+07	7.89
D2	23/06/2010	5.15	9.5E+05	5.98	1.5E+08	8.18
D3	07/07/2010	4.83	7.0E+05	5.85	1.3E+08	8.12
D4	17/08/2010	5.08	3.8E+06	6.58	3.4E+07	7.53
D5	22/09/2010	5.52	4.7E+06	6.67	1.0E+07	7.02

The data from Table 6.6.4 is represented graphically in Figure 6.6.4.

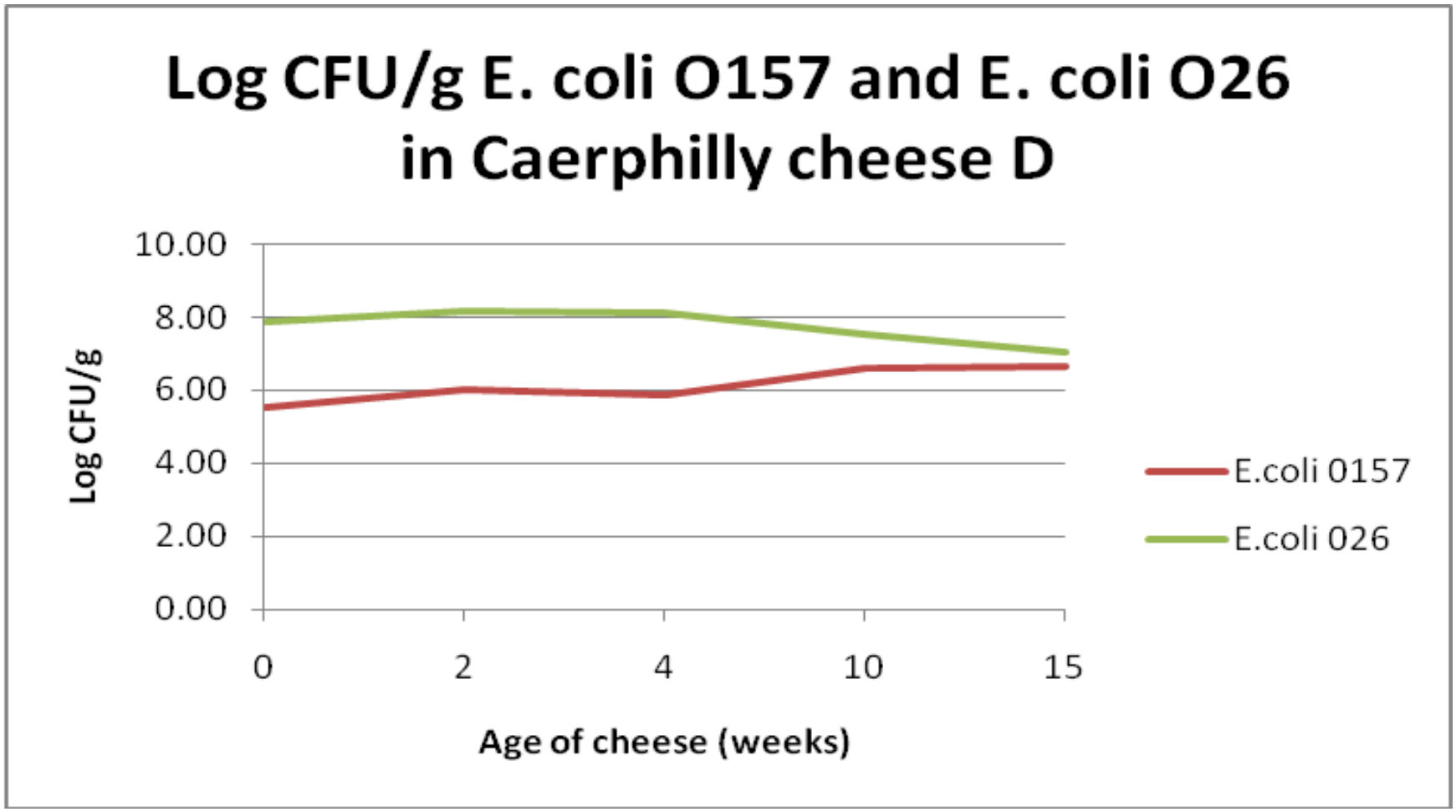


Figure 6.6.4 Persistence of VTEC microorganisms in Caerphilly Cheese D / 13 over its life at 10-12°C

7. End of life analysis

At the end of storage of the cheese, it was irradiated so that its chemical characteristics could be measured. The irradiation was carried out by Isotron Ltd. (now Synergy Health Sterilisation UK Ltd.), Swindon, and a dose of 10 kgrays was used.

After irradiation, the cheese was tested for the presence of VTEC. Following clearance that no viable VTEC remained, the cheese was analysed for: acidity; fat; moisture; pH; and salt.

8. Discussion of results

During all four of the cheesemaking events where VTEC was inoculated into the milk, there was an increase in VTEC numbers, as illustrated below in Table 8.1.

Table 8.1 VTEC counts associated with Caerphilly cheeses at beginning and end of production.

Cheese	VTEC strain	Count inoculated into milk (log cfu/g)	Count present in the cheese curd at make (log cfu/g)	Increase (log cfu/g)
A / 10	O157 (1)	4.65	6.12	1.47
A / 10	O26	4.60	8.04	3.44
B / 11	O157 (2)	4.57	6.04	1.47
B / 11	O26	4.63	8.03	3.40
C / 12	O157 (1)	2.76	3.98	1.22
C / 12	O26	2.70	6.36	3.66
D / 13	O157 (2)	2.76	3.88	1.12
D / 13	O26	1.79	5.18	3.39

The average increase for strain O157 (1) (= Campden code 16244) was 1.345 logs, for O157 (2) (= Campden code 16414) it was 1.295. For strain O26 (Campden code 16485) it was 3.4725. The increase in count during the cheesemaking process itself for the O26 strain was 3 times that of either of the O157 strains.

The VTEC levels stayed quite constant throughout the storage period of the cheese. The low salt, low pH and high moisture may well have influenced this. However, even under these conditions there was a gradual decline in VTEC numbers (Figure 8.2). Interestingly the O26 numbers remained higher than either of the O157 strains in all the inoculated cheeses made.

Table 8.2 VTEC counts associated with Caerphilly cheeses at beginning and end of maturation.

Cheese	VTEC strain	Count in cheese at beginning of maturation (end of production) (log cfu/g)	Count in cheese at end of maturation (log cfu/g)	Maturation period (weeks)	Decrease in log count per week	D-value (time for 1 log decrease in count (weeks))
A / 10	O157 (1)	7.61	4.00	21	0.172	5.81
A / 10	O26	8.77	6.96	21	0.086	11.63
B / 11	O157 (2)	6.72	4.85 (8.00)	14	0.134	7.46
B / 11	O26	8.08	6.39	19	0.089	11.23
C / 12	O157 (1)	5.98	5.78	17	0.012	83.33
C / 12	O26	8.04	7.37	17	0.039	25.64
D / 13	O157 (2)	5.54	6.67	15	-0.075	NA
D / 13	O26	7.89	7.02	15	0.058	17.24

NA, not applicable

In general terms, it is not possible to draw any firm conclusions about the rate of decrease in count between the two strains tested. Of course, the inoculated levels are higher than one would ever anticipate in cheeses at point of production. However, if VTEC strains are able to grow during the cheese manufacture process, high levels could be potentially seen in newly made Caerphilly cheese. These levels would not drop off rapidly during the maturation of the cheese.

The findings that VTEC grew during the cheese-making process and died off gradually over the life of the cheese is mirrored by work carried out at the University of Vermont (D'Amico *et al.* 2010) on Gouda and stirred-curd Cheddar cheeses, and on Camembert cheese by Montet *et al.* (2009).

In the work carried out by D'Amico *et al.* (2010), cheeses were manufactured from unpasteurized milk experimentally contaminated with one of three strains of *E. coli* O157:H7 at an approximate population level of 20 cfu/ml. Samples of milk, whey, curd, and cheese were collected for enumeration of bacteria throughout the manufacturing and aging process. Overall, bacterial counts in both cheese types increased almost 10-fold from initial inoculation levels in milk to approximately 145 cfu/g found in cheeses on day 1. From this point, counts dropped significantly over 60 days to mean levels of 25 and 5 CFU/g in Cheddar and Gouda, respectively. Levels of *E. coli* O157:H7 fell and stayed below 5 CFU/g after an average of 94 and 108 days in Gouda and Cheddar, respectively, yet remained detectable after selective enrichment for more than 270 days in both cheese types. Changes in pathogen levels observed throughout manufacture and aging did not significantly differ by cheese type. The results from the work carried out by D'Amico *et al.* (2010), suggests that a 60-day aging requirement alone is insufficient to completely eliminate levels of viable *E. coli*

O157:H7 in Gouda or stirred-curd Cheddar cheese manufactured from raw milk contaminated with low levels of this pathogen.

Montet *et al.* (2009), investigated the growth and survival of acid-resistant (AR) and non-acid-resistant (NAR) shiga-toxin-producing *Escherichia coli* (STEC) strains during the manufacture and ripening of microfiltered milk Camembert cheeses. The induction of acid resistance of the STEC strains in cheeses was also studied. Six different mixtures of AR and/or NAR STEC strains were inoculated separately into microfiltered milk at a level of 10 cfu/ml. The STEC counts (AR and NAR) initially increased by 1 to 2 log cfu/ml during cheese-making. Thereafter, the populations stabilized during salting/drying and then decreased during the early stages of ripening. Exposing the STEC strains in artificially inoculated cheeses to simulated gastric fluid reduced the number of NAR strains to undetectable levels within 40 minutes, versus 120 minutes for the AR STEC strains. AR and NAR STEC were able to survive during the manufacture and ripening of Camembert cheese prepared from microfiltered milk with no evidence of induced acid tolerance in NAR STEC strains.

To determine what is accepted as characteristics for Caerphilly cheeses, the literature and a number of cheese making companies in addition to the SCA were consulted. A summary of findings are given in Table 8.3. Also in this Table are the characteristics of Caerphilly cheeses made at Campden BRI and Queens University Belfast during this project.

Table 8.3 A summary of characteristics of Caerphilly Cheeses

Reference	pH	Titratable Acidity	Moisture (g/100g)	Salt (g/100g)	Fat (g/100g)	Comments
1. CBRI Uninoculated						
1.1 Cheese 2	5.02	(0.73)[A]	48.4	1.4	26.0	
1.2 Cheese 3	5.16	(0.82)	49.2	1.6	21.5	
1.3 Cheese 5	4.88	(1.66)	47.9	0.8	26.7	
1.2 Cheese 6	5.12	(0.57)	50.0	1.0	20.7	
2. CBRI Inoculated						
2.1 Cheese 10A	5.39-6.57[B]	(0.48-0.92)	49.8-47.9	0.85	23.8	
2.2 Cheese 11B	6.02-6.95	(0.15-0.6)	50.5-51.3	0.95	21.5	
2.3 Cheese 12C	6.10-5.85	(0.15-1.02)	50.0-47.9	1.05	23.3	
2.4 Cheese 13D	6.13-5.76	(0.13-1.06)	49.6-53.6	0.90	22.1	
3. QUB Inoculated						
	5.25-5.60	0.24-0.30	40.23-43.04	1.51-2.04	28.16-31.59	(summary of 9 cheeses)
4. Randolph Hodgson, Specialist Cheesemakers Association	4.8	>0.3	~43	2.0		
5. Banks (2006)	4.6-6.2 5.3-5.4 (Table 11)	0.28-0.40	44-55[C]			[C]Semi-soft cheeses (e.g. Caerphilly)
6. Fundamentals of Cheese Science, Aspen Publications, 2000	5.4		41.8	1.5		
7. Company 1	4.7-5.2		38-44 (legal max. 46%)	0.90-1.50		
8. Company 2	5.35		33.92	1.51	34	
9. Company 3	4.6-5.3		38-46	1.0-1.7		

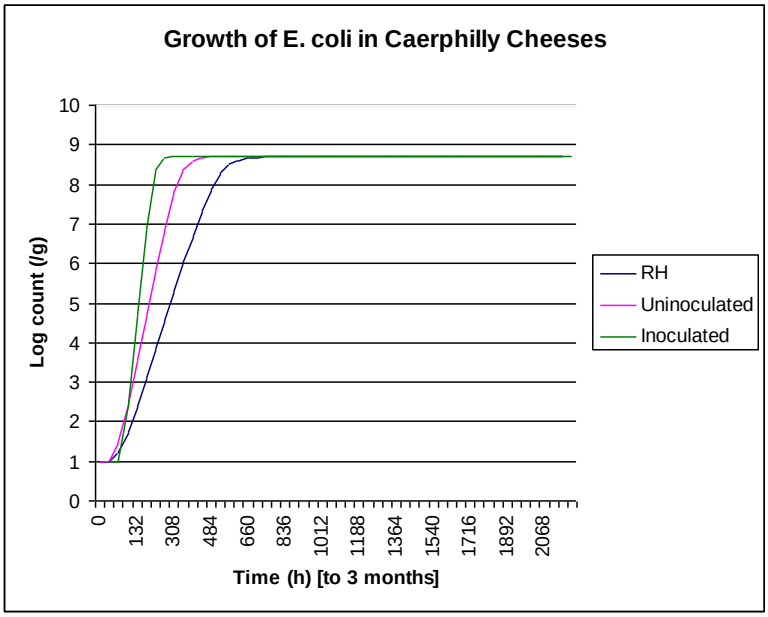
[A]Bracketed values are Acidity (Lactic) (g/100g)
[B]Value at start of cheese life and at end

Cheesemakers appear to use lower levels of salt and higher pHs than were stipulated by the SCA for Caerphilly cheeses. As a consequence of our findings, our uninoculated cheeses have an acceptable pH (although the cheeses took slightly longer to make than stipulated by SCA) and salt level. The moisture level however is too high. Interestingly, in our inoculated cheeses (and those of Queens), the pHs of the cheeses were higher than the accepted levels.

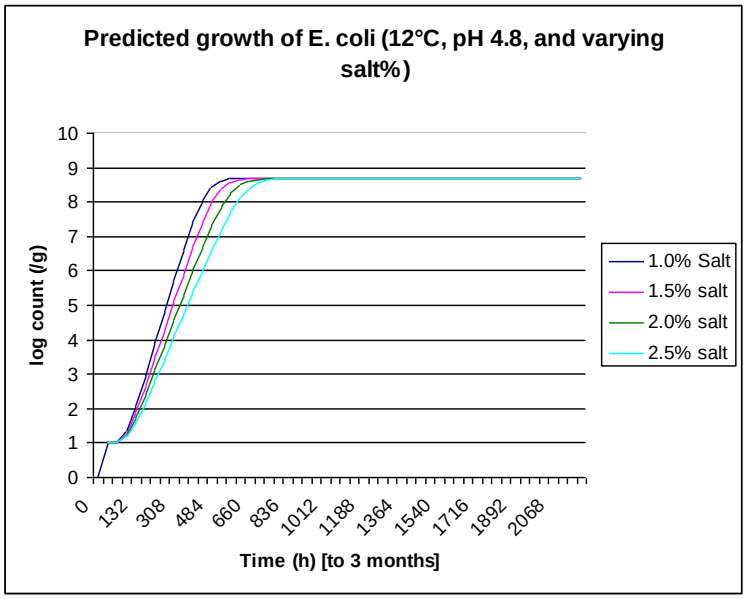
ComBase predictions were carried out to look at the impact of salt and pH on the growth of *E. coli* under conditions found in cheeses. Of course ComBase does not take into account the

impact of competing microflora (from the starter and natural flora of the raw milk), but it is useful to see how little the growth is impacted by either pH or salt.

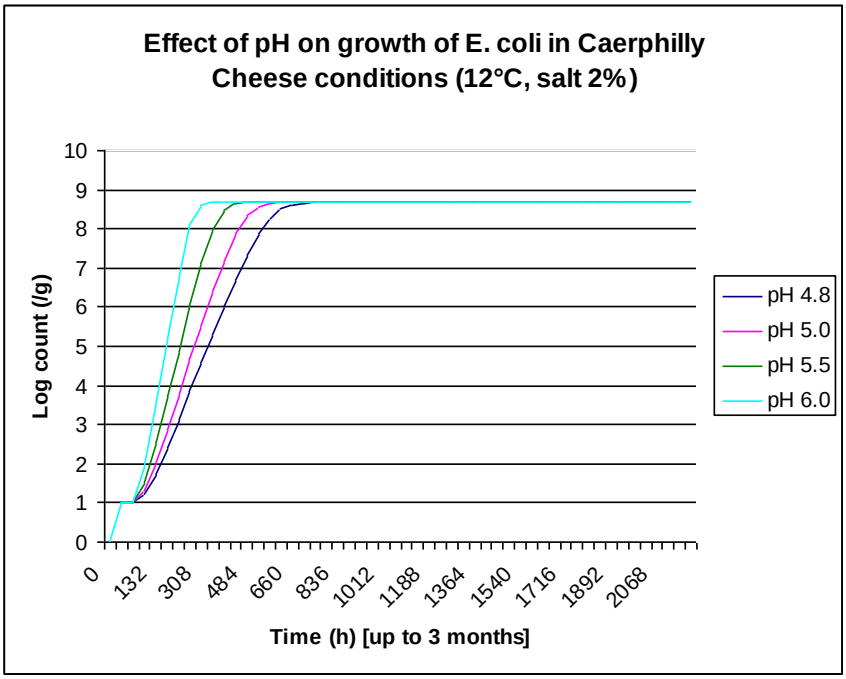
1. **Comparison of the behaviour of *E. coli* in conditions for Caerphilly cheese:**
    a. As suggested by SCA (RH) (pH 4.8, salt 2%)
    b. As found in Campden BRI uninoculated cheese pH 5.16, salt 1.6%
    c. As found in Campden BRI inoculated cheese pH 6.13, salt 0.9%



2. **Effect of salt on growth of *E. coli* under conditions found in Caerphilly Cheese**



3. **Effect of pH on growth of *E. coli* under conditions found in Caerphilly Cheese**



It is clear from the ComBase predictions illustrated above, that small changes in pH and salt levels in cheese would be expected to have a limited impact on preventing growth of VTECs in cheeses. However, it is important to keep in mind that there have been very few cases of VTEC food poisoning associated with raw milk cheese in the UK.

7. CONCLUSIONS AND RECOMMENDATIONS

A. There have been very few (1) cases of VTEC illness associated with raw milk cheese in the UK. Unfortunately, this is not true for other parts of the world such as the U.S.A.

B. The following detection and enumeration methods were identified as being most suitable to detect VTEC in cheeses.

Detection

(i) Enrichment in mTSB at 41.5°C
(ii) Immuno-magnetic separation (IMS) at 6h and/or 24h
(iii) Streak onto:
- ChromID O157 agar (VTEC O157 and VTEC O26)
- CTSMAC (VTEC O157)
- CT-RMAC (VTEC O26)

Enumeration

(i) Dilute in primary enrichment (mTSB) with sodium citrate.
(ii) Further dilutions if required are made in MRD.
(iii) Spread plates (0.1ml) are to be used when levels are high.
(iv) Pour plates are used when levels fall:
- 1ml in 90mm Petri dishes and/or
- 10ml in 140mm Petri dishes.

(v) ChromID O157 agar (VTEC O157/O26).
(iv) CT RMAC (VTEC O26).

C. From the evidence gathered from various sources, it appears that the accepted characteristics of 'Caerphilly' cheeses are variable. With the exception of moisture content (which was a little on the high side), the characteristics of the Caerphilly cheeses made at Campden BRI are were broadly within the accepted ranges.

Reasons for the high moisture content could be:

(i) inadequate pressing of the cheeses to remove sufficient whey; and
(ii) coating the cheese in wax, which would retain moisture, and which is not done to commercial Caerphilly.

D. With respect to pH, Campden BRI's uninoculated Caerphilly was at an acceptable pH and inoculation led to production of a cheese at a higher pH. This was also seen in the cheeses made at Queens University Belfast.

E. ComBase predictions (which don't take into account the impact of starter cultures, or naturally occurring Lactic Acid Bacteria) suggest that VTEC will grow in Caerphilly cheese.

F. At Campden BRI we found 'growth' (or rather increase in concentration of organisms in milk compared to organisms in curd) of VTEC during the cheesemaking process. However there is a general decline in number with time, which was also found in a U.S. study (D'Amico *et al.* 2010).

G. The increase in VTEC 'concentration' during cheese manufacture and the slow decline in count over the life of the Caerphilly cheese, would suggest that there is a potential for VTEC to cause food safety issues in Caerphilly Cheese made from raw milk. This

especially being so, if the cheese is eaten in the first week or so after production, when any presence of VTEC would be at its highest level.

H. The results presented here reiterate that VTEC is a potential issue with Caerphilly cheese made from unpasteurised milk. Since the moisture content of the cheese made was higher than what would be expected for Caerphilly cheese, it is not possible to say for sure that the results reflect what would happen in industry-made Caerphilly cheese.

8. SUGGESTIONS FOR FUTURE WORK

1. In order for the results from experimental work to be meaningful, the cheese produced has to be representative of what is made by industry. Any future work with Caerphilly or other cheeses would have to be done with this in mind.

2. The number of strains of VTEC included in the study was small. The number of these could be increased in future work.

3. The physiological state of the starter was not investigated in this study, but would be useful to look into in future work. The state of the starter would be very important in terms of its ability to inhibit any pathogens present in the raw milk used as a raw material for the cheese. In this light, the physiological state of the VTEC strains used would also be important.

4. In any future work, it would be useful to look at lower levels of VTEC inoculum. It would be anticipated that lower levels of pathogens would be more readily inhibited by starter organisms that higher ones, and any natural non-starter LABs present.

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APPENDIX 1 **Poster**

Philip A. Voysey, Christopher L. Baylis, Kerry J. Bridgwater and Rebecca A. Green
Laboratory-Scale Manufacture Of UK Semi-Hard (Caerphilly) Cheese From Raw Milk Contaminated With Verocytotoxin-Producing Escherichia coli (VTEC).

- Poster and poster abstract presented at Food Micro 2010, Copenhagen, Denmark 30/08/10 to 03/09/10.
- Poster and poster abstract presented at Society For Applied Microbiology (sfam), Summer Conference 2011, Dublin, Ireland, 4-7 July.

Laboratory-scale manufacture of UK semi-hard (Caerphilly) cheese

from raw mlk contaminated with verocytotoxin-producing *Escherichia coli* (VTEC)

Campden BRI
food and drink innovation

Philip A. Voysey, Christopher L. Baylis, Kerry J. Bridgwater, and Rebecca A. Green
Campden BRI, Microbiology Department, Chipping Campden

Introduction

Raw milk can be a potential source of foodborne pathogens. There have been a number of food-poisoning outbreaks associated with direct consumption of raw milk and cheeses made from it, including from Verocytotoxin-producing *E. coli* (VTEC)[1]. Despite this, there is interest in production and consumption of cheeses made from raw milk.

Work described here set out to increase knowledge regarding the persistence of VTEC strains during the production and storage of raw milk cheese. A protocol for manufacturing inoculated cheese in the laboratory, and methods for isolating and enumerating VTEC strains from the cheese were devised and are described here.

The cheese studied was Caerphilly, a semi-hard cheese which has a relatively short maturation period (typically up to 3-4 months).

Since VTEC are categorised as Hazard Group 3 Biological Agents, all the cheese-making procedures were modified to satisfy the stringent health and safety criteria required. At the same time, the cheese was made using methods recognisable to the cheesemaking industry.

Methods

(i) Cheesemaking protocol

1. Heat unpasteurised milk (10L) in vat to 32°C, add VTEC inoculum (a cocktail of *E. coli* O157 and *E. coli* O26 strains)
2. Add **starter** (MT36, Barbers, Somerset, England), allow the milk to ripen for 1 hour
3. Add **rennet** (Naturen Plus 175, Chr Hansen's) and stop stirring after 3 mins. Look for flocculation and development of "bouncy" curd
4. Using a sharp knife, **cut** curds into 1 cm^3 cubes. Stir for 10 mins
5. **Scald** the curds by heating to 34°C in 10 mins and hold at this temperature for 20-50 mins whilst gently stirring
6. Stop stirring and leave the curds to **pitch** for 10 minutes
7. Allow the whey to slowly **drain** from the vat. Gently squeeze the curd to remove the last of the whey
8. Cut the curd into large blocks and turn until the pH reaches less than 6.0. Once this is achieved, cut the curd into 2.5 cm^3 cubes and mix (mill) salt into the curd
9. After lining with cheese cloth, fill the cheese **mould** with curds
10. **Press** the curds into cheese over 16 hours at 22-25°C
11. Coat the cheese in **wax** and mature at 12°C

An important factor for deciding when the next step in the process should be started was the measurement of the pH and/or titratable acidity, which was done.

(ii) Isolation and enumeration of VTEC from cheese

Raw milk cheese contains a natural bacterial flora in addition to starter bacteria. The challenge was to isolate and enumerate VTEC strains from cheese containing high levels of background microflora. A variety of VTEC media and incubation temperatures were evaluated and the best combination of enrichment and plating media established.

Results

Each of the steps in the manufacture of Caerphilly cheese was simulated in a laboratory. The cheesemaking manipulations were adapted so that they could be carried out in the confines of a biological safety cabinet. Caerphilly cheeses were produced from raw milk inoculated with VTEC O157 and O26 strains of *E. coli*. Methods for determining the persistence of these strains during and post-production of the cheese have been selected. These are:

Detection	Enumeration
• Enrichment in modified Tryptone Soya Broth (mTSB) at 41.5°C	• Primary dilution in sodium citrate solution
• Immuno-magnetic separation (IMS) at 6 and/or 24 hours	• Further dilutions in MRD
• Streak onto:	• Spread plates (0.1ml) when levels are high
- ChromID O157 agar (bioMérieux) (O157 & O26)	• Pour plates (when levels fall)
- CTSMAC (O157)	• ChromID O157:H7 agar (O157/O26)
- CT-RMAC (O26)	• CT-RMAC (O26)

ChromID O157:H7 agar (37 & 41.5°C)
(*E. coli* O157: green colonies)

Conclusions

A procedure for investigating the persistence of VTEC through the Caerphilly cheese production process has been devised. The technique can be adapted to investigate the behaviour of other microorganisms in a range of cheese types.

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Food Standards Agency
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Reference

1 Baylis, C. L (2009) Review: Raw milk and raw milk cheeses as vehicles for infection by Verocytotoxin-producing *Escherichia coli*. *International Journal of Dairy Technology*, 62, 8, 293-307.

Campden BRI, Station Road, Chipping Campden, Gloucestershire, UK, GL55 6LD, Tel: +44(0)1386 842000 Fax: +44(0)1386 842100
www.campden.co.uk

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PART 2 WORK CARRIED OUT AT QUEENS UNIVERSITY BELFAST ON *M. bovis*

Survival of *Mycobacterium bovis* in UK-made raw milk cheeses.

1 Introduction

Human tuberculosis (HTB) is an insidious disease with nine million new cases occurring in the world every year resulting in an estimated two million deaths. In the UK the rates of HTB continue to increase and are at their highest for nearly thirty years, exceeding 9,000 per year with a rate of 40 per 100,000 or more in major urban areas. This is compounded by an increasing incidence of multidrug resistant cases approaching 2% in London, for example.

Although the vast majority of HTB is caused by *Mycobacterium tuberculosis*, up to 1% of non-pulmonary HTB is caused by *M. bovis*: both these organisms are Hazard Group 3 (HG3) pathogens. This 1% of HTB is likely to be an underestimate since HTB due to *M. tuberculosis* is clinically and pathologically similar to that caused by *M. bovis* and those laboratories that attempt culture from clinical samples routinely use media supplemented with glycerol, as the main carbon and energy source, which suppresses the growth of *M. bovis* (Table 1).

Table 1: Discriminatory phenotypic characteristics of *M. bovis* and *M. tuberculosis*[a]

	M. tuberculosis	***M. bovis***
Colony morphology and growth	Heaped up ('eugonic')	Flat ('dysgonic')
Oxygen metabolism	Aerobic	Microaerophilic
Growth enhanced by glycerol	Yes	No (suppressed)
Growth enhanced by pyruvate	No	Yes
Nitrate reduction	Yes	No
Niacin synthesis	Yes	No
Sensitivity to thiophene-2-carboxylic acid hydrazide (TCH)	No	Yes[b]
Sensitivity to isolicotinic acid hydrazide (INH)	No	Yes
Nicotinamidase	Positive	Negative
Natural resistance to pyrazinamide (PZA)	No	Yes[c]

[a] From de la Rua-Domenech 2006
[b] Goat adapted clones ('*M. bovis* subsp. *Caprae*') are resistant to low concentrations of TCH.
[c] Goat-adapted clones are susceptible to PZA.

Historically, the main route of transmission of *M. bovis* from animals to humans is through the consumption of raw milk or milk products. This is in the context that regulations to introduce pasteurization were promulgated in the 1940s and the time temperature combination was predicated to ensure lethality for *M. bovis* in a milk menstruum. This was later made more severe to take account of *Coxiella burnetii*, the causative agent of Q-fever. The risks however of contracting HTB through the consumption of raw milk or milk products still remain. A number of recent cases of culture positive HTB in the USA have been attributed to *M. bovis* (Fujamura Leite *et al.,* 2003; Centers Disease Control and Prevention, 2005). In both instances, epidemiological investigations indicated that the consumption of unpasteurized dairy products, including soft cheese originating from Mexico, may have accounted for these cases. A follow-up survey for the presence of *M. bovis* detected the viable organism in a panela-style cheese (n=204; Harris *et al.,* 2007; Kinde *et al.,* 2007). Hegarty *et al.,* (2002) also highlighted the risk associated with unpasteurized milk/cheese, citing 11 human cases of *M. bovis* HTB in Ireland in 1999 likely to be related to unpasteurized milk consumption. In addition, no doubt in order to clarify the position of the competent authority as a result of a legal challenge, the Food Safety Authority of Ireland (FSAI) has stated in a report that upon detection of tuberculosis in a herd, all cheese made from raw milk originating from that herd since the most recent inspection or negative herd tuberculin test should be regarded as unfit for human consumption (FSAI, 2008).

In the United Kingdom the incidence of bovine tuberculosis (BTB) is increasing with 4,894 new BTB cases recorded in England and Wales during 2011, a 3.6% increase on 4,723 cases during 2010 as reported by the annual zoonoses report (Department for Environment, Food and Rural Affairs, 2012). This is against a background of an increasing interest in artisan cheeses, some of which are made from raw milk (Kupiec and Revell, 1998). Currently approximately 250 artisan cheeses are manufactured for sale in the UK (FSA, 2006). This must be viewed in the context of a lack of published information of the survival kinetics of *M. bovis* during manufacture, ripening and storage of such cheeses and the absence of recognized culture or molecular methods for the detection of the organism from dairy products in general (FSAI, 2008). More recently the Advisory Committee on the Microbiological Safety of Food (ACMSF) was asked by the FSA to consider the risk posed to human health associated with *M. bovis* and raw milk, and raw milk products. In response a risk assessment was produced indicating that the risk of HTB acquired from raw milk and raw milk products had changed with the increased cases of BTB. However, it was assessed that that the risk to human health from *M. bovis* contamination in raw milk and raw milk products is very low (ACMSF, 2011).

There is limited published information on the number of *M. bovis* cells secreted in the milk or faeces of clinical or sub-clinical animals, and the maximum level of *M. bovis* in naturally contaminated milk is reported to be 10^4 cfu ml^{-1} (Kells & Lear, 1960). A parallel is therefore another member of the genus, *M. avium* subsp. *paratuberculosis* (MAP), which produces a disease of cattle called Johne's disease. In this case the number secreted directly in the milk is low (< 100 cfu ml^{-1}), but very high in the faeces of clinical animals (> 10^6 cfu g^{-1}). The human infective dose for *M. bovis* is regarded as low (10-100) for the respiratory route of infection but high (>10^6) for the gastrointestinal route.

2. Current evidence that *M. bovis* survives in cheese.

Mycobacterium bovis has been shown to be very resistant to acids and alkali and hence likely to survive in cheese (Rowe and Donaghy, 2008). Cheese naturally contaminated with the tuberculous agent was found to be still infective after 220, 305 and 120 days with Cheddar,

Tilsit and Bulgarian white cheese respectively and three months with Camembert (International Dairy Federation, 1980). Lafont and Lafont (1981) also reported the presence of viable *M. bovis* after three to four months ripening of blue-veined cheese artificially contaminated (1×10^4 cfu ml^{-1}) with the organism, despite a rapid reduction in numbers in the first fourteen days post-manufacture. It should be recognized that the origin of most of this data is somewhat dated i.e. before quantitative culture methods for *M. bovis* from dairy matrices had been properly developed and the taxonomic status of *M. bovis* and *M. tuberculosis* resolved.

3. Project objectives

It was considered important that the FSA acquire an evidence base for the survival kinetics of *M. bovis* during the manufacture and ripening of raw milk cheeses. This was to enable the FSA to provide cogent advice to Environmental Health Officers, artisan cheese manufacturers and the general public should a herd, producing milk destined for raw milk cheese production, have evidence of *M. bovis* infection.

Essentially the tasks were as follows:

a) Select strains of *M. bovis* that would allow maximum extrapolation of the results generated within a UK scenario.
b) Devise a medium or media to allow the enumeration of *M. bovis* from a milk menstruum and cheese matrix without an initial decontamination stage. Decontamination it was considered would induce too many injured organisms which would not grow with the selective agents present in the enumeration media leading to an under-estimate of the true viable, and hence infective, *M. bovis* cells present. The difficulty of the task was not under-estimated at the outset because of the more varied and probably larger background bacterial and fungal populations likely to be present as a result of the absence of pasteurization.
c) Devise protocols for the safe laboratory scale production of raw milk cheese within the constraints of category 3 containment conditions. In addition, that the resulting cheeses have physicochemical properties commensurate with commercially produced raw milk cheeses.
d) Design and execute the minimum number of experimental runs that would generate sufficient data for valid statistical analysis that could withstand subsequent peer review.

4. Approach adopted

It should be recognized that the Queen's University research group had previous experience of producing laboratory-scale cheese with MAP a Hazard Group 2 (HG2) organism (Donaghy *et al.*, 2004). The PSG was formed of artisan cheesemakers and other representatives of the UK dairy industry to provide credibility for the experimental design and execution, advice and facilitate dissemination of the results generated.

5. Experimental plan

The choice of Cheddar and Caerphilly was decided by the PSG to represent a hard and medium hard cheese chosen to partly overlap with the *E. coli* O157 element of the project who were also using Caerphilly in their work. The brining stage for the Caerphilly cheese was omitted to reduce risks for the researchers, after consultation with representatives of the PSG. Although it would have been desirable, for statistical reasons, to completely randomize both the cheesemaking i.e. order of Cheddar and Caerphilly and sequence of strains this was not possible because of time constraints on the project. Cheddar cheese takes considerably

longer to ripen than Caerphilly and hence the Cheddar cheeses were made initially. The cheeses, once made, were sealed in wax rather than vacuum packed in plastic film to facilitate sampling and for safety reasons. This was with the approval of the PSG. After receiving statistical advice from the Biometrics Department of AFBI it was considered that there should be three replicates of each cheese and strain combination making a total of eighteen experimental runs. It was also decided that these should use as high an initial inoculum as possible ($\approx 10^6$ cfu ml^{-1}) so that as many data points as possible could be gained during the ripening process before the viable count fell below the level of sensitivity of the counting method employed. Experiments using this level of inoculum will be referred to as high-inoculum investigations. The pH value of the raw milk was measured initially after collection and again after sixteen hours incubation at 22^{o}C. Microbiological testing was also performed on the raw milk viz total viable count, coliform count and count of lactic acid bacteria (LAB) in addition to a test for antibiotic residues. No raw milk sample showed evidence of antibiotic residues. The question of antibiotics was considered important since, even though the cheese starter cultures added would also be inhibited, it could be argued that the decline in viable *M. bovis* in the cheese was partly due to such residues and not the other inimical factors such as acid, salt and background microflora that pertain in cheese. The cheese was also subjected to proximate chemical analysis on Day 1.

6. Strains chosen and preparation of inoculum.
The three *M. bovis* isolates used during this investigation were

1. *M. bovis* AF2122/97
2. *M. bovis* VNTR0024(SB0130)
3. *M. bovis* VNTR001(SB0140)

These isolates were selected as being representative of the *M. bovis* genotypes responsible for herd breakdowns in the UK and ROI.

6.1 Nomenclature and description of *M. bovis* isolates
Both *M. bovis* AF2122/97 and VNTR001(SB0140) have the spoligotype SB0140 (also known in GB as VLA9). The third isolate *M. bovis* VNTR0024(SB0130) has a spoligotype SB0130. All spoligotypes are named according to an agreed international convention (www.mbovis .org), and spoligotyping is a method of *M. bovis* typing based on strain-dependent hybridization patterns (Kamerbeek *et al.,* 1997). It is believed that the SB0140 is descended from the SB0130 spoligotype although it is unclear whether this was due to the loss of four spoligo spacers in one event or the loss of single spacers on four separate occasions.

Further discrimination between spoligotypes can be achieved by use of multi-locus variable number tandem repeat (VNTR) profiling (Skuce *et al.,* 2010). For example, the three isolates have three different VNTR profiles AF2122/97, VNTR0024 and VNTR001. The spoligotype is retained within the nomenclature to give the genotype names VNTR0024(SB0130) and VNTR001(SB0140). The shorter local designations used in previous reports during this investigation G24.130 and G1.140 relate to VNTR0024(SB0130) and VNTR001(SB0140) respectively. For convenience the shorter local designations AF2122, G24.130 and G1.140 will be used for subsequent sections of this report.

6.1.1 *M. bovis* AF2122/97

M. bovis AF2122/97 was first isolated in 1997 in the south west of England, and was fully sequenced in 2002 (Garnier *et al.,* 2003). As mentioned above AF2122/97 has a SB0140 spoligotype which is the most common GB spoligotype representing 37% of isolates in a structured GB population survey (2005). The AF2122/97 VNTR profile was detected at 2% making it the 4th most common VNTR type in the UK.

6.1.2 *M. bovis* VNTR0024(SB0130)
M. bovis VNTR0024(SB0130) was isolated in Northern Ireland (NI) from an animal imported from the Republic of Ireland (ROI). The SB0130 spoligotype was detected at 15% prevalence in a ROI survey (2005). However, the most common spoligotype in the ROI, is again SB0140 at 60% of *M. bovis* detected. The VNTR0024 profile was present at 8% of the *M. bovis* detected in the ROI and <1% in Northern Ireland when surveyed (2005).

6.1.3 *M. bovis* VNTR001(SB0140)
The SB140 spoligotype constituted 56% of the *M. bovis* isolates in NI (2005). *M. bovis* VNTR001(SB0140) was the most common genotype (26%) when this nomenclature was first applied to *M. bovis* in 2003. The proportion of VNTR001(SB0140) has now fallen to 9% (2010).

6.2 Preparation of inoculum
The working culture was treated to disrupt clumps of cells and leave a suspension of individual planktonic organisms which, it was considered, would equate better during the challenge test to the survival of individual cells. Inoculum stock for the three *M. bovis* genotypes was prepared from mid-log phase cultures grown on bi-phasic medium of 7H11 agar, overlaid with 7H9 broth supplemented with 10% acid–dextrose complex and 0.05% Tween-80. Once the cultures had reached mid-log phase, the cells were fully suspended in the 7H9 broth. The *M. bovis* suspensions in 7H9 broth were then centrifuged and the resultant pellets washed three times in 200 ml of PBS. After washing the pellets were resuspended in a final volume of 500 ml PBS. This suspension was then passed through a 5 µm pore size syringe filter to remove clumps and cords of bacilli before storage of 10 ml aliquots at -80°C. Method used was adapted from Rodgers *et al.,* (2007).

7. Methods
The methods for media preparation, cheese production and *M. bovis* enumeration including the standard operating procedures can be found in Appendix 1.

8. Results and discussion.
8.1 Evaluation of *M. bovis* selective media for use with cheese
One of the objectives of the project was to identify a medium or media that would maximize enumeration of *M. bovis* from the two cheese matrices, without recourse to an initial decontamination phase. There is currently no recognized medium for this purpose. Two main media types are available for the culture of Mycobacteria viz. egg based and agar albumin based media. After consideration no egg based media was included in the trial due to the more complex method of media production and problems due to inactivation of antimicrobials during inspissation (Mitchison *et al.,* 1972); inspissation is a heating step of 70 – 80 °C and is responsible for the coagulation of the egg albumin in egg based media.

The agar based media selected for further investigation were:

1. Middlebrook 7H11 agar

Derived by the addition of casein hydrolysate to Middlebrook 7H10 mycobacterial medium (Cohn *et al.*, 1968). This agar is often used for the isolation *M. bovis* from clinical samples (Rowe & Donaghy, 2008).

2. Gallaghers medium

A modification of Middlebrook 7H11 agar improved for the growth of *M. bovis* from clinical samples by the addition 10% (v/v) bovine calf serum, 0.5% (v/v) lysed defibrinated sheep blood and 0.0035% (w/v) malachite green (Gallagher & Horwill, 1977).

Two different antimicrobial supplement combinations were used in conjunction with these media: commercially available PANTA (Becton Dickenson) and laboratory prepared PCAT. The concentrations of antimicrobials in PANTA and PCAT had been carefully assessed previously empirically for their effect on growth of mycobacteria and their ability to reduce non-mycobacterial contamination (Siddiqi *et al.*,1986, Mitchison *et al.*, 1972). Furthermore, in conjuction with Middlebrook 7H10, PANTA had already been evaluated for recovery of MAP from Cheddar cheese (Donaghy *et al.*, 2003). As the two agars and the antimicrobial combinations PANTA/PCAT had been developed for mycobacteria, or specifically for *M. bovis*, it was felt these presented the best options for diagnostic media that would maximize recovery from *M. bovis* contaminated cheese.

The main focus of this preliminary investigation of media was to determine how the individual types would perform when challenged with non-mycobacterial contaminants. This was achieved by producing cheeses inoculated with the three individual *M. bovis* types. Furthermore, production of these cheeses allowed method refinement before commencing the main challenge test investigation. Each *M. bovis* isolate was also enumerated on non-selective and selective media. The growth of *M. bovis* AF2122, G24.130 and G1.140 inoculum on non-selective and selective Middlebrook 7H11 and Gallaghers media is shown in Fig. 1(a).

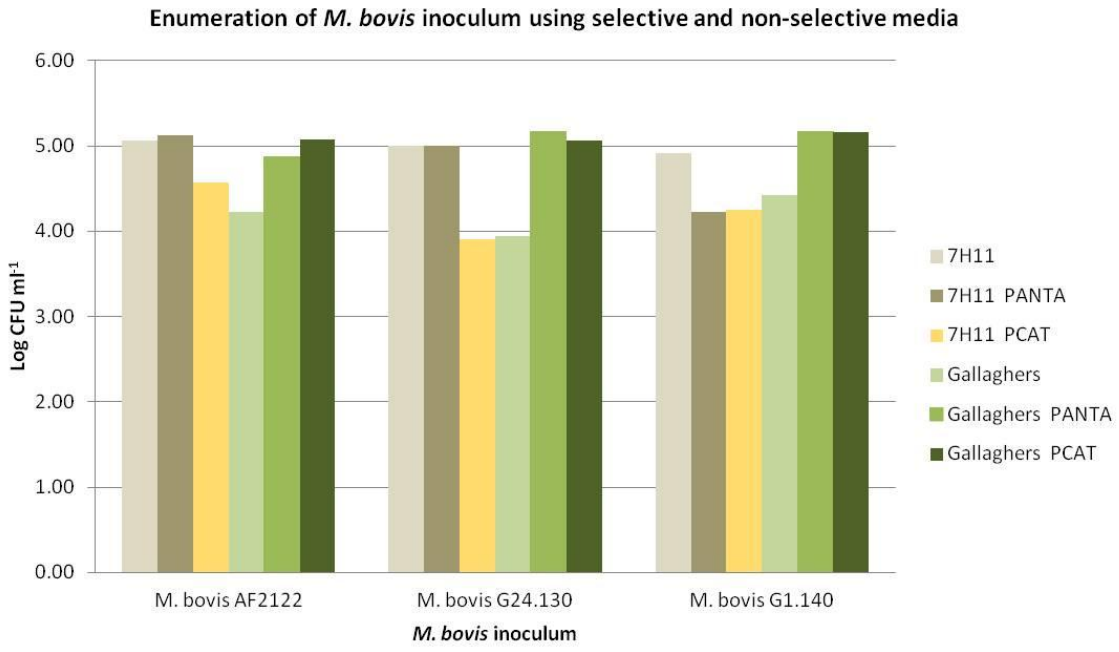

Fig. 1(a). Isolation of *M. bovis* AF2122, G24.130 and G1.140 inoculum using non-selective 7H11 and Gallaghers media and selective 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA, and Gallaghers PCAT media.

These results demonstrated that the selective media supported the growth of the target *M. bovis* isolates producing typical colony morphology. Non-selective Gallaghers medium was outperformed by selective Gallaghers media whilst only 7H11 PCAT appeared to have a consistent inhibitory effect on target *M. bovis* when compared to the non-selective media 7H11.

Subsequently the selective media types were challenged with liquid samples obtained during the cheesemaking process i.e. homogenized cheese samples obtained after Day 1 of maturation and further maturation samples taken at regular intervals. These samples contained not only the *M. bovis* target organism, but also contaminants and competitors originating from the raw milk and cheese starter. It was considered that this properly assessed the ability of the selective media to prevent growth of the raw milk, whey and cheese microflora whilst permitting the growth of *M. bovis*. Using enumeration data of *M. bovis* inocula obtained on selective media it was possible to estimate recoverable numbers when a known volume of *M. bovis* culture was used for the subsequent inoculation of the cheese vats containing eight litres of raw milk.

The results of isolation of *M. bovis* AF2122, G24.130 and G1.140 from raw milk are shown in Fig. 1(b) and from whey are shown in Fig. 1(c)

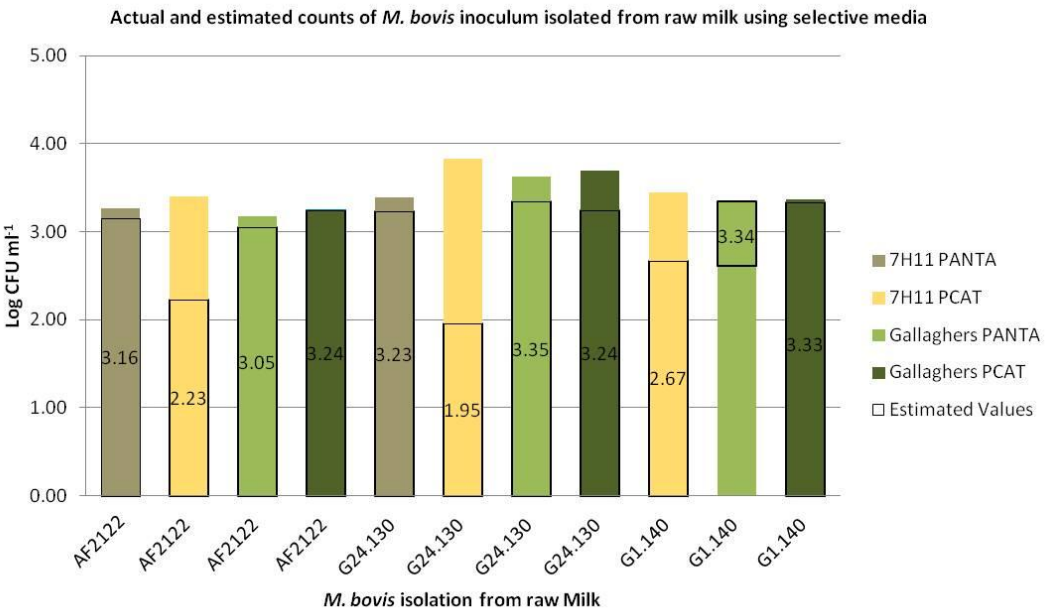

Fig. 1(b). Isolation of *M. bovis* AF2122, G24.130 and G1.140 from raw milk used for Cheddar cheese production using selective media: 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA and Gallaghers PCAT. The estimated values are represented by the black boundary and data labels.

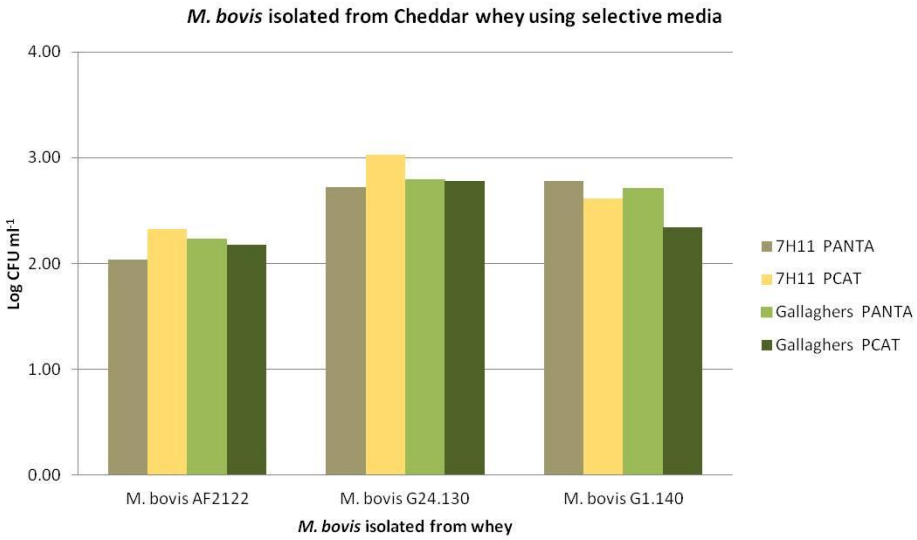

Fig. 1(c). Isolation of *M. bovis* AF2122, G24.130 and G1.140 from whey obtained from Cheddar cheese production using selective media: 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA and Gallaghers PCAT.

The identity of *M. bovis* isolates was confirmed by colony morphology, Ziehl-Neelsen staining and VNTR molecular typing. In all raw milk *M. bovis* samples, except *M. bovis* G1.140 isolated using Gallaghers PANTA medium, the actual recovery of the target organism was equal to or exceeded the estimated values. This would suggest that presence of contaminants and competitor microflora in the raw milk and starter culture do not inhibit the isolation of *M. bovis* from raw milk using the media types described above. Furthermore, when isolating *M. bovis* from whey samples, growth of contaminants was limited and easily distinguished from *M. bovis* by colony morphology.

Overall enumeration of *M. bovis* inocula and isolation of *M. bovis* from raw milk and whey demonstrated that the selective media types allowed good recovery of *M. bovis* in the presence of contaminating microflora without serious inhibition of the target organism. When the selective media were challenged with Day 1 Cheddar cheese homogenate containing *M. bovis* no medium consistently outperformed the others. However, after approximately Day 40 differences in media performance became apparent when the isolation of *M. bovis* on 7H11 PANTA and 7H11 PCAT were superior to Gallaghers PANTA and Gallaghers PCAT, see Fig. 2(a) to Fig. 2(c) below.

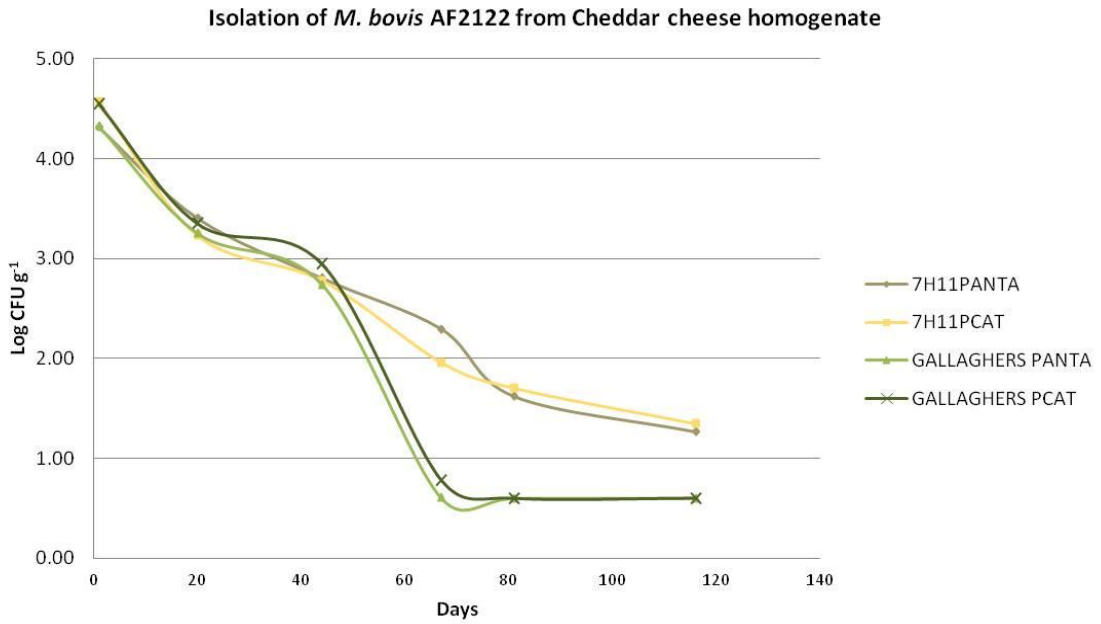

Fig. 2(a). Isolation of *M. bovis* AF2122 from Cheddar cheese homogenate from Day 1 to Day 116. *M. bovis* AF2122 was isolated using 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA, Gallaghers PCAT.

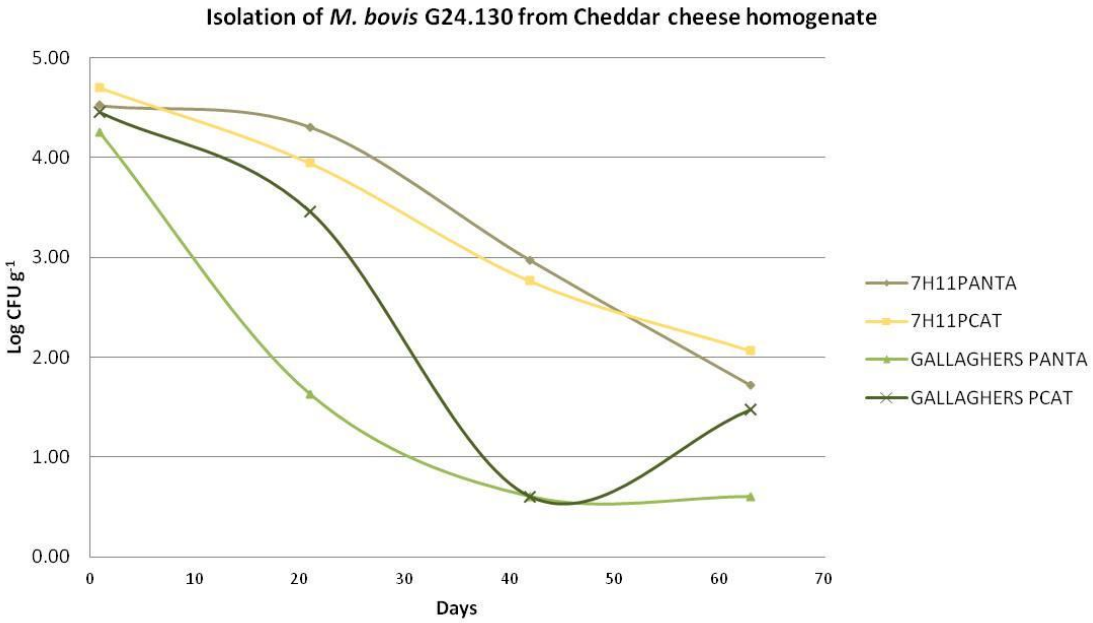

Fig. 2(b). Isolation of *M. bovis* G24.130 from Cheddar cheese homogenate from Day 1 to Day 63. *M. bovis* AF2122 was isolated using 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA, Gallaghers PCAT.

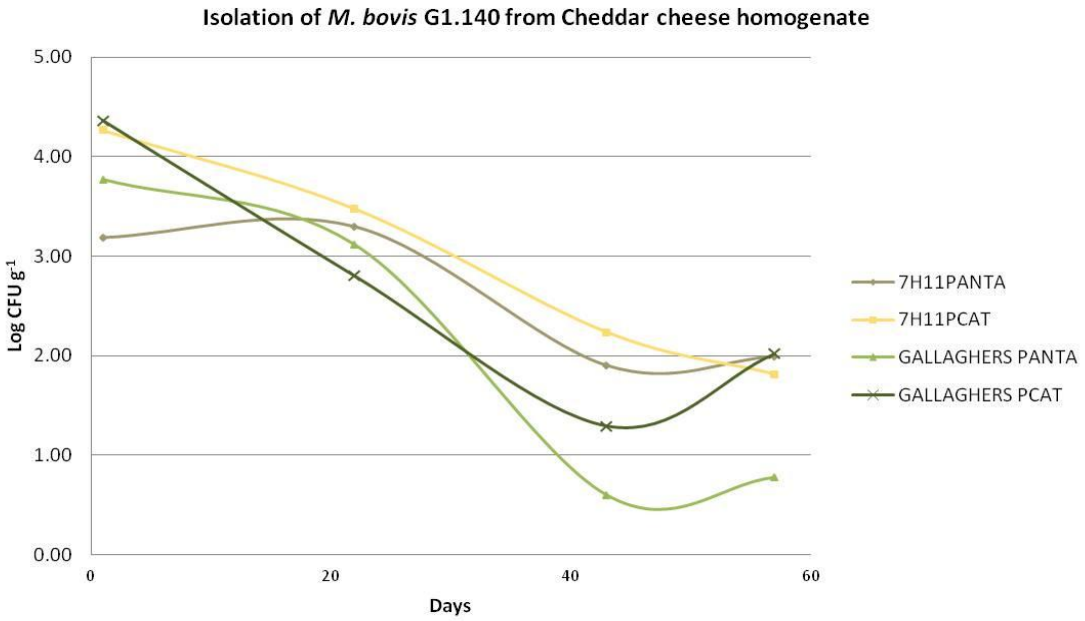

Fig. 2(c). Isolation of *M. bovis* G1.140 from Cheddar cheese homogenate from Day 1 to Day 57. *M. bovis* AF2122 was isolated using 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA, Gallaghers PCAT.

M. bovis enumeration data obtained from each cheese homogenate from Day 1 onwards was statistically analyzed by repeated measures analysis using REML on GenStat Release 14.2 for Windows 7 (The correlation between the points was assessed using an antidependence model of order one). The results of the analysis are presented in Table 2 below and a full output of the statistical analysis can be found in Appendix 2

Table 2: Results of Repeated Measure Analysis of *M. bovis* enumeration data obtained from Cheddar cheese homogenates using various selective Media - Selective Media Predicted Means, Standard Error of differences and *P* value.

Media Type	Predicted Means for Media (log cfu g^{-1})	Average Standard Errors of Differences	*P* value
7H11 PANTA	2.677	0.2342	<0.001
7H11 PCAT	2.721		
Gallaghers PANTA	1.877		
Gallaghers PCAT	2.345		

Statistical analysis of the enumeration data indicates that there is a significant difference in *M. bovis* recovery between the selective media types. Furthermore predictive mean values of 2.677 log cfu g^{-1} for 7H11 PANTA and 2.721 log cfu g^{-1} for 7H11 PCAT are greater than the predicted means for Gallaghers PANTA 1.877 log cfu g^{-1} and Gallaghers PCAT 2.345 log cfu g^{-1}. These predicted means values for media are indicative of a selective medium's ability to culture *M. bovis* with higher values associated with greater recovery of the target organism. Therefore the statistical analysis supports the observation made above that 7H11 PANTA and 7H11 PCAT outperform Gallaghers PANTA and Gallaghers PCAT in the isolation of *M. bovis* from cheese homogenate.

Gallaghers medium is produced from Middlebrook 7H11 by the addition of three components, two encouraging *M. bovis* growth (bovine calf serum & sheep blood) with the

third malachite green an antimicrobial agent acting against contaminants. It was felt that the increased quantity of the inhibitory compound malachite green could be responsible for the lower recovery of *M. bovis* using Gallaghers PANTA and Gallaghers PCAT. Malachite green was therefore omitted and the new medium designated 7H11++ before testing against further Cheddar cheese homogenate samples. Elevated recovery of *M. bovis* was subsequently observed using 7H11++ PANTA and 7H11++ PCAT without apparent loss in selectivity as shown in Fig. 3(a) to Fig. 3(c).

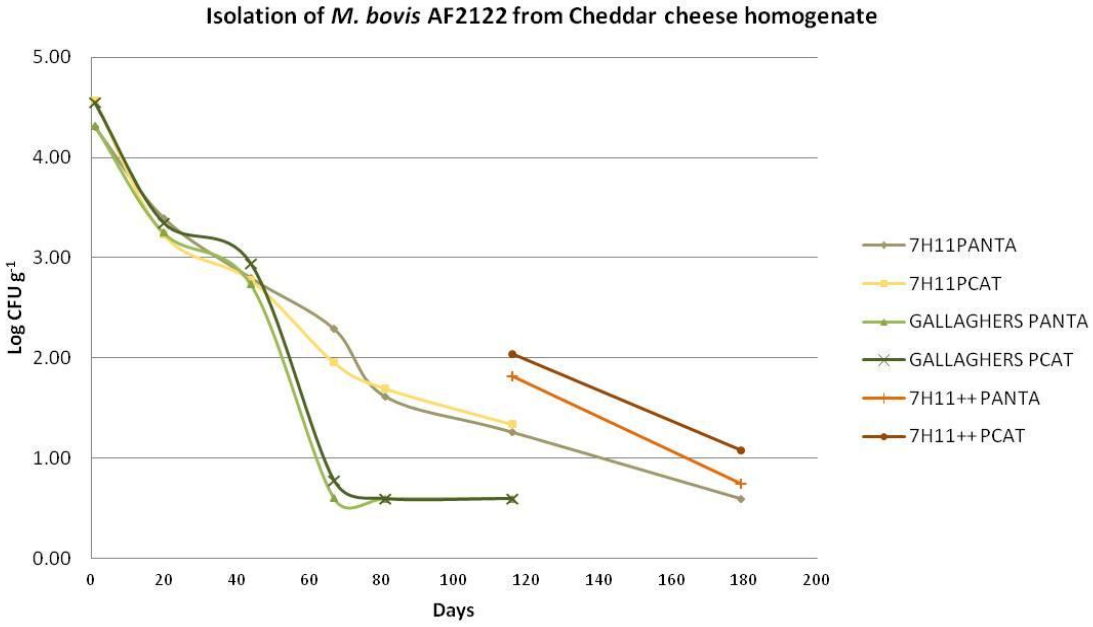

Fig. 3(a). Isolation of *M. bovis* AF2122 from Cheddar cheese homogenate Day 1 to Day 179. *M. bovis* AF2122 was isolated from Day 1 to Day 120 using 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA and Gallaghers PCAT. From Day 120 to Day 179 *M. bovis* AF2122 was isolated using 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

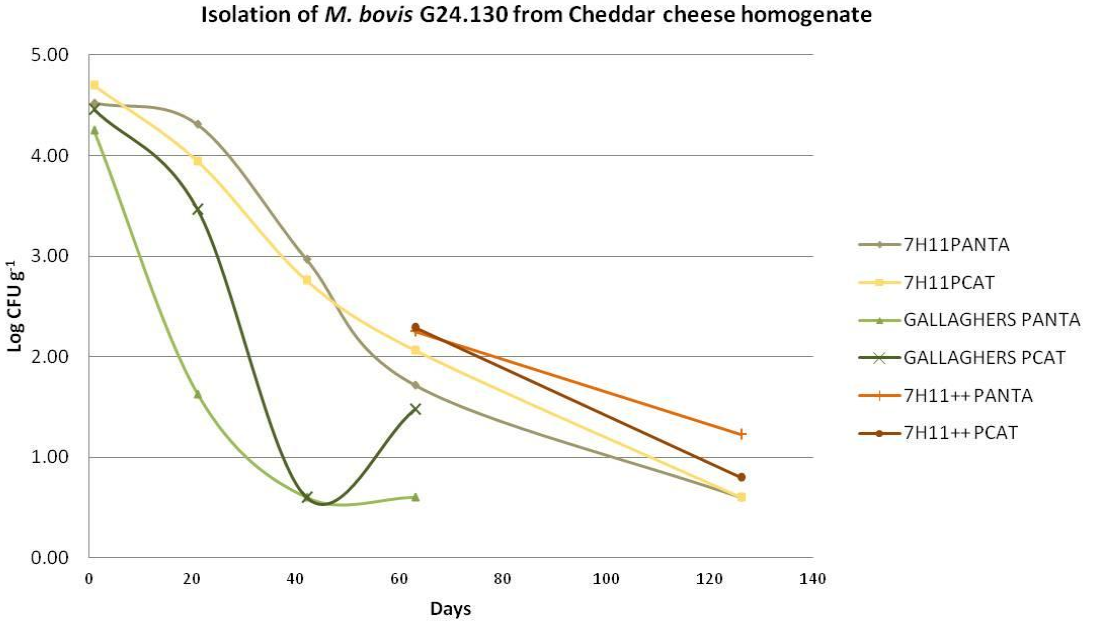

Fig. 3(b). Isolation of *M. bovis* G24.130 from Cheddar cheese homogenate Day 1 to Day 126. *M. bovis* G24.130 was isolated from Day 1 to Day 63 using 7H11 PANTA, 7H11 PCAT, Gallaghers

PANTA and Gallaghers PCAT. From Day 63 to Day 126 *M. bovis* G24.130 was isolated using 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

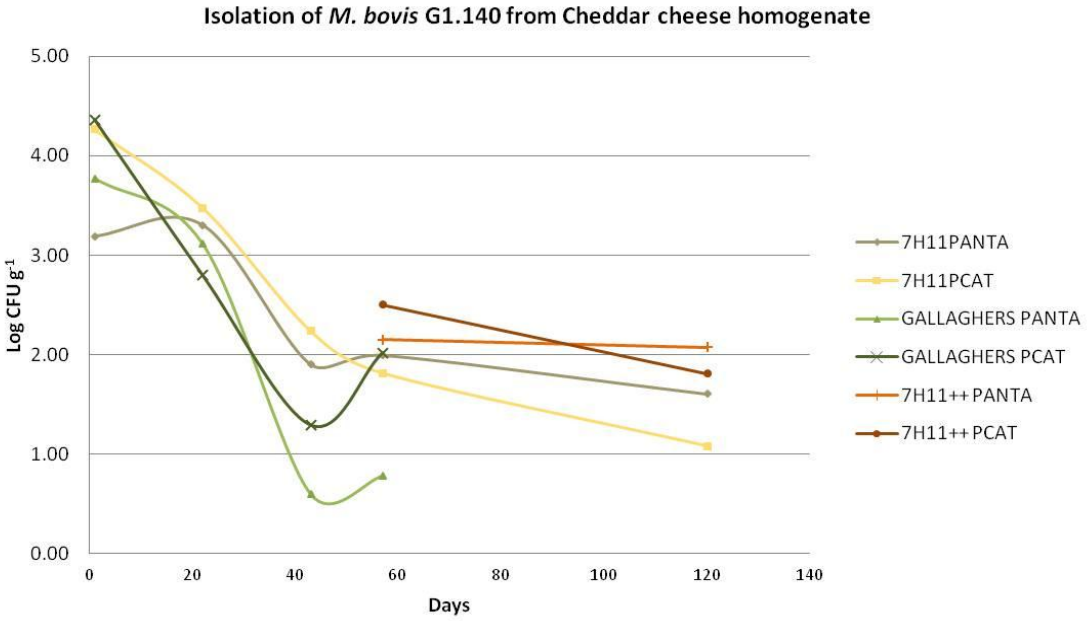

Fig. 3(c). Isolation of *M. bovis* G1.140 from Cheddar cheese homogenate Day 1 to Day 120. *M. bovis* G1.140 was isolated from Day 1 to Day 57 using 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA and Gallaghers PCAT. From Day 57 to Day 120 *M. bovis* G1.140 was isolated using 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

The growth of *M. bovis* AF2122, G24.130 and G1.140 inocula was also assessed on non-selective and selective 7H11++ as shown below Fig. 4(a). These results again demonstrated that the selective media supported the growth of the target *M. bovis* isolates producing typical colony morphology. However, selective 7H11++ PCAT medium appeared to be less productive than non-selective 7H11++ and selective 7H11++ PANTA.

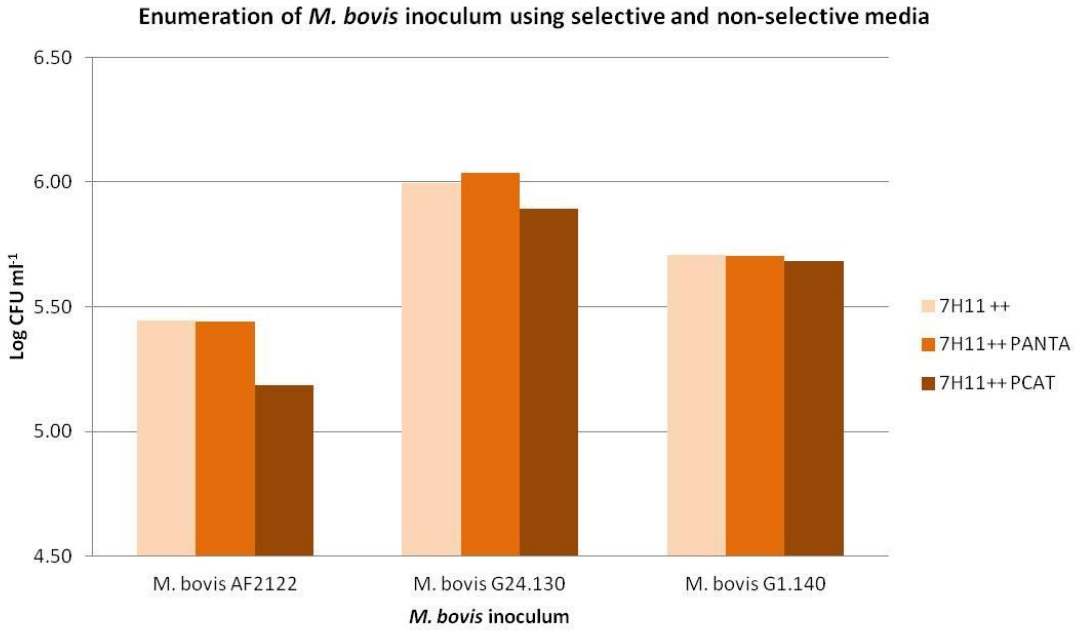

Fig. 4(a). Isolation of *M. bovis* AF2122, G24.130 and G1.140 inoculum using non-selective 7H11++ and selective 7H11++ PANTA and 7H11++ PCAT media.

Isolation of *M. bovis* from Cheddar cheese homogenate demonstrated that selective Middlebrook 7H11 media performed better than selective Gallaghers media. As a result the media assessment exercise using Caerphilly cheese was completed using only selective Middlebrook 7H11 media and selective 7H11++ media.

Selective media were assessed with raw milk and whey samples obtained during Caerphilly laboratory-scale production. Again it was possible to estimate *M. bovis* recovery for comparison with actual numbers isolated from raw milk used in cheesemaking. The results of isolation of *M. bovis* AF2122, G24.130 and G1.140 from raw milk are shown in Fig. 4(b) and from whey Fig. 4(c).

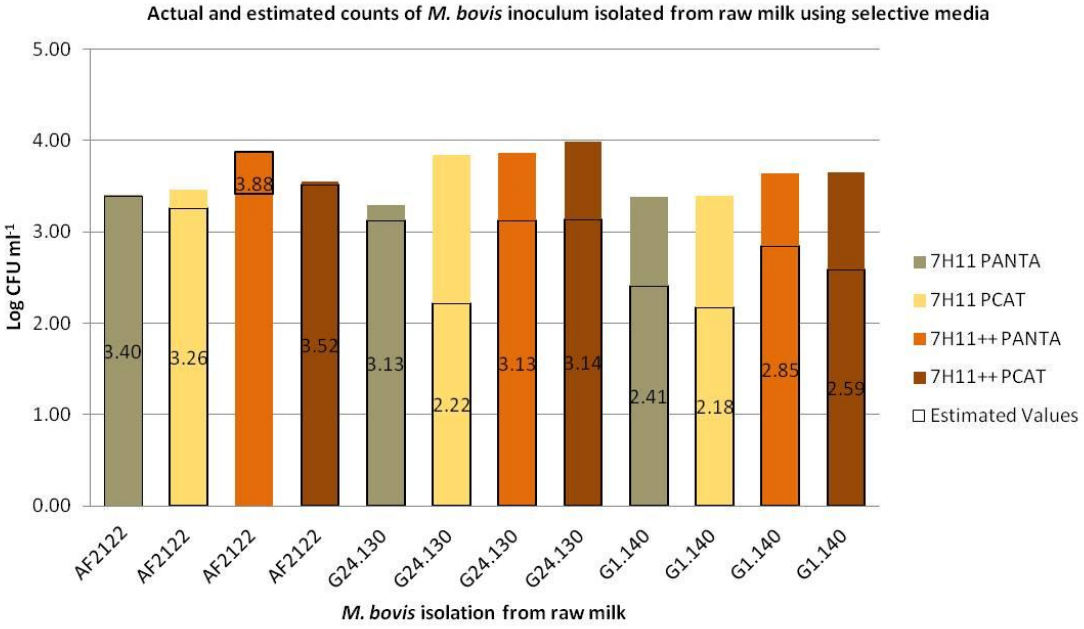

Fig. 4(b). Isolation of *M. bovis* AF2122, G24.130 and G1.140 from raw milk used for Caerphilly cheese production using selective media: 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT. The estimated values are represented by the black boundary and data labels.

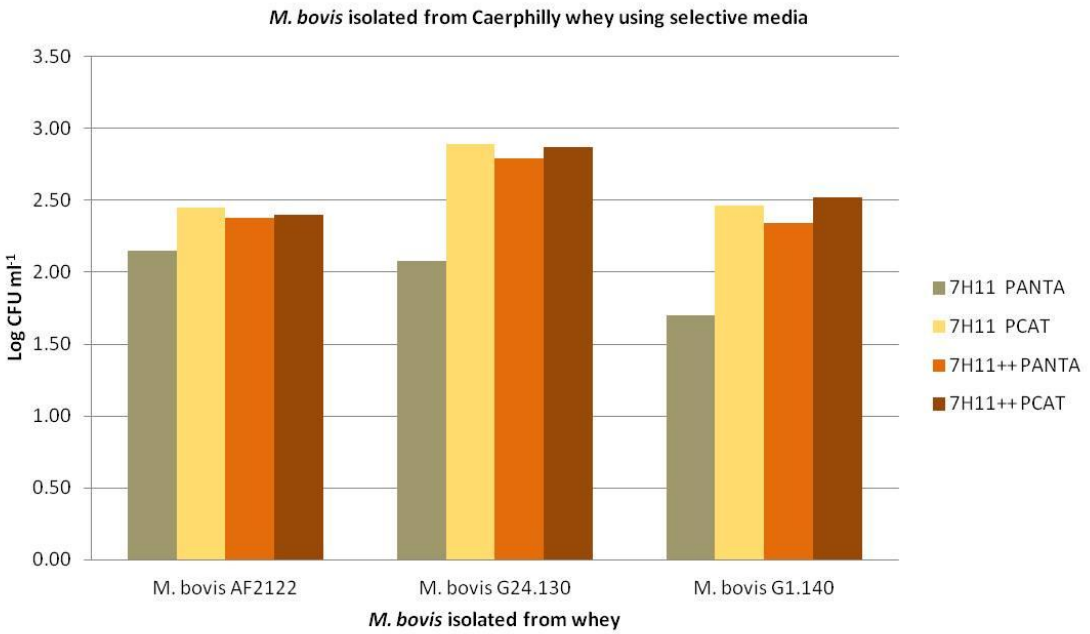

Fig. 4(c). Isolation of *M. bovis* AF2122, G24.130 and G1.140 from whey obtained from cheddar cheese production using selective media: 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

The identity of *M. bovis* isolates was confirmed by colony morphology, Ziehl-Neelsen staining and VNTR molecular typing. When sampling raw milk and *M. bovis*, 7H11++ selective media appeared to marginally outperform selective Middlebrook 7H11. For all selective media types actual recovered numbers of *M. bovis* from raw milk were consistently equal to or greater than the estimated values as indicated by the black border and data labels in Fig. 4(b). The only exception was *M. bovis* AF2122 isolated using 7H11++ PANTA which did not achieve the estimated value of 3.88 log cfu ml^{-1}.

Sampling of Caerphilly whey samples (Fig. 4(c)) allowed good recovery of target *M. bovis* with minimal contamination. However, it appeared that the Middlebrook 7H11 PANTA had a slightly inhibitory effect on all *M. bovis* types compared to the other selective media. It should be noted that significant levels of the three *M. bovis* isolates were detected in the whey fraction for both Cheddar and Caerphilly. When calculated from cheeses made for the media trial the average percentage loss, across the three *M. bovis* types, for Cheddar was 13.2% and 7.4% for Caerphilly. Hence the destination and use of the whey component should also be taken into account should a consignment of raw milk cheese be suspected of containing viable *M. bovis.*

Selective Middlebrook 7H11 and 7H11++ media were also tested for the isolation of *M. bovis* AF2122, G24.130 and G1.140 from Caerphilly cheese homogenates obtained after Day 1 of maturation up to approximately Day 87. Fig. 5(a) to Fig. 5(c) below show the isolation of *M. bovis* from Caerphilly cheese homogenates using Middlebrook 7H11 PANTA, Middlebrook 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

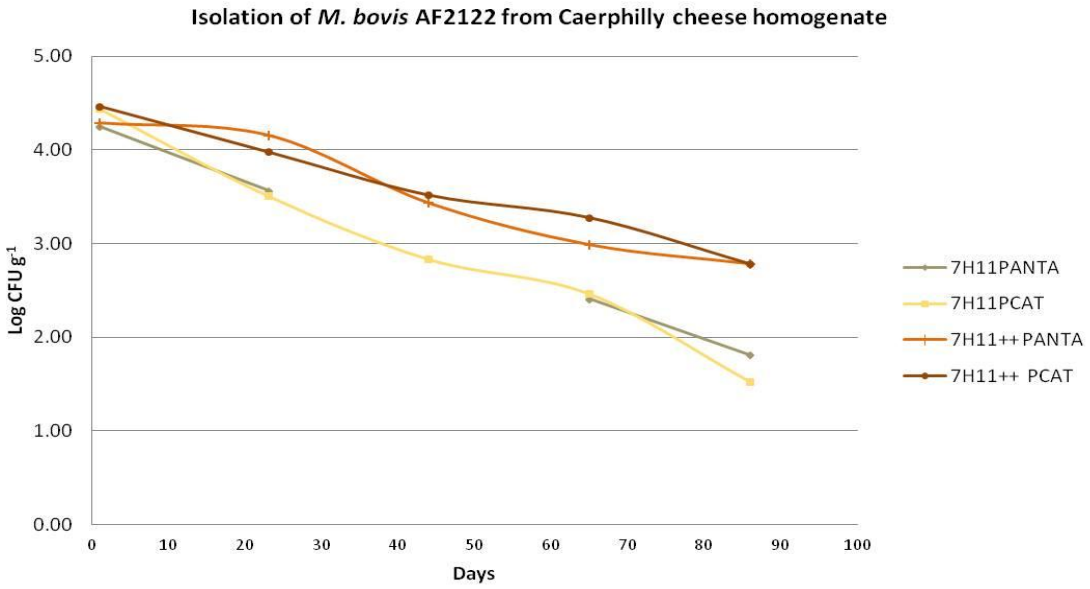

Fig. 5(a). Isolation of *M. bovis* AF2122 from Caerphilly cheese homogenate Day 1 to Day 86. *M. bovis* AF2122 was isolated using 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

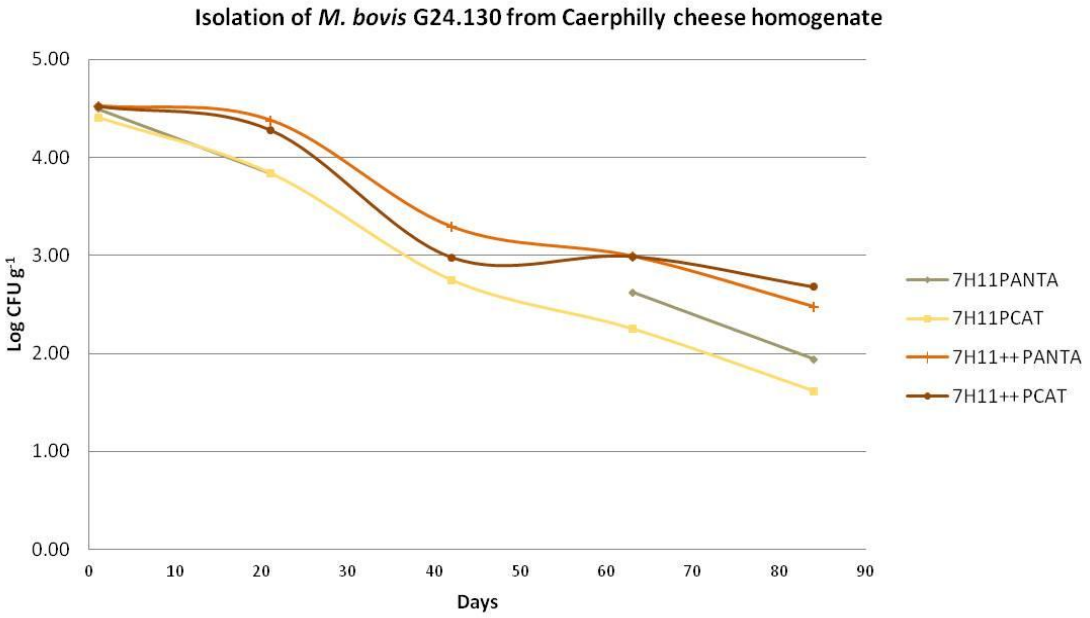

Fig. 5(b). Isolation of *M. bovis* G24.130 from Caerphilly cheese homogenate Day 1 to Day 84. *M. bovis* G24.130 was isolated using 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

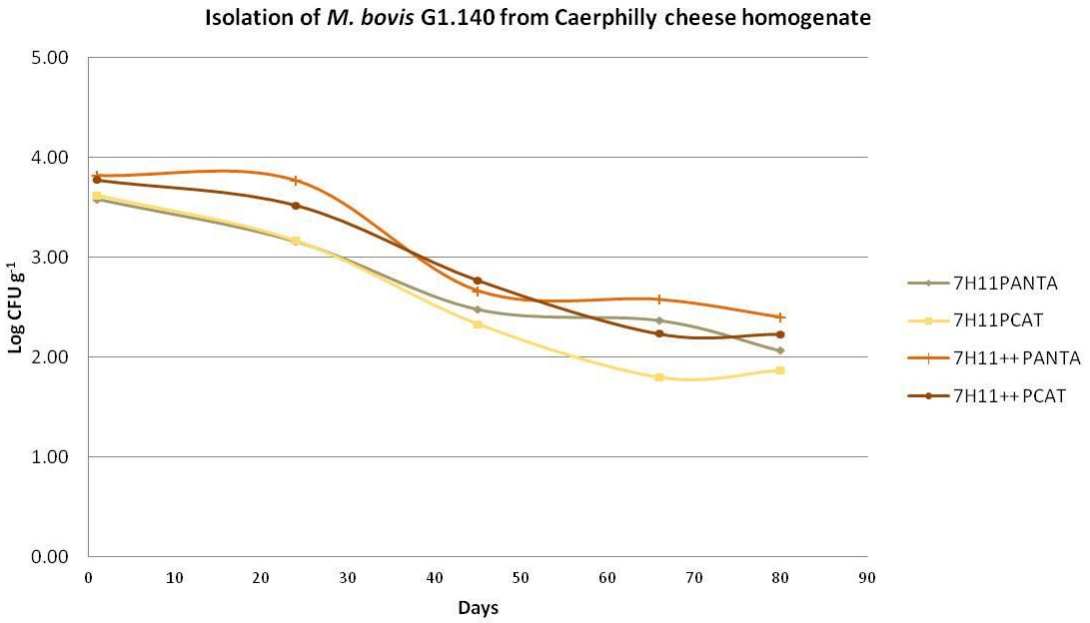

Fig. 5(c). Isolation of *M. bovis* G1.140 from Caerphilly cheese homogenate Day 1 to Day 80. *M. bovis* G1.140 was isolated using 7H11 PANTA, 7H11 PCAT, 7H11++ PANTA and 7H11++ PCAT.

The *M. bovis* enumeration data obtained from each Caerphilly cheese homogenate was statistically analyzed as before. The full output of the statistical analysis can be found in Appendix 2 and the results of the repeated measures analysis are presented in Table 3 below.

Table 3: Results of Repeated Measure Analysis of *M. bovis* enumeration data obtained from Caerphilly cheese homogenates using various selective Media - Selective Media Predicted Means, Standard Error of differences and *P* value.

Media Type	Predicted Means for Media (log cfu g^{-1})	Average Standard Errors of Differences	*P* value
7H11 PANTA	2.943	0.1259	<0.001
7H11 PCAT	2.826		
7H11++ PANTA	3.368		
7H11++ PCAT	3.332		

From *M. bovis* enumeration data presented in Fig. 5(a) to Fig. 5(c) it appeared that 7H11++ PANTA and 7H11++ PCAT outperformed selective Middlebrook 7H11 media types. This was confirmed by the statistical analysis, which revealed a significant difference between media types, and identified 7H11++ PANTA and 7H11++ PCAT as having the highest predicted means for isolation of *M. bovis* from Caerphilly cheese homogenate.

It was therefore clear that 7H11++ PANTA and 7H11++ PCAT were successful in suppressing the growth of competitor bacteria present in the raw milk and cheese whilst enabling good growth of target *M. bovis*. A final analysis was conducted of the six media types using cheese homogenate obtained from three *M. bovis* inoculated Cheddars. *M. bovis* AF2122 was chosen as the sequenced strain since the SB0140 spoligotype is representative of the most common GB spoligotype. Each cheese was sampled on three occasions on Day 1 and approximately Day 55 and Day 110. Fig. 6 below plots mean values (log cfu g^{-1}) of *M. bovis* AF2122 isolated from each Cheddar homogenate using the six selective media types.

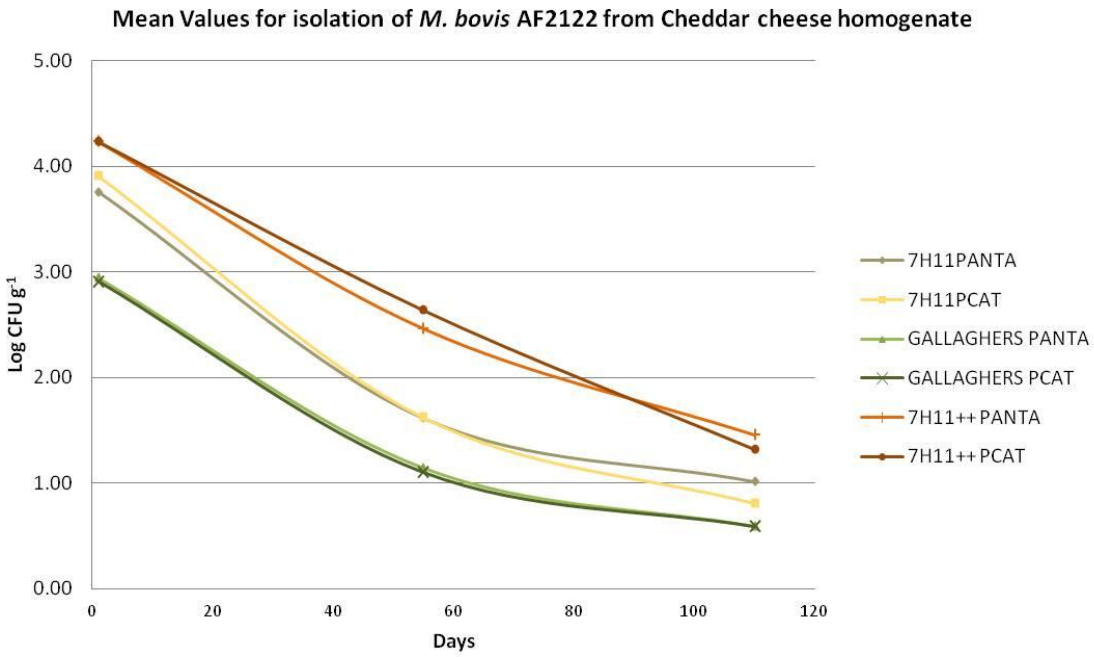

Fig. 6. Isolation of *M. bovis* AF2122 from Cheddar cheese homogenate Day 1, Day 55 and Day 110. *M. bovis* AF2122 was isolated using 7H11 PANTA, 7H11 PCAT, Gallaghers PANTA, Gallaghers PCAT, 7H11++ PANTA and 7H11++ PCAT.

The enumeration data obtained from the three *M. bovis* AF2122 inoculated Cheddars was also analyzed using repeated measures analysis (Table 4). The full output of the statistical analysis can be found in Appendix 3

Table 4: Results of Repeated Measure Analysis of *M. bovis* AF2122 enumeration data obtained from Cheddar cheese homogenates using various selective Media - Selective Media Predicted Means, Standard Error of differences and *P* value.

Media Type	Predicted Means for Media (log cfu g^{-1})	Standard Errors of Differences	*P* value
7H11 PANTA	2.127	0.1145	<0.001
7H11 PCAT	2.114		
Gallaghers PANTA	1.560		
Gallaghers PCAT	1.534		
7H11++ PANTA	2.717		
7H11++ PCAT	2.730		

This analysis confirms, that in addition to providing the best recovery of *M. bovis* from Caerphilly, 7H11++ media offers the greatest recovery from Cheddar cheese homogenates. 7H11++ PANTA and 7H11++ PCAT provided similar predictive means of 2.717 log cfu g^{-1} and 2.730 log cfu g^{-1}, outperforming the next best media 7H11 by a minimum of 0.590 log cfu g^{-1}. It was observed, that often when non *M. bovis* contaminants inhibited *M. bovis* enumeration from a sample using 7H11++ PANTA or PCAT, an identical sample applied to 7H11++ containing the alternative selection combination would be contamination free, and allow successful *M. bovis* enumeration. On this basis it was determined that both

7H11++PANTA and 7H11++PCAT selective media would be used during the challenge test phase of the investigation.

Using laboratory prepared Cheddar and Caerphilly provided multiple cheese homogenate samples. This allowed media selectivity to be assessed during cheese maturation when the cheese microflora and hence competitor bacteria profile develops and changes. *M. bovis* will not grow in the cheese matrix, therefore it is axiomatic that target cells isolated from cheese homogenates will have originated from the *M. bovis* inoculum introduced at the time of cheesemaking. During the cheesemaking and maturation phase *M. bovis* may become stressed or damaged. This may provide an explanation for the performance of some selective media, which initially allowed good recovery of target *M. bovis* that subsequently diminished following longer periods of cheese ripening. When cheese homogenate was applied to selective Gallaghers media the presence of additional malachite green produced a medium which was overly stringent and therefore had limited efficacy after a period of time. Also Middlebrook 7H11 PANTA and 7H11 PCAT lacked the addition factors for promotion of *M. bovis* growth (bovine calf serum & sheep blood). This, it was considered, would have diminished the ability of the 7H11 media to recover stressed *M. bovis* that remained viable under the more favourable growth conditions of 7H11++.

8.2 Cheddar cheese manufacture

Raw milk was collected from the dairy on the day before cheese production. It was suggested, by the members of the PSG, that the raw milk be assessed for the potential of the natural microflora to play a role in acidification of the milk during cheesemaking. The method followed is used by traditional cheesemakers for this purpose. Samples of the raw milk were removed to 10 ml screw cap test tubes and the pH measured before and after sixteen hours incubation at 22°C. The average milk pH prior to incubation was 6.63 and post incubation the average pH was 5.68, thus indicating that the natural microflora could play a positive role during the milk ripening step.

On the morning of cheese production and prior to the addition of *M. bovis* an aliquot of uncontaminated milk was removed for microbiological analysis. Milk was assessed for TVC, coliforms, LAB and antimicrobial compounds. The bacterial counts and raw milk measurement are shown in Table 5. Each of the nine Cheddars was named according to the *M. bovis* strain with which it was inoculated and numbered in sequence eg AF2122 CH1, AF2122 CH2, etc.

Table 5: Microbiological assessment and pH measurement of raw milk prior to Cheddar cheesemaking. All enumeration values are recorded as cfu ml^{-1}.

Cheese	AF2122 CH1	AF2122 CH2	AF2122 CH3	G24.130 CH1	G24.130 CH2	G24.130 CH3	G1.140 CH1	G1.140 CH2	G1.140 CH3
TVC	1.5×10^6	6.6×10^4	1.6×10^6	7.5×10^4	1.6×10^7	1.5×10^5	1.0×10^7	5.8×10^5	1.3×10^7
Coliforms	1.0×10^5	2.4×10^3	9.9×10^3	5.0×10^3	1.9×10^5	1.6×10^4	2.0×10^5	3.8×10^3	1.3×10^4
LAB	1.2×10^4	1.9×10^4	1.3×10^4	5.2×10^3	6.2×10^4	3.1×10^4	1.4×10^6	4.1×10^3	1.2×10^4
pH Milk	6.63	6.69	6.58	6.73	6.75	6.82	6.85	6.81	6.75

Titratable acidity (TA), recorded as percentage lactic acid, was determined during cheesemaking as a method of monitoring acid development. TA was measured from milk before addition of starter, after the ripening step and from the whey after cutting at various

points until the curd was filled into the cheese mould. The TA results for each high-inoculum Cheddar cheese are recorded in Table 6 below.

Table 6: TA Monitoring during raw milk Cheddar cheesemaking. All values recorded as percentage (%) lactic acid.

Target TA	TA Sample	AF2122 CH1	AF2122 CH2	AF2122 CH3	G24.130 CH1	G24.130 CH2	G24.130 CH3	G1.140 CH1	G1.140 CH2	G1.140 CH3
0.16	**TA Milk**	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
0.18	**TA before Rennet**	0.18		0.18	0.17	0.17	0.17	0.18	0.17	0.17
0.1 - 0.12	**TA After Cutting**	0.12	0.12	0.12	0.12	0.12	0.12	0.13	0.13	0.13
0.14	**TA Max Scald**	0.16	0.15	0.15	0.14		0.15	0.15		
0.17-0.18	**TA Pitching**	0.26	0.26	0.19	0.22	0.21	0.25	0.25	0.23	0.22
0.22-0.25	**Curd handling**									
	1kg	0.37	0.35	0.26	0.30	0.28	0.35	0.39	0.3	0.3
	2kg			0.32					0.35	
	4kg				0.34		0.41			
	8kg					0.46			0.39	0.4
	16kg	0.51	0.55		0.45		0.50	0.49	0.45	0.47
	16kg			0.61					0.55	0.53
>4.5	**TA Into Mould**	0.72	0.77	0.74		0.61	0.72	0.65	0.66	0.64

The chemical analysis data of each Cheddar (one day old) is given in Table 7. This was presented to the PSG and the values were considered indicative of commercial raw milk Cheddar.

Table 7: Chemical analysis of one day old raw milk Cheddars. All values except pH recorded as percentage (%).

Parameter	AF2122 CH1	AF2122 CH2	AF2122 CH3	G24.130 CH1	G24.130 CH2	G24.130 CH3	G1.140 CH1	G1.140 CH2	G1.140 CH3
Moisture	32.66	30.56	30.59	30.82	31.93	32.19	32.9	33.65	33.86
Ash	3.59	3.69	4.14	3.69	3.67	3.51	3.51	3.55	3.57
Total Fat	33.73	34.93	35.01	34.7	34.57	34.69	35.27	35.84	35.78
Nitrogen	4.04	4.06	4.18	4.01	4.09	4.09	4.2	4.08	4.03
Crude Protein	25.28	25.38	26.15	25.08	25.59	25.58	26.27	25.49	25.17
Sodium Chloride	1.83	1.77	2.0	1.75	1.76	1.64	1.76	1.64	1.63
pH	5.54	5.44	5.69	5.56	5.59	5.48	5.36	5.34	5.57
% Salt/moisture	5.6	5.8	6.5	5.7	5.5	5.1	5.3	4.9	4.8
% moisture: nonfatsubstance	49.3	47.0	47.1	47.2	48.8	49.3	50.8	52.4	52.7
% Fat/dry matter	50.1	50.3	50.4	50.2	50.8	51.2	52.6	54.0	54.1

The average percentage loss of *M. bovis* in the whey fraction across the *M. bovis* types for high-inoculum Cheddars was 11%. In addition there was an average 5.8-fold increase in the concentration of numbers of viable *M. bovis* during transition of the organism from raw milk to cheese curd. This has also been observed with MAP (Donaghy *et al.*, 2004) and is probably due to the high lipid content of the mycobacterial cell wall which aids partition into the cream fraction of milk. The identity of *M. bovis* isolates was confirmed by colony morphology, Ziehl-Neelsen staining and VNTR molecular typing.

DMFit web edition (Institute of Food Research, Reading, UK) was used to fit the Baranyi model (Baranyi and Roberts, 1994) to mean log values of *M. bovis* recovered from cheese samples taken at 28 day intervals using 7H11++ PANTA and 7H11++PCAT media. The inactivation curves of *M. bovis* log cfu g^{-1} against time in days for each high-inoculum Cheddar cheese are shown in Fig. 7(a) to Fig. 7(i). Viable numbers of all three strains were observed to decline in linear phase during three – four months maturation followed by a 'tailing effect' were the rate of inactivation declined and viable cells were detected at a lower level for the remainder of the sampling period. Such a tailing effect would have been expected as it has been previously observed in survivor curves of MAP when subjected to heat treatments (Rowe *et al.*, 2000). Use of DMFit allowed the different phases of *M. bovis* inactivation to be objectively differentiated. The *D* value represents the time in days for a one log reduction in viable numbers and is derived from the slope of the linear portion of the inactivation curve described in DMFit as the Maximum rate. The *D* values for the high-inoculum Cheddars can be found in Table 8 Section 8.4 Statistical Analysis of high-inoculum Cheddar and Caerphilly.

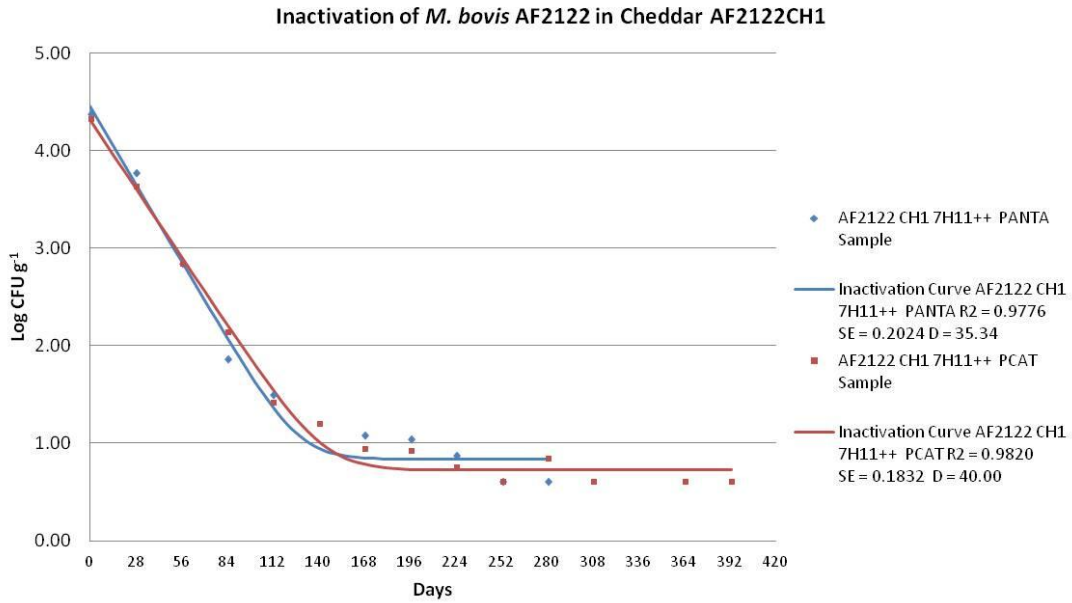

Fig. 7(a). Inactivation curve of *M. bovis* AF2122 from Cheddar AF2122 CH1. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar AF2122 CH1 up to 393 days.

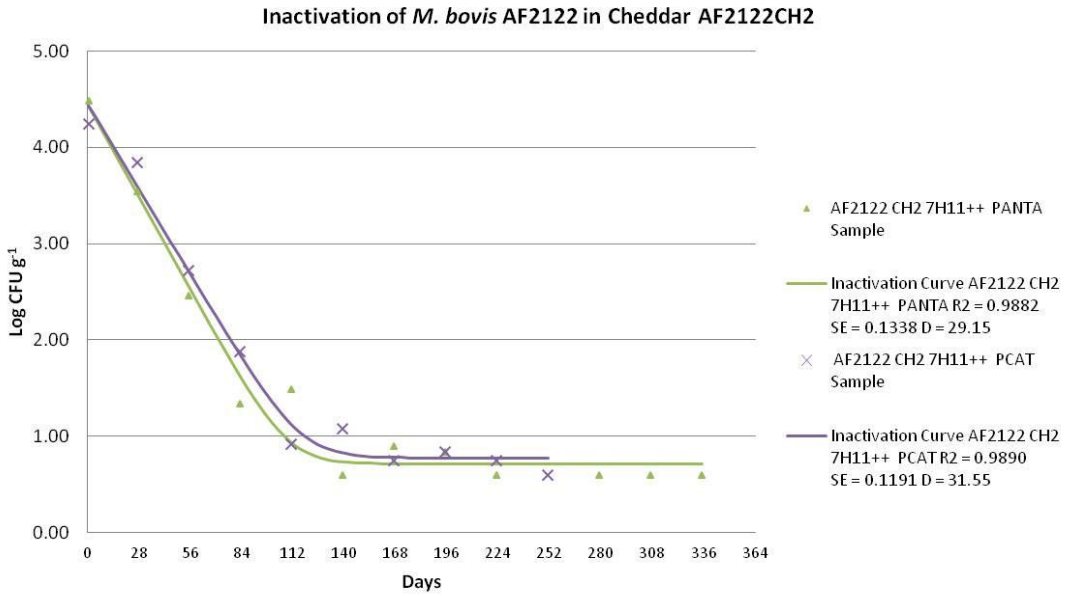

Fig. 7(b). Inactivation curve of *M. bovis* AF2122 from Cheddar AF2122 CH2 *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar AF2122 CH2 up to 335 days.

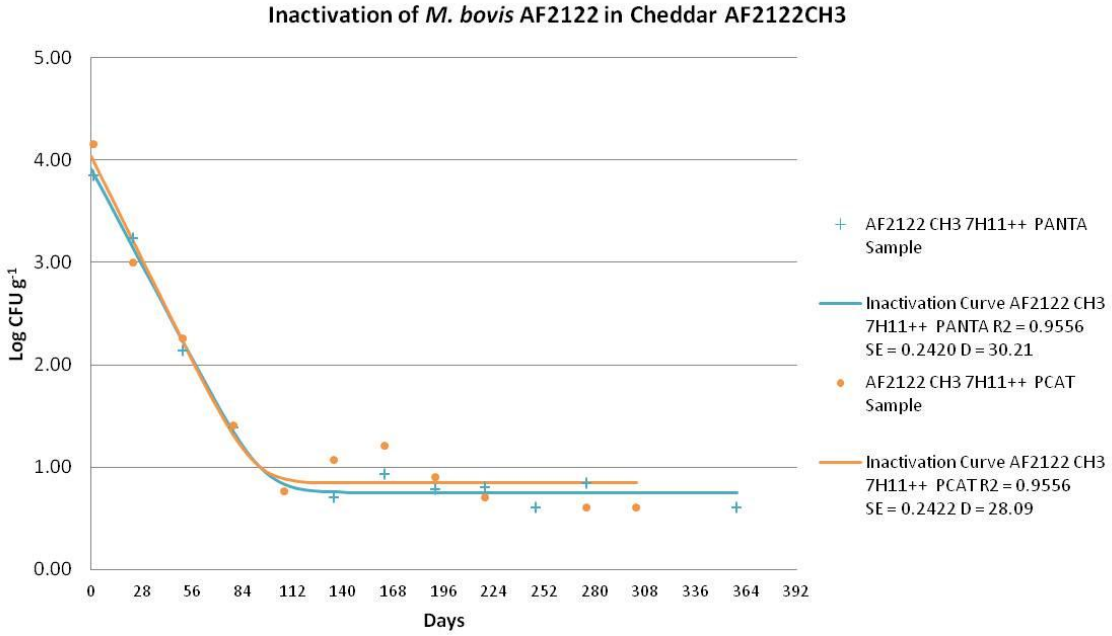

Fig. 7(c). Inactivation curve of *M. bovis* AF2122 from Cheddar AF2122 CH3. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar AF2122 CH3 up to 359 days.

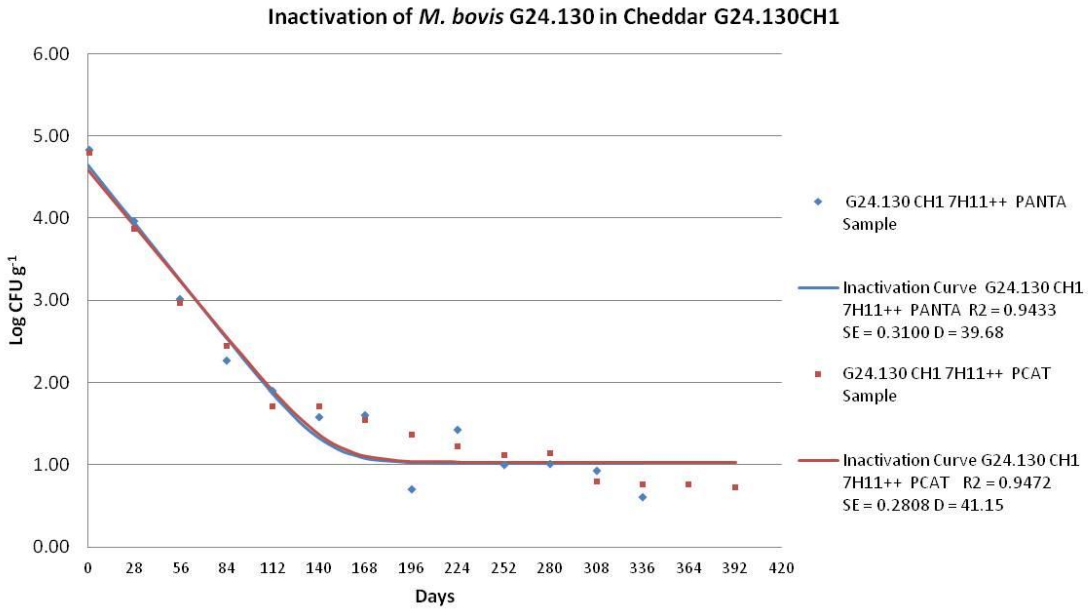

Fig. 7(d). Inactivation curve of *M. bovis* G24.130 from Cheddar G24.130 CH1. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar G24.130 CH1 up to 392 days.

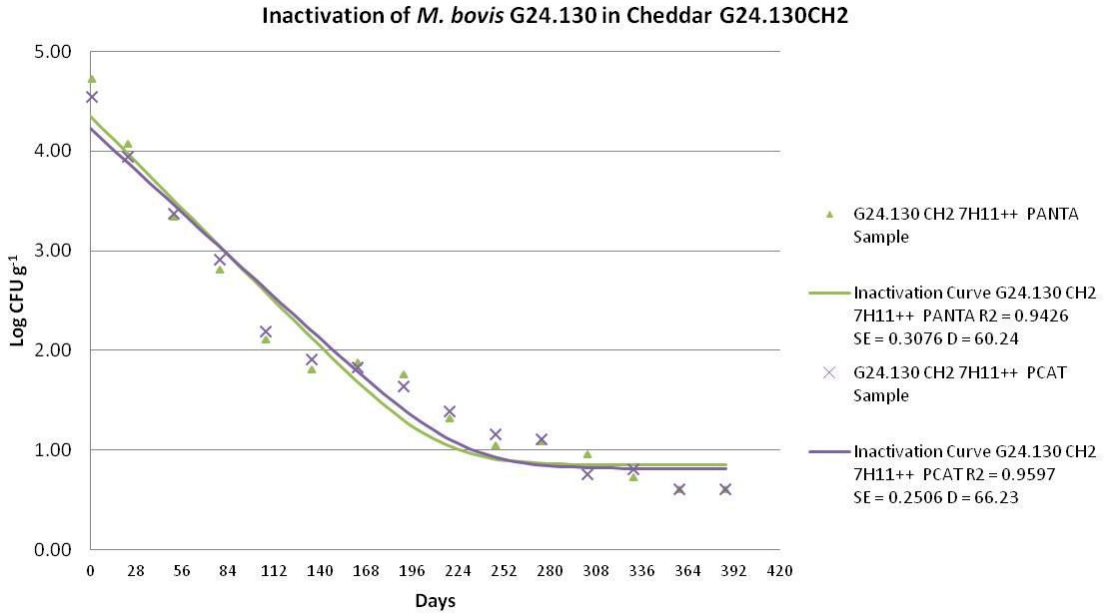

Fig. 7(e). Inactivation curve of *M. bovis* G24.130 from Cheddar G24.130 CH2. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar G24.130 CH2 up to 387 days.

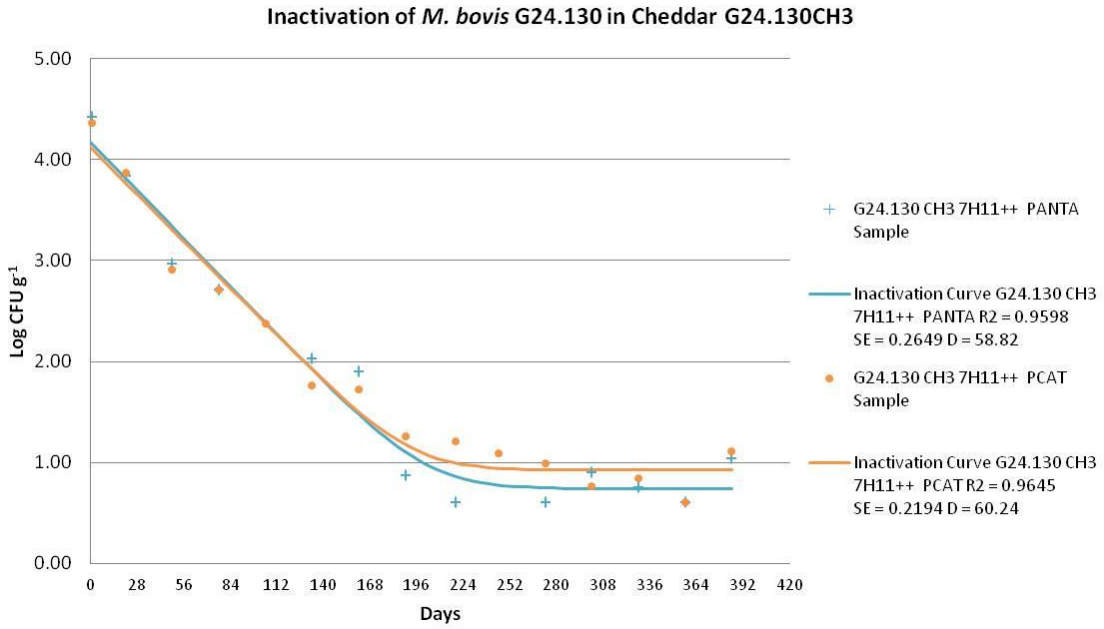

Fig. 7(f). Inactivation curve of *M. bovis* G24.130 from Cheddars G24.130 CH3. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar G24.130 CH3 up to 385 days.

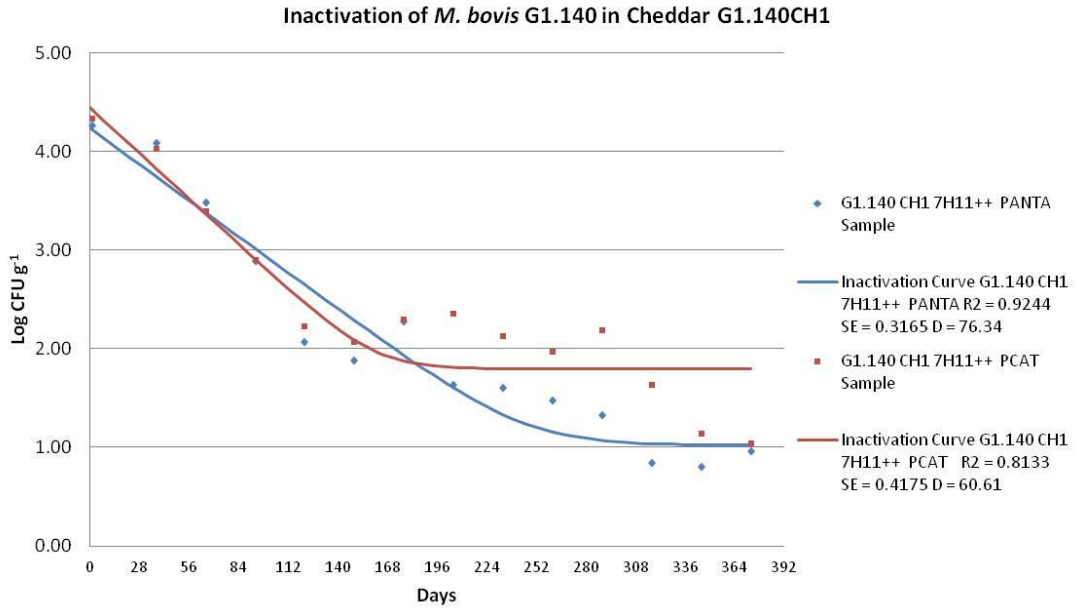

Fig. 7(g). Inactivation curve of *M. bovis* G1.140 from Cheddars G1.140 CH1. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar G1.140CH1 up to 401 days.

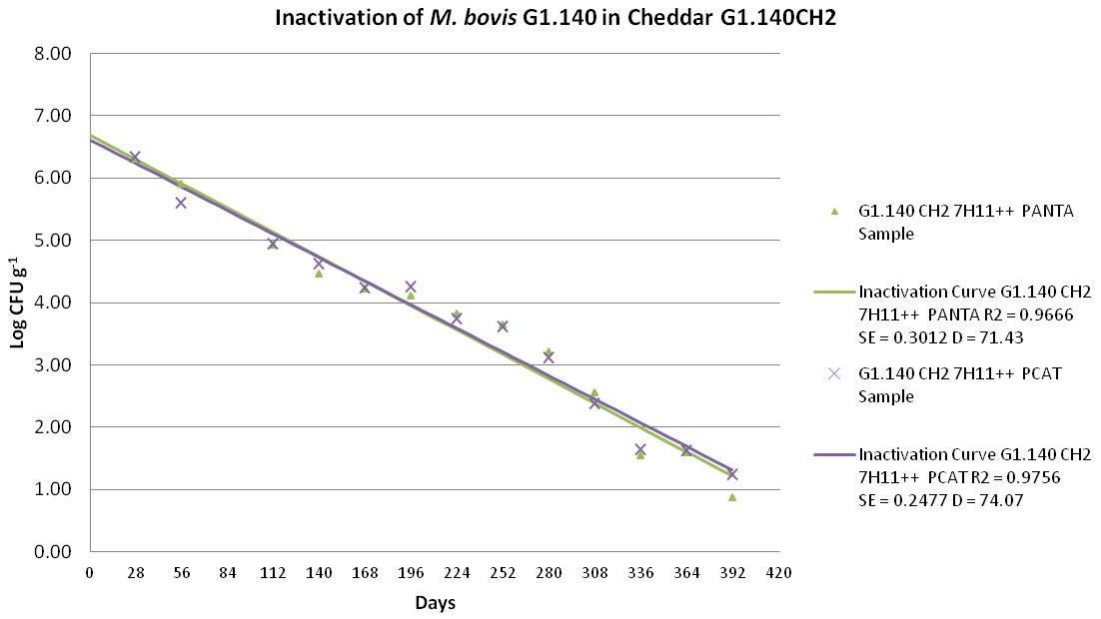

Fig. 7(h). Inactivation curve of *M. bovis* G1.140 from Cheddars G1.140 CH2. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar G1.140CH2 up to 391 days.

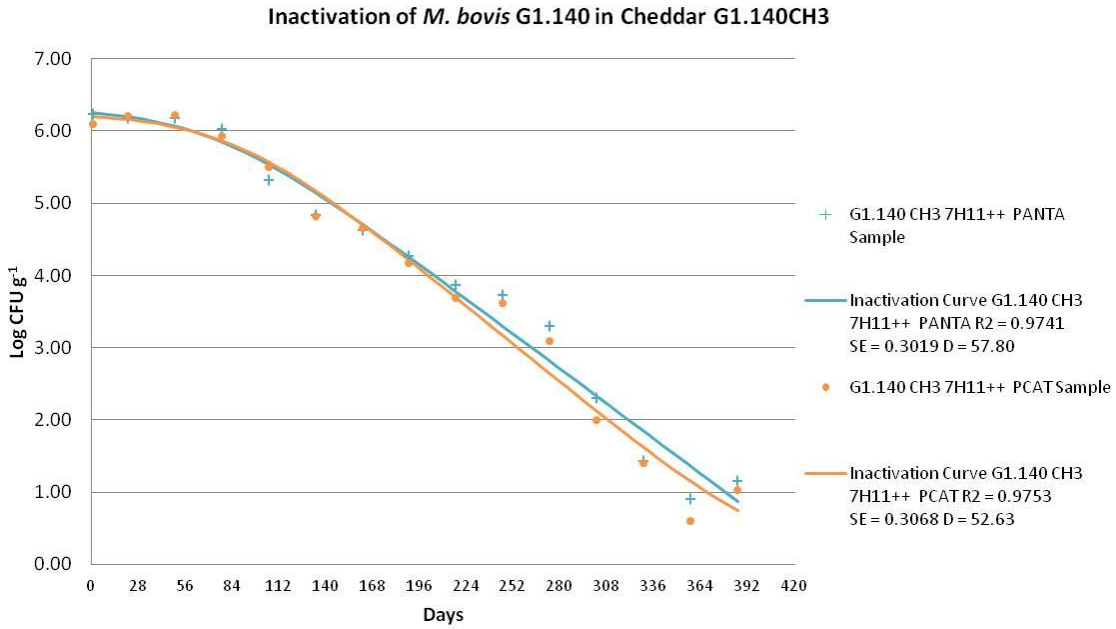

Fig. 7(i). Inactivation curve of *M. bovis* G1.140 from Cheddars G1.140 CH3. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar G1.140 CH3 up to 386 days.

8.3 Caerphilly cheese manufacture

As with the Cheddar investigation each Caerphilly cheese was named according to the *M. bovis* strain with which it was inoculated and numbered in sequence eg AF2122 CA1, AF2122 CA2, etc. The raw milk was once more collected from the dairy on the day before cheese production. Samples of the raw milk were removed and the pH measured before and after sixteen hours incubation at 22°C. The average milk pH prior to incubation was 6.79 and post incubation was 6.24. The absence of antibiotic residues was again confirmed, and the results of microbiological analyses performed and pH measurement of raw milk are shown in Table 8. TA was also recorded during Caerphilly cheesemaking as a method of monitoring

acid development and constituted the main method of process control. The TA results for each high-inoculum Caerphilly cheese are recorded in Table 9 below.

Table 8: Microbiological assessment and pH measurement of raw milk prior to Caerphilly cheesemaking. All enumeration values are recorded as cfu ml^{-1}.

Cheese	AF2122 CA1	AF2122 CA2	AF2122 CA3	G24.130 CA1	G24.130 CA2	G24.130 CA3	G1.140 CA1	G1.140 CA2	G1.140 CA3
TVC	1.3×10^7	7.4×10^3	7.9×10^6	1.3×10^4	3.1×10^6	6.5×10^6	2.4×10^6	2.4×10^5	2.3×10^7
Coliforms	9.1×10^5	4.2×10^2	2.5×10^6	3.1×10^2	8.8×10^4	1.0×10^6	9.2×10^2	3.5×10^3	1.2×10^4
LAB	2.7×10^5	2.5×10^3	9.5×10^5	4.5×10^4	2.2×10^5	7.3×10^5	1.5×10^4	4.2×10^4	5.4×10^4
pH Milk	6.88	6.82	6.72	6.76	6.77	6.74	6.82	6.82	6.84

Table 9: TA Monitoring during raw milk Caerphilly cheesemaking. All values recorded as percentage (%) lactic acid.

Target TA	TA Sample	AF2122 CA1	AF2122 CA2	AF2122 CA3	G24.130 CA1	G24.130 CA2	G24.130 CA3	G1.140 CA1	G1.140 CA2	G1.140 CA3
0.16	**TA Milk**	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.16
0.18	**TA before Rennet**	0.18	0.18	0.17	0.17	0.17	0.17	0.18	0.18	0.18
0.1 - 0.12	**TA After Cutting**	0.12	0.12	0.12	0.13	0.12	0.13	0.13	0.13	0.13
0.14	**TA Max Scald**	0.13	0.13	0.13	0.13		0.15	0.14		0.14
0.16	**TA Pitching**	0.15	0.15	0.15	0.15	0.15	0.18	0.16	0.16	0.16
0.19	**Curd handling**	0.18	0.18	0.18	0.15	0.16	0.21	0.17		0.18
		0.20	0.20	0.2	0.18	0.2	0.26	0.2	0.21	0.2
		0.24		0.21	0.2	0.21	0.27	0.23	0.23	0.21
		0.25		0.24	0.22	0.22		0.26	0.26	0.23
				0.26		0.25		0.29	0.28	0.25
										0.28
>0.3	**TA Into Mould**	0.3		0.27	0.24	0.27	0.3	0.3	0.3	0.29

Chemical analysis data for the nine one day old Caerphilly cheeses is given in Table 10.

Table 10: Chemical analysis of one day old raw milk Caerphilly. All values except pH recorded as percentage (%).

Parameter	AF2122 CA1	AF2122 CA2	AF2122 CA3	G24.130 CA1	G24.130 CA2	G24.130 CA3	G1.140 CA1	G1.140 CA2	G1.140 CA3
Moisture	40.76	43.04	41.76	41.3	41.68	40.23	40.5	40.84	41.09
Ash	3.8	4.06	3.67	4.03	3.71	3.72	3.84	3.52	3.65
Total Fat	29.9	28.89	29.69	28.16	29.27	30.84	31.59	30.59	30.58
Nitrogen	3.74	3.57	3.64	3.8	3.61	3.63	3.6	3.66	3.61
Crude Protein	23.38	22.33	22.74	23.75	22.57	22.71	22.53	22.88	22.57
Sodium Chloride	1.85	1.68	1.67	2.04	2.00	1.76	1.91	1.51	1.7
pH	5.25	5.6	5.38	5.49	5.39	5.4	5.42	5.44	5.44

Although the pH of Caerphilly on Day 1 post pressing was higher than the normal 4.8 pH for Caerphilly the overall measurements were deemed satisfactory by the PSG as commensurate

with commercially produced raw milk Caerphilly cheeses. Once more loss of *M. bovis* in the whey, and an increase in *M. bovis* numbers during the transition from milk to curd was observed: the average loss in the whey fraction was 2.7%, while the average increase in the concentration of viable *M. bovis* numbers was determined as 2.0-fold . The identity of *M. bovis* isolates was confirmed by colony morphology, Ziehl-Neelsen staining and VNTR molecular typing.

DMFit web edition was again used to generate inactivation curves. This model was applied to mean log values of *M. bovis* recovered using 7H11++ PANTA and 7H11++PCAT from cheese samples taken at 21 day intervals. The inactivation curves of *M. bovis* log cfu g^{-1} against time in days on 7H11++ PANTA and 7H11++PCAT media for each Caerphilly are shown in Fig. 8(a) to Fig. 8(i). These inactivation curves illustrate the inactivation of *M. bovis* over a four month maturation period (four months was the maximum maturation period for commercial Caerphilly as advised by the PSG).

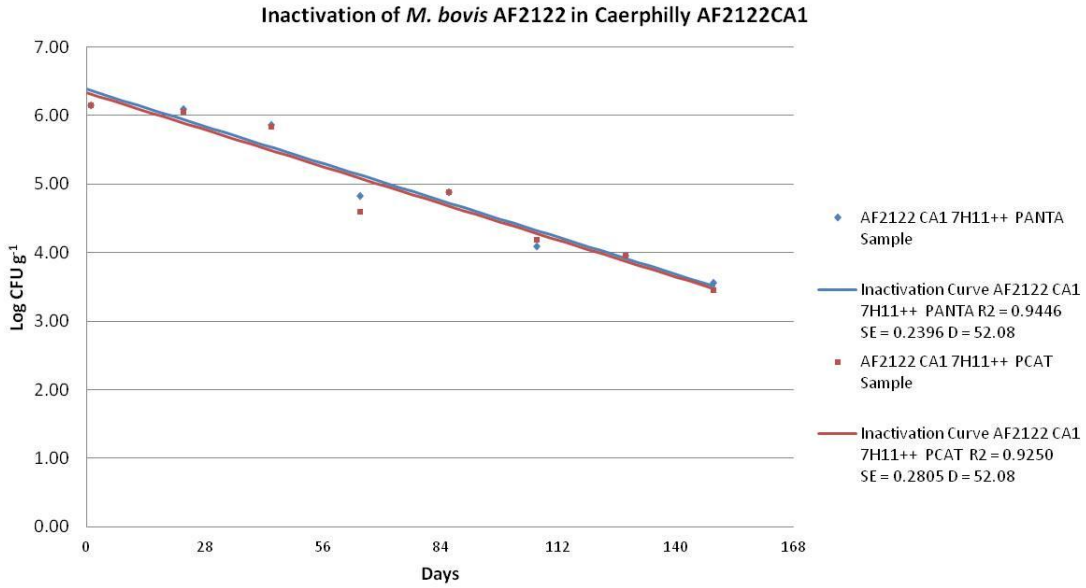

Fig. 8(a). Inactivation curve of *M. bovis* AF2122 from Caerphilly AF2122 CA1. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 CA1 up to 149 days.

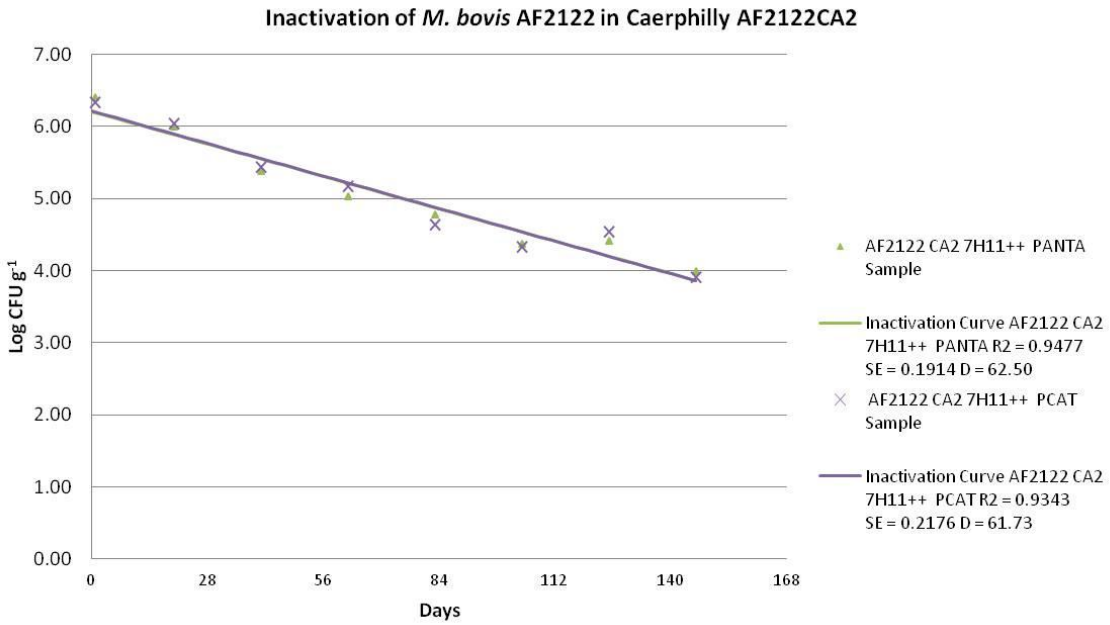

Fig. 8(b). Inactivation curve of *M. bovis* AF2122 from Caerphilly AF2122 CA2. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 CA2 up to 146 days.

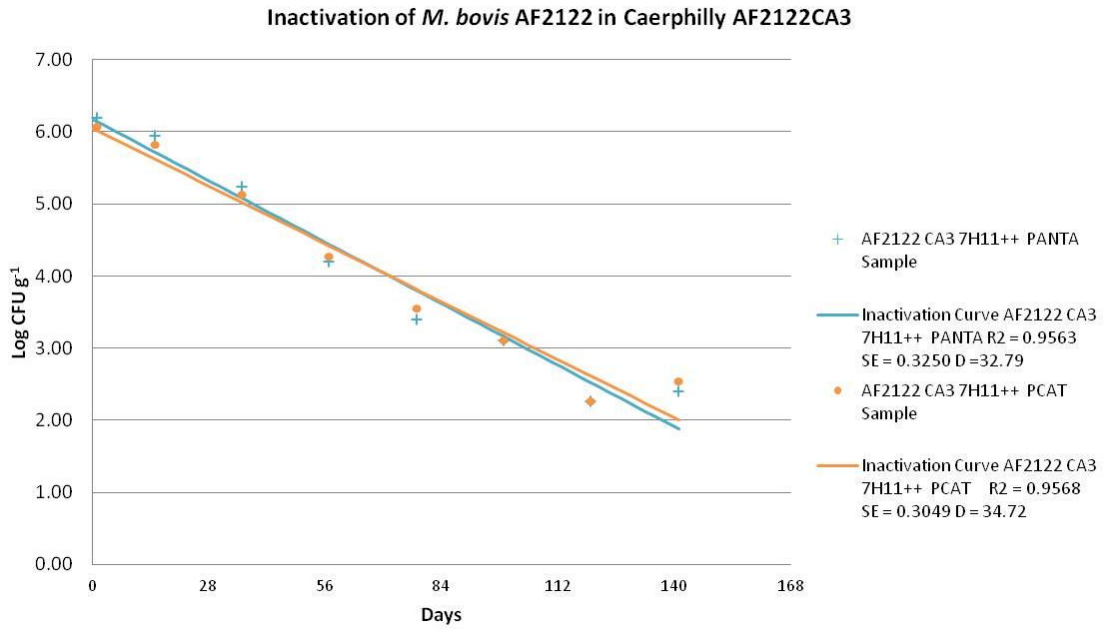

Fig. 8(c). Inactivation curve of *M. bovis* AF2122 from Caerphilly AF2122 CA3. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 CA3 up to 141 days.

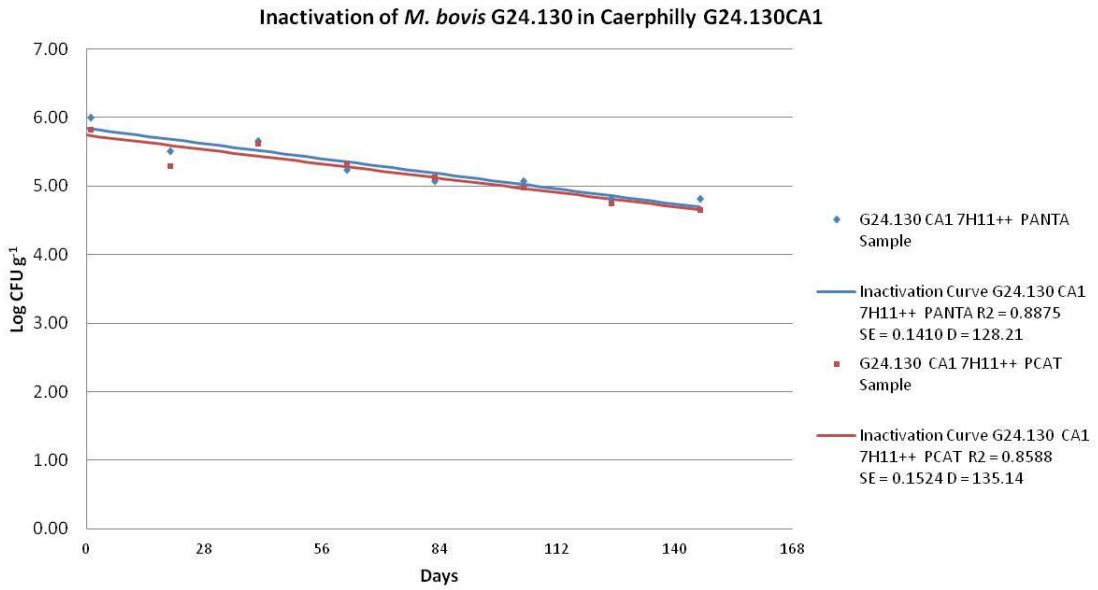

Fig. 8(d). Inactivation curve of *M. bovis* G24.130 from Caerphilly G24.130 CA1. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA1 up to 146 days.

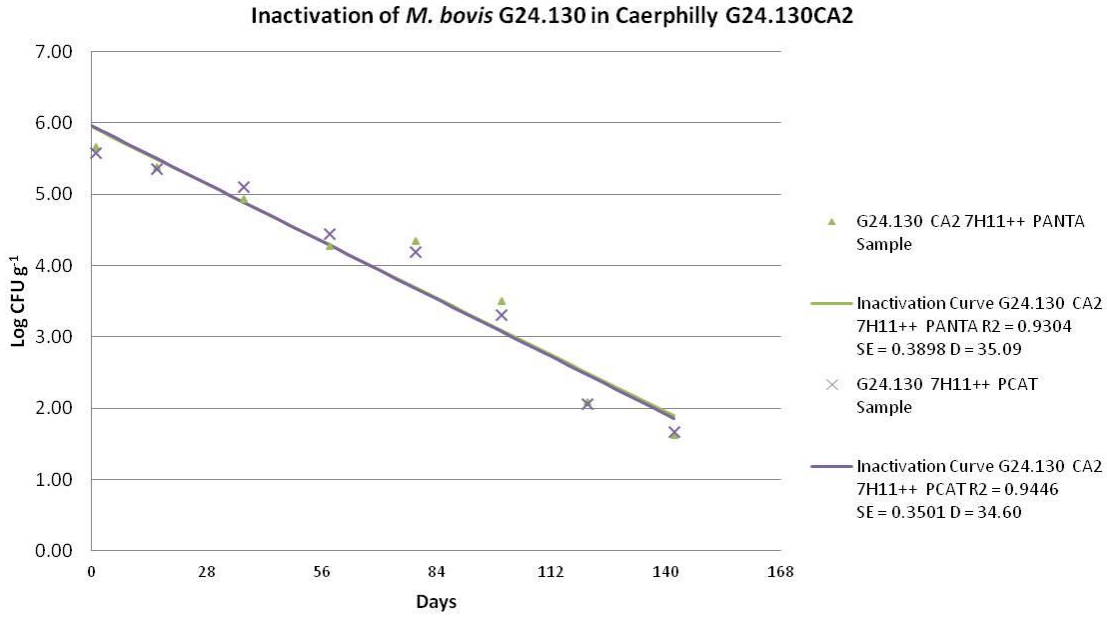

Fig. 8(e). Inactivation curve of *M. bovis* G24.130 from Caerphilly G24.130 CA2. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA2 up to 142 days.

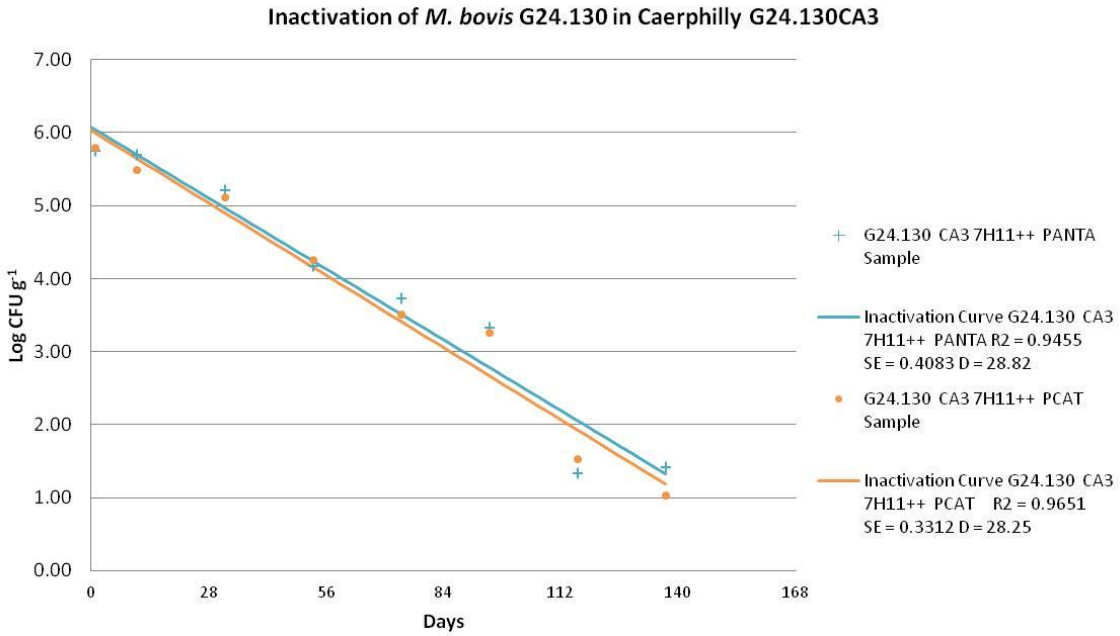

Fig. 8(f). Inactivation curve of *M. bovis* G24.130 from Caerphilly G24.130 CA3. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA3 up to 137 days.

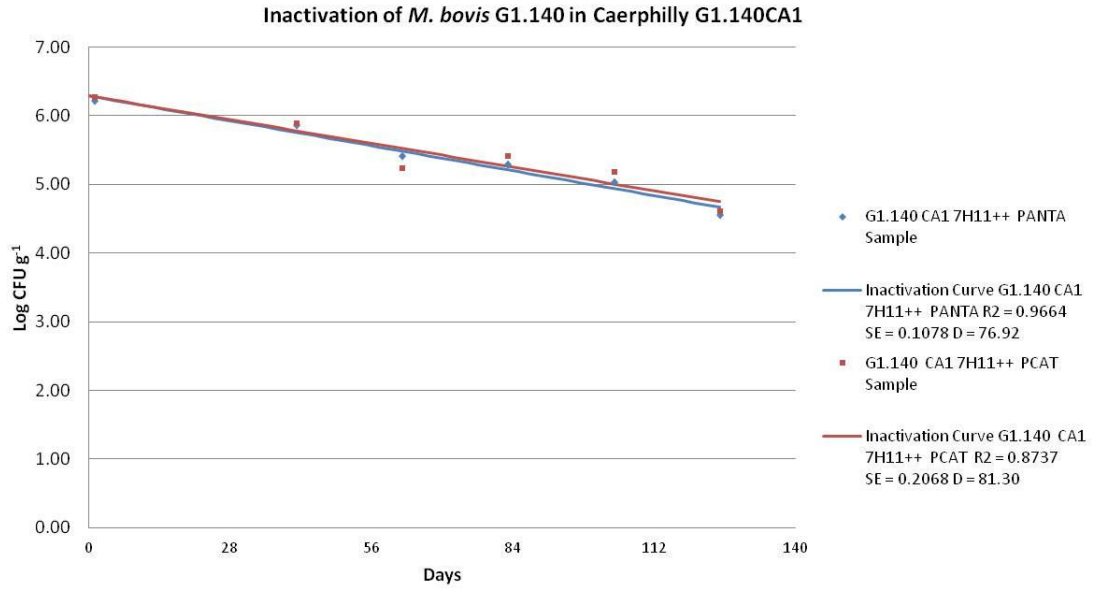

Fig. 8(g). Inactivation curve of *M. bovis* G1.140 from Caerphilly G1.140 CA1. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G1.140 CA1 up to 125 days.

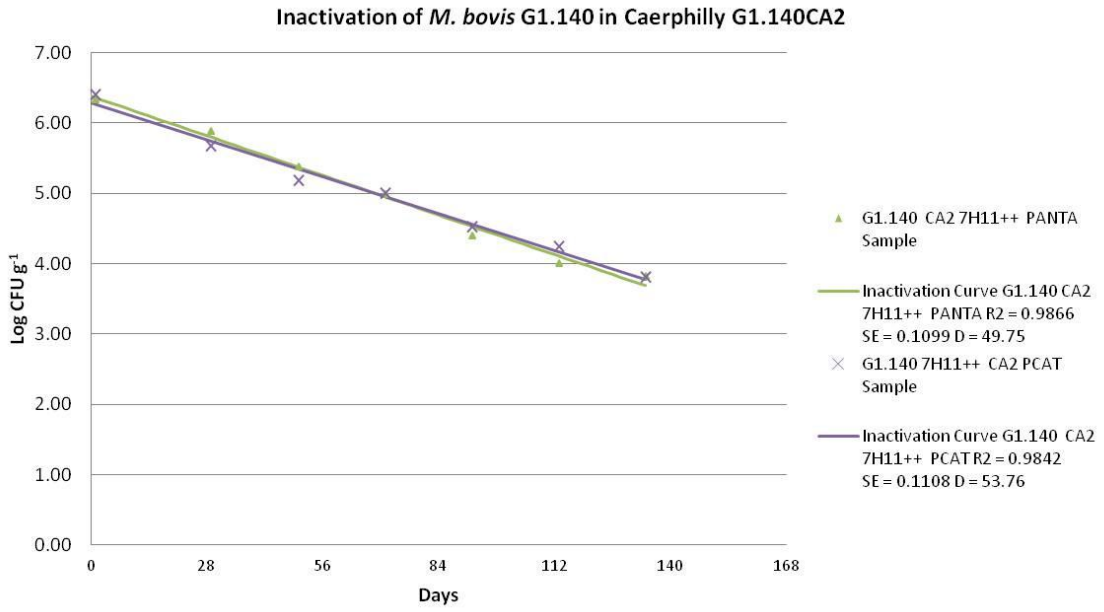

Fig. 8(h). Inactivation curve of *M. bovis* G1.140 from Caerphilly G1.140 CA2. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G1.140 CA2 up to 134 days.

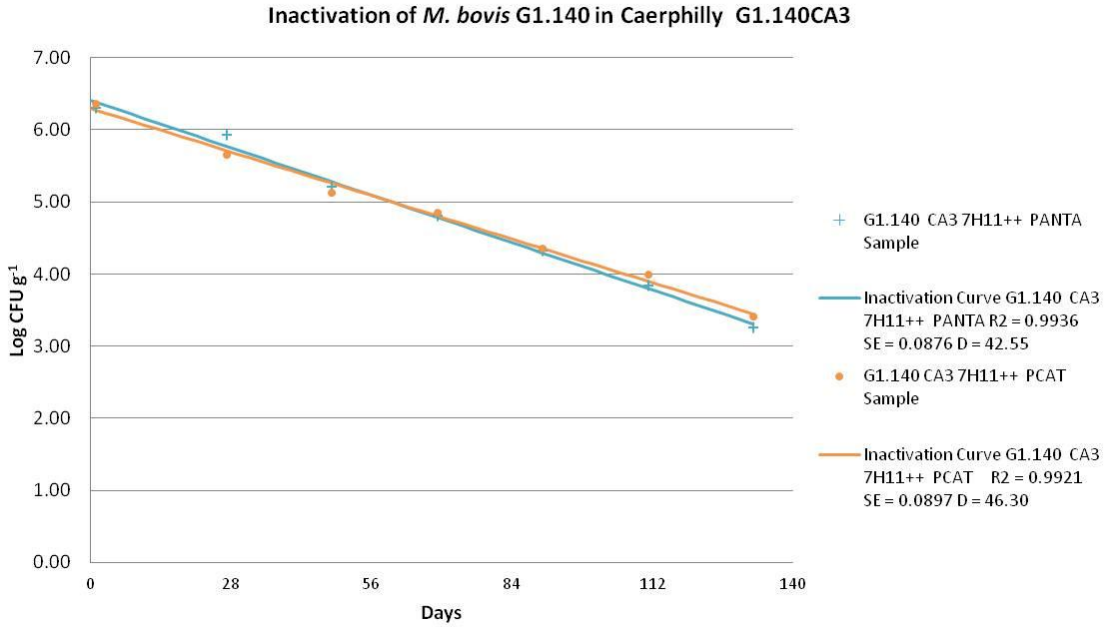

Fig. 8(i). Inactivation curve of *M. bovis* G1.140 from Caerphilly G1.140 CA3. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA3 up to 132 days.

These inactivation curves demonstrate that the log cfu g^{-1} values of *M. bovis* remained high even after four months maturation. This four month maturation period would be critical when considering the inactivation of *M. bovis* in commercially produced Caerphilly. Further sampling of *M. bovis* from Caerphilly cheeses was conducted beyond four months maturation. This maximized the inactivation data obtained from Caerphilly cheese and would indicate if a linear inactivation rate was maintained as the cheese moved beyond the recommended maturation time. Inactivation curves produced from data obtained from this extended maturation period can be found in Appendix 4. The *D* values calculated using inactivation curves for high-inoculum Caerphilly after four months maturation and after continuing maturation up to eight months can be found below in Table 12(a) and Table 12(b) Section 8.4 Statistical analysis of high-inoculum Cheddar and Caerphilly.

8.4 Statistical analysis of high-inoculum Cheddar and Caerphilly

Statistical analyses of high-inoculum Cheddar and Caerphilly *D* values were performed using GenStat Release 14.2 (for windows 7). The *D* values calculated for each high-inoculum cheese on media 7H11++ PANTA and 7H11++ PCAT are recorded for Cheddar in Table 11, Caerphilly after four months maturation in Table 12(a) and Caerphilly after eight months maturation in Table 12(b). The full output of the statistical analysis can be found in Appendix 5.

Table 11: *D* Value in Days for Cheddar Cheese.

Media Type	AF2122 CH1	AF2122 CH2	AF2122 CH3	G24.130 CH1	G24.130 CH2	G24.130 CH3	G1.140 CH1	G1.140 CH2	G1.140 CH3
7H11++ PANTA	35.34	29.15	30.21	39.68	60.24	58.82	76.34	71.43	57.80
7H11++ PCAT	40.00	31.55	28.09	41.15	66.23	60.24	60.61	74.07	52.63
Mean *D* Value	37.67	30.35	29.15	40.42	63.24	59.53	68.48	72.75	55.22

Table 12(a): *D* Value in days for Caerphilly Cheese after four month Maturation.

Media Type	AF2122 CA1	AF2122 CA2	AF2122 CA3	G24.130 CA1	G24.130 CA2	G24.130 CA3	G1.140 CA1	G1.140 CA2	G1.140 CA3
7H11++ PANTA	52.08	62.50	32.79	128.21	35.09	28.82	76.92	49.75	42.55
7H11++ PCAT	52.08	61.73	34.72	135.14	34.60	28.25	81.30	53.76	46.30
Mean *D* Value	52.08	62.12	33.76	131.68	34.85	28.54	79.11	51.76	44.43

Table 12(b): *D* Value in days for Caerphilly Cheese after eight month Maturation.

Media Type	AF2122 CA1	AF2122 CA2	AF2122 CA3	G24.130 CA1	G24.130 CA2	G24.130 CA3	G1.140 CA1	G1.140 CA2	G1.140 CA3
7H11++ PANTA	53.19	55.87	34.01	181.82	35.34	28.57	64.94	48.78	43.29
7H11++ PCAT	52.91	56.50	37.74	196.08	34.72	27.86	66.67	52.91	46.95
Mean D Value	53.05	56.19	35.88	188.95	35.03	28.22	65.81	50.85	45.12

An analysis of variance (ANOVA) was used to examine differences between mean *D* values of *M. bovis* types, cheese types and media types for Cheddar (Table 11) and Caerphilly after four month maturation (Table 12(a)). In addition the interaction between *M. bovis* type/media, cheese/media and *M. bovis* type/Cheese/media was considered. The mean, standard error of difference of means and *P* value for each source of variation are recorded in the Tables 13(a) to Table 13(e) below. The Grand Mean *D* value for all eighteen high-inoculum cheeses was calculated to be 54.17 Days.

Table 13(a): ANOVA results Mean, Standard Error of differences of Mean and *P* value for factors - *M. bovis*, Cheese and Media.

Factor		Mean	S.E.D. Means	*P* value
M. bovis Type	AF2122	40.85	15.162	0.344
	G24.130	59.71		
	G1.140	61.96		
Cheese Type	Cheddar	50.75	12.379	0.591
	Caerphilly	57.59		
Media Type	7H11++ PANTA	53.76	1.073	0.461
	7H11++PCAT	54.58		

Table 13(b): ANOVA results Mean, Standard Error of differences of Mean and *P* value for interaction of - *M. bovis*/Cheese.

	Mean			
***M. bovis* Type**	**Cheddar**	**Caerphilly**	**S.E.D. Means**	***P* value**
AF2122	32.39	49.32	21.442	0.721
G24.130	54.39	65.02		
G1.140	65.48	58.43		

Table 13(c): ANOVA results Mean, Standard Error of differences of Mean and *P* value for interaction of - *M. bovis*/Media.

	Mean			
***M. bovis* Type**	**7H11++ PANTA**	**7H11++ PCAT**	**S.E.D. Means[a]**	***P* value**
AF2122	40.35	41.36	15.219	0.439
G24.130	58.48	60.93		
G1.140	62.47	61.45		

a = S.E.D. of means 1.859 when comparing means of the same *M. bovis* type

Table 13(d): ANOVA results Mean, Standard Error of differences of Mean and *P* value for interaction of - Cheese/Media.

	Mean			
Cheese Type	**7H11++ PANTA**	**7H11++ PCAT**	**S.E.D. Means[a]**	***P* value**
Cheddar	51.00	50.51	12.426	0.245
Caerphilly	56.52	58.65		

a = S.E.D. of means 1.518 when comparing means of the same Cheese type

Table 13(e): ANOVA results Mean, Standard Error of differences of Mean and *P* value for interaction of - *M. bovis*/Cheese/Media.

	Cheddar		Caerphilly			
***M. bovis* Type**	**7H11++ PANTA**	**7H11++ PCAT**	**7H11++ PANTA**	**7H11++ PCAT**	**S.E.D. Means[a]**	***P* value**
AF2122	31.57	33.21	49.12	49.51	21.522	0.085
G24.130	52.92	55.87	64.04	66.00		
G1.140	68.52	62.44	56.41	60.45		

a = S.E.D. of means 2.629 when comparing means of the same *M. bovis*/Cheese type

The first ANOVA examining Cheddar and Caerphilly matured for four months demonstrates that there is no significant difference between *M. bovis* types, Cheese types or media types Table 13(a). Furthermore none of the interactions examined proved to be significant Table 13(b) to Table 13(e). This allows consideration of a mean *D* value for each *M. bovis* type AF2122, G24.130 and G1.140 by consolidating *D* values for each cheese type or alternatively a mean *D* value for Cheddar or Caerphilly by consolidating *D* values for each *M. bovis* type.

The mean *D* value for Cheddar and Caerphilly cheeses taking account of all three strains and the nine experimental runs was 50.75 days and 57.59 days respectively Table 13(a). In order to properly interpret this data there are a number of considerations:

1. *Mycobacterium bovis* forms 'cords' in milk so there is probably not a homogeneous dispersion of *M. bovis* cells in milk which impacts on the dilution effect of bulking milk from healthy and infected animals.
2. There is approximately a ten-fold concentration of *M. bovis* during transition from milk to curd.
3. *Mycobacterium bovis* is a slow growing organism, even within the confines of its hosts, and therefore growth in milk as opposed to survival can be considered negligible.
4. Excluding those individuals most at risk of infection by the aerosol route e.g. abattoir workers and veterinarians the main route of human *M. bovis* infection is via unpasteurized milk and dairy products.
5. By any route, the infectious dose for humans is unknown but is estimated to be in the tens to hundreds by the respiratory route and in the millions by the gastrointestinal route. On the basis of animal models and outbreak investigations it is known that the infectious dose is influenced by the species of host (potentially higher for humans than cattle), other host factors (immune status), route of infection (higher for ingestion) and strain of *M. bovis*. It should however be recognized that the existence of secondary tuberculosis means that any intervention, or lack of intervention, which increases exposure of the public to *M. bovis* may not have an immediate impact and only manifest itself in 20 to 30 years time. This may therefore not make control of human exposure to *M. bovis* an immediate political imperative.

M. bovis AF2212 gave the lowest *D* value for both Cheddar 32.39 days and Caerphilly 49.32 days. The combined *D* value for *M. bovis* AF2122 was 40.85 days. This lower *D* value may have been expected as *M. bovis* AF2212 as the sequenced strain has probably received more sub-culturing than the other *M. bovis* isolates and hence is less likely to be as resistant to environmental stresses. Equally it could be argued that *M. bovis* G24.130 and G1.140 have greater resistance to inactivation as they have been isolated directly from bovine tissue.

Attention should be drawn to the calculated *D* value for Caerphilly G24.130 CA1 of 131.68 days as it is at complete variance with the calculated values for the other two Caerphilly cheeses G24.130 CA2 34.85 days and G24.130 CA3 28.54 days. This was not due to skewing of one or two datum points but was consistent over the whole experimental run. Given the resistance of *M. bovis* to acid and alkali it is unlikely that the pH environment of the cheese contributed greatly to *M. bovis* inactivation. Furthermore, recording milk pH prior to production and pH of day old cheese gives an indication of milk and cheese quality. Milk pH for G24.130 CA1, G24.130 CA2 and G24.130 CA3 was 6.76, 6.77 and 6.74 respectively. It is improbable that conditions responsible for small differences in milk pH could contribute to such a disparity in *D* value.

When considering factors important for pathogen inactivation during cheese ripening such as percentage salt and cheese pH the values for G24.130 CA1 were 2.04% and 5.49 respectively. This is the highest percentage salt of any high-inoculum Caerphilly and it is doubtful if an excess of salt over the recommended 2% could contribute to the much higher *D* value observed for G24.130 CA1. Resistance of *M. bovis* to acid makes it unlikely that a pH of 5.49 had a diminished role in the inactivation *M. bovis* G24.130 compared to the pH 5.39, G24.130 CA2 and pH 5.40, G24.130 CA3. Furthermore pH 5.49, although higher than the other G24.130 Caerphilly cheeses, is less than the pH 5.60 of AF2122 CA2 which had a *D* value of 62.12 days.

Only when examining TA (Table 9) could any discrepancy between G24.130 CA1 and other high-inoculum Caerphilly cheeses be observed. The TA of whey expelled from G24.130 CA1 curd and loaded into the cheese mould was 0.24% compared to the target TA of 0.3%. In addition to determining the volume of alkali needed for acid neutralization in a given volume of milk or whey TA acts as an indicator of how the fermentation responsible for cheese production is progressing. The dairy starter cultures responsible for this fermentation are predominantly composed of LAB (Suskcovic *et al.* 2010). The major function of LAB is the synthesis of lactic acid which has three main roles as it aids rennet activity, increases the expulsion of whey thus reducing moisture and inhibiting growth of unwanted bacteria (Fox *et al*., 2000). The antimicrobial properties of LAB are not limited to competition for nutrients and production of organic acids including lactic and acetic acid, but also synthesis of hydrogen peroxide and bacteriocins (Reis *et al*., 2012). Bacteriocins are generally defined as a heterogeneous group of ribosomally synthesized, extracellularly released, bioactive peptides or proteins displaying antimicrobial activity against other bacteria (Beshkova and Frengova, 2012).

It is possible that, the lower than desired, starter culture activity in G24.130 CA1 resulted in a curd matrix unconducive to *M. bovis* inactivation. This would, in turn, have manifested itself as a much higher *D* value. It is interesting to note that milk micro analysis of the raw milk, used for Caerphilly G24.130 CA1, revealed that this batch of milk had amongst the lowest TVC, coliform and LAB counts in comparison with the other raw milk batches used for Caerphilly cheese. Had the counts been high this may have presented an explanation for the poorer performance of the starter culture as a result of competition. Instead low milk microflora counts combined with poor starter culture activity may have contributed little to the inactivation potential of the cheese resulting in an environment suitable for the persistence of *M. bovis* in Caerphilly G24.130 CA1.

A possible association between starter culture activity and *D* value would, in the authors' opinion, warrant further investigation. If starter culture activity and *D* value are linked this places additional importance on TA and pH records, not only for process control, but also as a guide to those assessing raw milk cheese produced from possibly *M. bovis* contaminated milk. For example, a batch of cheese produced with a perfect acid development profile may pose a lower risk than cheese where the acid development had been slow or had not reached the target value. Furthermore, there may be potential to identify specific bacteriocin producing LAB from a starter culture that could be incorporated into other cheese starters to enhance cheese properties for the inactivation of *M. bovis*.

A second ANOVA was completed for the mean *D* values of Caerphilly cheese matured for four months (Table 12(a)) and the *D* values for the same Caerphilly cheese matured up to eight months (Table 12(b)). The results of the analysis for Caerphilly maturation time and the interaction between maturation time/*M. bovis* type can be found below in Table 13(f) and Table 13(g).

Table 13(f): ANOVA results Mean, Standard Error of differences of Mean and *P* value for Caerphilly Maturation time - Four months, Eight months

Factor		Mean	S.E.D. Means	*P* value
Maturation time	Four Month	57.79	4.674	0.352
	Eight Month	62.12		

Table 13(g): ANOVA results Mean, Standard Error of differences of Mean and *P* value for interaction of - *M. bovis*/Maturation time.

	Maturation time			
M. bovis Type	Four Month	Eight Month	S.E.D. Means[a]	*P* value
AF2122	49.32	48.37	36.647	0.127
G24.130	65.02	84.06		
G1.140	58.43	53.92		

[a]S.E.D. Means = 8.095 when comparing means of the same *M. bovis* type

The second ANOVA revealed no significant difference for *D* values between Caerphilly cheeses matured for four month and the same cheese matured for eight months. Also there were no significant interactions between *M. bovis* type and maturation time. This suggests that if Caerphilly cheese was to have an extended maturation period the further development of cheese characteristics would not yield a significant improvement in *M. bovis* inactivation. Also the mean *D* value for Caerphilly G24.130CA1 increased from 131.68 days after four months maturation to 188.95 days after eight months maturation. The other Caerphilly cheeses displayed a modest change with a maximum increase in *D* value of 2.12 days by Caerphilly AF2122CA3 and a maximum decrease in *D* value of 13.31 days by Caerphilly G1.140CA1. Given that the *D* value for G24.130 increased by 57.28 days this would provide further evidence that the inactivation of *M. bovis*, in this individual cheese replicate, is uncharacteristic compared to the other Caerphilly cheeses.

8.5 Low-inoculum cheese manufacture

The project extension granted to facilitate twelve month sampling of high-inoculum Cheddar cheeses provided time which was used for an investigation using a reduced inoculum of *M. bovis*. This work was undertaken at the request of the PSG in an attempt to reflect what may happen, in practice, if there was an incident of *M. bovis* contamination of raw milk cheese. The average inoculum level of 2.72 log cfu ml^{-1} of milk was 2 – 3 logs lower than what had been used for the previous high-inoculum investigations. Experiments using this lower level of inoculum will be referred to as part of the low-inoculum investigation.

A statistical analysis determined no significant difference between *M. bovis* isolates, and it was decided, because of time constraints, only one *M. bovis* isolate would be used during the low-inoculum investigation. *M. bovis* AF2122 was selected for use in low-inoculum cheese production as it is the sequenced *M. bovis* strain and is an SB0140 spoligotype, the most common GB spoligotype. Cheeses were designated with an L to distinguish them from the high-inoculum Cheddar and Caerphilly eg. AF2122 LCA1, AF2122 LCH1 etc. Once more samples of raw milk were removed and retained before commencement of cheesemaking for microbiological analyses, pH measurement (Table 14) and confirmation of the absence of antibiotic residues. The average milk pH prior to incubation was pH 6.86 and post incubation was pH 6.31.

Table 14: Microbiological assessment and pH measurement of raw milk prior to low level cheesemaking. All enumeration values are recorded as cfu ml^{-1}.

Cheese	AF2122 LCA1	AF2122 LCA2	AF2122 LCA3	AF2122 LCH1	AF2122 LCH2	AF2122 LCH3
TVC	4.0×10^4	2.6×10^5	1.1×10^5	8.7×10^4	1.7×10^5	6.3×10^6
Coliforms	9.8×10^2	4.3×10^2	1.6×10^3	9.9×10^2	6.4×10^3	1.6×10^4
LAB	2.0×10^3	2.1×10^3	1.1×10^4	4.9×10^3	4.2×10^3	4.2×10^5
pH Milk	6.84	6.87	6.97	6.84	6.82	6.79

As mentioned above it was observed that the pH of the Caerphilly cheeses, post pressing, was higher than the desired value of 4.8 pH. After discussion with members of the PSG it was suggested that the delay between cheesemaking, sample irradiation and the chemical analysis may have allowed an increase in cheese pH. Following these discussions adjustments were made to the Caerphilly procedure.

1) The volume of bulk Caerphilly starter was doubled from 160 ml to 320 ml to facilitate more rapid acid development.
2) pH of the starter culture would be recorded prior to use in cheesemaking.
3) Reading of pH and temperature would be taken in addition to the measurement of TA.
4) A mellowing step of 30 minutes was introduced between milling and salting. This would allow LAB more time for acid production before the addition of salt which would inhibit their activity.

The TA results and pH after pressing for each low-inoculum Caerphilly and Cheddar cheese are recorded in Table 15(a) and Table 15(b) below. Chemical analysis data for the six one day old low-inoculum cheeses is given in Table 16.

Table 15(a): TA Monitoring of low-inoculum raw milk Caerphilly. All values recorded as percentage (%) lactic acid.

Target TA	TA Sample	AF2122 LCA1	AF2122 LCA2	AF2122 LCA3
0.16	TA Milk	0.16	0.16	0.16
0.18	TA before Rennet	0.18	0.18	0.18
0.1 - 0.12	TA After Cutting	0.14	0.15	0.14
0.14	TA Max Scald	0.14		0.15
0.16	TA Pitching	0.22	0.20	0.20
0.19	Curd handling	0.27	0.28	0.25
		0.30	0.30	0.3
		0.34	0.35	0.34
>0.3	TA Into Mould	0.51		0.46

Table 15(b): TA Monitoring of low-inoculum raw milk Cheddar. All values recorded as percentage (%) lactic acid.

Target TA	TA Sample	AF2122 LCH1	AF2122 LCH2	AF2122 LCH3
0.16	TA Milk	0.16	0.16	0.15
0.18	TA before Rennet	0.18	0.18	0.17
0.1 - 0.12	TA After Cutting	0.13	0.13	0.13
0.14	TA Max Scald	0.15	0.14	0.15
0.17-0.18	TA Pitching		0.30	0.25
0.22-0.25	Curd handling 1kg	0.37		0.35
	2kg	0.42	0.44	0.44
	4kg		0.55	0.56
	8kg	0.61	0.65	
	16kg			
	16kg			
>4.5	TA Into Mould	0.69	0.7	0.64

Table 16: Chemical analysis of one day old raw milk low-inoculum Caerphilly and Cheddar. All values except pH recorded as percentage (%).

Parameter	AF2122 LCA1	AF2122 LCA2	AF2122 LCA3	AF2122 LCH1	AF2122 LCH2	AF2122 LCH3
Moisture	38.78	37.4	40.07	32.78	33.76	34.34
Ash	3.17	3.31	3.02	3.77	3.28	3.61
Total Fat	32.51	33.13	31.62	34.74	33.88	33.74
Nitrogen	3.71	3.82	3.65	4.05	4.34	4.22
Crude Protein	23.17	23.88	22.81	25.33	27.15	26.35
Sodium Chloride	1.68	2.03	1.41	2.07	2.02	2.17
PH - Starter	4.42	4.46	4.47	4.79	4.76	4.86
pH - Pressing	4.86	4.6	4.7	4.85	4.85	4.83
pH - Time of Chem. Analysis	5.13	5.41	5.18	5.54	5.87	5.73

An average increase in *M. bovis* concentration from milk to cheese was calculated as 3.4-fold for low-inoculum Cheddar and 5.1-fold for low-inoculum Caerphilly. Also calculated were an average 9.2% loss of *M. bovis* in the whey fraction from low-inoculum Caerphilly and an average 3.8% loss of *M. bovis* from low-inoculum Cheddar.

The TA (Table 15(a)) from low-inoculum Caerphilly would indicate that alterations to the Caerphilly cheese make had improved acid development compared to high-inoculum Caerphilly (Table 9). It should be noted that despite the pH values of the low-inoculum Caerphilly being closer to the target pH 4.8, the pH at time of chemical analysis was still higher than desired. This would appear to support the suggestion that the delay between cheesemaking, sample irradiation and the chemical analysis allowed an increase in cheese pH.

The inactivation curves shown in Fig. 9(a) to Fig. 9(f) were generated using DMFit web edition. The model was applied to mean log values of *M. bovis* (log cfu g^{-1}) for each low-inoculum Caerphilly and Cheddar against time in days on 7H11++ PANTA and 7H11++PCAT media. Sampling of Cheddar and Caerphilly was conducted at 7 Day intervals up to 63 Days maturation.

The reduced culture volume used for low-inoculum cheeses meant that *M. bovis* would be recovered on spread plates at dilutions of 10^{-1} and 10^{-2}. This increased incidence of plate contamination as there was less dilution of the background microflora, in contrast to the high-inoculum investigation. Due to this contamination *M. bovis* was sometimes not recovered using either 7H11++PANTA or 7H11++PCAT. Furthermore, when *M. bovis* was recovered, often due to competing background microflora counts of *M. bovis* were less than would have been expected. This could be attributed to the inhibitory effect of contaminants on the growth of *M. bovis*. As a result some of the inactivation curves produced from the low-inoculum cheeses are not as complete as those produced during the high-inoculum Cheddar and Caerphilly studies.

The *D* values were calculated from the Maximum rate figure produced by DMFit. *M.bovis* AF2122 *D* values from each low-inoculum Caerphilly and Cheddar cheese are recorded below in Table 17, Section 8.6 Statistical analysis of low-inoculum Cheddar and Caerphilly.

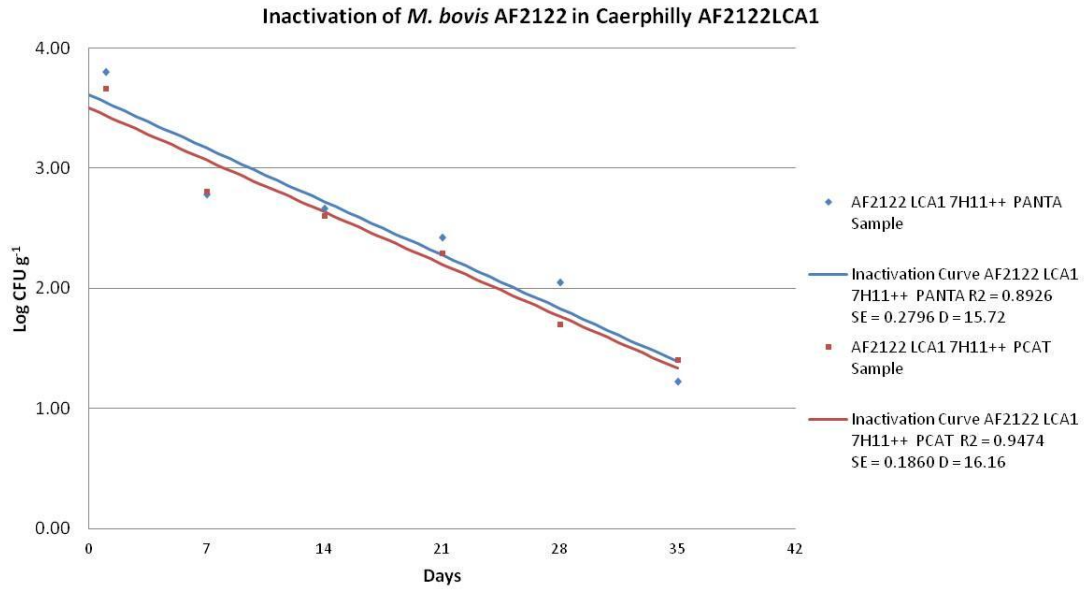

Fig. 9(a). Inactivation curve of *M. bovis* AF2122 from low-inoculum Caerphilly AF2122 LCA1. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 LCA1 up to 35 days.

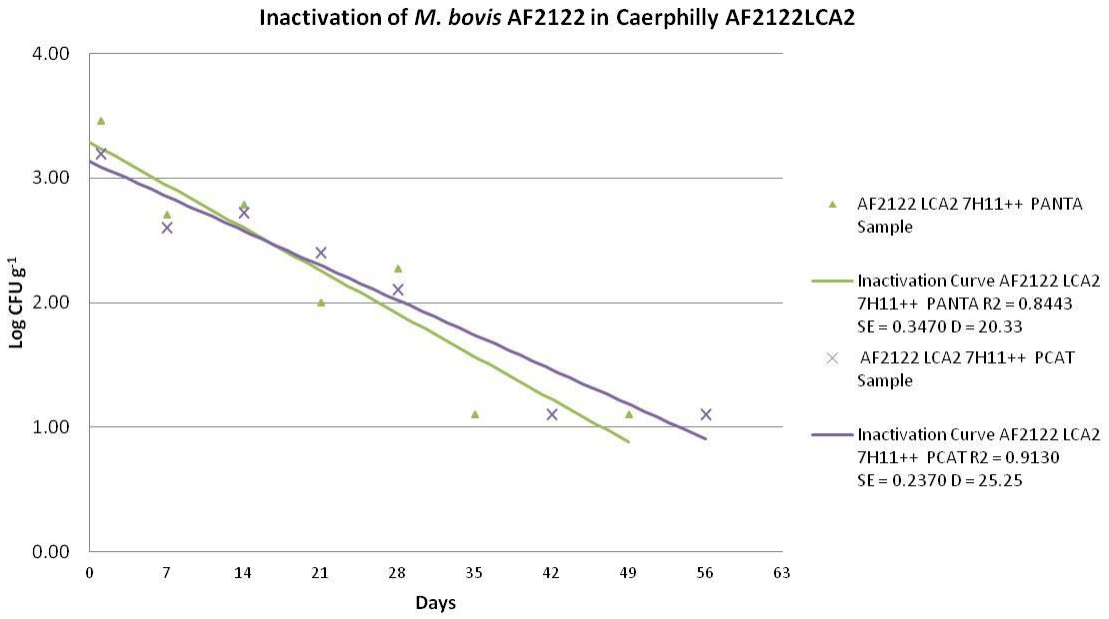

Fig. 9(b). Inactivation curve of *M. bovis* AF2122 from low-inoculum Caerphilly AF2122 LCA2. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 LCA2 up to 56 days.

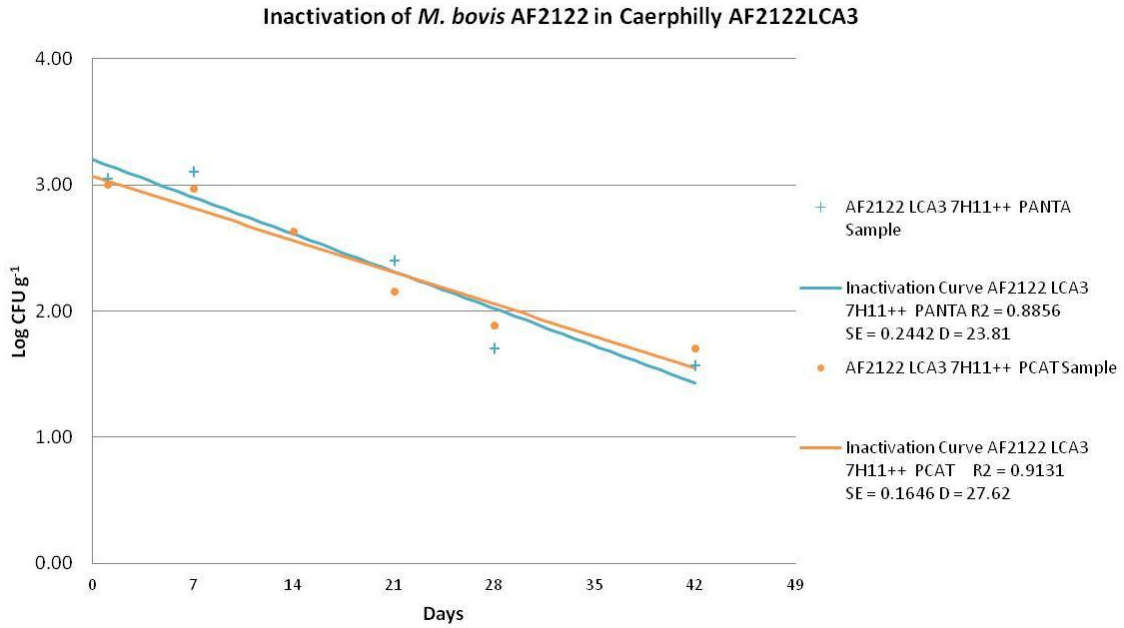

Fig. 9(c). Inactivation curve of *M. bovis* AF2122 from low-inoculum Caerphilly AF2122 LCA3. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 LCA3 up to 42 days.

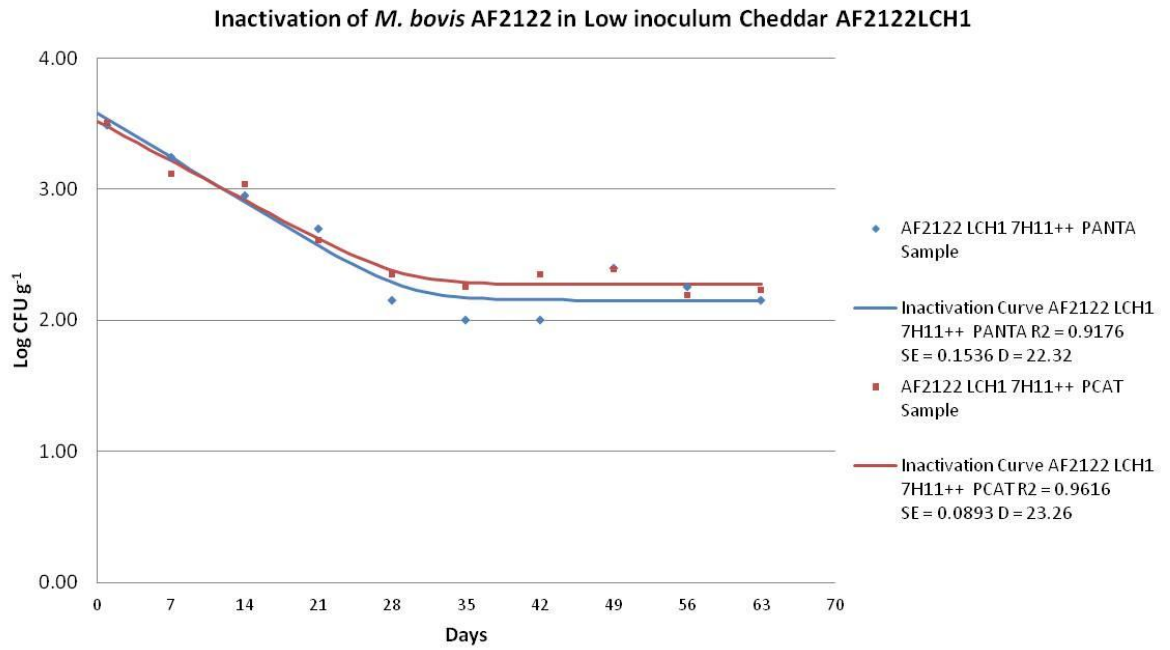

Fig. 9(d). Inactivation curve of *M. bovis* AF2122 from low-inoculum Cheddar AF2122 LCH1. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar AF2122 LCH1 up to 63 days.

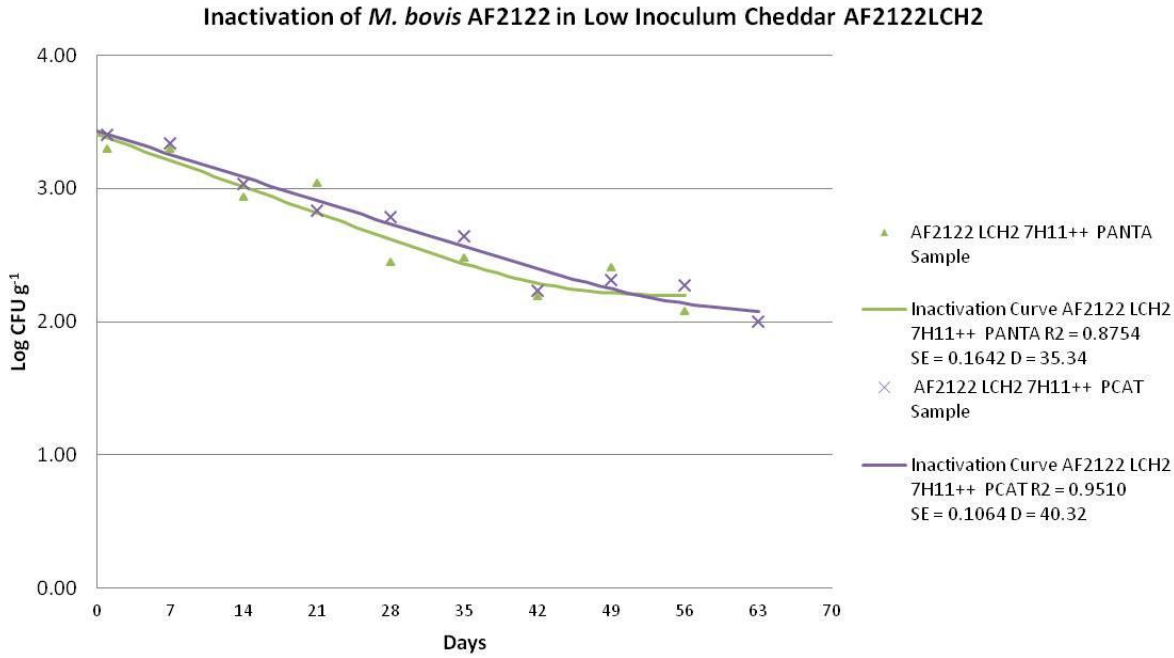

Fig. 9(e). Inactivation curve of *M. bovis* AF2122 from low-inoculum Cheddar AF2122 LCH2. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar AF2122 LCH2 up to 63 days.

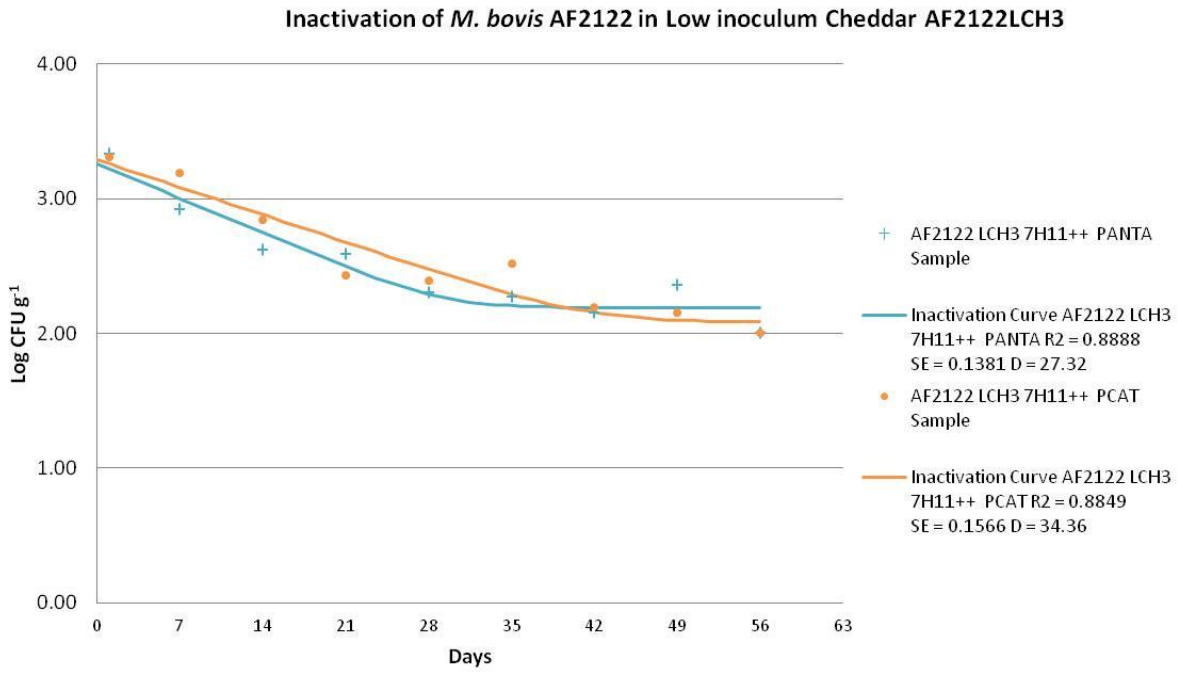

Fig. 9(f). Inactivation curve of *M. bovis* AF2122 from low-inoculum Cheddar AF2122 LCH3. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Cheddar AF2122 LCH3 up to 56 days.

8.6 Statistical analysis of low-inoculum Cheddar and Caerphilly

Statistical analyses of low-inoculum Cheddar and Caerphilly *D* values were performed using GenStat Release 14.2 (for windows 7). The *D* values obtained for each low-inoculum cheese using media 7H11++ PANTA and 7H11++ PCAT are recorded in Table 17. The full output of the statistical analysis can be found in Appendix 5.

Table 17: *D* values low-inoculum Caerphilly and Cheddar.

	Caerphilly Cheese *D* Values (Days)			Cheddar Cheese *D* Values (Days)		
Media Type	**AF2122 LCA1**	**AF2122 LCA2**	**AF2122 LCA3**	**AF2122 LCH 1**	**AF2122 LCH 2**	**AF2122 LCH 3**
7H11++ PANTA	15.72	20.33	23.81	22.32	35.34	27.32
7H11++ PCAT	16.16	25.25	27.62	23.26	40.32	34.36
Mean D Value	15.94	22.79	25.72	22.79	37.83	30.84

The first ANOVA examined the mean *D* values obtained from Cheddar and Caerphilly cheese when prepared using a low-inoculum of *M. bovis* AF2122 for differences between cheese types and media types. The interaction between cheese type and media type was also considered. The mean, standard error of difference of means and *P* value for each source of variation are recorded in the Tables 18(a) and Table 18(b) below.

Table 18(a): ANOVA results Mean, Standard Error of differences of Mean and *P* value for factors - *M. bovis*, Cheese and Media.

Factor		Mean	S.E.D. Means	*P* value
Cheese Type	Cheddar	30.49	5.223	0.160
	Caerphilly	21.48		
Media Type	7H11++ PANTA	24.14	1.123	0.030
	7H11++PCAT	27.83		

Table 18(b): ANOVA results Mean, Standard Error of differences of Mean and *P* value for interaction of - Cheese/Media.

	Mean			
Cheese Type	**7H11++ PANTA**	**7H11++ PCAT**	**S.E.D. Means[a]**	***P* value**
Caerphilly	19.95	23.01	5.342	0.604
Cheddar	28.33	32.65		

a = S.E.D. of means 1.588 when comparing means of the same Cheese type

The above ANOVA results demonstrate that, as with the high-inoculum investigation, there is no significant difference between cheese types Table 18(a). However, a significant difference was detected between the media types 7H11++ PANTA and 7H11++ PCAT. The mean *D* value for *M. bovis* AF2122 in low inoculum Caerphilly was 21.48 days and for low inoculum Cheddar cheese was 30.49 days. The results of the analysis also revealed that there was no significant interaction between cheese type and media type Table 18(b).

A further analysis was conducted for comparison of results from the low-inoculum cheeses with those obtained from high-inoculum cheeses prepared using *M. bovis* AF2122. This included the interaction between the level of inoculum and cheese type. The mean, standard error of difference of means and *P* value for each source of variation are recorded in the Tables 18(c) and Table 18(d) below

Table 18(c): ANOVA results Mean, Standard Error of differences of Mean and P value for factors - *M. bovis* AF2122 inoculum level, Cheese and Media.

Factor		Mean	S.E.D. Means	*P* value
M. bovis AF2122 inoculum	High	40.85	5.082	0.019
	Low	25.98		
Cheese Type	Cheddar	35.40	5.082	0.458
	Caerphilly	31.44		
Media Type	7H11++ PANTA	32.24	0.777	0.016
	7H11++PCAT	34.60		

Table 18(d): ANOVA results Mean, Standard Error of differences of Mean and *P* value for interaction - *M. bovis* AF2122 inoculum Level/Cheese Type.

	Cheese Type			
Inoculum Level	**Caerphilly**	**Cheddar**	**S.E.D. Means**	***P* value**
High	49.32b	32.39a	7.187	0.034
Low	21.48a	30.49a		

A significant difference was observed between mean *D* values at high and low *M. bovis* AF2122 inoculum levels and between media types. There was no significant difference between cheese types (see Table 18(c)), whilst the combined ANOVA determined an interaction between cheese type and inoculum level. This interaction was further resolved by assessing pair wise differences between the means using Fisher's least significant difference (LSD) test. The results of this test are indicated by the letters adjacent to mean *D* values in Table 18(d), and indicate that there is no significant difference when comparing mean *D* values identified by the same letter. The full output from the Fisher's LSD test can be found in Appendix 6.

The Fisher's LSD test reveals that there is no significant difference when comparing low-inoculum *M. bovis* AF2122 Caerphilly with *M. bovis* AF2122 Cheddars made using high- or low-inoculum. Furthermore the comparison of low-inoculum Cheddar with high-inoculum Cheddar reveals that there is no significant difference between the mean *D* values. This suggests that high-inoculum data obtained from Cheddar could be used to reliably assess the inactivation of *M. bovis* when initial contamination is much lower. This observation could be advanced by conducting a further low-inoculum investigation using the *M. bovis* isolates G24.130 and G1.140 to test for strain differences. It would be reasonable to expect similar findings using these *M. bovis* isolates given that no significant difference between *M. bovis* types was found at high-inoculum levels or when comparing high- and low-inoculum *M. bovis* AF2122 Cheddar. However, it should be understood that these *M. bovis* types are representative of only two *M. bovis* spoligotypes, and the possibility that different *M. bovis* isolates, representing alternative spoligotypes, could be a factor relating to increased or decreased *M. bovis* inactivation, during cheese ripening, cannot be discounted. In addition future investigation of low-inoculum cheese should allow for extended sampling until *M. bovis* is no longer recoverable. This would provide for improved characterization of the 'tailing effect' observed in low-inoculum cheeses. DMFit allowed the linear phase and 'tailing effect' of *M. bovis* inactivation to be objectively differentiated. Without the use of DMFit the reduced sampling period (up to only 63 days) presented difficulties in distinguishing between the phases.

It is interesting to note that the 'tailing effect' is observed at both the high- and low-inoculum level. As such, it may be appropriate to consider the inactivation of *M. bovis* in Cheddar as two phases viz. an initial linear decline followed by a slower decline or 'tailing effect'. The shortened sampling period meant that the 'tailing effect' in the low-inoculum Cheddars was under characterized, and it would be interesting to determine to what extent the length of tail is related to initial level of *M. bovis* contamination.

When comparing mean *D* values of low-inoculum Cheddar or Caerphilly with high-inoculum Caerphilly a significant difference was observed. The average *D* value of 21.48 days for *M.*

bovis AF2122 as a low-level Caerphilly inoculum was significantly different from the 49.32 days *D* value determined for high-level Caerphilly. It is conceivable that the 21.48 days *D* value is associated with greater starter culture activity indicated by greater TA and lower pH values. This result would appear to support the observation of a possible link between TA/starter culture activity and *M. bovis D* value described above.

Another factor that should be considered is that higher initial *M. bovis* AF2122 numbers contribute to greater *D* values. It has been observed in milk that the presence of high numbers of MAP can aid survival in a laboratory pasteurization process. Investigation of this phenomenon found that the clumped aggregates of MAP offered greater protection than a de-clumped cell suspension (Rowe *et al.,* 2000). Also, in the same paper it was proposed that clumping of MAP would offer greater protection than the typical chord structure of *M. bovis,* thus explaining the disparity in heat resistance between MAP and *M. bovis*. Despite this *M. bovis* chords or chord fragments could offer some protection in the less stringent environment of the cheese matrix. When the *M. bovis* inoculum was filtered to produce a single cell suspension a number of cell aggregates may have remained intact or re-formed after the filtering procedure. These chord fragments would have been more prevalent at the high-inoculum level and may have afforded *M. bovis* cells with greater protection during cheese manufacture and maturation producing greater *D* values. Alternatively, at a lower inoculum level there would be an expectation of fewer chord fragments and this diminished number would be reflected in a lower *D* value.

9 Overall discussion and conclusion

The project required that media be identified for enumerating *M. bovis* from cheese in the absence of a sample decontamination step. Subsequently 7H11++ media was modified from a panel of *M. bovis* selective media previously developed for use without decontamination. 7H11++ PANTA and 7H11++ PCAT proved to be satisfactory for use during the challenge test phase of the investigation, allowing good recovery of the target organism. Problems of contamination became more prevalent during the investigation of low-inoculum Cheddar and Caerphilly cheese. Unfortunately, time constraints prevented further assessment of 7H11++ PANTA and 7H11++ PCAT media in isolating low numbers of *M. bovis*, amongst a proportionally increased population of non-target cheese microflora. Further media assessment would be beneficial: including adjustment of the *M. bovis* growth promoters, sheep blood and bovine calf serum, and the PANTA/PCAT antimicrobial cocktail. It should be noted however that 7H11++ PANTA and 7H11++ PCAT represent a good starting point for the development of media that could be used for the screening of *M. bovis* from industrially produced cheese and it is hoped that this finding will be ratified in a peer reviewed journal in due course.

The critical period for *M. bovis* inactivation comes after cheese production and is during the maturation/ripening phase. It was not the intention of this project to arrive at a value for a minimum maturation time (MMT), but to monitor *M. bovis* levels over a period of time typical for Cheddar and Caerphilly ripening. Any risk assessment or efforts to determine an MMT should take account of additional factors. The maximum level of *M. bovis* in naturally contaminated milk is reported to be 10^4 cfu ml^{-1} (Kells & Lear, 1960). This must be seen in the context that the number of cattle with TB lesions in their udders is believed to be very small, at less than 1% of infected animals (Collins, 2000). It must be recognized that when a herd looses its TB free status, due to one or more reactor cows, this will not always result in reactor animals shedding *M. bovis* in their milk. During cheese production there will also be a dilution factor, as any contaminated milk used, would be diluted with milk from cattle not

actively shedding *M. bovis.* As a result, levels of *M. bovis* contamination may never reach levels of 10^4 cfu ml^{-1} in milk used for cheese production.

Although not reported here, an opportunity presented itself twice for cheesemaking using milk from an individual reactor cow. On one occasion milk was obtained from a cow which had marked induration of one quarter and had been linked to positive skin tests from young calves fed using its milk. However, no *M. bovis* could be recovered from the Day 1 cheese or subsequent samples at a detection limit of <6 cfu g^{-1}. This gives some indication that *M. bovis* may be shed at low levels, yet there is still further potential for dilution of *M. bovis* should contaminated milk be mixed with non-contaminated milk destined for raw milk cheesemaking. Therefore, the influence of herd size and batch volume of cheese production, on any dilution factor is an important consideration; there is still scope for further investigations using low-inoculum *M. bovis* cultures thus simulating this scenario. It may be beneficial, if required, when assessing an individual raw milk cheese stock, with respect to *M. bovis,* if the herd looses TB free status, that *M. bovis* levels in bulk milk be determined, and compared with the pathology of the animals once slaughtered. In particular sampling of milk obtained from animals with clinical signs of TB mastitis would also be advantageous in contributing towards a risk assessment. In addition, it should be understood that there is potential for anergic cattle: cows in contact with reactor animals that have become infected, whilst remaining undetected using tuberculin testing (Liebana *et al.,* 2008). The latent role played by these animals, in *M. bovis* contamination of raw milk for cheesemaking, should be considered before and after detection of the first tuberculin positive cow.

These factors will combine to dictate the potential final level of *M. bovis* contamination in bulk milk and subsequently the cheese. Based on this information and the *D* values calculated from Cheddar and Caerphilly challenge tests, it may be possible to determine a maturation time that would ensure complete *M. bovis* elimination from a given cheese by intelligent extrapolation from the results generated in this study. It is important for the feasibility of maturation as an inactivation mechanism, for *M. bovis,* that cheese type and appropriate ripening time be accounted for. For example, it would be otiose when assessing a particular cheese variety if the maturation time, enabling *M. bovis* elimination, was well beyond when the cheese is ripe and ready for sale. Using the cheeses produced during this investigation, as a reference, clearly Caerphilly with a short ripening time, of up to four months, would have to be matured much longer to ensure total *M. bovis* inactivation. Therefore extended maturation would be commercially unfeasible for some cheese varieties.

It would be important to establish by further studies that the prescribed period of maturation successfully obtained complete inactivation, before employing ripening time, as an appropriate measure, in dealing with *M. bovis*. As such a preliminary study of the shedding of *M. bovis* in milk would be beneficial: to better define the levels of *M. bovis* contamination caused by reactor cows. The *D* values produced during this study, Cheddar 50.75 Days and Caerphilly 57.75 Days, could then be used to calculate a maturation time, based on specified contamination levels. It was not requested by the PSG to monitor the cheese microflora during cheese production and ripening. However, this additional data would have proved useful for analyses of *M. bovis* inactivation and the role, if any, played by this microflora. Therefore, it is considered that, any future study should include microflora enumeration, particularly of LAB, as it would corroborate the observation that improved starter culture activity, as indicated by greater TA values in some cheeses, produced improved *M. bovis* inactivation.

If an association between cheese microflora and *M. bovis* inactivation could be established, microflora enumeration and particularly of identified types could serve as a tool to aid risk assessment, of a suspect raw milk cheese, with respect to *M. bovis*. In addition, a link between starter culture and subsequent inactivation of *M. bovis* could influence development of bulk starter cultures and their preparation procedures: with the objective of maximizing the potential for inactivation in the event of possible *M. bovis* contamination.

Critical to the success of this investigation were the production equipment, and adaptation of methods for hard and semi-hard cheesemaking. It was essential that equipment and methods satisfied local health and safety regulations. Equally methods had to produce a cheese that had physicochemical properties equivalent to commercially available product. As a result, most of the apparatus was devised and fabricated, for each stage of the cheesemaking process, including cheese vats, Cheddaring equipment, cheese moulds and cheese press. The value of this element of the project, beyond allowing the incorporation of *M. bovis* during Cheddar and Caerphilly cheesemaking, within the prevailing safety constraints, should not be underestimated. Indeed, all the equipment and methods could be easily adapted for investigation using alternative pathogens (HG2 & HG3) or cheese types. Using these methods it was possible to produce replicates, of both Cheddar and Caerphilly cheese types, to generate sufficient data for valid statistical analysis as evidenced by the results. Should contaminated milk be used for raw milk cheese production, it was established that the manufacturing process alone is an insufficient hurdle to prevent *M. bovis* presence in the finished cheese. Following the challenge test experiments an increase in concentration of *M. bovis* was observed: calculated for all cheeses there was on average a 4.1-fold increase in the concentration of *M. bovis* from milk to curd. Furthermore an average 7.9% loss of *M. bovis* in the whey indicated that how the whey fraction is used or disposed of, as a contaminated by product, should be considered. Finally if *M. bovis,* bearing in mind initial contamination levels and strain type, survives the cheesemaking process the cheese starter and raw milk microflora in conflation with maturation time, appear to, produce the cheese properties responsible for at least diminishing viable *M. bovis* numbers.

It should be noted that a parallel investigation, not funded as part of this project, allowed the development of a real-time PCR assay for *M. bovis* from cheese which will be subjected to publication in a peer reviewed journal in due course. This it is hoped will provide useful screening tool for suspect UK and imported raw milk cheeses. Also poster presentations were made at European Society of Mycobacteriology summer conference ESM 2008, European symposium of the International Association of Food Protection IAFP 2009 and Society for Applied Microbiology summer conference SFAM 2011; these can be found Appendix 7. It is anticipated that in addition to these posters and the review already published (Rowe and Donaghy, 2008), there will be at least two further papers in peer reviewed journals viz.

1. Development of media that allow enumeration of *M. bovis* from cheese matrices.
2. Survival kinetics of *M. bovis* in Cheddar and Caerphilly cheeses.

In addition, a presentation of the results was presented to the Advisory Committee on the Microbiological Safety of Foods to provide an evidence base to inform advice to local council environmental health officers in the event of an incident and also future legislation, should that be deemed required.

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Appendix 1.1

Method: preparation of media

Preparation of Middlebrook 7H11 (*M. bovis*)

- Suspend 4.2g Middlebrook 7H11 Agar (Difco 283810) in 180 ml of distilled water in a 250 ml glass Schott bottle.
- Sterilise at 121°C for 10 min 7H10 Agar or 121°C for 15 min 7H11 Agar.
- Cool to 56°C[FMBDR003] and add the following supplements

1. OADC enrichment: - To each 180 ml volume aseptically add 20ml Middlebrook OADC enrichment and Swirl to mix.
2. Add appropriate antibiotic supplements as PANTA, PCAT, VAN.

PANTA

Concentrations reconstituted PANTA

Polymyxin B	10000 u
Amphotericin B	1000 µg
Nalidixic acid	4000 µg
Trimethroprim	1000 µg
Azlocillin	1000 µg

Add 5 ml BACTEC reconstitution fluid to each PANTA vial and add 1 vial per 180 ml 7H11 Agar base

Final concentration PANTA in Media

Polymyxin B	50 u
Amphotericin B	5 µg
Nalidixic acid	20 µg
Trimethroprim	5 µg
Azlocillin	5 µg

PCAT

The antibiotics were prepared as described below. Add 1ml stock per 180 ml Agar base

Antibiotic	Sigma Product Code	Solvent	Stock Concentration mg ml^{-1}	Final Concentration[a]
Polymyxin B	P-1004	UPW	5	(200)
Carbenicillin	C-1389	UPW	20	100
Amphotericin B	A-4888	DMSO	10	50
Trimethroprim	T-7883	DMSO	4	20

[a] All concentrations are in µg ml^{-1} except those in parentheses which are units ml^{-1}

Preparation of Middlebrook Gallaghers media (For the isolation of *M. bovis* from cheese) (Modified from Gallagher & Horwill 1977 The Journal of Hygiene 79: 155 – 160.)

- Suspend 4.2 g (Middlebrook 7H11 Agar (Difco 283810) in 180 ml of distilled water in a 250 ml glass Schott bottles.

- Boil to dissolve the agar completely.

- Cool to 56^{o}C[FMBDR003] and add the following supplements

3. OADC enrichment: - To each 180 ml volume aseptically add 20ml Middlebrook OADC enrichment and Swirl to mix.
4. 2 ml 0.2% (w/v) Malachite green solution

Dissolve 2g of malachite green powder in 100 ml distilled water by gentle heating. Filter sterilise.

5. 20ml Fresh bovine serum(final concentration (10% v/v)

PAA Laboratories Cat No. B11-008.

6. 1ml Lysed defibrinated sheep blood

Sheep blood defibrinated TCS Biosciences Ltd Cat No. SB054. Blood cells lysed by 4 successive freeze thawing at 50°C and -20°C.

7. Add appropriate antibiotic supplements as PANTA, PCAT, VAN.

PANTA

Concentrations reconstituted PANTA

Polymyxin B	10000 u
Amphotericin B	1000 µg
Nalidixic acid	4000 µg
Trimethroprim	1000 µg
Azlocillin	1000 µg

Add 5 ml BACTEC reconstitution fluid to each PANTA vial and add 1 vial per 180 ml 7H11 Agar base

Final concentration PANTA in Media (ml^{-1})

Polymyxin B	50 u
Amphotericin B	5 µg
Nalidixic acid	20 µg
Trimethroprim	5 µg
Azlocillin	5 µg

PCAT

The antibiotics were prepared as described below. Add 1ml stock per 180 ml 7H11 Agar base

Antibiotic	Sigma Product Code	Solvent	Stock Concentration mg ml^{-1}	Final Concentration[a]
Polymyxin B	P-1004	UPW	5	(200)
Carbenicillin	C-1389	UPW	20	100
Amphotericin B	A-4888	DMSO	10	50
Trimethroprim	T-7883	DMSO	4	20

[a] All concentrations are in µg ml^{-1} except those in parentheses which are units ml^{-1}

Polymyxin B
Polymyxin B = 8070u/mg
5 mg ml^{-1} Polymyxin B = 8070u/mg x 5 = 40350 u/ml

Preparation of 7H11++ media (For the isolation of *M. bovis* from cheese)

- Suspend 4.2g Middlebrook 7H11 Agar (Difco 283810) in 180 ml of distilled water in a 250 ml glass Schott bottle. For larger volumes suspend 8.4g Middlebrook 7H11 Agar in 360ml of distilled water in a 500 ml glass Schott bottle.

- Sterilise at 121^{o}C for 15 min.

- Cool to 56^{o}C[FMBDR003] and add the following supplements

8. *OADC enrichment: - To each 180 ml volume aseptically add 20ml Middlebrook OADC enrichment and Swirl to mix.
9. *20ml Fresh bovine serum(final concentration (10% v/v)
PAA Laboratories Cat No. B11-008.
10. *1ml Lysed defibrinated sheep blood
Sheep blood defibrinated TCS Biosciences Ltd Cat No. SB054. Blood cells lysed by 4 successive freeze thawing at 50°C and -20°C.
11. *Add appropriate antibiotic supplements as PANTA, PCAT.

*NB Double quantities of supplement for 360 ml 7H11 Agar base.

PANTA

Concentrations reconstituted PANTA

Polymyxin B	10000 u
Amphotericin B	1000 µg
Nalidixic acid	4000 µg
Trimethroprim	1000 µg
Azlocillin	1000 µg

Add 5 ml BACTEC reconstitution fluid to each PANTA vial and add 1 vial per 180 ml 7H11 Agar base or 2 vials per 360 ml 7H11 Agar base.

Final concentration PANTA in Media (ml^{-1})

Polymyxin B	50 u
Amphotericin B	5 µg
Nalidixic acid	20 µg
Trimethroprim	5 µg
Azlocillin	5 µg

PCAT

The antibiotics were prepared as described below. Add 1ml stock per 180 ml 7H11 Agar base or 2ml stock per 360 ml 7H11 Agar base.

Antibiotic	Sigma Product Code	Solvent	Stock Concentration mg ml^{-1}	Final Concentration[a]
Polymyxin B	P-1004	UPW	5	(200)
Carbenicillin	C-1389	UPW	20	100
Amphotericin B	A-4888	DMSO	10	50
Trimethroprim	T-7883	DMSO	4	20

[a] All concentrations are in µg ml^{-1} except those in parentheses which are units ml^{-1}

Polymyxin B
Polymyxin B = 8070u/mg
5 mg ml^{-1} Polymyxin B = 8070u/mg x 5 = 40350 u/ml

Appendix 1.2

Method: preparation of inoculated Cheddar cheese.

SOP prep. of Cheddar cheese containing the HG3 organism *Mycobacterium bovis.*
Ingredients

3.1 Raw milk (Farmview, Castlereagh).
3.2 Cheese starter culture MT24 (AJ & RG Barber Ltd)
3.3 Animal Rennet (Naturen Rennet, CHR Hansen)
3.4 *Mycobacterium bovis* filtered culture.

1. Equipment/Reagents

Table 1: Stainless steel cheese making equipment

	Stainless steel cheese making equipment
1.1	Cheese vat
1.2	Paddle
1.3	Flat mesh tool
1.4	Curved mesh tool
1.5	Large spoon
1.6	Horizontal cheese knife
1.7	Vertical cheese knife
1.8	Large palette knife
1.9	Cheddaring Plate
1.10	Cheddaring equipment support
1.11	Cheese Mould
1.12	Potato Chipper
1.13	Fish Slice
1.14	Tongs
1.15	Forceps x 4
1.16	Laboratory jacks x 2
1.17	Retort Pole x 3

Table 2: General equipment for cheese making

	General cheese making equipment
2.1	Masses 2 x 1Kg, 2 x 2Kg, 2 x 5Kg
2.2	Wired bung x 2
2.3	15 L Lowboy Carboy (Nalgene)- whey collection
2.4	15 L Lowboy Carboy (Nalgene) - 10% (v/v) Trigene
2.5	Modified lid assembly for Carboy
2.6	0.3 m Silicone Tubing Bore 25.4 mm Wall 3.2 mm
2.7	0.5 m Silicone Tubing Bore 25.4 mm Wall 3.2 mm
2.8	5 cm Silicone Tubing Bore 25.4 mm Wall 3.2 mm
2.9	1.5 m PVC Tubing Bore 25.4 mm Wall 3.2 mm
2.10	Inspection mirror
2.11	1 L wide neck jar x 2
2.12	27 L Polypropylene dunk tank 10% (v/v) Trigene
2.13	27 L Polypropylene dunk tank 200PPM Chloros
2.14	150 mm Polypropylene funnel
2.15	500 ml Pyrex Basin x 2
2.16	Semi-burette for titratable acdity
2.17	260 x 160 x 80 mm Polypropylene basin & Lid
2.18	300 x 200 x 100 mm Polypropylene basin & Lid
2.19	Boss head x 6
2.20	Clamps x 3
2.21	Long chipper support rod
2.22	Short chipper support rod
2.23	25 – 35 mm Jubilee clips x 4
2.24	27 mm Rubber Bung
2.25	33 mm Rubber Bung x 4
2.26	Heat resistant silicone gloves
2.27	Thermometer holder
2.28	Autoclave boxes

Table 3: Mains powered cheese making equipment

	Mains powered equipment
3.1	Cheese vat speed controller
3.2	Temperature - controlled water bath (Thermo-Haake)
3.3	Hot Plate
3.3	Baumatic Low Temperature cooler

Table 4: Consumable cheese making equipment

	Consumable cheese making equipment
4.1	Disposable hockey sticks
4.2	1000 µl tips
4.3	Kimtech process wipers
4.4	Polythene oversleeves
4.5	150 mm Polypropylene funnel
4.6	Nitrile gloves Regular Length
4.7	Nitrile gloves Xtra Length 350mm
4.8	Light polyethylene gloves
4.9	Cheese cloth (50 cm2 squares - autoclaved)
4.10	vacuum packing bags
4.11	Autoclave bags
4.12	Sharpsafe boxes
4.13	10ml Serology Pipettes
4.14	50ml Serology Pipettes
4.15	50ml centrifuge Tubes
4.16	50 ml weighing boats
4.17	200 ml weighing boats

Table 5: Cheese making chemicals/Reagents

	Chemicals/Reagents
5.1	8L Raw milk
5.2	MT24 (AJ & RG Barber Ltd)
5.3	Vegetarian rennet
5.4	Sodium Chloride
5.5	Sodium hydroxide Sol^{n} (M/9)
5.6	Phenylthalein solution (Reagecon, IPTOH5H)
5.7	Chloros solution
5.8	Trigene solution

2. Methodology

Preparation of Cheese vat, accessories and utensils

On the afternoon before the cheese is to be produced

- Prepare 20 L 200 PPM chloros solution. Fill the chloros tank to the 20 L marker with tap water before adding 40 ml sodium hypochlorite 100000 PPM.
- Prepare 20 L 10% (v/v) Trigene in the Dunk Tank. Add 2 L of Trigene to the Tank before filling the tank to the 20 L marker with tap water. Prepare a further 15 L of 10% (v/v) Trigene in 3 x 5 L Trigene containers (500 ml in 4.5 L).
- The cheese vat is connected to the temperature controlled water bath and the water bath turned on (water in at bottom - water out at top). Both the cheese vat and water bath should be positioned outside the cabinet at this point.
- Place the paddle in the cheese vat.
- Attach Retort Pole 1 with a clamp to the motor side of the cheese vat (Position A) and Retort Pole 2 to the valve end of the cheese vat (Position B).

- To disinfect the cheese vat and paddle pour approximately 10 L of the chloros solution from the chloros tank into the cheese vat.

- Using the clamp attached to Retort Pole 1 suspend the thermometer over the vat co that the thermometer bulb is approximately 20 mm below the liquid surface. Endure the thermometer does not interfere with the movement of the vat paddle
- Using a jubilee clip attach 1.5 m of PVC tubing to the outlet of the cheese vat.

Preparation of safety cabinet

On the afternoon before the cheese is to be produced

- Open the front door of the cabinet to allow better access to the cabinet.
- Cover work surface of Class I cabinet with Kimtech process wipers.
- Place the 20 L 10% (v/v) Trigene dunk tank to the immediate left of the cabinet. The Trigene dunk tank should be rested on four 35 mm rubber bungs.
- Place 2 polypropylene trays (400 x 150 mm) along the back wall of the cabinet. Above the trays position suction cups with small and large hooks. These are used to hang contaminated equipment over the polypropylene trays between manipulations e.g cheese making utensils and the paddle.
- Other receptacles for the disposal of contaminated waste should be placed at the right hand side of the cabinet.
    - Autoclave bag
    - Sharpsafe box
    - 1 L wide neck jar 500ml (20% v/v) Trigene
- Fill the burette reservoir bottle with M/9 NaOH and bring the burette to the 0 mark before placing it to the right hand side of the cabinet in the Class I cabinet. Secure burette to the cabinet wall using a suction cup hook and cable ties.
- Close the front door of the cabinet.

Preparation of *M. bovis* culture

On the afternoon before the cheese is to be produced

- For high-inoculum Caerphilly

Remove 10 x 10 ml frozen aliquots of *M. bovis* culture from storage at -80°C to a biopot. Use some scrunched up white roll to keep the tubes upright inside the biopot before transferring the biopot to the chilled cabinet in G61.

- For low-inoculum Caerphilly

Remove 1 x 10 ml frozen aliquots of *M. bovis* culture from storage at -80°C to a biopot. Use some scrunched up white roll to keep the tube upright inside the biopot before transferring the biopot to the chilled cabinet in G61.

Preparation of Cheese Vat accessories and utensils

On the morning of cheese production

- Immerse the equipment listed below in the chloros solution.

    Horizontal cheese knife
    Vertical cheese knife
    Flat stainless steel mesh strainer
    Curved stainless steel mesh strainer
    Stainless steel spoon
    Wired bung for blockage of cheese vat drain x 2
    Cheese cloth
    Cheddaring Plate
    Cheese Mould
    260 x 160 x 80 mm Polypropylene basin & Lid
    300 x 200 x 100 mm Polypropylene basin

- Using the attached tubing empty the chloros from the cheese vat back into the chloros tank.
- Loosen the jubilee clip and detach the large tubing from the cheese vat.
- Turn off the circulating pump and detach the cheese vat from the water bath. Stopper both tubes using 13 mm rubber bungs.
- Lift the cheese vat and carry to the sink.
- Direct the closed cheese vat valve over the sink.
- Using tap water rinse the cheese vat, paddle and thermometer with water until approximately 1/3 full. Open the valve and continue to rinse with tap water whilst water drains. Close the valve and repeat the rinsing procedure.
- The cheese vat is now ready for transfer to the safety cabinet.

Placement and preparation of cheese vat inside the safety cabinet

- Open the front door of the cabinet to allow better access to the cabinet.
- Soak work surface of Class I cabinet covered by Kimtech process wipers by pouring n approx. 1 L 5% (v/v) Trigene.
- Position the cheese vat to the immediate right of the dunk tank.
- Pass power cable from cheese vat motor behind the trigene dunk tank.
- The cheese vat is connected to the temperature controlled water bath located on the adjacent bench. Pass the tubes through the opening of the cabinet. Unstopper the tubes and connect the outlet of the water bath to bottom inlet of the cheese vat and secondly the inlet of the water bath to upper outlet of the cheese vat.
- Turn on water bath.
- Connect cheese vat motor to the power supply and speed controller also located on the adjacent. Pass cable through the opening of the cabinet.
- Remove the wired bung from the chloros dunk tank and rinse with distilled water. **Insert the wired bung to block the cheese vat drain.** Clamp the wire end of the wired bung using a large tube clamp. In turn using a boss head secure the tube clamp to Retort Pole 2. Ensure that the bung wire is close to the cheese vat wall and will not inhibit the movement of the cheese vat paddle.
- Check the main vat valve is closed.
- Add 8 Litres raw milk (3.1) to the cheese vat. Once the milk has been added remove the plastic containers from the cabinet and dispose in autoclave bags outside the cabinet.
- **Record time milk is added.**
- Connect paddle to the cheese vat electric motor by connection of the drive arm to the bottom connection hole of the cheese vat paddle.
- Turn on motor by pressing RUN on the speed control unit. Adjust paddle speed by turning the frequency control knob until the digital display shows 23.
- Close the front door of the cabinet turn on fan.

Set up apparatus for Tirtrateable acidity (TA) and determining TA from a milk or whey sample

- TA will be determined in 50 ml weighing boats.
- Use an empty sealed pipette tip box to elevate the weighing boat thus reducing the distance between the burette tip and the weighing boat.
- When determining TA from milk add 1 ml Phenylphthalein indicator (Reagecon, IPTOH5H) to the weighing boat before introducing the weighing boat to the cabinet.

- Withdraw a 10 ml sample of milk or whey using a pipette and transfer to the weighing boat. Titrate quickly with M/9 NaOH from the burette, while stirring continuously using a disposable hockey stick. Note volume of NaOH titrated.
- Calculate and record the titratable acidity as % lactic acid by volume; titre/10 = % lactic acid.
- Before inoculating the milk with starter or *M. bovis* the milk TA should be determined. All pipettes used should be disposed of in the sharp safe box inside the cabinet.
- The waste liquid from determining TA should be disposed of in the 1 L wide neck jar.
- The used weighing boats and disposable hockey stick should be disposed of in the autoclave bag inside the cabinet.

Inoculation of 8 Litres raw milk in cheese Vat

- Transfer the biopot containing the inoculum tubes to the Class I cabinet. Also have 10 ml pipettes ready for the transfer of inoculum from the tubes to the vat.
- Once *M. bovis* culture has been introduced to the cabinet all future manipulations whilst wearing Nitrile Xtra Length 350mm gloves.
- A second pair of regular length gloves should be worn over the long gloves and removed after each manipulation inside the cabinet. Short gloves should be disposed of in the autoclave bag inside the cabinet.
- Typically for high level cheesemaking, 100 ml of *M. bovis* culture (10^6 CFU/ml) will be added to the 8 Litres of raw milk - (a final volume of 8 Litres containing 10^4 CFU/ml).
- Carefully loosen the lids of the 15 ml centrifuge tubes whilst they are in the biopot.
- Taking one tube at a time first remove the lid completely and carefully transfer 10 ml thawed *M. bovis* inoculum to the cheese vat using a 10 ml pipette.
- Repeat until all 10 of the *M. bovis* inoculum have been added.
- Use the biopot to store and transfer samples of inoculum, inoculum & milk and whey to the adjacent cabinet for enumeration. Discard each empty centrifuge tube and lid to the autoclave bag in the cabinet.
- For low level cheesemaking make a -1 serial dilution (FMSOP24). Add 1 ml of this -1 serial dilution to the 8 Litres of raw milk.
- **This represents a large volume of milk contaminated with a high level of a Hazard Group 3 pathogen. CARE MUST be exercised from this point onwards to avoid splashes and spillages from the vat.**
- Equilibrate milk temp (approx 30mins) to 32ºC, monitoring temperature using the thermometer.

Preparation and addition of starter

- The cheese starter culture is MT24 (AJ & RG Barber Ltd). A pint bottle of MT24 starter was thawed and aliquoted into 10 ml volumes in 15 ml polypropylene centrifuge tubes. These 10 ml aliquots were then stored at -80 ºC.
- Before use the starter is bulked up to 160 ml. 2 days before cheesemaking remove a 10 ml aliquot from storage at -80 ºC. Place the starter aliquot in a 22ºC water bath for 16 Hours incubation (Into water bath 16:00). On the day before cheesemaking after 16 Hours incubation remove the starter aliquot from the water bath and store at 4 ºC. That afternoon transfer 1 ml of the starter to 160 ml of pasteurised milk in a honey jar (Into water bath 17:00). Incubate at 22ºC for a further 16 Hours.
- On the morning of cheese making when the milk has reached 32 °C remove the 160 ml of bulk MT24 starter from the 22ºC incubator and add evenly over the surface of the cheese vat to the 8 L of milk. Rinse the honey jar out with 50 ml uninoculated raw milk from and 50 ml centrifuge tube.

- Discard the honey jar and 50 ml centrifuge tube to the autoclave bag inside the cabinet described previously.
- **Record time and quantity of starter culture added.**
- Allow milk to 'ripen' with continued agitation (via paddle) for 1 hour at 32ºC.
- **Determine and record the TA after 1 hours ripening as described previously.**
- A significant increase in titratable acidity should have occurred (10%).

Addition of Rennet

- Start to prepare rennet for addition to the cheese vat approx 10 mins before the end of ripening period.
- Pipette 1.6 ml of rennet into a 30 ml universal.
- Immediately before the rennet is to be added dilute with approx 14.4 ml sterile distillled water in the 30 ml universal by filling the universal to just above the 15 ml line.
- Introduce 30 ml universal to the safety cabinet. Remove the lid and discard in the autoclave bag inside the cabinet.
- Gently pour the contents of the sample evenly over the milk surface.
- The 30 ml universal should be disposed of in the autoclave bag inside the cabinet.
- **Record time of addition of Rennet and quantity added.**
- Stir the rennetted milk for 3 min.
- Stop paddle agitation by disconnecting the drive arm from the paddle.
- Rest the moving drive arm on the left hand side vat handle.
- Remove the central paddle from the cheese vat. Allow excess milk to run off the paddle into the vat by holding paddle at an angle over the vat (approx 30 secs).
- Using the suction cups and larger hooks hang the paddle over the temporary storage tray.
- Place cheese vat lid over cheese vat during coagulum formation.
- Allow coagulum formation to proceed for 45 minutes.
- Approx. 5 mins before renneting is completed remove the following equipment from the from the chloros dunk tank.

  Horizontal cheese knife
  Vertical cheese knife
  Large palette knife

- Rinse equipment over the sink using tap water.
- Once equipment has been rinsed place items inside the safety cabinet. The palette knife should be hung over the temporary storage trays. Rest the remaining items on the temporary storage trays so that surfaces that will be in contact with coagulated milk are not contaminated with trigene disinfectant.

Cutting

- Remove the cheese vat lid and store behind in gap behind trigene dunk tank.
- Test the set of the milk by making a slight cut in the surface using the large palette knife. Subsequently insert the knife at the start of the cut at a 45 ° angle approximately 50 mm into the milk. Lift the palette knife so that the coagulated milk is also raised. The coagulated milk should split cleanly along the line of the original incision and yellow whey should be observed in the cut.
- If a clean split is observed the milk is ready for cutting if not return the palette knife to a hook over the temporary storage tray and wait a further 5 – 10 mins before repeating the test.

- A standard procedure of cutting is used to ensure uniformity in the size of the curd pieces produced. The vertical knife is first drawn lengthways along the vat and then across it.
- Once the vertical knife has been used person B should raise the lid of the trigene dunk tank.
- Person A should allow curd pieces/whey to drip into the vat before gently transferring the vertical knife to the trigene dunk tank.
- Repeat the cutting and disposal process with the horizontal cheese knife.
- **Determine and record whey TA also record time and temperature.**
- Once cutting has been completed allow the curd to heal for 5 minutes.
- The central paddle is replaced in the vat and agitation continued as described previously 15 rev/min.

Scalding

- In the absence of stirring during the coagulation step the temperature of curd/whey may have fallen below 32 ºC
- Immediately after agitation has been resumed increase the temperature of the circulating water bath by 1 ºC.
- **Record the time, target temperature of the circulating water bath and the thermometer reading of the cheese vat.**
- Use the temperature control unit to raise the temperature of curd/whey from 32 ºC to 40 ºC in a linear manner over a period of 1 hour (1 ºC ~ every 7 minutes).
- **Every 7 mins record the time and temperature.**
- **When curd/whey has reached Max scald temperature (40 ºC) record time also determine and record TA.**
- Curd/whey is held at 40 ºC for 1 hour with continuous agitation.
- **After 1 hour of scalding at 40 ºC record time also determine and record TA**.
- The curd pieces if squeezed against the wall of the cheese vat using the large spoon should be firm and retain their shape.
- Check the temperature frequently during this period and also ensure that the curd does not settle on the bottom of the vat. Use the stainless steel spoon and palette knife to dislodge curd from the walls and bottom corners of vat. Between mixes place the palette knife and stainless steel spoon on the temporary storage tray.
- Approx. 5 mins before scalding is completed remove the following equipment from the from the chloros dunk tank.

  Flat stainless steel mesh strainer
  Curved stainless steel mesh strainer
  150 mm Plastic Funnel - shortened spout

- Rinse equipment over the sink using tap water.
- Once equipment has been rinsed place items inside the safety cabinet. The Curved stainless steel mesh strainer should be hung over the temporary storage trays. Rest the flat stainless steel mesh strainer on the temporary storage trays so that surfaces that will be in contact with coagulated milk are not contaminated with trigene disinfectant.

Pitching

- Stop paddle agitation by pressing STOP on the speed control unit.
- Detach the drive arm from the paddle and remove the central paddle from the cheese vat. Allow excess milk to run off the paddle into the vat by holding paddle at an angle over the vat (approx 30 secs).
- Using the suction cups and larger hooks hang the paddle over the temporary storage tray.
- After 5 mins remove the thermometer and clamp (Person B). Place the clamp to the left of the cheese vat in front of the trigene dunk tank. The thermometer should be placed on the temporary storage tray.
- Use the flat mesh strainer to push the curd back from the vat outlet about 1/3 of the way along the cheese vat (Person A). The flat mesh strainer arm should be at the motor side of the vat pointing away from the outlet. After the strainer has been positioned clamp in place using Retort Pole 1 (Person A).
- Detach the tube clamp of the wired bung from Retort Pole 2. Temporarily secure the tube clamp of the wired bung by tucking it through the handle of the cheese vat.
- With the large spoon transfer any curd pieces on the outlet side of the flat mesh strainer to the other side of the strainer. Return the large spoon to its position over the temporary storage tray.
- Unclamp the tube clamp of the wired bung from below the cheese vat handle.
- Grasp the tube clamp of the wired bung in the left hand using a pair of forceps in the right grasp the wire.
- Place left wrist on the edge of the cheese vat wall (cabinet door side) move hand backwards away from the outlet until the wire is taut. Once wire is taut slowly pull the wire only using a wrist action. The bung should be released at this point. Do not pull using an arm motion as this can cause the bung to be released suddenly producing a splash. Still holding the wired bung by the handle and wire transfer carefully to the temporary storage tray.
- Position the curved stainless steel mesh strainer around the cheese vat outlet.
- At this point prepare the funnel for collecting a fresh whey sample obtained by curd squeezing.
- Clamp the funnel over the cheese vat using retort Pole 3 (Position C Cabinet Door side). The funnel should drain into the vat outlet side of the flat mesh strainer.
- After the curds have been pitched for 10 mins obtain a fresh whey sample by transferring a sample of curd using the stainless steel spoon into the 150 mm funnel held over the cheese vat.
- Allow the whey to drain from the funnel into the vat for a few seconds before pressing the curd mass in the funnel using the stainless steel spoon (Person A).
- Person B - hold a 200 ml weighing boat below the funnel and collect the whey until enough for a 2 x 10 ml sample has been collected place the 200ml weighing boat and whey sample beside the TA apparatus.
- Person A - unclamp the funnel and lower the wide funnel end towards the surface of the whey. Using the spoon ease the curd sample out of the funnel and into the whey.
- Person A - hang up the stainless steel spoon and place the funnel on the temporary storage tray.
- Person A – Detach Retort Pole 3 from the cheese vat.
- The cheese vat is now ready to be raised.

Whey Run

- Person A – lift using cheese vat handles ensuring that the vat is kept level (Approx. 50 – 100 mm).
- Person B – Quickly place laboratory jacks below the cheese vat.
- Person A – Immediately rest the cheese vat on the lab jacks.
- Person A – Screw lab jack knobs to elevate the cheese vat. Proceed until the cheese vat has reached maximum elevation.
- Person B – Whilst the cheese vat is being elevated the vented 15 L low boy carboy containing 700 ml of Trigene should be introduced to the safety cabinet.
- Trigene is used as a disinfectant as whey may contain *M. bovis* cells. Insert the carboy into the cabinet with the spigot opening pointing away from the cheese vat. The larger opening should be directed away from the cabinet and into the lab. Once the carboy has been inserted into the cabinet as far as the larger opening rotate the carboy in a clockwise direction. The rest of the carboy should slip easily into the cabinet.
- Person B – Remove the lid from larger carboy opening and set on top of the carboy.
- Person B – **Ensure the spigot closure of the 15 L carboy is tightly closed.**
- Person B – Screw modified lid assembly and attached silicone tubing on to carboy opening. Ensure that when the assembly is tightened the tubing is directed towards the cheese vat outlet.
- Person A – Once the cheese vat has been sufficiently elevated connect silicone tubing to the cheese vat outlet and secure using a jubilee clip. Set screw driver to the left of the cheese in front of the trigene dunk tank.
- Person A & B – Before commencing drain the jack positions should be checked if necessary move the jack at the motor end of the cheese vat towards the back of the cabinet so that it better supports the cheese vat.
- Person A – Lift cheese vat.
- Person B – Position motor end lab jack.
- Person B – **Determine and record whey TA of sample obtained at the end of pitching.**
- Person A – Open cheese vat outlet slightly and allow the whey to drain slowly into the carboy. **Record Time.** The progress of draining can be checked by using the inspection mirror. Draining should take 10 mins, but the speed of drainage should be increased if the TA was high at the time of pitching.
- When drainage is nearing completion slightly lower the jack at the outlet end of the cheese vat. Insert two door wedges between the cheese vat and the jack at the motor end so that draining can be continued.
- Person A – Lift cheese vat.
- Person B – Position door wedges between the cheese vat and the jack at the motor end.
- This produces a gradient in the cheese vat allowing further whey drainage.
- Once drainage has slowed again the cheese vat should be lowered.
- Person A – Close cheese vat valve and commence lowering of the cheese vat.
- Person B – Detach silicone tubing from the cheese vat outlet. Return screw driver to the left of the cheese vat. The lid assembly should be detached from the carboy and passed to person A for disposal in the Trigene dunk tank. Do not put the jubilee clip into the dunk tank. The carboy lid should be used to reseal the carboy.
- The sharp safe box, 1 L wide neck jar and autoclave box should be brought forward and the carboy placed upright in the immediate right hand corner of the cabinet.
- Person A – Once lowered lift cheese vat (Approx. 50 – 100 mm).

- Person B – Quickly remove laboratory jacks from below the cheese vat and place between the cheese vat and the temporary storage trays.
- Person A – Immediately rest the cheese vat on the floor of the safety cabinet. Unclip the curved mesh strainer. Allow whey to drip from strainer before transferring to the trigene dunk tank. Unclamp the flat mesh strainer briefly allow whey to drip into cheese before transferring to the trigene dunk tank. Remove boss head from Retort Pole 1 and set to the left of the cheese vat in front of the trigene dunk tank.
- During the cheddaring process further whey will be released by the curd mass. To ease collection of the wired bung should be reinserted in the cheese vat outlet.
- Person A – Position door wedges below the cheese vat at the valve end.
- Person B – Prepare several pairs of light polyethylene gloves.
- The light polyethylene gloves are to be worn as a third pair of gloves over the other pairs of blue nitrile gloves.
- Person A – Put on a pair of polyethylene gloves and retrieve the wired bung from the temporary storage tray and pass tube clamp of the wired bung below the right hand vat handle as before.
- Person A – Insert wired bung in the outlet of the cheese vat. Hands should be held over the cheese vat until Person B is ready to remove polyethylene gloves.
- Person B – Remove gloves from Person A using forceps and immediately dispose of in autoclave bag inside the cabinet.
- Person A – Reposition door wedges below the cheese vat at the motor end.
- A significant quantity (>30 ml) of whey may still remain in the cheese vat. This whey can be extracted by pipetting using a 50 ml pipette.
- Person B – Using a 50 ml pipette withdraw whey. Transfer enough for 2 TA samples to a 50 ml weighing boat and a further 50 ml in a labelled 50 ml centrifuge tube to the biopot.
- The remaining whey in the pipette can be disposed of in the 1 L wide neck jar. Repeat the process until a small volume of whey remains (10 – 20 ml). All pipettes used should be disposed of in the sharp safe box inside the cabinet.
- Person A – At this point all liquid samples have been taken and the biopot is ready for transfer to the adjacent cabinet for enumeration procedures. Wipe down the surface of the biopot with 10% Trigene and pass to Person B outside the cabinet who can complete the transfer to the adjacent cabinet.
- **Determine and record whey TA and time.**

Cheddaring

The development of the fibrous curd structure is dependent on curd flow and the manual operations of cutting and stacking curd. For curd masses <1 kg a system is used in which pressure is applied to the curd in a controlled and incremental manner.

- Person B – remove the following equipment from the chloros dunk tank and rinse.
  Cheddaring Plate
  Fish Slice.
- Person B – Attach the Cheddaring Plate to the cheddaring equipment support. Pass the assembled cheddaring equipment to Person A.

- Person A – Rest the Cheddaring Plate on the temporary storage tray so that surfaces that will be in contact with the curd mass are not contaminated with trigene disinfectant. Using the palette knife cut a channel in the curd mass to allow more whey to flow towards the valve end of the vat. Use the fish slice to separate the curd mass from the walls of the cheese vat and then place one half of the curd mass on top of the other. Move stacked curd mass to the centre of the vat where it will fall directly below the cheddaring plate. Hang fish slice over temporary storage tray.
- Person A – Reattach Retort Pole 3 and secure the cheddaring equipment between Retort Poles 1 and 3 by tightening attached boss heads. Ensure the curd mass is positioned directly below the cheddaring plate. If necessary the curd mass can be positioned using the fish slice.
- Person A – Place a 1 kg mass on the chedddaring equipment. **Record time.**
- Person A – After 15 minutes remove the 1 Kg mass set to the right outlet side of the cheese vat. Loosen the boss heads were they secure the cheddaring equipment support cross bar. This allows the chedddaring equipment to be rotated out the way and rested against the floor of the cheese vat. Repeat the stacking and cutting procedure described previously using the fish slice. Position the curd mass and rotate the chedddaring equipment so that it is again situated over the curd mass. Resecure the boss heads to cheddaring equipment support cross bar. The fish slice can be stored in cheese vat during cheddaring.
- Person A – Place a 1 kg and 2 kg masses on the chedddaring equipment. **Record time.**
- Person B – Whilst Person A performs curd handling Person B can pass 2 kg & 5 kg masses as required and can carry out sampling procedures (FMBDR070) in the adjacent cabinet.
- **If sufficient whey has been produced during the 15 mins cheddaring periods take a 10 ml sample and determine and record TA as previously described.**
- Repeat this process until 16 kg has been placed on the cheddaring equipment as described in Table 6. A target TA of 0.45% la should be achieved by the end of the cheddaring process.

Table 6

Cheddaring Time (min)	Mass in Cheddaring Box (Kg)
0 - 15	0
15 - 30	2
30 - 45	4
45 - 60	8
60 - 75	16
75 - 90	16

- Person B – Once the target TA has been achieved remove the masses and stack carefully to the right of the cheese vat.

- Person A – Loosen the boss heads were they secure the cheddaring equipment support cross bar. Rotate the cheddaring equipment so that the cheddaring plate is raised out of the cheese vat where it can be detached from the cheddaring equipment support. Carefully place the cheddaring plate in the Trigene dunk tank. Completely release boss heads securing the cheddaring equipment between Retort Pole 1 and 3. Store the cheddaring equipment in the right hand corner of the cabinet behind the Trigene/Whey carboy. Rehang the fish slce over the temporary storage tray.

Weighing
Before the cheese is salted the mass of cheese produced must be determined.

- Person A – Remove Retort Pole 2 & 3.
- Person B – Remove the following equipment from the chloros dunk tank and rinse. Shake excess water from basins and dry with a piece of white role.

  260 x 160 x 80 mm Polypropylene basin & Lid
  300 x 200 x 100 mm Polypropylene basin
  Tongs.
  Potato Chipper

- Person B – Set the Potato Chipper on a the bench to the left hand side of the Safety cabinet along with the Pass long chipper support bar and short chipper support bar. Pass the tongs to Person A inside the cabinet.
- Person A – Place tongs on the temporary storage tray.
- Person B – Set the smaller basin inside the larger basin before placing the lid on each basin. Tare both basins on the balance and record mass. Place both basins on transport trolley so that they are convenient to the cabinet. Stick 4 pieces of tape around the smaller lid. These pieces of tape will be used to secure the lid to the basin.
- Person B – Hold the polypropylene basin (left hand) and lid (right hand) inside the cabinet do not allow the basin to touch any surfaces inside the cabinet.
- Person A – Using the tongs transfer the curd mass from the cheese vat to the polypropylene basin held by Person B. Tongs can be stored in the cheese vat at this point.
- Person B – Once the curd mass has been transferred immediately put the lid on the smaller basin. Then remove the smaller basin from the cabinet and carefully place inside the larger basin on the transport trolley. Immediately lid the larger basin and secure in place with the tape.
- Person A – Reattach Retort poles 2 & 3.
- Person B – Weigh basins and curd mass.
- Person B – Pass long chipper support bar, short chipper support bar and the potato chipper to Person A.
- Person A –. Assemble milling equipment. Secure the long chipper support rod between Retort Poles 1 and 3 approx. 50 mm above the top edge of the cheese vat.
- Person B – Determine the mass of curd. From this value calculate the quantity of salt required to add salt at 2.4% (w/w) (The final target for salt is 2%).
- Person B – Remove the larger basin lid and set outside the cabinet pass both basins to Person A inside the cabinet.
- Person A – Set both basins inside the cheese vat before removing the small basin and setting to the immediate right of the cheese vat valve. In turn remove the small basin lid and set adjacent to the right hand side of the small basin.

- Person A – Attach short chipper support bar to Retort Pole 2 so that it is level with and parallel to the long chipper support rod. Attach the potato chipper to the short support rod by sliding the rod between the rear lip and the square base of the chipper. The front of the chipper can now be lowered on to the long support rod.

Milling

- Person A – In the smaller basin cut the curd mass using the pizza cutter into 20 x 40 mm cubes. Transfer to potato chipper for further milling to 10 x 10 mm cubes.
- Person B – Operate potato chipper only 1 – 2 large cubes at a time and move the build up below the chipper to the rear of the basin using the fish slice.
- Continue milling process until all the curd mass has been cubed.
- Person A – After completion of milling detach the potato chipper. Remove the metal cutting baldes and plastic part from the potato chipper. Transfer the main body of the potato chipper to the trigene dunk tank. Remove pieces of curd from the cutters and plastic component using forceps. Discard these curd pieces in the autoclave bag. Set the cutter and plastic component on the temporary storage tray. Remove both chipper support rods and place at the left hand side of the cheese vat.

Salting

- Add salt in two portions to the milled curd inside the large polypropylene basin mix the curd using the fish slice after the addition of salt.
- Allow the curd to mellow and the salt to dissolve for 15 minutes, stir twice during this period.
- Person B - Whilst the curd pieces are mellowing remove cheese cloth from the chloros and rinse using tap water before passing to Person A. Subsequently remove the cheese mould components from the chloros tank and using the alum key tighten the grub screw ensure the cylindrical mould is tightened against the cheese mould base locators.
- Person A – Use the cheese cloth to line the small polypropylene basin.
- Person B – Place the cheese mould in the cabinet on top of the lid from the small basin. Also transfer the 2 large nuts for the cheese mould screw rods to the cabinet.
- Person A – Position the cheese mould on the lid. The lid will serve as a tray for the collection of whey during cheese moulding.

Mould Filling

- Person B – Prepare several pairs of light polyethylene gloves. Pass a pair of light polyethylene gloves to Person A. Remove the large polypropylene basin from the cheese vat. Pour about 1/3 – 1/2 the salted curd pieces into the cheese cloth lined small polypropylene basin. Return the large polypropylene basin to the cheese vat.
- Person A – Put on a pair of polyethylene gloves. Gather up the cheese cloth into a parcel containing the salted curd and transfer to the cheese mould. Ensure all the cheese cloth is tucked inside the mould.
- Person B – Using the follower and handle push down on the curd pieces to create more space in the cheese mould. Then remove follower and screw from the cheese mould and rest on the bottom of the cabinet so that the follower is not in contact with trigene.
- Person A – Whilst wearing the polyethylene gloves reopen the cheese cloth and pull over the edge of the cheese mould. Move to the left of the cabinet so as not to obstruct Person B as they fill the mould.
- Person B – Use the large stainless steel spoon to transfer curd pieces into the cheese mould until the mould has been filled to the top edge.

- Person A – Whilst wearing the polyethylene gloves tuck the cheese cloth back inside the cheese mould.
- Person B – Using the follower and screw push down on the curd pieces to create more space in the cheese mould. If there is still salted curd in the large polypropylene basin repeat these steps until all the curd has been transferred to the cheese mould.
- Person B – Once the filling of the mould has been completed remove the light polyethylene gloves from Person A using forceps. Dispose of the gloves in the autoclave bag.
- Person A – Rotate the screw until the rectangular plate and circular follower are as close as possible to each other. Line up the holes in the rectangular plate with the screw rods of the base of the cheese mould. Rest follower on top of the curd pieces and cheese cloth.
- Person A & B – Tighten the 2 nuts down on to the rectangular plate until the plate rests on top of the cheese cylindrical mould.
- Person B – Hold the cheese mould securely by the screw rods.
- Person A – Tighten the screw handle until it cannot be rotated any further. This presses the curd pieces into the shape of the cheese mould.
- Person A – After about 10 minutes loosen the screw handle slightly (3 or 4) turns. Using the alum key loosen the grub screw. .
- Person B – Once the screw handle has been loosened completely remove both nuts from the large screw rods.
- Person A – remove the rectangular plate and screw handle and rest on the bottom of the cabinet in front of the Trigene dunk tank.
- Person A – Put on a pair of polyethylene gloves and remove cylindrical cheese mould from the base plate.
- Person B – Hold the neck of a thick polypropylene bag inside the cabinet.
- Person A – Whilst wearing the polyethylene gloves place cylindrical cheese mould inside the polypropylene bag.
- Person B – Role up the sides of the bag and twist the neck of the bag shut. Immediately transfer the bagged cheese mould to the adjacent cabinet.
- The cheese is now ready for pressing in the adjacent cabinet.

Pressing

- Have the rinsed stainless steel followers available before starting to press the cheese and prepare the vacuum pack bags by slicing with a scalpel along the bottom and one of the sides.
- Open up the vacuum pack bag and lay over the top of a piece of Kimtech process wiper. This provides a uncontaminated surface on which the cheese can be manipulated. Use another piece of Kimtech process wiper cut to the appropriate size to cover the back brace and base of the cheese press. This soaks up contaminated whey expelled during pressing.
- Person A – Whilst wearing polyethylene gloves remove the cheese mould from the bag. Set the mould on top of the opened vacuum pack bag. Remove the cheese from the mould. There will be parts of the cheese where the cheese cloth was caught between curd pieces during mould filling. Carefully unwrap the cheese and pull the cheese cloth up the sides ensuring that the cheesecloth is freed from between the curd pieces. Gather the cheese cloth round the top of the cheese and carefully reinsert to the cheese mould. Set the cheese mould on top of the 10 mm follower and use the 2 mm follower to cover the other end. With both hands lift the mould and followers together and rest in position on top of the kimtech wiper protecting the cheese press.

- Person A – The 10 mm follower should be towards the back brace of the cheese press.
- Person B – Whilst Person A holds the cheese mould and followers in place attach the pressing rod and adapter to the cheese press pneumatic ram.
- Person B – Again whilst Person A holds the cheese mould and followers in place Pump the handle of the Enerpac Pump until 0.7 kN is observed on the dial. This pressure will drop as whey is exuded to approximately 0.5 kN.
- The cheese pressing is allowed to proceed for 1 hour. During this period commence cleanup. Consult cleanup procedure below.
- After 1 hour initial pressing the cheese is ready for turning.
- Person B – Open the pressure release valve gradually to return the follower to its initial position.
- Person A – Whilst wearing polyethylene gloves hold the followers in place and turn the cheese mould through 180°.
- Person B – Whilst person A holds the cheese mould and followers in place pump the handle of the Enerpac Pump until 3.5 kN is observed on the dial this will fall to 2.5 kN as further whey is expelled. Leave the cheese pressing overnight with the cabinet running.
- Person A – On the next morning after 16 hours pressing slowly open the pressure release valve to return the pneumatic ram to its original position. Lay a cut vacuum pack bag flat on the bottom of the cabinet. Remove the cheese mould whilst wearing polyethylene gloves. Push the 2 mm follower against the cheese inside the mould so that the cheese is forced from the mould. Rest the cheese on the surface of the open vacuum pack bag. Return the empty cylinder to position on the cheese press.
- Person B – Whilst wearing polyethylene gloves use a pair of sterile forceps peel the cheese cloth from the top and sides of the cheese.
- Person A – Lift and separate the cheese from the cheese cloth. Return the cheese to the vacuum pack bag and discard the cheese cloth and polyethylene gloves to an autoclave bag inside the cabinet.
- Allow the cheese surface to dry for several 10 – 15 minutes before waxing turning at regular intervals.
- Before waxing - slice a 1 cm thick section from one end of the cheese this provides a sample for *M. Bovis* enumeration and the remainder can be used for chemical analysis.

Waxing

Table 7

	Wax Type	Number of coats	Quantity (g)	Temp °C
Layer 1	Translucent	1	110	110
Layer 2	Red	2	150	95

Waxing - Preparation

- Before preparing the wax turn on the hot plate set to 146 °C inside safety cabinet (the temp reading of thermometer in the wax should reach 110 °C).
- Lay white roll over the Kimtech wiper adjacent to the hot plate this will prevent the Kimtech roll being melted by the pryrex dish during waxing or cooling.
- Weigh the required amount of each wax into clean Pyrex basins.

- First melt the translucent wax on another hot plate outside the cabinet. Gently swirl the wax whilst holding the basin using heat resistant silicone gloves.
- Once the wax is completely molten transfer to the hotplate inside the cabinet.
- Using a thermometer determine the temperature of the molten wax. If the temperature reading is >110ºC remove the basin from the hot plate and gently swirl on the white roll. If the temperature is <110ºC gently swirl the basin on the hot plate until the target temperature is reached.
- Once the desired temp is reached the cheese should be dipped immediately. This part of the process requires two people.

Waxing - Dipping

- Person B – Remove thermometer from the basin. Keep the thermometer in hand to monitor wax temperature between first and second dipping.
- Person A – Whilst wearing polyethylene gloves grasp the cheese at one end. Lift the cheese over the molten wax and in a careful vertical motion submerge the cheese in the wax. Continue downwards until the wax has travelled as far as possible up the walls of the cheese and basin without overflowing the basin. Then remove the cheese from the basin and rest the unwaxed end of the cheese on the open vacuum pack bag. Allow the wax to solidify for 1 min. Discard the polyethylene gloves to an autoclave bag inside the cabinet.
- Person B – Return the Pyrex basin to the hotplate and maintain the temp at 110ºC.
- Person A –Grasp the cheese by the waxed end and repeat the process as described previously. Take care as the waxed surface has less purchase than the unwaxed cheese surface. Any small unwaxed areas can be filled by molten wax applied from the tip of the thermometer.
- If excess wax will not be needed for waxing other cheddar cheese of the same *M. bovis* type it should be poured whilst still molten into a 200 ml weighing boat and discarded once solidified in a autoclave bag inside the cabinet.
- For Layer 2 of red wax repeat the process as described above and in Table 7. Build up several layers of the red wax (4-5) and allow a longer drying time 15 - 20 minutes before waxing the other end of the cheese. Test a small area of wax with a finger to ensure it is well set before waxing the other end.
- Once the waxing is completed label the wax coat on the circular wall with the date made, cheese type, Cheese number.
- The empty Pyrex dishes should be placed inside an autoclave bag. Twist the neck of the autoclave bag shut and fasten using a cable tie.

If another cheese is to be produced using the same *M. Bovis* isolate before the cabinet is fumigated the wax in the basins can be reused.

Cheese Sampling

See cheese sampling protocol FMBDR070

Preparation for storage and maturation

- Person A – Place the waxed cheese inside a labelled cheese bag (Cheese & *M. bovis* isolate) . Seal the neck of the bag with a cable tie. Wipe the exterior of the bag with 5% (v/v) Trigene and remove from the cabinet to a labelled outer cheese bag. Twist the neck of the autoclave bag shut and fasten using a cable tie. Transfer the bagged cheese to the Baumatic low temperature cooler for maturation at 12ºC.

Clean Up

Immediately following Cheese production Cabinet 7

- After cheese mould has been transferred to the adjacent cabinet commence cleanup procedure.
- Remove large curd piece from the basins and lids by dunking in the Trigene dunk tank. Then transfer large basin to the space to the right of the cheese vat. Place the smaller basin inside the larger basin.
- Using a jubilee clip secure a 50 mm piece of silicone tubing (25.4 mm Bore) to the cheese vat outlet. Stopper the silicone tubing using a 27 mm rubber bung.
- Open the cheese vat valve and remove wired bung from the cheese vat outlet. The wired bung should remain in the cheese vat for Trigene disinfection.
- Using a paint scraper and piece of sponge rub the larger pieces of curd from the walls and bottom of the cheese vat before they dry in. Remove larger pieces of curd to the autoclave bag using forceps and the paint scraper.
- Part fill the cheese vat with 10% (v/v) Trigene run from the exterior of the cabinet using the 0.5 m piece of silicone tubing and funnel. After approx 2 L 10% (v/v) Trigene has been added lift the cheese vat at the left hand side to free air trapped in the cheese vat outlet this can be observed in the silicone tubing. Once the air has been freed raise the cheese vat up slightly by positioning on 4 door stops. A
- Add the remaining 8 L of 10% (v/v) Trigene to the cheese vat.
- Fill the larger basin with 10% (v/v) Trigene using the same tubing and funnel. Ensure that the smaller basin is submerged inside the larger basin.
- Rinse the cheese making utensils and potato chipper components in the trigene dunk tank before transferring to steep the cheese vat. Also submerge the temporary storage trays in the cheese vat.
- Place the sharp safe inside an autoclave bag and seal the neck with a cable tie.
- Reattach the retort polls to the cheese vat. Attach a bosshead to Retort Pol 2 approximately half way up the retort pole. Use Poles 1 and 3 to support the 2 Kg masses. Use Pole 3 to support the 2 x 1 Kg masses.
- Set the 5 Kg masses on their narrow edge to maximise the surface exposed during fumigation.
- Use a cable tie to seal the neck of the autoclave waste bag.
- Once the contents have been prepared fumigate the cabinet according to CL3 SOP 04.

After fumigation Cabinet 7 (Day 2 cheesemaking)

It is important to complete the cleanup first thing in the morning so that equipment can be autoclaved and the cabinet given a 6 hr fumigation. This allows the equipment and cabinet to be made ready for a cheese making the following day.

- Open and purge cabinet according to CL3 SOP 04.
- Check the lid is tightly secured on the 15 L lowboy and the 1 L wide neck jar.
- Open down the front of the cabinet and remove the Trigene dunk tank from the cabinet immediately to the autoclave
- Remove 15 L lowboy from the safety cabinet to a square autoclave box. The 1 L wide neck jar should be removed a standard autoclave box
- Autoclave the dunk tank, 15 L lowboy and 1 L wide neck jar on a liquid media cycle 121°C for 30 minutes. This takes a long time so it is important to have this run loaded early in the morning so that the equipment can be rinsed and cleaned in the afternoon.
- Remove waste bag for disposal CL3 SOP 16
- Remove Masses and cheddaring equipment from safety cabinet.

- Remove the sharp safe box from the cabinet to standard autoclave box.
- Close up the cabinet front.
- Detach retort poles from the vat and remove from the cabinet any other boss heads and clamps can also be removed.
- Remove the cheese making utensils submerged in the cheese vat and wipe to remove any pieces of curd using white roll. Hang the utensils on the hooks at the rear of the cabinet.
- Close the cheese vat valve and remove the bung from the silicone tubing. Outside the cabinet position the 10% (v/v) Trigene collection Carboy. Connect the tubing of carboy to the silicone tubing of the cheese vat using a copper elbow and secure using a jubilee clip.
- Open the valve and drain 2 – 3 L of 10% (v/v) Trigene into the Carboy then close the valve.
- Lift the Cheese vat and reposition on the lab jacks ensuring the cheese vat valve is above the bottom lip of the safety cabinet. Drain the remaining 10% (v/v) Trigene from the cheese vat.
- Pour the 10% (v/v) Trigene from the basins into the cheese vat and drain to the 10% (v/v) Trigene collection Carboy. Wipe down the basins using white roll and set to the back right hand corner of the cabinet. Dispose of the white roll in an autoclave bag inside the cabinet.
- Remove potato chipper components and wired bung from the cheese vat. Rub down these components with white roll and a small piece of kitchen sponge cleaner to remove traces of cheese curd. Dispose of the white roll in an autoclave bag inside the cabinet.
- Lift any remaining lumps of curd from the cheese vat using the paint scraper and white roll and dispose of in an autoclave bag inside the cabinet. Clean down the cheese vat removing any traces of contaminated curd using white roll and a piece of kitchen sponge cleaner cleaner.
- Close the cheese vat valve and dry down the vat interior. Dispose of contaminated cleaning materials in the autoclave bag.Loosen the jubilee clip and disconnect the copper elbow from the silicone tubing connected to the cheese vat. Stopper the copper elbow using an 18 mm rubber bung. Remove the tubing of the 10% (v/v) Trigene collection Carboy from the safety cabinet and place inside an autoclave bag. Secure the end of the bag using a cable tie.
- Remove the silicone tubing from the cheese vat and open the valve.
- Remove the cheese vat from the lab jacks and reposition on the 4 doorstops. Position the lab jacks upright to the right hand side of the cheese vat.
- Seal the autoclave waste bag using a cable tie.
- Fumigate the cabinet according to CL3 SOP 04.

After 6 hr fumigation cheesemaking Cabinet 7 (C 7) /Autoclaving trigene dunk tank (Day 2 cheesemaking)

- Remove the cheese vat from the cabinet and rinse down with warm water and washing up liquid. If cheese is to be produced the following day, prepare the cheese vat as described previously.
- Remove the waste bag from the cabinet.
- Remove and wash cheesemaking utensils.
- Remove the remaining items from the cabinet which is now ready for cheesemaking setup as described previously.

- After autoclaving the trigene dunk tank pour of the trigene and rinse the equipment in soapy water. Place cheesemaking utensils and equipment on a piece of kimtech cloth to dry.

Method: preparation of inoculated Caerphilly cheese.

SOP prep. of Caerphilly cheese containing the HG3 organism *Mycobacterium bovis.*

1. Equipment/Reagents

Table 1: Stainless steel cheese making equipment

	Stainless steel cheese making equipment
1.1	Cheese vat
1.2	Paddle
1.3	Flat mesh tool
1.4	Curved mesh tool
1.5	Large spoon
1.6	Horizontal cheese knife
1.7	Vertical cheese knife
1.8	Large palette knife
1.9	Cheese Mould
1.10	Fish Slice
1.11	Tongs
1.12	Forceps x 4
1.13	Laboratory jacks x 2
1.14	10 mm Follower
1.15	3 mm Follower

Table 2: General equipment for cheese making

	General cheese making equipment
2.1	Wired bung x 1
2.2	15 litre Lowboy Carboy (Nalgene) for whey collection
2.3	15 litre Lowboy Carboy (Nalgene) for 10% (v/v) Trigene
2.4	20 litre Carboy (Nalgene) for 10% (v/v) Trigene collection
2.5	Modified lid assembly for Carboy
2.6	0.3 m Silicone Tubing Bore 25.4 mm Wall 3.2 mm
2.7	0.5 m Silicone Tubing Bore 25.4 mm Wall 3.2 mm
2.8	5 cm Silicone Tubing Bore 25.4 mm Wall 3.2 mm
2.9	1.5 m PVC Tubing Bore 25.4 mm Wall 3.2 mm
2.10	Inspection mirror
2.11	1 L wide neck jar x 2
2.12	27 L Polypropylene dunk tank 10% (v/v) Trigene
2.13	27 L Polypropylene dunk tank 200PPM Chloros
2.14	150 mm Polypropylene funnel x 2
2.15	Semi-burette for titratable acdity
2.17	300 x 200 x 100 mm Polypropylene basin & Lid
2.18	Boss head x 6
2.19	Clamps x 3
2.20	Tube clamp
2.21	25 – 35 mm Jubilee clips x 4
2.22	27 mm Rubber Bung
2.23	33 mm Rubber Bung x 4
2.24	Polypropylene Potato masher
2.25	Autoclave boxes

Table 3: Mains powered cheese making equipment

	Mains powered equipment
3.1	Cheese vat speed controller
3.2	Temperature - controlled water bath (Thermo-Haake)
3.3	Bench top vacuum packer
3.3	Baumatic Low Temperature cooler

Table 4: Consumable cheese making equipment

	Consumable cheese making equipment
4.1	Disposable hockey sticks
4.2	1000 µl tips
4.3	Kimtech process wipers
4.4	Polythene oversleeves
4.5	Nitrile gloves Regular Length
4.6	Nitrile gloves Xtra Length 350mm
4.7	Light polyethylene gloves
4.8	Cheese cloth (50 cm2 squares)
4.9	vacuum packing bags
4.10	Autoclave bags
4.11	Sharpsafe boxes
4.12	10ml Serology Pipettes
4.13	50ml Serology Pipettes
4.14	50ml centrifuge Tubes
4.15	50 ml weighing boats
4.16	200 ml weighing boats

Table 5: Cheese making chemicals/Reagents

	Chemicals/Reagents
5.1	8L Raw milk
5.2	Starter Culture MT36 (AG & RJ Barber)
5.3	Vegetarian rennet
5.4	Sodium Chloride
5.5	Sodium hydroxide Sol^{n} (M/9)
5.6	Phenylthalein solution (Reagecon, IPTOH5H)
5.7	Chloros solution
5.8	Trigene solution

2. Methodology

Preparation of Cheese vat, accessories and utensils

On the afternoon before the cheese is to be produced

- Prepare 20 L 200 PPM chloros solution. Fill the chloros tank to the 20 L marker with tap water before adding 40 ml sodium hypochlorite 100000 PPM.
- Prepare 20 L 10% (v/v) Trigene in the Dunk Tank. Add 2 L of Trigene to the Tank before filling the tank to the 20 L marker with tap water. Prepare a further 15 L of 10% (v/v) Trigene in 3 x 5 L Trigene containers (500 ml in 4.5 L).
- The cheese vat is connected to the temperature controlled water bath and the water bath turned on (water in at bottom - water out at top). Both the cheese vat and water bath should be positioned outside the cabinet at this point.
- Place the paddle in the cheese vat.
- Attach Retort Pole 1 with a clamp to the motor side of the cheese vat (Position A) and Retort Pole 2 to the valve end of the cheese vat (Position B).
- To disinfect the cheese vat and paddle pour approximately 10 L of the chloros solution from the chloros tank into the cheese vat.
- Using the clamp attached to Retort Pole 1 suspend the thermometer over the vat co that the thermometer bulb is approximately 20 mm below the liquid surface. Endure the thermometer does not interfere with the movement of the vat paddle.
- Using a jubilee clip attach 1.5 m of PVC tubing to the outlet of the cheese vat.

Preparation of safety cabinet

On the afternoon before the cheese is to be produced

- Open the front door of the cabinet to allow better access to the cabinet.
- Cover work surface of Class I cabinet with Kimtech process wipers.
- Place the 20 L 10% (v/v) Trigene dunk tank to the immediate left of the cabinet. The Trigene dunk tank should be rested on four 35 mm rubber bungs.
- Place 2 polypropylene trays (400 x 150 mm) along the back wall of the cabinet. Above the trays position suction cups with small and large hooks. These are used to hang contaminated equipment over the polypropylene trays between manipulations e.g cheesemaking utensils and the paddle.
- Other receptacles for the disposal of contaminated waste should be placed at the right hand side of the cabinet.
  - Autoclave bag
  - Sharpsafe box
  - 1 L wide neck jar 500ml (20% v/v) Trigene
- Fill the burette reservoir bottle with M/9 NaOH and bring the burette to the 0 mark before placing it to the right hand side of the cabinet in the Class I cabinet. Secure burette to the cabinet wall using a suction cup hook and cable ties.
- Close the front door of the cabinet.

Preparation of *M. bovis* culture

On the afternoon before the cheese is to be produced

- For high-inoculum Caerphilly

Remove 10 x 10 ml frozen aliquots of *M. bovis* culture from storage at -80°C to a biopot. Use some scrunched up white roll to keep the tubes upright inside the biopot before transferring the biopot to the chilled cabinet in G61.

- For low level inoculum Caerphilly

Remove 1 x 10 ml frozen aliquots of *M. bovis* culture from storage at -80°C to a biopot. Use some scrunched up white roll to keep the tube upright inside the biopot before transferring the biopot to the chilled cabinet in G61.

Preparation of Cheese Vat accessories and utensils

On the morning of cheese production

- Immerse the equipment listed below in the chloros solution.

  Horizontal cheese knife
  Vertical cheese knife
  Flat stainless steel mesh strainer
  Curved stainless steel mesh strainer
  Stainless steel spoon
  Wired bung for blockage of cheese vat drain x 2
  Cheese cloth
  Cheese Mould
  260 x 160 x 80 mm Polypropylene basin & Lid
  300 x 200 x 100 mm Polypropylene basin

- Using the attached tubing empty the chloros from the cheese vat back into the chloros tank. Loosen the jubilee clip and detach the large tubing from the cheese vat.
- Turn off the circulating pump and detach the cheese vat from the water bath. Stopper both tubes using 13 mm rubber bungs.
- Lift the cheese vat and carry to the sink.

- Direct the closed cheese vat valve over the sink.
- Using tap water rinse the cheese vat, paddle and thermometer with water until approximately 1/3 full. Open the valve and continue to rinse with tap water whilst water drains. Close the valve and repeat the rinsing procedure.
- The cheese vat is now ready for transfer to the safety cabinet.

Placement and preparation of cheese vat inside the safety cabinet

On the morning of cheese production

- Open the front door of the cabinet to allow better access to the cabinet.
- Soak work surface of Class I cabinet covered by Kimtech process wipers by pouring n approx. 1 L 5% (v/v) Trigene.
- Position the cheese vat to the immediate right of the dunk tank.
- Pass power cable from cheese vat motor behind the trigene dunk tank.
- The cheese vat is connected to the temperature controlled water bath located on the adjacent bench. Pass the tubes through the opening of the cabinet. Unstopper the tubes and connect the outlet of the water bath to bottom inlet of the cheese vat and secondly the inlet of the water bath to upper outlet of the cheese vat.
- Turn on water bath.
- Connect cheese vat motor to the power supply and speed controller also located on the adjacent. Pass cable through the opening of the cabinet.
- Remove the wired bung from the chloros dunk tank and rinse with distilled water. **Insert the wired bung to block the cheese vat drain.** Clamp the wire end of the wired bung using a large tube clamp. In turn using a boss head secure the tube clamp to Retort Pole 2. Ensure that the bung wire is close to the cheese vat wall and will not inhibit the movement of the cheese vat paddle.
- Check the main vat valve is closed.
- Add 8 Litres raw milk (3.1) to the cheese vat. Once the milk has been added remove the plastic containers from the cabinet and dispose in autoclave bags outside the cabinet.
- **Record time milk is added.**
- Connect paddle to the cheese vat electric motor by connection of the drive arm to the bottom connection hole of the cheese vat paddle.
- Turn on motor by pressing RUN on the speed control unit. Adjust paddle speed by turning the frequency control knob until the digital display shows 23.
- Close the front door of the cabinet turn on fan.

Set up apparatus for Tirtrateable acidity (TA) and determining TA from a milk or whey sample

- TA will be determined in 50 ml weighing boats.
- Use an empty sealed pipette tip box to elevate the weighing boat thus reducing the distance between the burette tip and the weighing boat.
- When determining TA from milk add 1 ml Phenylphthalein indicator (Reagecon, IPTOH5H) to the weighing boat before introducing the weighing boat to the cabinet.
- Withdraw a 10 ml sample of milk or whey using a pipette and transfer to the weighing boat. Titrate quickly with M/9 NaOH from the burette, while stirring continuously using a disposable hockey stick. Note volume of NaOH titrated.
- Calculate and record the titratable acidity as % lactic acid by volume; titre/10 = % lactic acid.
- Before inoculating the milk with starter or *M. bovis* the milk TA should be determined. All pipettes used should be disposed of in the sharp safe box inside the cabinet.
- The waste liquid from determining TA should be disposed of in the 1 L wide neck jar.

- The used weighing boats and disposable hockey stick should be disposed of in the autoclave bag inside the cabinet.

Inoculation of 8 Litres raw milk in cheese Vat

- Transfer the biopot containing the inoculum tubes to the Class I cabinet. Also have 10 ml pipettes ready for the transfer of inoculum from the tubes to the vat.
- Once *M. bovis* culture has been introduced to the cabinet all future manipulations whilst wearing Nitrile Xtra Length 350mm gloves.
- A second pair of regular length gloves should be worn over the long gloves and removed after each manipulation inside the cabinet. Short gloves should be disposed of in the autoclave bag inside the cabinet.
- Typically for high level cheesemaking, 100 ml of *M. bovis* culture (10^6 CFU/ml) will be added to the 8 Litres of raw milk - (a final volume of 8 Litres containing 10^4 CFU/ml).
- Carefully loosen the lids of the 15 ml centrifuge tubes whilst they are in the biopot.
- Taking one tube at a time first remove the lid completely and carefully transfer 10 ml thawed *M. bovis* inoculum to the cheese vat using a 10 ml pipette.
- Repeat until all 10 of the *M. bovis* inoculum have been added.
- Use the biopot to store and transfer samples of inoculum, inoculum & milk and whey to the adjacent cabinet for enumeration. Discard each empty centrifuge tube and lid to the autoclave bag in the cabinet.
- For low level cheesemaking make a -1 serial dilution (FMSOP24). Add 1 ml of this -1 serial dilution to the 8 Litres of raw milk.
- **This represents a large volume of milk contaminated with a high level of a Hazard Group 3 pathogen. CARE MUST be exercised from this point onwards to avoid splashes and spillages from the vat.**
- Equilibrate milk temp (approx 30mins) to 32ºC, monitoring temperature using the thermometer.

Preparation and addition of starter

- The cheese starter culture is MT36 (AJ & RG Barber Ltd). A pint bottle of MT36 starter was thawed and aliquoted into 10 ml volumes in 15 ml polypropylene centrifuge tubes. These 10 ml aliquots were then stored at -80 ºC.
- Before use the starter is bulked up to 160 ml. 2 days before cheesemaking remove a 10 ml aliquot from storage at -80 ºC. Place the starter aliquot in a 22ºC water bath for 16 Hours incubation (Into water bath 16:00). On the day before cheesemaking after 16 Hours incubation remove the starter aliquot from the water bath and store at 4 ºC. That afternoon transfer 1 ml of the starter to 160 ml of pasteurised milk in a honey jar (Into water bath 15:00). Incubate at 22ºC for a further 18 Hours. If more starter is required (320 ml) increase number of inoculated 160 ml volumes.

- On the morning of cheesemaking when the milk has reached 32 °C remove the 160 ml of bulk MT36 starter from the 22ºC incubator and add evenly over the surface of the cheese vat to the 8 L of milk. Rinse the honey jar out with 50 ml uninoculated raw milk from and 50 ml centrifuge tube.
- Discard the honey jar and 50 ml centrifuge tube.to the autoclave bag inside the cabinet described previously.
- **Record time and quantity of starter culture added.**
- Allow milk to 'ripen' with continued agitation (via paddle) for 1 hour at 32ºC.
- **Determine and record the TA after 1 hours ripening as described previously.**
- A significant increase in titratable acidity should have occurred (10%).

Addition of Rennet

- Start to prepare rennet for addition to the cheese vat approx 10 mins before the end of ripening period.
- Pipette 1.6 ml of rennet into a 30 ml universal.
- Immediately before the rennet is to be added dilute with approx 14.4 ml sterile distillled water in the 30 ml universal by filling the universal to just above the 15 ml line.
- Introduce 30 ml universal to the safety cabinet. Remove the lid and discard in the autoclave bag inside the cabinet.
- Gently pour the contents of the sample evenly over the milk surface.
- The 30 ml universal should be disposed of in the autoclave bag inside the cabinet.
- **Record time of addition of Rennet and quantity added.**
- Stir the rennetted milk for 3 min.
- Stop paddle agitation by disconnecting the drive arm from the paddle.
- Rest the moving drive arm on the left hand side vat handle.
- Remove the central paddle from the cheese vat. Allow excess milk to run off the paddle into the vat by holding paddle at an angle over the vat (approx 30 secs).
- Using the suction cups and larger hooks hang the paddle over the temporary storage tray.
- Place cheese vat lid over cheese vat during coagulum formation.
- Allow coagulum formation to proceed for 45 minutes.
- Approx. 5 mins before renneting is completed remove the following equipment from the from the chloros dunk tank.

  Horizontal cheese knife
  Vertical cheese knife
  Large palette knife

- Rinse equipment over the sink using tap water.
- Once equipment has been rinsed place items inside the safety cabinet. The palette knife should be hung over the temporary storage trays. Rest the remaining items on the temporary storage trays so that surfaces that will be in contact with coagulated milk are not contaminated with trigene disinfectant.

Cutting

- Remove the cheese vat lid and store behind in gap behind trigene dunk tank.
- Test the set of the milk by making a slight cut in the surface using the large palette knife. Subsequently insert the knife at the start of the cut at a 45 ° angle approximately 50 mm into the milk. Lift the palette knife so that the coagulated milk is also raised. The coagulated milk should split cleanly along the line of the original incision and yellow whey should be observed in the cut.
- If a clean split is observed the milk is ready for cutting if not return the palette knife to a hook over the temporary storage tray and wait a further 5 – 10 mins before repeating the test.
- A standard procedure of cutting is used to ensure uniformity in the size of the curd pieces produced. The vertical knife is first drawn lengthways along the vat and then across it.
- Once the vertical knife has been used person B should raise the lid of the trigene dunk tank.
- Person A should allow curd pieces/whey to drip into the vat before gently transferring the vertical knife to the trigene dunk tank.

- Repeat the cutting and disposal process with the horizontal cheese knife.
- **Determine and record whey TA also record time and temperature.**
- Once cutting has been completed allow the curd to heal for 5 minutes.
- The central paddle is replaced in the vat and agitation continued as described previously 15 rev/min

Scalding

- In the absence of stirring during the coagulation step the temperature of curd/whey may have fallen below 32 ºC
- Immediately after agitation has been resumed increase the temperature of the circulating water bath by 1 ºC.
- **Record the time, target temperature of the circulating water bath and the thermometer reading of the cheese vat.**
- Use the temperature control unit to raise the temperature of curd/whey from 32 ºC to 34 ºC in a linear manner over a period of 15 mins (1 ºC ~ every 7 minutes).
- **Every 7 mins record the time and temperature.**
- **When curd/whey has reached Max scald temperature (34 ºC) record time also determine and record TA.**
- Curd/whey is held at 34 ºC for 30 mins with continuous agitation.
- **After 30 mins of scalding at 34 ºC record time also determine and record TA**.
- The curd pieces if squeezed against the wall of the cheese vat using the large spoon should be firm and retain their shape.
- Check the temperature frequently during this period and also ensure that the curd does not settle on the bottom of the vat. Use the stainless steel spoon and palette knife to dislodge curd from the walls and bottom corners of vat. Between mixes suspend the palette knife and stainless steel spoon over the temporary storage tray.
- Approx. 5 mins before scalding is completed remove the following equipment from the from the chloros dunk tank.

  Flat stainless steel mesh strainer
  Curved stainless steel mesh strainer
  150 mm Plastic Funnel - shortened spout

- Rinse equipment over the sink using tap water.
- Once equipment has been rinsed place items inside the safety cabinet. The Curved stainless steel mesh strainer should be hung over the temporary storage trays. Rest the flat stainless steel mesh strainer on the temporary storage trays so that surfaces that will be in contact with coagulated milk are not contaminated with trigene disinfectant.

Pitching

- Stop paddle agitation by pressing STOP on the speed control unit.
- Detach the drive arm from the paddle and remove the central paddle from the cheese vat. Allow excess milk to run off the paddle into the vat by holding paddle at an angle over the vat (approx 30 secs).
- Using the suction cups and larger hooks hang the paddle over the temporary storage tray.
- After 5 mins remove the thermometer and clamp (Person B). Place the clamp to the left of the cheese vat in front of the trigene dunk tank. The thermometer should be placed on the temporary storage tray.

- Use the flat mesh strainer to push the curd back from the vat outlet about 1/3 of the way along the cheese vat (Person A). The flat mesh strainer arm should be at the motor side of the vat pointing away from the outlet. After the strainer has been positioned clamp in place using retort pole 1 (Person A).
- Detach the tube clamp of the wired bung from Retort Pole 2. Temporarily secure the tube clamp of the wired bung by tucking it through the handle of the cheese vat.
- With the large spoon transfer any curd pieces on the outlet side of the flat mesh strainer to the other side of the strainer. Return the large spoon to its position over the temporary storage tray.
- Unhook the wired bung handle from below the cheese vat handle.
- Grasp the tube clamp of the wired bung in the left hand using a pair of forceps in the right grasp the wire.
- Place left wrist on the edge of the cheese vat wall (cabinet door side) move hand backwards away from the outlet until the wire is taut. Once wire is taut slowly pull the wire only using a wrist action. The bung should be released at this point. Do not pull using an arm motion as this can cause the bung to be released suddenly producing a splash. Still holding the wired bung by the handle and wire transfer carefully to the temporary storage tray.
- Position the curved stainless steel mesh strainer around the cheese vat outlet.
- At this point prepare the funnel for collecting a fresh whey sample obtained by curd squeezing.
- Remove a 150 mm funnel with a shortened spout from from the chloros dunk tank and rinse with distilled water.
- Clamp the funnel over the cheese vat using Retort Pole 3 (Position C Cabinet Door side). The funnel should drain into the vat outlet side of the flat mesh strainer.
- After the curds have been pitched for 10 mins obtain a fresh whey sample by transferring a sample of curd using the stainless steel spoon into the 150 mm funnel held over the cheese vat.
- Allow the whey to drain from the funnel into the vat for a few seconds before pressing the curd mass in the funnel using the stainless steel spoon (Person A).
- Person B - hold a 200 ml weighing boat below the funnel and collect the whey until enough for a 2 x 10 ml sample has been collected place the 200ml weighing boat and whey sample beside the TA apparatus.
- Person A - unclamp the funnel and lower the wide funnel end towards the surface of the whey. Using the spoon ease the curd sample out of the funnel and into the whey.
- Person A - hang up the stainless steel spoon and place the funnel on the temporary storage tray.
- Person A – Detach Retort Pole 3 from the cheese vat.
- The cheese vat is now ready to be raised.

Whey Run

- Person A – lift using cheese vat handles ensuring that the vat is kept level (Approx. 50 – 100 mm).
- Person B – Quickly place laboratory jacks below the cheese vat.
- Person A – Immediately rest the cheese vat on the lab jacks.
- Person A – Screw lab jack knobs to elevate the cheese vat. Proceed until the cheese vat has reached maximum elevation.

- Person B – Whilst the cheese vat is being elevated the vented 15 L low boy carboy containing 700 ml of Trigene should be introduced to the safety cabinet. Trigene is used as a disinfectant as whey may contain *M. bovis* cells. Insert the carboy into the cabinet with the spigot opening pointing away from the cheese vat. The larger opening should be directed away from the cabinet and into the lab. Once the carboy has been inserted into the cabinet as far as the larger opening rotate the carboy in a clockwise direction. The rest of the carboy should slip easily into the cabinet.
- Person B – Remove the lid from larger carboy opening and set on top of the carboy.
- Person B – **Ensure the spigot closure of the 15 L carboy is tightly closed.**
- Person B – Screw modified lid assembly and attached silicone tubing on to carboy opening. Ensure that when the assembly is tightened the tubing is directed towards the cheese vat outlet.
- Person A – Once the cheese vat has been sufficiently elevated connect silicone tubing to the cheese vat outlet and secure using a jubilee clip. Set screw driver to the left of the cheese in front of the trigene dunk tank.
- Person A & B – Before commencing drain the jack positions should be checked if necessary move the jack at the motor end of the cheese vat towards the back of the cabinet so that it better supports the cheese vat.
- Person A – Lift cheese vat.
- Person B – Position motor end lab jack.
- Person B – **Determine and record whey TA of sample obtained at the end of pitching.**
- Person A – Open cheese vat outlet slightly and allow the whey to drain slowly into the carboy. **Record Time.** The progress of draining can be checked by using the inspection mirror. Draining should take 10 mins, but the speed of drainage should be increased if the TA was high at the time of pitching.
- When drainage is nearing completion slightly lower the jack at the outlet end of the cheese vat. Insert two door wedges between the cheese vat and the jack at the motor end so that draining can be continued.
- Person A – Lift cheese vat.
- Person B – Position door wedges between the cheese vat and the jack at the motor end.
- This produces a gradient in the cheese vat allowing further whey drainage.
- Once drainage has slowed again the cheese vat should be lowered
- Person A – Close cheese vat valve and commence lowering of the cheese vat.
- Person B – Detach silicone tubing from the cheese vat outlet. Return screw driver to the left of the cheese vat. The lid assembly should be detached from the carboy and passed to person A for disposal in the Trigene dunk tank. Do not put the jubilee clip into the dunk tank.
- The carboy lid should be used to reseal the carboy. The sharp safe box, 1 L wide neck jar and autoclave box should be brought forward and the carboy placed upright in the immediate right hand corner of the cabinet.
- Person A – Once lowered lift cheese vat (Approx. 50 – 100 mm).
- Person B – Quickly remove laboratory jacks from below the cheese vat and place between the cheese vat and the temporary storage trays.
- Person A – Immediately rest the cheese vat on the floor of the safety cabinet. Unclip the curved mesh strainer. Allow whey to drip from strainer before transferring to the trigene dunk tank. Unclamp the flat mesh strainer briefly allow whey to drip into cheese before transferring to the trigene dunk tank.

- During the curd handling process further whey will be released by the curd mass. To ease collection of the wired bung should be reinserted in the cheese vat outlet.
- Person A – Position door wedges below the cheese vat at the valve end.
- Person B – Prepare several pairs of light polyethylene gloves.
- The light polyethylene gloves are to be worn as a third pair of gloves over the other pairs of blue nitrile gloves.
- Person A – Put on a pair of polyethylene gloves and retrieve the wired bung from the temporary storage tray and pass tube clamp of the wired bung below the right hand vat handle as before.
- Person A – Insert wired bung in the outlet of the cheese vat. Hands should be held over the cheese vat until Person B is ready to remove polyethylene gloves.
- Person B – Remove gloves from Person A using forceps and immediately dispose of in autoclave bag inside the cabinet.
- Person A – Reposition door wedges below the cheese vat at the motor end.
- A significant quantity (>30 ml) of whey may still remain in the cheese vat. This whey can be extracted by pipetting using a 50 ml pipette.
- Person B – Using a 50 ml pipette withdraw whey. Transfer enough for 2 TA samples to a 50 ml weighing boat and a further 50 ml in a labelled 50 ml centrifuge tube to the biopot.
- The remaining whey in the pipette can be disposed of in the 1 L wide neck jar. Repeat the process until a small volume of whey remains (10 – 20 ml). All pipettes used should be disposed of in the sharp safe box inside the cabinet.
- Person A – At this point all liquid samples have been taken and the biopot is ready for transfer to the adjacent cabinet for enumeration procedures. Wipe down the surface of the biopot with 10% Trigene and pass to Person B outside the cabinet who can complete the transfer to the adjacent cabinet.
- **Determine and record whey TA and time.**

Curd handling

- Curd handling should take no longer than 15 – 20 min.
- Have the rinsed potato masher, fish slice and tongs ready for use before starting this stage.
- Make 2 curved cuts in the curd from the front to the back of the cheese vat. Remove the cut curd from the rest of the curd mat and distribute equally on either side of the vat.
- Carry out additional curved cuts on either side of the curd mass until no further cuts can be made without disturbing the stacked curd.
- Using the potato masher squeeze down on the stacked curd this should bind it together better before turning. After the curd on both sides of the cheese vat has been pressed with the potato masher use the fish slice to the turn over the stacked curd.
- Repeat the process of squeezing the curd with the potato masher on the other side of the stacked curd.
- Using the fish slice make a cut in the middle of the curd pieces parallel with shorter side of the rectangular curd piece.
- Use the fish slice to stack one cut curd piece on top of the other so there is again only 2 curd piles. Repeat the squeezing of the curd piles before again turning and squeezing.
- Stack one curd piece on top of the other and squeeze. Turn the curd pieces before a further squeezing.
- Repeat the process of cutting, stacking, turning and squeezing for 15 – 20 min.

- During this period collect whey samples to determine the TA.

Weighing
Before the cheese is salted the mass of cheese produced must be determined.

- Person A – Remove Retort Pole 2 & 3.
- Person B – Remove the following equipment from the chloros dunk tank and rinse. Shake excess water from basins and dry with a piece of white role.

  260 x 160 x 80 mm Polypropylene basin & Lid
  300 x 200 x 100 mm Polypropylene basin
  Tongs.

- Person B – Pass the tongs to Person A inside the cabinet.
- Person A – Place tongs on the temporary storage tray.
- Person B – Set the smaller basin inside the larger basin before placing the lid on each basin. Tare both basins on the balance and record mass. Place both basins on transport trolley so that they are convenient to the cabinet. Stick 4 pieces of tape around the smaller lid. These pieces of tape will be used to secure the lid to the basin.
- Person B – Hold the polypropylene basin (left hand) and lid (right hand) inside the cabinet do not allow the basin to touch any surfaces inside the cabinet.
- Person A – Using the tongs transfer the curd mass from the cheese vat to the polypropylene basin held by Person B. Tongs can be stored in the cheese vat at this point.
- Person B – Once the curd mass has been transferred immediately put the lid on the smaller basin. Then remove the smaller basin from the cabinet and carefully place inside the larger basin on the transport trolley. Immediately lid the larger basin and secure in place with the tape.
- Person B – Weigh basins and curd mass.
- Person B – Determine the mass of curd. From this value calculate the quantity of salt required to add salt at 4.5% (w/w) (The final target for salt is 2%).
- Person B – Remove the larger basin lid and set outside the cabinet pass both basins to Person A inside the cabinet.
- Person A – Set both basins inside the cheese vat before removing the small basin and setting to the immediate right of the cheese vat valve. In turn remove the small basin lid and set adjacent to the right hand side of the small basin.

Milling
- Person A – In the smaller basin cut the curd mass using the fish slice and tongs into 20 x 40 mm cubes.
- Person A – Transfer the curd cubes to the larger basin inside the cheese vat.
- Person B – Should determine and weigh out the required quantity of salt.

Salting

- Add salt in two portions to the milled curd inside the large polypropylene basin mix the curd using the fish slice after the addition of salt.
- Allow the curd to mellow and the salt to dissolve for 15 minutes, stir twice during this period. Additional whey should be collected using a 10 ml pipette and transferred to a 50 ml centrifuge tube in a rack inside the cabinet. The 10 ml pipette should be disposed of in the sharp safe box inside the cabinet.
- Person B - Whilst the curd pieces are mellowing remove cheese cloth from the chloros and rinse using tap water before passing to Person A. Subsequently remove the cheese mould components from the chloros tank and using the alum key tighten the grub screw ensure the cylindrical mould is tightened against the cheese mould base locators.
- Person A – Use the cheese cloth to line the small polypropylene basin.
- Person B – Pass cheese mould a medium polypropylene bag to Person A.
- Person A – Place the lid from the small basin inside the cabinet and position the cheese mould on the lid. Use the lid as a tray for the cheese during moulding. Set the medium polypropylene bag to the right side of the cabinet.

Mould Filling

- Person B – Prepare several pairs of light polyethylene gloves. Pass a pair of light polyethylene gloves to Person A. Remove the large polypropylene basin from the cheese vat. Pour about 1/3 the salted curd pieces into the cheese cloth lined small polypropylene basin. Return the large polypropylene basin to the cheese vat.
- Person A – Put on a pair of polyethylene gloves. Gather up the cheese cloth into a parcel containing the salted curd and transfer to the cheese mould. Ensure all the cheese cloth is tucked inside the mould.
- Person B – Using the follower and handle push down on the curd pieces to create more space in the cheese mould. Then remove follower and screw from the cheese mould and rest on the bottom of the cabinet so that the follower is not in contact with trigene.
- Person A – Whilst wearing the polyethylene gloves reopen the cheese cloth and pull over the edge of the cheese mould. Move to the left of the cabinet so as not to obstruct Person B as they fill the mould.
- Person B – Use the large stainless steel spoon to transfer curd pieces into the cheese mould until the mould has been filled to the top edge.
- Person A – Whilst wearing the polyethylene gloves tuck the cheese cloth back inside the cheese mould. It may be necessary to manipulate some of the curd pieces by hand before the cheese cloth can be tucked into the mould.
- Person B – Using the follower and screw push down on the curd pieces to create more space in the cheese mould. If there is still salted curd in the large polypropylene basin repeat these steps until all the curd has been transferred to the cheese mould.
- Person B – Once the filling of the mould has been completed remove the light polyethylene gloves from Person A using forceps. Dispose of the gloves in the autoclave bag.
- Person A – Rotate the screw until the rectangular plate and circular follower are as close as possible to each other. Line up the holes in the rectangular plate with the screw rods of the base of the cheese mould. Rest follower on top of the curd pieces and cheese cloth.
- Person A & B – Tighten the 2 nuts down on to the rectangular plate until the plate rests on top of the cheese cylindrical mould.

- Person B – Hold the cheese mould securely by the screw rods.
- Person A – Tighten the screw handle until it cannot be rotated any further. This presses the curd pieces into the shape of the cheese mould.
- Person A – After about 10 minutes loosen the screw handle slightly (3 or 4) turns. Using the alum key loosen the grub screw. .
- Person B – Once the screw handle has been loosened completely remove both nuts from the large screw rods.
- Person A – remove the rectangular plate and screw handle and rest on the bottom of the cabinet in front of the Trigene dunk tank.
- Person A – Put on a pair of polyethylene gloves and remove cylindrical cheese mould from the base plate.
- Person B – Hold the neck of a thick polypropylene bag inside the cabinet.
- Person A – Whilst wearing the polyethylene gloves place cylindrical cheese mould inside the polypropylene bag.
- Person B – Role up the sides of the bag and twist the neck of the bag shut. Immediately transfer the bagged cheese mould to the adjacent cabinet.
- The cheese is now ready for pressing in the adjacent cabinet.
- Person B – Once the mould has been transferred remove the light polyethylene gloves from Person A using forceps. Dispose of the gloves in the autoclave bag.

Pressing

- Have the rinsed stainless steel followers available before starting to press the cheese and prepare the vacuum pack bags by slicing with a scalpel along the bottom and one of the sides.
- Open up the vacuum pack bag and lay over the top of a piece of Kimtech process wiper. This provides a uncontaminated surface on which the cheese can be manipulated. Use another piece of Kimtech process wiper cut to the appropriate size to cover the back brace and base of the cheese press. This soaks up contaminated whey expelled during pressing.
- Person A – Whilst wearing polyethylene gloves remove the cheese mould from the bag. Set the mould on top of the opened vacuum pack bag. Remove the cheese from the mould. There will be parts of the cheese where the cheese cloth was caught between curd pieces during mould filling. Carefully unwrap the cheese and pull the cheese cloth up the sides ensuring that the cheesecloth is freed from between the curd pieces. Gather the cheese cloth round the top of the cheese and carefully reinsert to the cheese mould. Set the cheese mould on top of the 10 mm follower and use the 2 mm follower to cover the other end. With both hands lift the mould and followers together and rest in position on top of the kimtech wiper protecting the cheese press.
- Person A – The 10 mm follower should be towards the back brace of the cheese press.
- Person B – Whilst Person A holds the cheese mould and followers in place attach the pressing rod and adapter to the cheese press pneumatic ram.
- Person B – Again whilst Person A holds the cheese mould and followers in place Pump the handle of the Enerpac Pump until 0.7 kN is observed on the dial. This pressure will drop as whey is exuded to approximately 0.5 kN.
- The cheese pressing is allowed to proceed for 1 hour. During this period commence cleanup. Consult cleanup procedure below.
- After 1 hour initial pressing the cheese is ready for turning.

- Person B – Open the pressure release valve gradually to return the follower to its initial position.
- Person A – Whilst wearing polyethylene gloves hold the followers in place and turn the cheese mould through 180°.
- Person B – Whilst person A holds the cheese mould and followers in place pump the handle of the Enerpac Pump until 1.3 kN is observed on the dial this will fall to 0.7 kN as further whey is expelled. Leave the cheese pressing overnight with the cabinet running.
  Person A – On the next morning after 16 hours pressing slowly open the pressure release valve to return the pneumatic ram to its original position. Lay a cut vacuum pack bag flat on the bottom of the cabinet. Remove the cheese mould whilst wearing polyethylene gloves. Push the 2 mm follower against the cheese inside the mould so that the cheese is forced from the mould. Rest the cheese on the surface of the open vacuum pack bag. Return the empty cylinder to position on the cheese press.
- Person B – Whilst wearing polyethylene gloves use a pair of sterile forceps peel the cheese cloth from the top and sides of the cheese.
- Person A – Lift and separate the cheese from the cheese cloth. Return the cheese to the vacuum pack bag and discard the cheese cloth and polyethylene gloves to an autoclave bag inside the cabinet.
- Allow the cheese surface to dry for several 10 – 15 minutes before waxing turning at regular intervals.
- Before waxing - slice a 1 cm thick section from one end of the cheese this provides a sample for *M. Bovis* enumeration and the remainder can be used for chemical analysis.

Waxing

Table 6

	Wax Type	Number of coats	Quantity (g)	Temp °C
Layer 1	Translucent	1	110	110
Layer 2	Yellow	2	150	95

Waxing - Preparation

- Before preparing the wax turn on the hot plate set to 146 °C inside safety cabinet (the temp reading of thermometer in the wax should reach 110 °C).
- Lay white roll over the Kimtech wiper adjacent to the hot plate this will prevent the Kimtech roll being melted by the pryrex dish during waxing or cooling.
- Weigh the required amount of each wax into clean Pyrex basins.
  - First melt the translucent wax on another hot plate outside the cabinet. Gently swirl the wax whilst holding the basin using heat resistant silicone gloves.
  - Once the wax is completely molten transfer to the hotplate inside the cabinet.
  - Using a thermometer determine the temperature of the molten wax. If the temperature reading is >110ºC remove the basin from the hot plate and gently swirl on the white roll. If the temperature is <110ºC gently swirl the basin on the hot plate until the target temperature is reached.
  - Once the desired temp is reached the cheese should be dipped immediately. This part of the process requires two people.

Waxing - Dipping

- Person B – Remove thermometer from the basin. Keep the thermometer in hand to monitor wax temperature between first and second dipping.
- Person A – Whilst wearing polyethylene gloves grasp the cheese at one end. Lift the cheese over the molten wax and in a careful vertical motion submerge the cheese in the wax. Continue downwards until the wax has travelled as far as possible up the walls of the cheese and basin without overflowing the basin. Then remove the cheese from the basin and rest the unwaxed end of the cheese on the open vacuum pack bag. Allow the wax to solidify for 1 min. Discard the polyethylene gloves to an autoclave bag inside the cabinet.
- Person B – Return the Pyrex basin to the hotplate and maintain the temp at 110ºC.
- Person A –Grasp the cheese by the waxed end and repeat the process as described previously. Take care as the waxed surface has less purchase than the unwaxed cheese surface. Any small unwaxed areas can be filled by molten wax applied from the tip of the thermometer.
- If excess wax will not be needed for waxing other Caerphilly cheese of the same *M. bovis* type it should be poured whilst still molten into a 200 ml weighing boat and discarded once solidified in a autoclave bag inside the cabinet.
- For Layer 2 of yellow wax repeat the process as described above and in Table 6. Build up several layers of the yellow wax (4-5) and allow a longer drying time 15 - 20 minutes before waxing the other end of the cheese. Test a small area of wax with a finger to ensure it is well set before waxing the other end.
- Once the waxing is completed label the wax coat on the circular wall with the date made, cheese type, Cheese number.
- The empty Pyrex dishes should be placed inside an autoclave bag. Twist the neck of the autoclave bag shut and fasten using a cable tie.

If another cheese is to be produced using the same *M. Bovis* isolate before the cabinet is fumigated the wax in the basins can be reused.

Cheese Sampling

See cheese sampling protocol FMBDR070

Preparation for storage and maturation

- Person A – Place the waxed cheese inside a labelled cheese bag (Cheese & *M. bovis* isolate) . Seal the neck of the bag with a cable tie. Wipe the exterior of the bag with 5% (v/v) Trigene and remove from the cabinet to a labelled outer cheese bag. Twist the neck of the autoclave bag shut and fasten using a cable tie. Transfer the bagged cheese to the Baumatic low temperature cooler for maturation at 12ºC.

Clean Up

Immediately following Cheese production Cabinet 7

- After cheese mould has been transferred to the adjacent cabinet commence cleanup procedure.
- Remove large curd piece from the basins and lids by dunking in the Trigene dunk tank. Then transfer large basin to the space to the right of the cheese vat. Place the smaller basin inside the larger basin.
- Using a jubilee clip secure a 50 mm piece of silicone tubing (25.4 mm Bore) to the cheese vat outlet. Stopper the silicone tubing using a 27 mm rubber bung.

- Open the cheese vat valve and remove wired bung from the cheese vat outlet. The wired bung should remain in the cheese vat for Trigene disinfection.

- Using a paint scraper and piece of sponge rub the larger pieces of curd from the walls and bottom of the cheese vat before they dry in. Remove larger pieces of curd to the autoclave bag using forceps and the paint scraper.
- Part fill the cheese vat with 10% (v/v) Trigene run from the exterior of the cabinet using the 0.5 m piece of silicone tubing and funnel. After approx 2 L 10% (v/v) Trigene has been added lift the cheese vat at the left hand side to free air trapped in the cheese vat outlet this can be observed in the silicone tubing. Once the air has been freed raise the cheese vat up slightly by positioning on 4 door stops. A
- Add the remaining 8 L of 10% (v/v) Trigene to the cheese vat.
- Fill the larger basin with 10% (v/v) Trigene using the same tubing and funnel. Ensure that the smaller basin is submerged inside the larger basin.
- Rinse the cheese making utensils and potato chipper components in the trigene dunk tank before transferring to steep the cheese vat. Also submerge the temporary storage trays in the cheese vat.
- Place the sharp safe inside an autoclave bag and seal the neck with a cable tie.
- Reattach the retort polls to the cheese vat. Attach a bosshead to Retort Pol 2 approximately half way up the retort pole. Use Poles 1 and 3 to support the 2 Kg masses. Use Pole 3 to support the 2 x 1 Kg masses.
- Set the 5 Kg masses on their narrow edge to maximise the surface exposed during fumigation.
- Use a cable tie to seal the neck of the autoclave waste bag.
- Once the contents have been prepared fumigate the cabinet according to CL3 SOP 04.

After fumigation of the cheesemaking Cabinet (C7) - Day 2 cheesemaking
It is important to complete the cleanup first thing in the morning so that equipment can be autoclaved and the cabinet given a 6 hr fumigation. This allows the equipment and cabinet to be made ready for cheesemaking the following day.

- Open and purge cabinet according to CL3 SOP 04.
- Check the lid is tightly secured on the 15 L lowboy and the 1 L wide neck jar.
- Open down the front of the cabinet and remove the Trigene dunk tank from the cabinet immediately to the autoclave
- Remove 15 L lowboy from the safety cabinet to a square autoclave box. The 1 L wide neck jar should be removed a standard autoclave box
- Autoclave the dunk tank, 15 L lowboy and 1 L wide neck jar on a liquid media cycle 121°C for 30 minutes. This takes a long time so it is important to have this run loaded early in the morning so that the equipment can be rinsed and cleaned in the afternoon.
- Remove waste bag for disposal CL3 SOP 16
- Remove the sharp safe box from the cabinet to standard autoclave box.
- Close up the cabinet front.
- Detach retort poles from the vat and remove from the cabinet any other boss heads and clamps can also be removed.
- Remove the cheesemaking utensils submerged in the cheese vat and wipe to remove any pieces of curd using white roll. Hang the utensils on the hooks at the rear of the cabinet.
- Close the cheese vat valve and remove the bung from the silicone tubing. Outside the cabinet position the 10% (v/v) Trigene collection Carboy. Connect the tubing of

carboy to the silicone tubing of the cheese vat using a copper elbow and secure using a jubilee clip.

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Appendix 1.3

- Open the valve and drain 2 – 3 L of 10% (v/v) Trigene into the Carboy then close the valve.
- Lift the Cheese vat and reposition on the lab jacks ensuring the cheese vat valve is above the bottom lip of the safety cabinet. Drain the remaining 10% (v/v) Trigene from the cheese vat.
- Pour the 10% (v/v) Trigene from the basins into the cheese vat and drain to the 10% (v/v) Trigene collection Carboy. Wipe down the basins using white roll and set to the back right hand corner of the cabinet. Dispose of the white roll in an autoclave bag inside the cabinet.
- Lift any remaining lumps of curd from the cheese vat using the paint scraper and white roll and dispose of in an autoclave bag inside the cabinet. Clean down the cheese vat removing any traces of contaminated curd using white roll and a piece of kitchen sponge cleaner. Dispose of the white roll in an autoclave bag inside the cabinet.
- Close the cheese vat valve and dry down the vat interior. Dispose of contaminated cleaning materials in the autoclave bag.
- Loosen the jubilee clip and disconnect the copper elbow from the silicone tubing connected to the cheese vat. Stopper the copper elbow using an 18 mm rubber bung. Remove the tubing of the 10% (v/v) Trigene collection Carboy from the safety cabinet and place inside an autoclave bag. Secure the end of the bag using a cable tie.
- Remove the silicone tubing from the cheese vat and open the valve.
- Remove the cheese vat from the lab jacks and reposition on the 2 doorstops. Position the lab jacks upright to the right hand side of the cheese vat.
- Seal the autoclave waste bag using a cable tie.
- Fumigate the cabinet according to CL3 SOP 04.

After 6 hr fumigation cheesemaking Cabinet (C 7) /Autoclaving trigene dunk tank (Day 2 cheesemaking)

- Remove the cheese vat from the cabinet and rinse down with warm water and washing up liquid. If cheese is to be produced the following day, prepare the cheese vat as described previously.
- Remove the waste bag from the cabinet.
- Remove and wash cheesemaking utensils.
- Remove the remaining items from the cabinet which is now ready for cheesemaking setup as described previously.
- After autoclaving the trigene dunk tank pour of the trigene and rinse the equipment in soapy water. Place cheesemaking utensils and equipment on a piece of kimtech cloth to dry.

Appendix 1.4

Method: enumeration of *Mycobacterium bovis* from cheese.

SOP for the Enumeration of the HG3 organism *Mycobacterium bovis* from cheese.

1. Equipment/Reagents

Table 1: Stainless steel cheese sampling equipment

	Stainless steel cheese making equipment
1.1	Sterile Cork borer
1.2	Sterile forceps
1.3	Cork borer plunger

Table 2: General equipment for cheese sampling

	General cheese sampling equipment
2.1	Trimmed vaccum pack bag
2.2	Sterile scalpel
2.3	Kimtech process wipers
2.4	Wire cutters x 2
2.5	Stomacher bags x 2 per sample
2.6	50 ml centrifuge tubes x 2 per sample
2.7	2 ml syringe x 1 per sample
2.8	90 mm Petri dish x 1 per sample
2.9	10 ml syringe x 1 per sample
2.10	Duraseal film
2.11	Cable ties
2.12	Cheese bag
2.13	Plate bags
2.14	10 ml pippettes
2.15	1 L trigene liquid waste discard jar x 2
2.16	Timer
2.17	50 ml wax in a 100 ml Schott bottle.
2.18	Kitchen cleaning sponge (cut to size)
2.18	Disposable honey Jar

Table 3: Cheese Sampling Reagents

	Cheese Sampling Reagents
3.1	Cheese diluent 45 ml per sample
3.2	7H11++ PANTA plates
3.3	7H11++ PCAT plates

Table 4: Electrical equipment

	Electrical equipment
4.1	Cheese vat speed controller
4.2	Temperature - controlled water bath (Grant)
4.3	Hot Plate
4.4	Pipette controller
4.5	Electric balance.

2. Methodology

Sampling of liquids

All pipettes used should be disposed of in the discard jar used specifically for pipette diposal inside the cheese making cabinet.

Samples in labelled centrifuge tubes stored in a biopot before transfer to the adjacent safety cabinet.

Appendix 1.4

Liquid samples

Inoculum

Retain inoculum (1 ml – 2 ml) added to the milk in the cheese vat

Milk & Inoculum

After addition of inoculum to the cheese vat remove a sample (5 ml – 10 ml) using a 10 ml pipette to a labelled centrifuge tube.

Whey post draining 5 ml – 10 ml

After draining and removing the jacks from below the cheese vat take a sample (5 ml – 10 ml) and store as described previously.

After all liquid samples have taken seal the lid of the biopot and wipe down with trigene before transfer to the adjacent cabinet.

Enumeration of *M. bovis* from liquid samples

The enumeration of *M. bovis* in liquids is performed by serial dilution (FMSOP24) into maximum recovery diluent (MRD) (9 ml).
100 µl of sample per plate

Inoculum

Dilutions -1, -2, -3, -4, -5.
Also plated out on Antibiotic free 7H11 and BL media

Milk & Inoculum

Dilutions N, -1.
2 x replicates.

Whey post cutting 5 ml – 10 ml

Dilutions N, -1.

Whey post draining 5 ml – 10 ml

Dilutions N, -1.

Storage and Incubation of plates

- Plates of the same sample and media should be stacked and sealed by wrapping in Duraseal.
- Once wrapped all sealed plates of the same sample type should be transferred from inside the cabinet to a labelled plate bag outside the cabinet. The bag should be labelled sample type, cheese type, *M. bovis* isolate and date.
- Seal the bag with a cable tie and trim of the excess length using wire clippers. Transfer the plates to a 37 °C incubator.

Sampling Cheese

Preparation of Sampling Materials

The number of each item required will be determined by the number of samples to be taken.

- Prepare the vacuum pack bags by slicing with a scalpel along the bottom and one of the sides.
- Prepare the 2 ml syringe by cutting the circular thumb push from the end of the syringe plunger this allows the plunger to pass through the cork borer.
- Label one 50 ml centrifuge tube with the cheese type and *M. bovis* isolate.
- Place one stomacher bag inside another so that stomached samples are double bagged. Label the outer stomacher with cheese type and *M. bovis* isolate.
- Measure 45 ml of cheese diluent into a second 50 ml centrifuge tube.
- Trim a fresh inner bag and label with the *M. bovis* isolate type. If the cheese is to be turned after sampling then indicate the direction in which cheese should be placed in the bag either sample side up or sample side down.

Cheese Sampling procedure

- Place the labelled bags, tubes, vacuum pack bags, sterile scalpels, sterile forceps, sterile cork borers, 90mm Petri dishes, 2ml syringes, 10 ml pipettes, pipette controller, autoclave bag and discard inside the cabinet.
- Spread out a piece of Kimtech process wiper on the bottom of the cabinet and open up the vacuum pack bag on top this provides the surface on which equipment can be rested and the cheese sample manipulated.
- Once this equipment is inside the cabinet commence the cheese sampling. If the cheese has only been waxed that day the cheese will already be inside the cabinet. If the cheese has been matured then remove it from the maturation incubator remove the cable tie from the outer bag using the wire cutters and place the inner bag and waxed cheese inside the cabinet. Remove the cable tie from the inner bag. Discard the inner bag into the autoclave bag and rest the cheese to the left hand side of the vacuum pack bag with the side were the samples have been taken previously facing upwards.
- Using a sterile cork borer mark the wax then cut a square hole in the wax surrounding the mark remove the piece of wax to reveal the cheese surface below.
- Discard contaminated wax to the honey jar.
- Slowly drive the cork borer into the cheese as far as possible whilst rotating. Then remove the borer slowly again whilst rotating. The sample should be inside the cork borer.
- Rest the cork borer on the base of the labelled petri dish and tear the corresponding labelled 50 ml tube on the balance inside the cabinet.
- Use the plunger from the 2 ml syringe to force the cheese sample through the cork borer if necessary use the cork borer plunger to push the syringe plunger further through the cork borer.
- Once the sample is in the Petri dish use the scalpel and forceps to slice it into smaller pieces. Then transfer to the tube on the balance and weigh out a 5 g sample.
- Once 5 g has been measured transfer the sample from the tube to the stomacher bag and add the 45 ml of cheese diluent.
- Stomach the sample for 4 minutes.
- Discard contaminated cheese and syringe plungers to the honey jar.

- Repeat these steps until all the samples have been taken. Submerge the samples in the waterbath inside the cabinet set to 37 °C for 1 hour.
- Melt wax (colour will be determined by cheese type) on the hot plate set at 90 °C.
- Use a 10 ml syringe to transfer molten wax to fill the hole produced in the cheese. Fill hole with wax to the the cheese surface. Allow to set before adding a further layer of wax to the cheese surface. Build up layers of wax until the wax covering the sample point is level with the surrounding wax of the cheese surface.
- After the wax covering the sample point has solidified using a scalpel mark the sample point with a roman numeral so the order in which the samples were taken can be distinguished II, III etc.
- Replace the cheese in the corresponding inner cheese bag and seal with a cable tie removing the excess length from the cable tie after tightening with the wire cutters.
- With assistance from someone outside the cabinet transfer the bagged cheese to the original outer bag outside the cabinet.
- Seal the outer bag with a cable tie and again remove the excess length with wire cutters.
- Return the cheese to the cheese maturation incubator.
- The cork borer, forceps and cork borer plunger should be rubbed down with a piece of Kitchen cleaning sponge wetted with 10% (v/v) Trigene to remove traces of contaminated cheese. To clean the inside of the cork borer use the plunger to force a small bit of sponge rough side out, down the borer barrel. Once cleaned this equipment should be placed in the 1 L trigene liquid waste discard jar filled with 10% (v/v) Trigene to complete the decontamination of the equipment. NB. Do not put liquid cheese waste in this Jar use the jar specifically for liquid cheese waste.
- The vacuum pack bag should be discarded to the autoclave bag.
- Remove the scalpel blade from the disposable handle using the wire cutters and discarded to the sharpsafe box specifically for sharps inside the cabinet. The handle can then be placed in the autoclave bag
- After the cheese sample has been incubated at 37 °C for 1 hour remove from the water bath and dry the exterior of the stomacher bag by resting against the kimtech cloth. Then stomach the sample for a second time for 4 minutes.
- Transfer the cheese suspension to the labelled 50 ml centrifuge tube using a 10 ml pipette.
- This cheese sample represents the -1 dilution and is ready for further serial dilution into 9 ml MRD to -2 and -3 (FMSOP24).
- Spread on selective media.
- Dispose of the contaminated pipette by placing it inside the contaminated stomacher bag before placing in a empty autoclave bag. Once all samples have been taken seal the bag with a cable tie. This prevents the being overfilled and avoids the pipettes puncturing the autoclave bag.

<u>Contaminated Cheese Day 1 to Day 60</u>
Dilutions -1, -2 -3.
2 x replicates.

Storage and Incubation of plates

- Plates of the same sample and media should be stacked and sealed by wrapping in Duraseal.
- Once wrapped all sealed plates of the same sample should be transferred from inside the cabinet to a labelled plate bag outside the cabinet. The bag should be labelled sample number, cheese type, *M. bovis* isolate and date.
- Seal the bag with a cable tie and trim of the excess length using wire clippers. Transfer the plates to a 37 °C incubator.

Concentration of contaminated cheese samples

- After 60 days cheese maturation there will have been a steady decline in *M. bovis* counts. Therefore it may be necessary to concentrate the -1 cheese suspension to increase sensitivity.
- The 50 ml tubes can be balanced against another sample or a water blank. The mass of the samples should be increased using MRD and the volume required can be determined using the balance inside the cabinet.
- Transfer the balanced tubes from the cabinet to the centrifuge buckets outside and immediately seal with centrifuge lids. Insure the tubes are in a matched pair of buckets and lids eg 1.1 & 1.2.
- Spin Samples at 3000 rpm for 15 mins.
- After centrifugation transfer the centrifuge buckets to the cabinet before opening.
- Open the buckets and decant the supernatant into the trigene discard pot.
- Resuspend the pellet in 1 ml MRD and transfer to a 20 ml universal labelled with x25, cheese type and *M. bovis* isolate. Using a further 900 - 1000 µl volume of MRD recover the remains of the sample and transfer to the universal. The normal 50 ml -1 dilution sample has been concentrated x 25.
- Make a further dilution to x 2.5.
- Spread on selective media.
- Dispose of contaminated tubes in autoclave bag. After all samples have been plated out and plates placed in the incubator discard contaminated liquid into the trigene discard pot.

<u>Contaminated Cheese Day 61 –</u>
Dilutions x 25, x 2.5,
2 x replicates.

Storage and Incubation of plates

As for liquid samples

Appendix 2

Statistical analysis of selective media: Cheddar and Caerphilly homogenates.

```
  14
  15  FOR
  16    PAGE
  17    SKIP [FILE=OUT] 2
  18    PRIN [IPR=*; SQ=Y] 'Received from:','Michael Rowe/Richard
Forgrave'; FIELD=39; JUST=L
  19    & 'Cost code:','44187'; FIELD=39; JUST=L
  20    & 'Statistical Analysis number:','S20120570'; FIELD=39; JUST=L
  21    & 'Folder:',Folder; FIELD=39; JUST=L
  22    & 'Data File(s):',InFile; FIELD=39; JUST=L
  23    & 'Output File:',OutFile; FIELD=39; JUST=L
  24    & 'Output files created by Alan Gordon:',TDATE; FIELD=39;
JUST=L
  25    & 'Title:',Title; FIELD=39; JUST=L
  26    PAGE
  27  ENDFOR

Received from:                          Michael Rowe/Richard Forgrave
Cost code:                              44187
Statistical Analysis number:            S20120570
Folder:
P:/Biometrics/Analysis/AlanG/Alan/Micro/Michael Rowe/M Bovis in Raw
Milk Cheese (44187)
Data File(s):                           Michael Rowe 121109 M bovis
Media Trial Stats Data.xlsx
Output File:                            Michael Rowe 121109 M bovis
Media Trial Stats Data.txt
Output files created by Alan Gordon:    22/11/12
Title:                                  M Bovis in Raw Milk Cheese
Analysis


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  29
  30  IMPORT InFile; SHEET='AF2122 Media'
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Appendix 2

```
Loading Spreadsheet File
------------------------
Catalogue of file
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Description: Data read from Michael Rowe 121109 M bovis Media Trial
Stats Data.xlsx [Cheddar]A2:R103
Sheet Type: vector
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      1            text     102   Bovis_Type
      2            text     102   Cheese_Ref
      3            text     102   Media
      4            text     102   Cheese_Type
      5         variate     102   Cheese_Rep
      6         variate     102   Day
      7         variate     102   Log_cfu_g_1
  76
  77  GROUP [RED=Y] Bovis_Type,Media,Day
  78  SUBSET [(Day.NI.!(81,179)).AND.(Media.NI.!T('7H11++
PANTA','7H11++ PCAT')); SET=Y] Bovis_Type,Media,Day,Log_cfu_g_1
  79  FACT [LEV=!(1,21,43,62,121)] Day2
  80  CALC Day2=NEWL(Day;!(1,3(21,43,62,121)))
  81  & NV=NLEV(Bovis_Type)*NLEV(Media)*NLEV(Day2)
  82  TABU [CL=Bovis_Type,Media,Day2] Log_cfu_g_1; MEAN=MEAN
  83  VARI [N=NV; MOD=Y] Log_cfu_g_1; VAL=MEAN
  84  DELE [RED=Y] MEAN
  85  FACT [N=NV; MOD=Y] Bovis_Type,Media,Day2
  86  GENE Bovis_Type,Media,Day2
  87  FACPRODUCT !P(Media,Bovis_Type); PROD=Subject
  88
  89  FOR
  90
  91    PAGE
  92    PRIN Bovis_Type,Media,Day2,Log_cfu_g_1; DEC=3(0),2
  93
  94    PAGE
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  99    MATR [ROW=NLEV(Day2); COL=NLEV(Subject);VALUE=#_resid ] _mresid
 100    SYMM [ROW=NLEV(Day2)] _vinit
 101    CALC _vinit=_mresid*+T(_mresid)/NLEV(Subject)
 102    VCOM [FIX=Media*Day2] RAN= Bovis_Type+Subject.Day2; CON=P
 103    VSTRUC[Subject.Day2]FACT=Day2;MOD=ANT;ORD=1;HET=N;
INIT=!(#_vinit)
 104    REML [PR=MOD,COM,MEAN,WALD; MVINC=E,Y] Log_cfu_g_1
 105    FCLASS [NT=3] Media*Day2; OUT=F[1...3]
 106    FOR FF=F[]
 107      VKEEP [WMETH=A] #FF; FSTAT=FSTAT; NDF=DF1; DDF=DF2
 108      CALC PROB=1-FPROB(FSTAT;DF1;DF2)
```

```
109      IF PROB.LT.0.05
110        VMCOMPARISON [PR=LET] #FF
111      ENDIF
112      DELE [RED=Y] DF1,DF2,FSTAT,PROB
```

Appendix 2

```
113    ENDFOR
114    VPLOT [GRAPH=L] FIT,NORM
115    DELE [RED=Y] _resid,_mresid,_vinit,F
116
117    PAGE
118  ENDFOR
```

Bovis_Type	Media	Day2	Log_cfu_g_1
AF2122	7H11 PANTA	1	4.31
AF2122	7H11 PANTA	21	3.40
AF2122	7H11 PANTA	43	2.80
AF2122	7H11 PANTA	62	2.29
AF2122	7H11 PANTA	121	1.26
AF2122	7H11 PCAT	1	4.57
AF2122	7H11 PCAT	21	3.23
AF2122	7H11 PCAT	43	2.78
AF2122	7H11 PCAT	62	1.96
AF2122	7H11 PCAT	121	1.34
AF2122	Gallaghers PANTA	1	4.32
AF2122	Gallaghers PANTA	21	3.25
AF2122	Gallaghers PANTA	43	2.74
AF2122	Gallaghers PANTA	62	0.60
AF2122	Gallaghers PANTA	121	0.60
AF2122	Gallaghers PCAT	1	4.55
AF2122	Gallaghers PCAT	21	3.35
AF2122	Gallaghers PCAT	43	2.95
AF2122	Gallaghers PCAT	62	0.78
AF2122	Gallaghers PCAT	121	0.60
G1.140	7H11 PANTA	1	3.19
G1.140	7H11 PANTA	21	3.30
G1.140	7H11 PANTA	43	1.90
G1.140	7H11 PANTA	62	1.99
G1.140	7H11 PANTA	121	1.60
G1.140	7H11 PCAT	1	4.27
G1.140	7H11 PCAT	21	3.48
G1.140	7H11 PCAT	43	2.24
G1.140	7H11 PCAT	62	1.81
G1.140	7H11 PCAT	121	1.08
G1.140	Gallaghers PANTA	1	3.77
G1.140	Gallaghers PANTA	21	3.11
G1.140	Gallaghers PANTA	43	0.60
G1.140	Gallaghers PANTA	62	0.78
G1.140	Gallaghers PANTA	121	*
G1.140	Gallaghers PCAT	1	4.37
G1.140	Gallaghers PCAT	21	2.80
G1.140	Gallaghers PCAT	43	1.29
G1.140	Gallaghers PCAT	62	2.02
G1.140	Gallaghers PCAT	121	*
G24.130	7H11 PANTA	1	4.52
G24.130	7H11 PANTA	21	4.31
G24.130	7H11 PANTA	43	2.97
G24.130	7H11 PANTA	62	1.72

G24.130	7H11 PANTA	121	0.60
G24.130	7H11 PCAT	1	4.70
G24.130	7H11 PCAT	21	3.94
G24.130	7H11 PCAT	43	2.76

G24.130	7H11 PCAT	62	2.06
G24.130	7H11 PCAT	121	0.60
G24.130	Gallaghers PANTA	1	4.25
G24.130	Gallaghers PANTA	21	1.63
G24.130	Gallaghers PANTA	43	0.60
G24.130	Gallaghers PANTA	62	0.60
G24.130	Gallaghers PANTA	121	*
G24.130	Gallaghers PCAT	1	4.46
G24.130	Gallaghers PCAT	21	3.46
G24.130	Gallaghers PCAT	43	0.60
G24.130	Gallaghers PCAT	62	1.48
G24.130	Gallaghers PCAT	121	*

REML variance components analysis
==========

Response variate: Log_cfu_g_1
Fixed model: Constant + Day2 + Media + Day2.Media
Random model: Bovis_Type + Subject.Day2
Number of units: 60

Subject.Day2 used as residual term with covariance structure as below
Sparse algorithm with AI optimisation
Units with missing data values included

Covariance structures defined for random model

Covariance structures defined within terms:

Term	Factor	Model	Order	No. rows
Subject.Day2	Subject	Identity	0	12
	Day2	Antedependence	1	5

Estimated variance components

Random term	component	s.e.
Bovis_Type	0.000	bound

Residual variance model

Term	Factor	Model(order)	Parameter	Estimate	s.e.
Subject.Day2			Sigma2	1.000	fixed
	Subject	Identity	-	-	-
	Day2	Antedependence(1)			
			dinv_1	6.077	3.051
			dinv_2	3.126	1.572
			dinv_3	1.418	0.709
			dinv_4	10.26	5.15
			dinv_5	5.591	3.953
			u_12	-0.3765	0.5046
			u_23	-0.6509	0.5249
			u_34	0.1758	0.1204
			u_45	-0.6599	0.7341

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Day2	424.00	4	77.40	6.9	<0.001
Media	40.84	3	13.60	12.5	<0.001
Day2.Media	20.70	12	1.23	7.6	0.401

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Day2.Media	20.70	12	1.23	7.6	0.401

* MESSAGE: denominator degrees of freedom for approximate F-tests are calculated using algebraic derivatives ignoring fixed/boundary/singular variance parameters.

Table of predicted means for Constant

2.405 Standard error: 0.0828

Table of predicted means for Day2

Day2	1	21	43	62	121
	4.272	3.272	2.018	1.507	0.956

Standard errors of differences
Average: 0.2410
Maximum: 0.3488
Minimum: 0.1588

Average variance of differences: 0.06175

Table of predicted means for Media

Media	
7H11 PANTA	2.677
7H11 PCAT	2.721
Gallaghers PANTA	1.877
Gallaghers PCAT	2.345

Standard errors of differences
Average: 0.2342
Maximum: 0.2442
Minimum: 0.2238

Average variance of differences: 0.05488

Appendix 2

Table of predicted means for Day2.Media

Media Day2	7H11 PANTA	7H11 PCAT	Gallaghers PANTA	Gallaghers PCAT
1	4.007	4.511	4.112	4.457
21	3.669	3.550	2.664	3.204
43	2.557	2.592	1.313	1.613
62	2.000	1.944	0.660	1.425
121	1.155	1.007	0.638	1.026

Standard errors of differences

Average:	0.4849
Maximum:	0.7530
Minimum:	0.2538

Average variance of differences: 0.2513

Standard error of differences for same level of factor:

	Day2	Media
Average:	0.4739	0.4799
Maximum:	0.7530	0.7390
Minimum:	0.2873	0.2538

Average variance of differences:

0.2525	0.2470

Fisher's unprotected least significant difference test

Comparisons between Media means

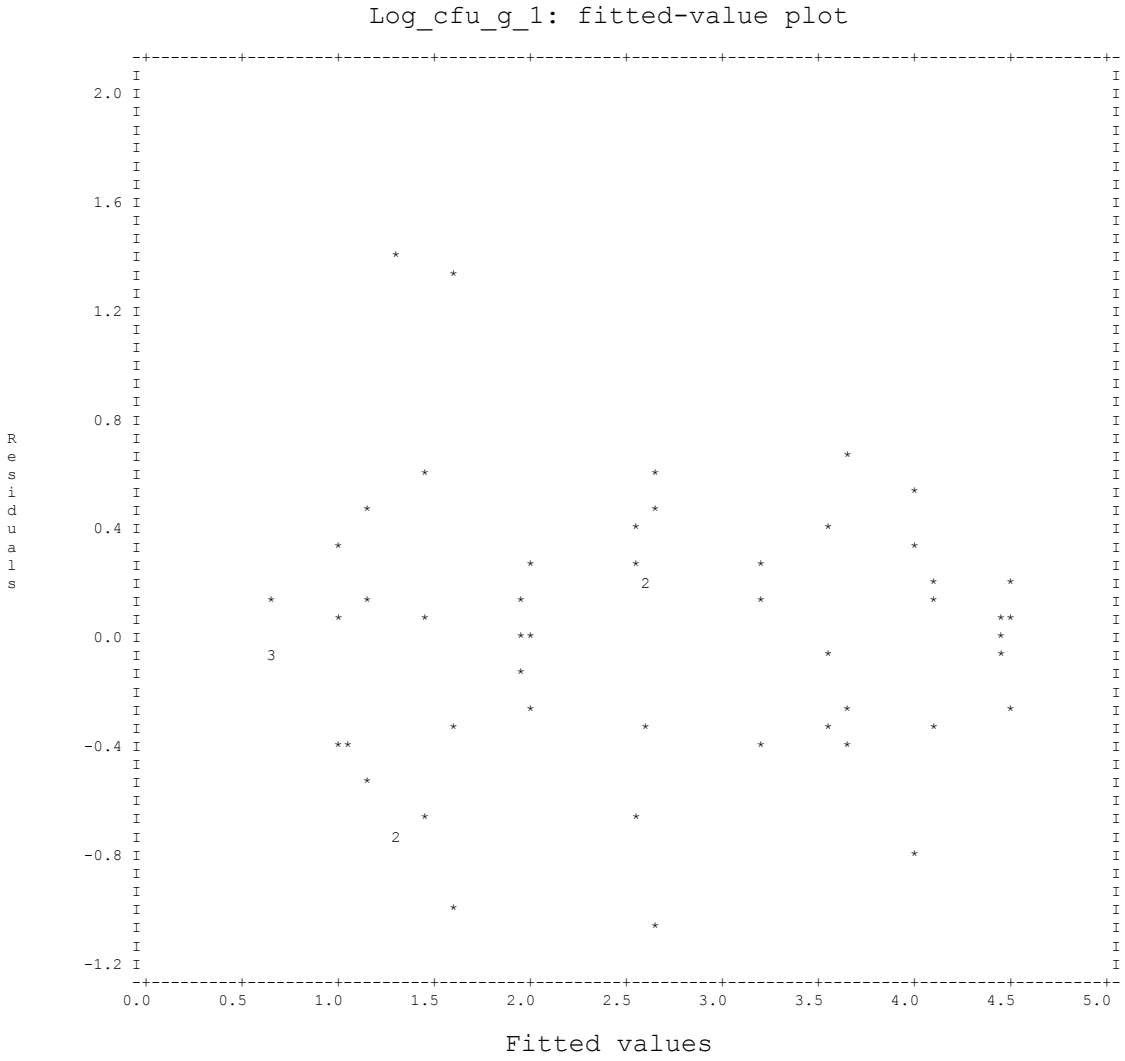
	Mean
Gallaghers PANTA	1.877 a
Gallaghers PCAT	2.345 ab
7H11 PANTA	2.677 b
7H11 PCAT	2.721 b

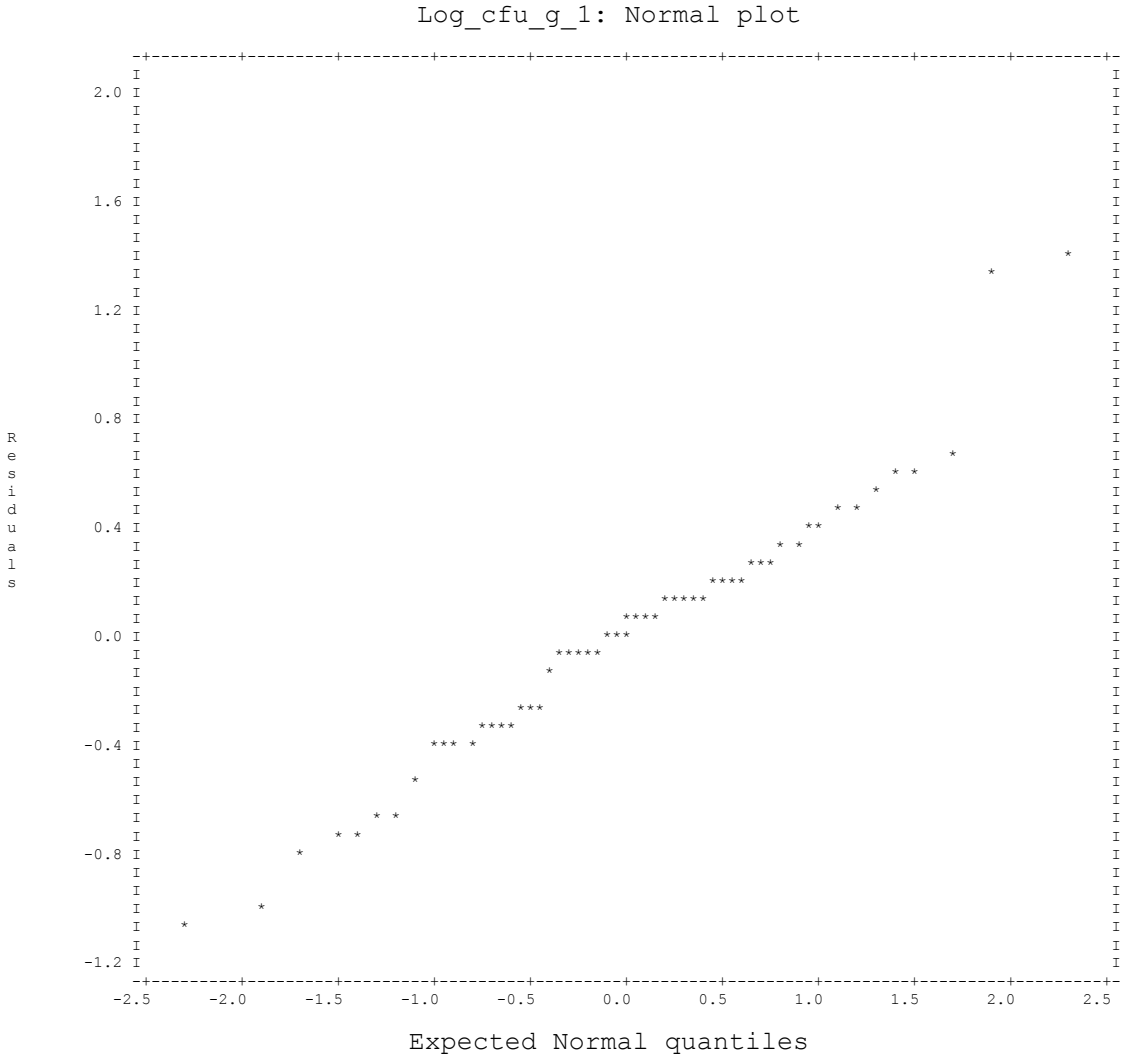
Fisher's unprotected least significant difference test

Comparisons between Day2 means

	Mean
121	0.956 a
62	1.507 b
43	2.018 b
21	3.272 c
1	4.272 d

Appendix 2


Log_cfu_g_1: fitted-value plot
Residuals
2.0
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Fitted values




```
 119
 120  DELE [RED=Y]
Bovis_Type,Cheese_Ref,Media,Cheese_Type,Cheese_Rep,Day,Log_cfu_g_1,Day2
,Subject
 121
 122  IMPORT InFile; SHEET='Caerphilly'; CELL='A1:G61'
```

Appendix 2

```
Loading Spreadsheet File
------------------------

Catalogue of file
C:\USERS\1006315\APPDATA\LOCAL\TEMP\GENSTAT\G142D9E.GWB

Sheet Title: Sheet1
Description: Data read from Michael Rowe 121109 M bovis Media Trial
Stats Data.xlsx [Caerphilly]A2:G61
Sheet Type: vector
  Index            Type    Nval   Name
      1            text      60   Bovis_Type
      2            text      60   Cheese_Ref
      3            text      60   Media
      4            text      60   Cheese_type
      5         variate      60   Cheese_Rep
      6         variate      60   Day
      7         variate      60   Log_cfu_g_1

 123
 124  GROUP [RED=Y] Bovis_Type,Media,Day
 125  FACT [LEV=!(1,23,44,65,86)] Day2
 126  CALC Day2=NEWL(Day;!(1,3(23,44,65,86)))
 127  & NV=NLEV(Bovis_Type)*NLEV(Media)*NLEV(Day2)
 128  TABU [CL=Bovis_Type,Media,Day2] Log_cfu_g_1; MEAN=MEAN
 129  VARI [N=NV; MOD=Y] Log_cfu_g_1; VAL=MEAN
 130  DELE [RED=Y] MEAN
 131  FACT [N=NV; MOD=Y] Bovis_Type,Media,Day2
 132  GENE Bovis_Type,Media,Day2
 133  FACPRODUCT !P(Media,Bovis_Type); PROD=Subject
 134
 135  FOR
 136
 137    PAGE
 138    PRIN Bovis_Type,Media,Day2,Log_cfu_g_1; DEC=3(0),2
 139
 140    PAGE
 141    VCOM [FIX=Media*Day2] CON=P
 142    REML [PR=*; MVINC=E,Y] Log_cfu_g_1; RES=_resid
 143    CALC _resid=MVREP(_resid;0)
 144    SORT [INDEX=Day2,Subject] _resid
 145    MATR [ROW=NLEV(Day2); COL=NLEV(Subject); VALUE=#_resid ]
_mresid
 146    SYMM [ROW=NLEV(Day2)] _vinit
 147    CALC _vinit=_mresid*+T(_mresid)/NLEV(Subject)
 148    VCOM [FIX=Media*Day2] RAN= Bovis_Type+Subject.Day2; CON=P
 149    VSTRUC [Subject.Day2] FACT=Day2; MOD=ANT; ORD=1; HET=N;
INIT=!(#_vinit)
 150    REML [PR=MOD,COM,MEAN,WALD; MVINC=E,Y] Log_cfu_g_1
 151    FCLASS [NT=3] Media*Day2; OUT=F[1...3]
 152    FOR FF=F[]
 153      VKEEP [WMETH=A] #FF; FSTAT=FSTAT; NDF=DF1; DDF=DF2
 154      CALC PROB=1-FPROB(FSTAT;DF1;DF2)
```

```
155      IF PROB.LT.0.05
156        VMCOMPARISON [PR=LET] #FF
157      ENDIF
```



```
158      DELE [RED=Y] DF1,DF2,FSTAT,PROB
159    ENDFOR
160    VPLOT [GRAPH=L] FIT,NORM
161    DELE [RED=Y] _resid,_mresid,_vinit,F
162
163    PAGE
164  ENDFOR
```

Bovis_Type	Media	Day2	Log_cfu_g_1
AF2122	7H11 PANTA	1	4.24
AF2122	7H11 PANTA	23	3.57
AF2122	7H11 PANTA	44	*
AF2122	7H11 PANTA	65	2.41
AF2122	7H11 PANTA	86	1.81
AF2122	7H11++ PANTA	1	4.29
AF2122	7H11++ PANTA	23	4.16
AF2122	7H11++ PANTA	44	3.44
AF2122	7H11++ PANTA	65	2.99
AF2122	7H11++ PANTA	86	2.79
AF2122	7H11++ PCAT	1	4.47
AF2122	7H11++ PCAT	23	3.98
AF2122	7H11++ PCAT	44	3.52
AF2122	7H11++ PCAT	65	3.28
AF2122	7H11++ PCAT	86	2.78
AF2122	7H11PCAT	1	4.44
AF2122	7H11PCAT	23	3.51
AF2122	7H11PCAT	44	2.83
AF2122	7H11PCAT	65	2.46
AF2122	7H11PCAT	86	1.52
G1.140	7H11 PANTA	1	3.58
G1.140	7H11 PANTA	23	3.15
G1.140	7H11 PANTA	44	2.48
G1.140	7H11 PANTA	65	2.36
G1.140	7H11 PANTA	86	2.06
G1.140	7H11++ PANTA	1	3.81
G1.140	7H11++ PANTA	23	3.76
G1.140	7H11++ PANTA	44	2.66
G1.140	7H11++ PANTA	65	2.57
G1.140	7H11++ PANTA	86	2.39
G1.140	7H11++ PCAT	1	3.77
G1.140	7H11++ PCAT	23	3.52
G1.140	7H11++ PCAT	44	2.77
G1.140	7H11++ PCAT	65	2.23
G1.140	7H11++ PCAT	86	2.23
G1.140	7H11PCAT	1	3.62
G1.140	7H11PCAT	23	3.16
G1.140	7H11PCAT	44	2.33
G1.140	7H11PCAT	65	1.79
G1.140	7H11PCAT	86	1.86
G24.130	7H11 PANTA	1	4.50
G24.130	7H11 PANTA	23	3.84
G24.130	7H11 PANTA	44	*
G24.130	7H11 PANTA	65	2.63
G24.130	7H11 PANTA	86	1.94
G24.130	7H11++ PANTA	1	4.52
G24.130	7H11++ PANTA	23	4.38
G24.130	7H11++ PANTA	44	3.29
G24.130	7H11++ PANTA	65	2.99
G24.130	7H11++ PANTA	86	2.48
G24.130	7H11++ PCAT	1	4.52
G24.130	7H11++ PCAT	23	4.28

G24.130	7H11++ PCAT	44	2.98
G24.130	7H11++ PCAT	65	2.99
G24.130	7H11++ PCAT	86	2.68

G24.130	7H11PCAT	1	4.41
G24.130	7H11PCAT	23	3.84
G24.130	7H11PCAT	44	2.75
G24.130	7H11PCAT	65	2.25
G24.130	7H11PCAT	86	1.62

REML variance components analysis
==========

Response variate: Log_cfu_g_1
Fixed model: Constant + Day2 + Media + Day2.Media
Random model: Bovis_Type + Subject.Day2
Number of units: 60

Subject.Day2 used as residual term with covariance structure as below

Sparse algorithm with AI optimisation
Units with missing data values included

Covariance structures defined for random model

Covariance structures defined within terms:

Term	Factor	Model	Order	No. rows
Subject.Day2	Subject	Identity	0	12
	Day2	Antedependence	1	5

Estimated variance components

Random term	component	s.e.
Bovis_Type	0.124	0.125

Residual variance model

Term	Factor	Model(order)	Parameter	Estimate	s.e.
Subject.Day2			Sigma2	1.000	fixed
	Subject	Identity	-	-	-
	Day2	Antedependence(1)			
			dinv_1	59.80	31.10
			dinv_2	755.4	458.4
			dinv_3	16.83	10.69
			dinv_4	33.07	18.82
			dinv_5	9.962	4.981
			u_12	0.2036	0.1273
			u_23	2.273	2.263
			u_34	-0.6388	0.2539
			u_45	-0.9013	0.4893

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Day2	508.96	4	94.43	7.4	<0.001
Media	751.56	3	250.38	6.6	<0.001
Day2.Media	29.50	12	1.76	8.4	0.209

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Day2.Media	29.50	12	1.76	8.4	0.209

* MESSAGE: denominator degrees of freedom for approximate F-tests are calculated using algebraic derivatives ignoring fixed/boundary/singular variance parameters.

Table of predicted means for Constant

3.118 Standard error: 0.2083

Table of predicted means for Day2

Day2	1	23	44	65	86
	4.181	3.762	2.885	2.580	2.181

Standard errors of differences
Average: 0.08716
Maximum: 0.1140
Minimum: 0.04614

Average variance of differences: 0.008048

Table of predicted means for Media

Media	
7H11 PANTA	2.943
7H11++ PANTA	3.368
7H11++ PCAT	3.332
7H11PCAT	2.826

Standard errors of differences
Average: 0.1259
Maximum: 0.1277
Minimum: 0.1242

Average variance of differences: 0.01586

Appendix 2

Table of predicted means for Day2.Media

Media Day2	7H11 PANTA	7H11++ PANTA	7H11++ PCAT	7H11PCAT
1	4.106	4.209	4.253	4.155
23	3.520	4.100	3.925	3.503
44	2.686	3.129	3.088	2.636
65	2.467	2.851	2.832	2.169
86	1.938	2.552	2.563	1.668

Standard errors of differences
Average: 0.1881
Maximum: 0.3142
Minimum: 0.03667

Average variance of differences: 0.03963

Standard error of differences for same level of factor:

	Day2	Media
Average:	0.1787	0.1735
Maximum:	0.3142	0.2610
Minimum:	0.03667	0.09228

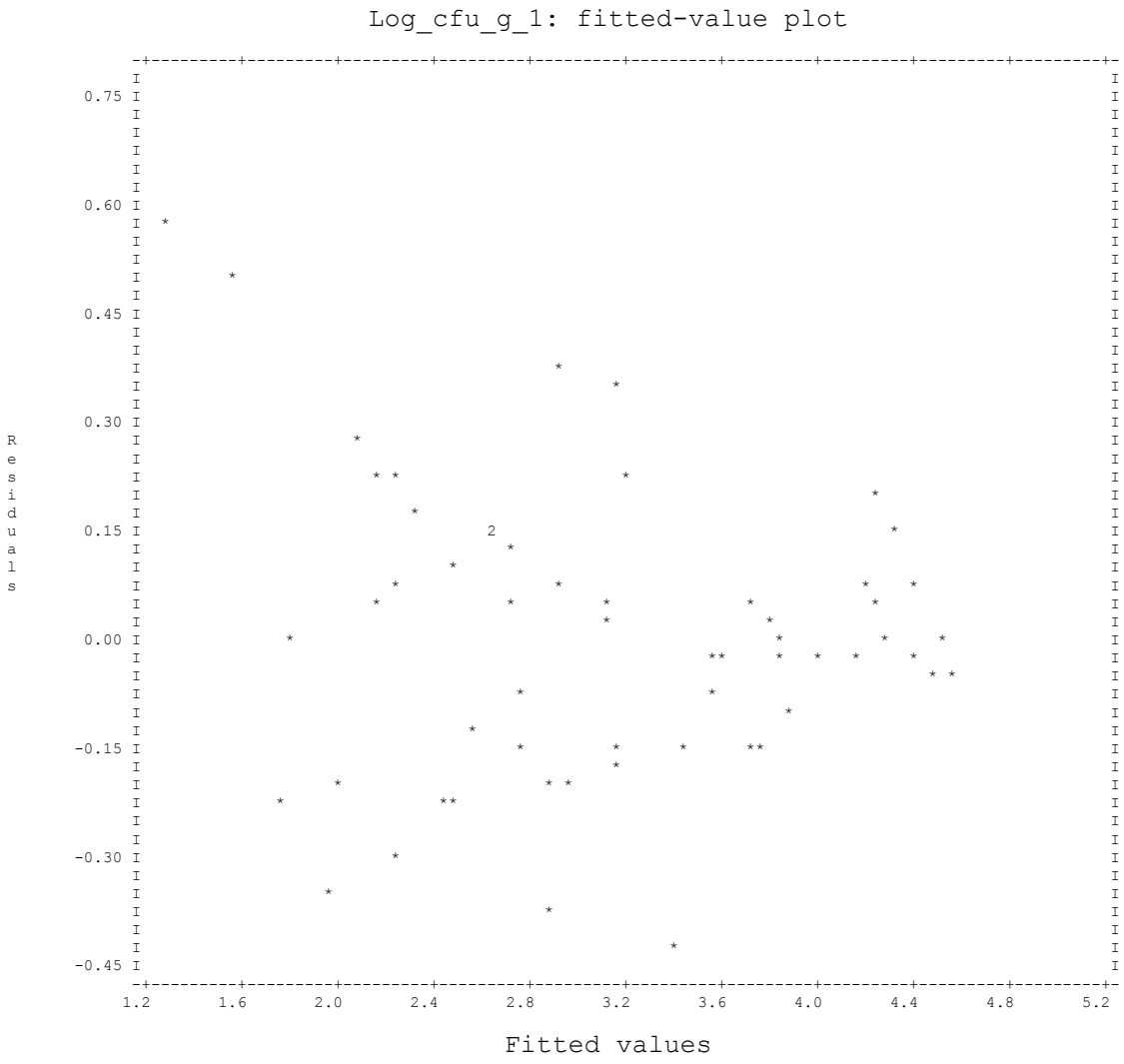
Average variance of differences:
0.04162 0.03219

Fisher's unprotected least significant difference test
Comparisons between Media means

	Mean
7H11PCAT	2.826 a
7H11 PANTA	2.943 a
7H11++ PCAT	3.332 b
7H11++ PANTA	3.368 b

Fisher's unprotected least significant difference test
Comparisons between Day2 means

	Mean
86	2.181 a
65	2.580 b
44	2.885 c
23	3.762 d
1	4.181 e


Log_cfu_g_1: fitted-value plot
Residuals
0.75
0.60
0.45
0.30
0.15
0.00
-0.15
-0.30
-0.45
1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
4.4
4.8
5.2
Fitted values

Appendix 3

Statistical analysis of selective media: Cheddar *M. bovis* AF2122 homogenates.

```
  14
  15  FOR
  16    PAGE
  17    SKIP [FILE=OUT] 2
  18    PRIN [IPR=*; SQ=Y] 'Received from:','Michael Rowe/Richard
Forgrave'; FIELD=39; JUST=L
  19    & 'Cost code:','44187'; FIELD=39; JUST=L
  20    & 'Statistical Analysis number:','S20130010'; FIELD=39; JUST=L
  21    & 'Folder:',Folder; FIELD=39; JUST=L
  22    & 'Data File(s):',InFile; FIELD=39; JUST=L
  23    & 'Output File:',OutFile; FIELD=39; JUST=L
  24    & 'Output files created by Alan Gordon:',TDATE; FIELD=39;
JUST=L
  25    & 'Title:',Title; FIELD=39; JUST=L
  26    PAGE
  27  ENDFOR

Received from:                          Michael Rowe/Richard Forgrave
Cost code:                              44187
Statistical Analysis number:            S20130010
Folder:
P:/Biometrics/Analysis/AlanG/Alan/Micro/Michael Rowe/M Bovis in Raw
Milk Cheese (44187)
Data File(s):                           Michael Rowe 130109 M bovis
Media Trial Stats Data V1.xlsx
Output File:                            Michael Rowe 130109 M bovis
Media Trial Stats Data V1.txt
Output files created by Alan Gordon:    11/01/13
Title:                                  M Bovis in Raw Milk Cheese
Analysis
  28
  29
  30  IMPORT InFile; SHEET='AF2122 Media'

Loading Spreadsheet File
------------------------
Catalogue of file
C:\USERS\1006315\APPDATA\LOCAL\TEMP\GENSTAT\G151D364892.GWB
Sheet Title: Sheet1
Description: Data read from Michael Rowe 130109 M bovis Media Trial
Stats Data V1.xlsx [AF2122 Media]A2:P109
Sheet Type: vector
  Index            Type    Nval   Name
      1            text     108   Bovis_Type
      2            text     108   Cheese_Ref
      3            text     108   Media
      4            text     108   Cheese_Type
      5         variate     108   Cheese_Rep
      6         variate     108   Day
      7         variate     108   Log_cfu_g_1

  31  GROUP [RED=Y] Media,Cheese_Rep,Day
  32  FACT [LEV=!(1,55,110)] Day2
  33  CALC Day2=NEWL(Day;!(1,3(55,110)))
```

```
  34  & NV=NLEV(Cheese_Rep)*NLEV(Media)*NLEV(Day2)
  35  TABU [CL=Cheese_Rep,Media,Day2] Log_cfu_g_1; MEAN=MEAN
  36  VARI [N=NV; MOD=Y] Log_cfu_g_1; VAL=MEAN
  37  DELE [RED=Y] MEAN
  38  FACT [N=NV; MOD=Y] Cheese_Rep,Media,Day2
  39  GENE Cheese_Rep,Media,Day2
  40  FACPRODUCT !P(Media,Cheese_Rep); PROD=Subject
  41
  42  FOR
  43
  44    PAGE
  45    PRIN Media,Cheese_Rep,Day2,Log_cfu_g_1; DEC=3(0),2
  46
  47    PAGE
  48    VCOM [FIX=Media*Day2] CON=P
  49    REML [PR=*; MVINC=E,Y] Log_cfu_g_1; RES=_resid
  50    CALC _resid=MVREP(_resid;0)
  51    SORT [INDEX=Day2,Subject] _resid
  52    MATR [ROW=NLEV(Day2); COL=NLEV(Subject); VALUE=#_resid ]
_mresid
  53    SYMM [ROW=NLEV(Day2)] _vinit
  54    CALC _vinit=_mresid*+T(_mresid)/NLEV(Subject)
  55    VCOM [FIX=Media*Day2] RAN=Cheese_Rep+Subject.Day2; CON=P
  56    VSTRUC [Subject.Day2] FACT=Day2; MOD=ANT; ORD=1; HET=N;
INIT=!(#_vinit)
  57    REML [PR=MOD,COM,MEAN,WALD; MVINC=E,Y] Log_cfu_g_1
  58    FCLASS [NT=3] Media*Day2; OUT=F[1...3]
  59    FOR FF=F[]
  60      VKEEP [WMETH=A] #FF; FSTAT=FSTAT; NDF=DF1; DDF=DF2
  61      CALC PROB=1-FPROB(FSTAT;DF1;DF2)
  62      IF PROB.LT.0.05
  63        VMCOMPARISON [PR=LET] #FF
  64      ENDIF
  65      DELE [RED=Y] DF1,DF2,FSTAT,PROB
  66    ENDFOR
  67    VPLOT [GRAPH=L] FIT,NORM
  68    DELE [RED=Y] _resid,_mresid,_vinit,F
  69
  70    PAGE
  71  ENDFOR
```

Appendix 3

Media	Cheese_Rep	Day2	Log_cfu_g_1
7H11 PANTA	1	1	4.19
7H11 PANTA	1	55	1.90
7H11 PANTA	1	110	1.05
7H11 PCAT	1	1	4.33
7H11 PCAT	1	55	1.77
7H11 PCAT	1	110	0.60
7H11++ PANTA	1	1	4.37
7H11++ PANTA	1	55	2.80
7H11++ PANTA	1	110	1.49
7H11++ PCAT	1	1	4.32
7H11++ PCAT	1	55	2.88
7H11++ PCAT	1	110	1.49
Gallaghers PANTA	1	1	3.03
Gallaghers PANTA	1	55	1.58
Gallaghers PANTA	1	110	0.59
Gallaghers PCAT	1	1	3.03
Gallaghers PCAT	1	55	1.29
Gallaghers PCAT	1	110	0.59
7H11 PANTA	2	1	4.08
7H11 PANTA	2	55	1.64
7H11 PANTA	2	110	1.38
7H11 PCAT	2	1	4.25
7H11 PCAT	2	55	1.75
7H11 PCAT	2	110	0.83
7H11++ PANTA	2	1	4.48
7H11++ PANTA	2	55	2.44
7H11++ PANTA	2	110	1.27
7H11++ PCAT	2	1	4.24
7H11++ PCAT	2	55	2.78
7H11++ PCAT	2	110	1.25
Gallaghers PANTA	2	1	3.20
Gallaghers PANTA	2	55	1.25
Gallaghers PANTA	2	110	0.59
Gallaghers PCAT	2	1	2.98
Gallaghers PCAT	2	55	1.02
Gallaghers PCAT	2	110	0.59
7H11 PANTA	3	1	3.00
7H11 PANTA	3	55	1.29
7H11 PANTA	3	110	0.60
7H11 PCAT	3	1	3.15
7H11 PCAT	3	55	1.34
7H11 PCAT	3	110	0.99
7H11++ PANTA	3	1	3.85
7H11++ PANTA	3	55	2.14
7H11++ PANTA	3	110	1.60
7H11++ PCAT	3	1	4.15
7H11++ PCAT	3	55	2.25
7H11++ PCAT	3	110	1.20
Gallaghers PANTA	3	1	2.60
Gallaghers PANTA	3	55	0.60
Gallaghers PANTA	3	110	0.59
Gallaghers PCAT	3	1	2.72
Gallaghers PCAT	3	55	0.99
Gallaghers PCAT	3	110	0.59

Appendix 3

```
REML variance components analysis
=================================
Response variate:  Log_cfu_g_1
Fixed model:       Constant + Day2 + Media + Day2.Media
Random model:      Cheese_Rep + Subject.Day2
Number of units:   54

Subject.Day2 used as residual term with covariance structure as below
Sparse algorithm with AI optimisation


Covariance structures defined for random model
----------------------------------------------
Covariance structures defined within terms:

Term                      Factor      Model                          Order
No. rows
Subject.Day2           Subject     Identity                        0        18
                       Day2        Antedependence                  1         3


Estimated variance components
-----------------------------
Random term                component        s.e.
Cheese_Rep                     0.062       0.065


Residual variance model
-----------------------
Term            Factor       Model(order)  Parameter        Estimate      s.e.
Subject.Day2                               Sigma2              1.000     fixed
                Subject      Identity      -                       -         -
                Day2         Antedependence(1)
                                           dinv_1              13.25      5.69
                                           dinv_2              45.23     20.83
                                           dinv_3              11.29      5.22
                                           u_12              -0.03599   0.17603
                                           u_23                0.3464    0.6245


Tests for fixed effects
-----------------------
Sequentially adding terms to fixed model

Fixed term                 Wald statistic  n.d.f.   F statistic  d.d.f.
F pr
Day2                           973.36       2        458.32    13.3  <0.001
Media                          378.05       5         75.61    10.2  <0.001
Day2.Media                      21.46      10          1.98    14.9   0.112

Dropping individual terms from full fixed model

Fixed term                 Wald statistic  n.d.f.   F statistic  d.d.f.
F pr
Day2.Media                          21.46      10          1.98    14.9
0.112

* MESSAGE: denominator degrees of freedom for approximate F-tests are
calculated using algebraic derivatives ignoring
```

```
fixed/boundary/singular variance parameters.

Table of predicted means for Constant
-------------------------------------
            2.130    Standard error:  0.1480

Table of predicted means for Day2
---------------------------------
Day2         1       55      110
         3.666    1.763    0.962

Standard errors of differences
Average:          0.08433
Maximum:          0.09679
Minimum:          0.07160

Average variance of differences: 0.007217

Table of predicted means for Media
----------------------------------
            Media
       7H11 PANTA    2.127
        7H11 PCAT    2.114
     7H11++ PANTA    2.717
      7H11++ PCAT    2.730
Gallaghers PANTA    1.560
 Gallaghers PCAT    1.534


Standard error of differences: 0.1145
Table of predicted means for Day2.Media
---------------------------------------
Media     7H11 PANTA     7H11 PCAT    7H11++ PANTA    7H11++ PCAT  Gallaghers PANTA
 Day2
    1          3.756         3.912          4.233           4.237             2.946
   55          1.613         1.621          2.462           2.637             1.145
  110          1.012         0.807          1.455           1.316             0.590

Media      Gallaghers PCAT
 Day2
    1               2.910
   55               1.103
  110               0.590

Standard errors of differences
Average:          0.2022
Maximum:          0.2466
Minimum:          0.1217

Average variance of differences: 0.04214

Standard error of differences for same level of factor:
                    Day2       Media
Average:          0.1976      0.2066
Maximum:          0.2466      0.2371
Minimum:          0.1217      0.1754
Average variance of differences:
            0.04199     0.04330
```

Appendix 3

Fisher's unprotected least significant difference test

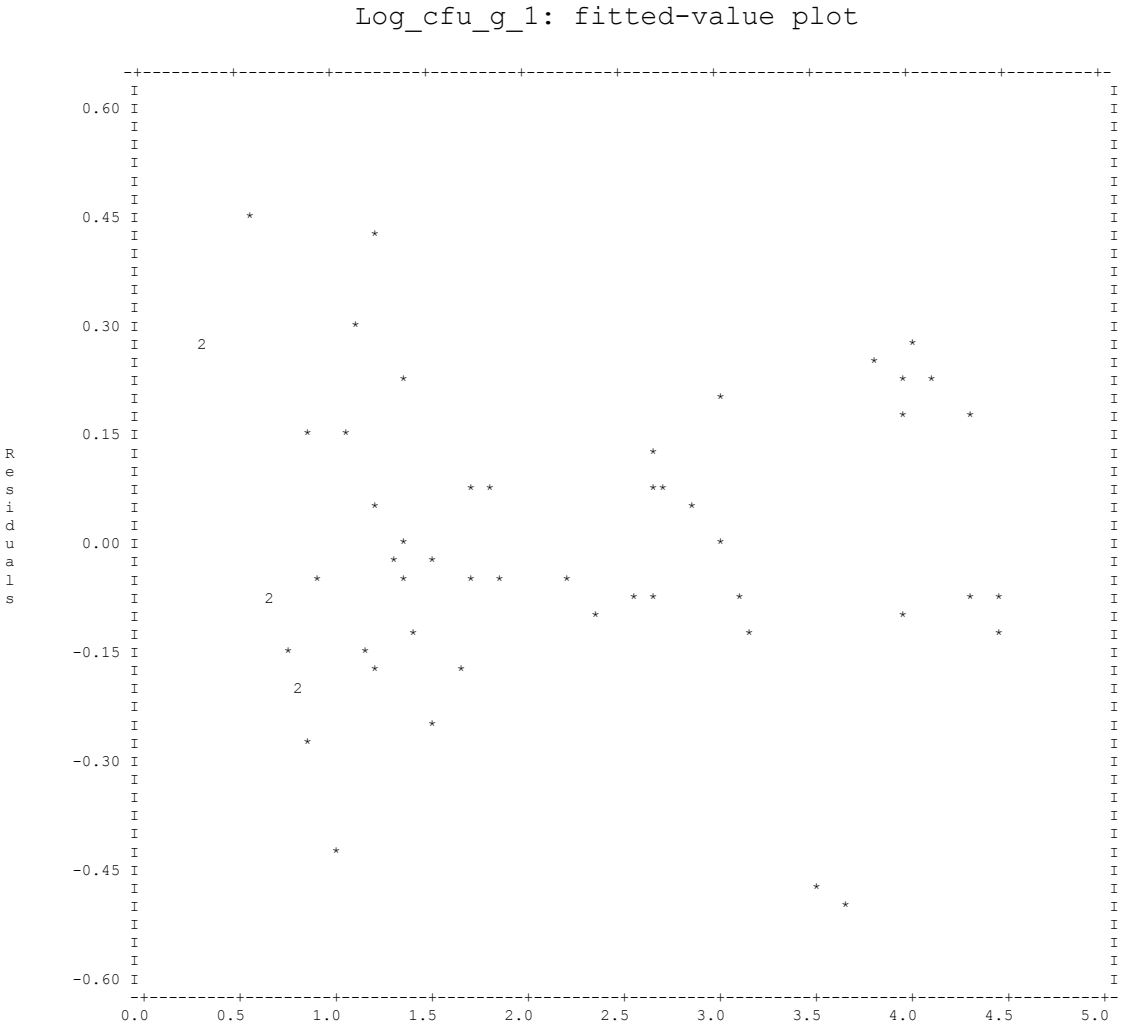
Comparisons between Media means

	Mean	
Gallaghers PCAT	1.534	a
Gallaghers PANTA	1.560	a
7H11 PCAT	2.114	b
7H11 PANTA	2.127	b
7H11++ PANTA	2.717	c
7H11++ PCAT	2.730	c

Fisher's unprotected least significant difference test

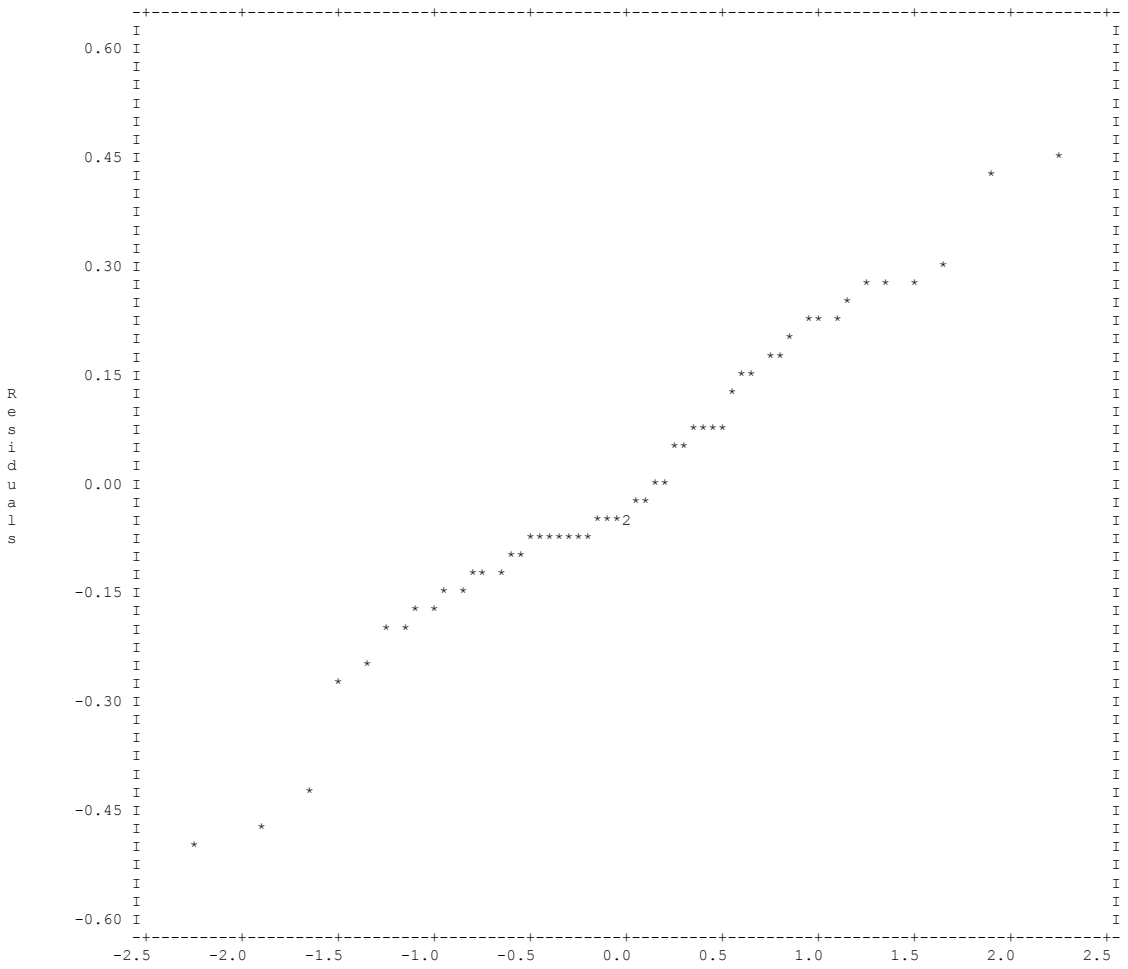
Comparisons between Day2 means

	Mean	
110	0.962	a
55	1.763	b
1	3.666	c



Appendix 3

Log_cfu_g_1: Normal plot

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  72  ENDJOB

********* End of job. Current data space: 16 blocks, peak usage 48% at
line 71.

GenStat 64-bit Release 15.1  (PC/Windows 7)       11 January 2013
11:54:57
Copyright 2012, VSN International Ltd.
Registered to: DARDNI

  73  STOP
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Appendix 4

Inactivation curves generated by extended sampling of Caerphilly: up to eight months.

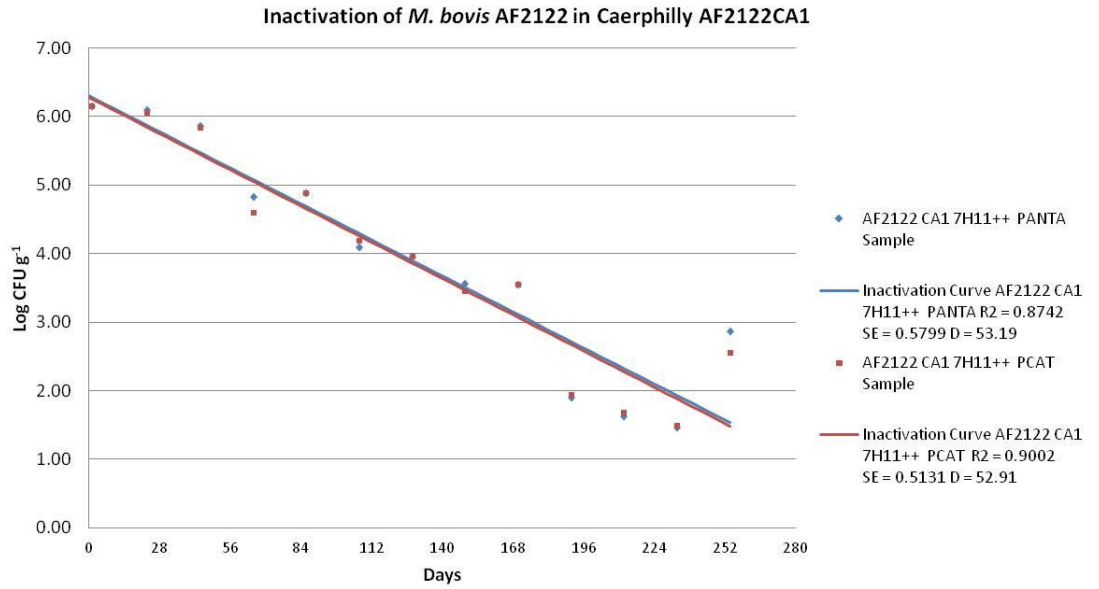

Fig. 1(a). Inactivation curve of *M. bovis* AF2122 from Caerphilly AF2122 CA1. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 CA1 up to 254 days.

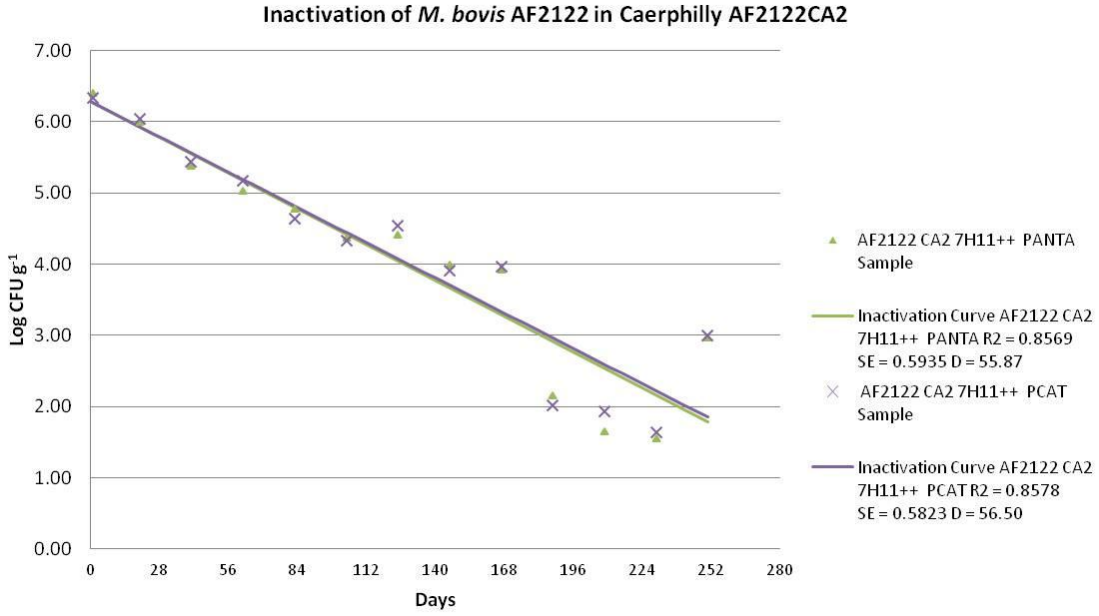

Fig. 1(b). Inactivation curve of *M. bovis* AF2122 from Caerphilly AF2122 CA2. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 CA2 up to 251 days.

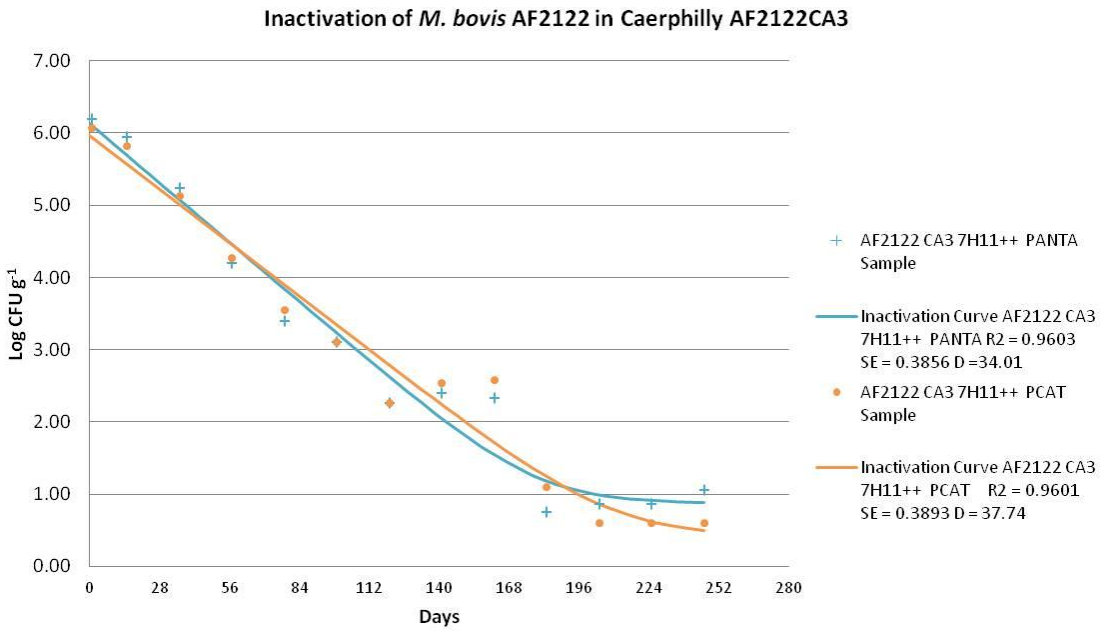

Fig. 1(c). Inactivation curve of *M. bovis* AF2122 from Caerphilly AF2122 CA3. *M. bovis* AF2122 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly AF2122 CA3 up to 246 days.

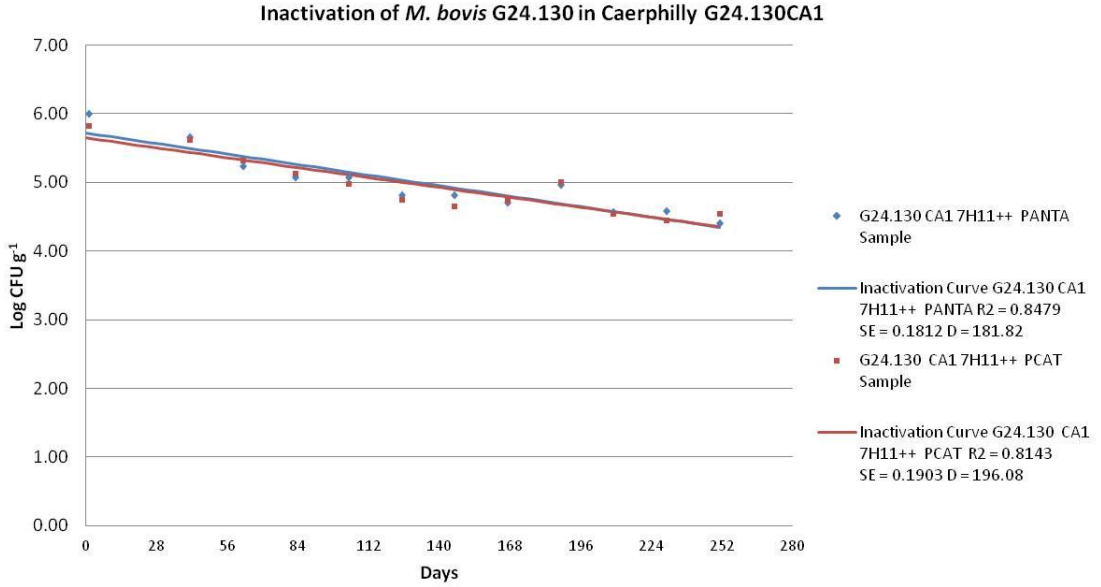

Fig. 1(d). Inactivation curve of *M. bovis* G24.130 from Caerphilly G24.130 CA1. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA1 up to 251 days.

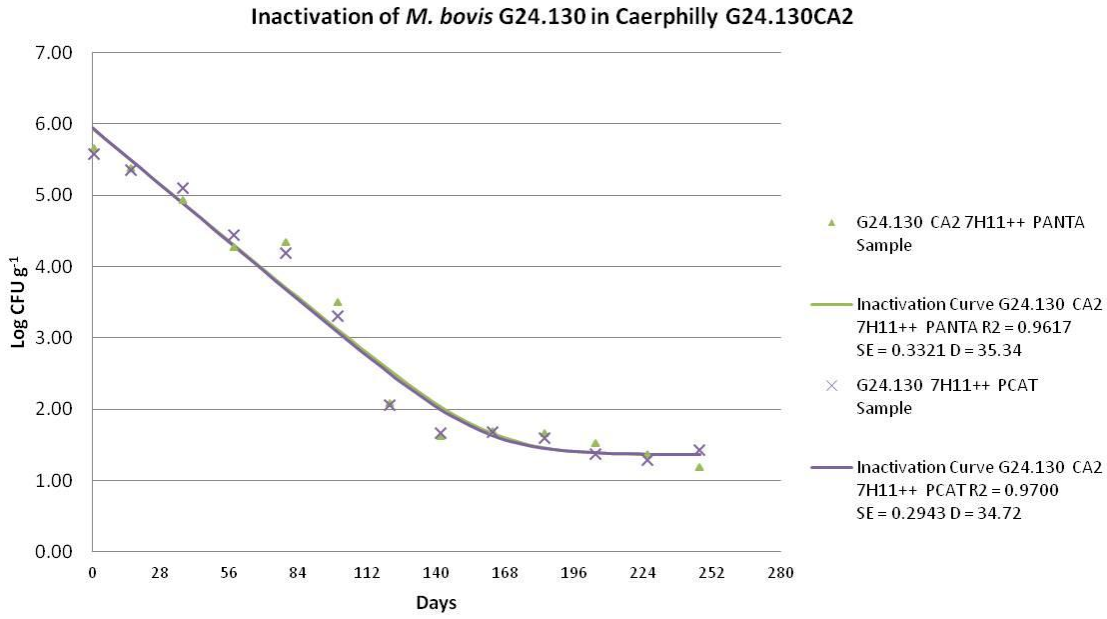

Fig. 1(e). Inactivation curve of *M. bovis* G24.130 from Caerphilly G24.130 CA2. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA2 up to 247 days.

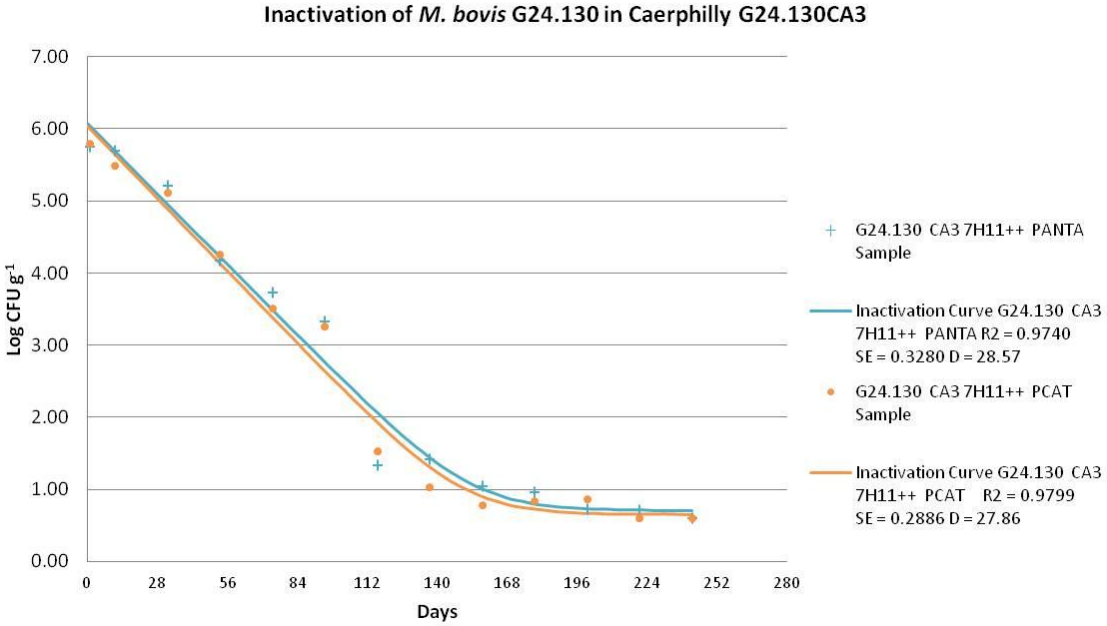

Fig. 1(f). Inactivation curve of *M. bovis* G24.130 from Caerphilly G24.130 CA3. *M. bovis* G24.130 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA3 up to 242 days.

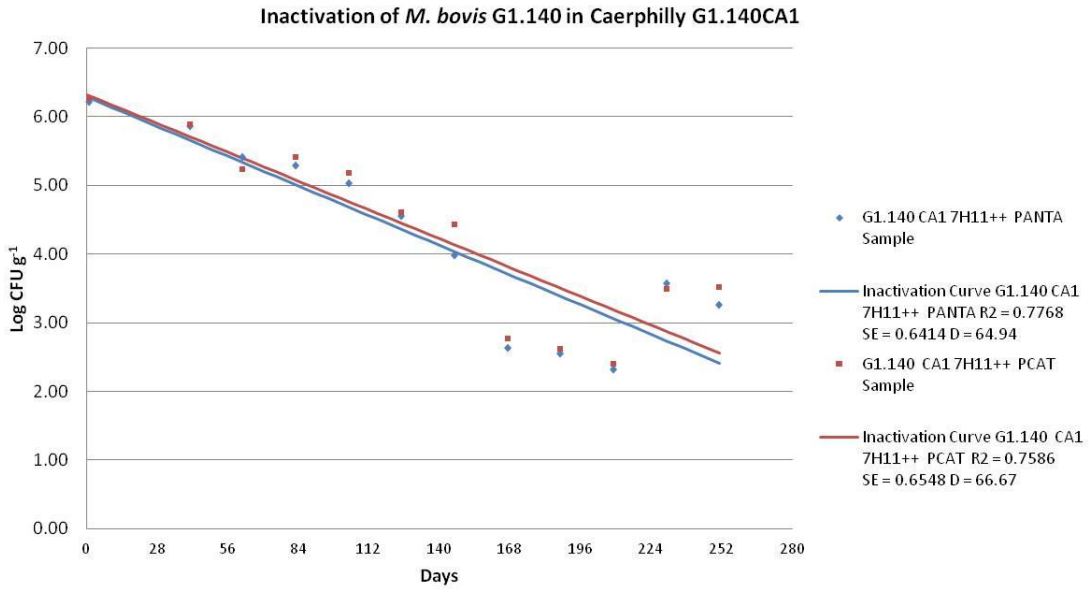

Fig. 1(g). Inactivation curve of *M. bovis* G1.140 from Caerphilly G1.140 CA1. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G1.140 CA1 up to 251 days.

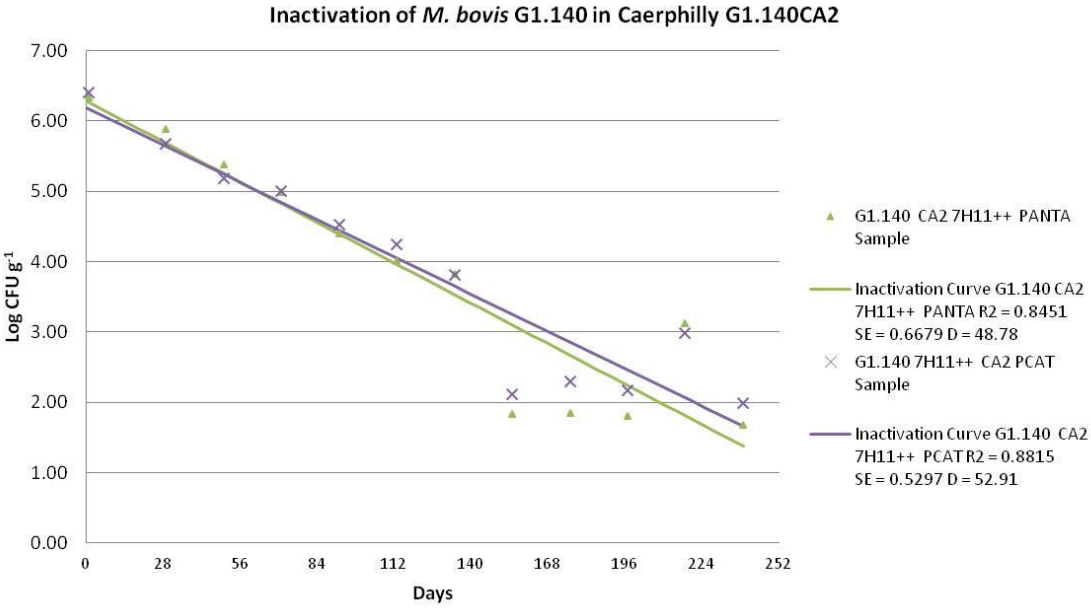

Fig. 1(h). Inactivation curve of *M. bovis* G1.140 from Caerphilly G1.140 CA2. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G1.140 CA2 up to 239 days.

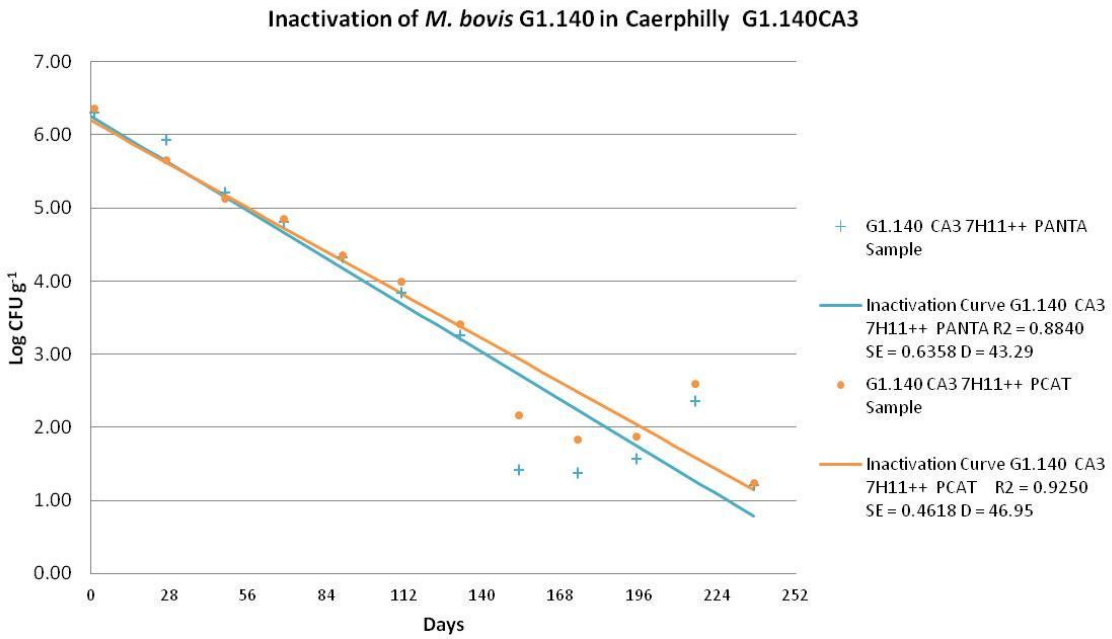

Fig. 7(i). Inactivation curve of *M. bovis* G1.140 from Caerphilly G1.140 CA3. *M. bovis* G1.140 was isolated using 7H11++PANTA and 7H11++PCAT from Caerphilly G24.130 CA3 up to 237 days.

Statistical analysis of high- and low-inoculum Cheddar and Caerphilly.

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  15  FOR
  16    PAGE
  17    SKIP [FILE=OUT] 2
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Forgrave'; FIELD=39; JUST=L
  19    & 'Cost code:','44187'; FIELD=39; JUST=L
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  24    & 'Output files created by Alan Gordon:',TDATE; FIELD=39;
JUST=L
  25    & 'Title:',Title; FIELD=39; JUST=L
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  27  ENDFOR

Received from:                          Michael Rowe/Richard Forgrave
Cost code:                              44187
Statistical Analysis number:            S20120501
Folder:
P:/Biometrics/Analysis/AlanG/Alan/Micro/Michael Rowe/M Bovis in Raw
Milk Cheese (44187)
Data File(s):                           Michael Rowe 121024 M bovis
stats data-DMfit.xlsx
Output File:                            Michael Rowe 121024 M bovis
stats data-DMfit.txt
Output files created by Alan Gordon:    25/10/12
Title:                                  M Bovis in Raw Milk Cheese
Analysis

  28
  29  IMPORT InFile; SHEET='High Level'; CELL='A2:F38'; ISAVE=Data

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      2            text      36   Media
      3            text      36   Cheese_Type
      4         variate      36   Cheese_Rep
      5         variate      36   Slope
      6         variate      36   D_Value

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      2            text      12   C2
      3            text      12   C3
      4         variate      12   C4
      5         variate      12   C5
      6         variate      12   C6

  31  & [IPREFIX='D'] InFile; SHEET='Hi lev C''philly 4 Month Mat   ';
CELL='A3:F20'; ISAVE=Data2

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      2            text      18   D_2
      3            text      18   D_3
      4         variate      18   D_4
      5         variate      18   D_5
      6         variate      18   D_6

  32  FOR DUM1=Data[]; DUM2=Data1[]; DUM3=Data2[]
  33    APPEND DUM1,DUM2,DUM3
  34  ENDFOR
  35  GROUP [RED=Y] Bovis_Type,Media,Cheese_Type,Cheese_Rep
  36  FACT [LAB=!T(High,Low,'4 Month Mat'); VAL=36(1),12(2),18(3)]
Level
  37  FACPRODUCT !P(Bovis_Type,Cheese_Type,Cheese_Rep,Level);
PROD=Block
  38  & !P(Bovis_Type,Cheese_Type,Cheese_Rep); PROD=Block1
  39
  40  FOR
  41
  42    PAGE
  43    PRIN
Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Value;
DEC=5(0),4,2
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  45    FOR I1=1...4; I2='High','Low','Both','High vs >4mths'
  46
  47      IF I1.LE.2
  48        CONC [NEW=TIT] OLD='Analysis for ',I2,' Level'
  49        REST
Block,Block1,Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Valu
e; Level.IN.I2
  50        BLOC Block
  51        IF I1.EQ.1
  52          TREA Bovis_Type*Cheese_Type*Media
  53        ELSE
  54          TREA Cheese_Type*Media
  55        ENDIF
  56      ELSIF I1.EQ.3
  57        CONC [NEW=TIT] OLD='Analysis for ',I2,' Levels'
  58        REST
Block,Block1,Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Valu
e;\
  59         (Bovis_Type.IN.'AF2122').AND.(Level.NI.'4 Month Mat')
  60        BLOC Block
  61        TREA Level*Cheese_Type*Media
  62      ELSE
  63        CONC [NEW=TIT] OLD='Analysis for ',I2
  64        REST
Block,Block1,Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Valu
e;\
  65         (Cheese_Type.IN.'Caerphilly').AND.(Level.NI.'Low')
  66        BLOC Block1/Media
  67        TREA Bovis_Type*Media*Level
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  70      PAGE
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  75      REST
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e
  76      DELE [RED=Y] TIT
  77
  78    ENDFOR
  79
  80    PAGE
  81  ENDFOR
```

Appendix 5

Bovis_Type	Media	Cheese_Type	Cheese_Rep	Level	Slope	D_Value
AF2122	7H11++ PANTA	Cheddar	1	High	-0.0283	35.34
AF2122	7H11++ PANTA	Cheddar	2	High	-0.0343	29.15
AF2122	7H11++ PANTA	Cheddar	3	High	-0.0331	30.21
AF2122	7H11++ PCAT	Cheddar	1	High	-0.0250	40.00
AF2122	7H11++ PCAT	Cheddar	2	High	-0.0317	31.55
AF2122	7H11++ PCAT	Cheddar	3	High	-0.0356	28.09
AF2122	7H11++ PANTA	Caerphilly	1	High	-0.0192	52.08
AF2122	7H11++ PANTA	Caerphilly	2	High	-0.0160	62.50
AF2122	7H11++ PANTA	Caerphilly	3	High	-0.0305	32.79
AF2122	7H11++ PCAT	Caerphilly	1	High	-0.0192	52.08
AF2122	7H11++ PCAT	Caerphilly	2	High	-0.0162	61.73
AF2122	7H11++ PCAT	Caerphilly	3	High	-0.0288	34.72
G24.130	7H11++ PANTA	Cheddar	1	High	-0.0252	39.68
G24.130	7H11++ PANTA	Cheddar	2	High	-0.0166	60.24
G24.130	7H11++ PANTA	Cheddar	3	High	-0.0170	58.82
G24.130	7H11++ PCAT	Cheddar	1	High	-0.0243	41.15
G24.130	7H11++ PCAT	Cheddar	2	High	-0.0151	66.23
G24.130	7H11++ PCAT	Cheddar	3	High	-0.0166	60.24
G24.130	7H11++ PANTA	Caerphilly	1	High	-0.0078	128.21
G24.130	7H11++ PANTA	Caerphilly	2	High	-0.0285	35.09
G24.130	7H11++ PANTA	Caerphilly	3	High	-0.0347	28.82
G24.130	7H11++ PCAT	Caerphilly	1	High	-0.0074	135.14
G24.130	7H11++ PCAT	Caerphilly	2	High	-0.0289	34.60
G24.130	7H11++ PCAT	Caerphilly	3	High	-0.0354	28.25
G1.140	7H11++ PANTA	Cheddar	1	High	-0.0131	76.34
G1.140	7H11++ PANTA	Cheddar	2	High	-0.0140	71.43
G1.140	7H11++ PANTA	Cheddar	3	High	-0.0173	57.80
G1.140	7H11++ PCAT	Cheddar	1	High	-0.0165	60.61
G1.140	7H11++ PCAT	Cheddar	2	High	-0.0135	74.07
G1.140	7H11++ PCAT	Cheddar	3	High	-0.0190	52.63
G1.140	7H11++ PANTA	Caerphilly	1	High	-0.0130	76.92
G1.140	7H11++ PANTA	Caerphilly	2	High	-0.0201	49.75
G1.140	7H11++ PANTA	Caerphilly	3	High	-0.0235	42.55
G1.140	7H11++ PCAT	Caerphilly	1	High	-0.0123	81.30
G1.140	7H11++ PCAT	Caerphilly	2	High	-0.0186	53.76
G1.140	7H11++ PCAT	Caerphilly	3	High	-0.0216	46.30
AF2122	7H11++ PANTA	Cheddar	1	Low	-0.0448	22.32
AF2122	7H11++ PANTA	Cheddar	2	Low	-0.0283	35.34
AF2122	7H11++ PANTA	Cheddar	3	Low	-0.0366	27.32
AF2122	7H11++ PCAT	Cheddar	1	Low	-0.0430	23.26
AF2122	7H11++ PCAT	Cheddar	2	Low	-0.0248	40.32
AF2122	7H11++ PCAT	Cheddar	3	Low	-0.0291	34.36
AF2122	7H11++ PANTA	Caerphilly	1	Low	-0.0636	15.72
AF2122	7H11++ PANTA	Caerphilly	2	Low	-0.0492	20.33
AF2122	7H11++ PANTA	Caerphilly	3	Low	-0.0420	23.81
AF2122	7H11++ PCAT	Caerphilly	1	Low	-0.0619	16.16
AF2122	7H11++ PCAT	Caerphilly	2	Low	-0.0396	25.25
AF2122	7H11++ PCAT	Caerphilly	3	Low	-0.0362	27.62
AF2122	7H11++ PANTA	Caerphilly	1	4 Month Mat	-0.0188	53.19
AF2122	7H11++ PANTA	Caerphilly	2	4 Month Mat	-0.0179	55.87
AF2122	7H11++ PANTA	Caerphilly	3	4 Month Mat	-0.0294	34.01
AF2122	7H11++ PCAT	Caerphilly	1	4 Month Mat	-0.0189	52.91
AF2122	7H11++ PCAT	Caerphilly	2	4 Month Mat	-0.0177	56.50
AF2122	7H11++ PCAT	Caerphilly	3	4 Month Mat	-0.0265	37.74
G24.130	7H11++ PANTA	Caerphilly	1	4 Month Mat	-0.0055	181.82
G24.130	7H11++ PANTA	Caerphilly	2	4 Month Mat	-0.0283	35.34
G24.130	7H11++ PANTA	Caerphilly	3	4 Month Mat	-0.0350	28.57

```
G24.130  7H11++ PCAT   Caerphilly    1   4 Month Mat -0.0051     196.08
G24.130  7H11++ PCAT   Caerphilly    2   4 Month Mat -0.0288      34.72
G24.130  7H11++ PCAT  Caerphilly     3   4 Month Mat -0.0359      27.86
G1.140 7H11++ PANTA  Caerphilly      1   4 Month Mat -0.0154      64.94
G1.140 7H11++ PANTA  Caerphilly      2   4 Month Mat -0.0205      48.78
G1.140 7H11++ PANTA  Caerphilly      3   4 Month Mat -0.0231      43.29
G1.140 7H11++ PCAT  Caerphilly       1   4 Month Mat -0.0150      66.67
G1.140  7H11++ PCAT  Caerphilly      2   4 Month Mat -0.0189      52.91
G1.140  7H11++ PCAT  Caerphilly      3   4 Month Mat -0.0213      46.95


Analysis for High Level

Analysis of variance
====================

Variate: D_Value

Source of variation        d.f.       s.s.     m.s.    v.r.  F pr.

Block stratum
Bovis_Type                    2   3223.02    1611.51    1.17  0.344
Cheese_Type                   1    420.30     420.30    0.30  0.591
Bovis_Type.Cheese_Type        2    926.88     463.44    0.34  0.721
Residual                     12 16551.18    1379.26  133.02

Block.*Units* stratum
Media                         1       6.02       6.02   0.58  0.461
Bovis_Type.Media              2      18.32       9.16   0.88  0.439
Cheese_Type.Media             1      15.50      15.50   1.49  0.245
Bovis_Type.Cheese_Type.Media  2      63.39      31.70   3.06  0.085
Residual                     12     124.43      10.37

Total                        35   21349.04


* MESSAGE: the following units have large residuals.

Block G24.130 Caerphilly 1 High         66.65   approx. s.e. 21.44

Block G1.140 Cheddar 1 High  *units* 1   4.82   approx. s.e. 1.86
Block G1.140 Cheddar 1 High  *units* 2  -4.82   approx. s.e. 1.86
Block G1.140 Cheddar 2 High  *units* 1  -4.37   approx. s.e. 1.86
Block G1.140 Cheddar 2 High  *units* 2   4.37   approx. s.e. 1.86
```

Appendix 5

Tables of means
==========

Variate: D_Value

Grand mean 54.17

Bovis_Type	AF2122	G1.140	G24.130
	40.85	61.96	59.71

Cheese_Type	Caerphilly	Cheddar
	57.59	50.75

Media	7H11++ PANTA	7H11++ PCAT
	53.76	54.58

Bovis_Type	Cheese_Type	Caerphilly	Cheddar
AF2122		49.32	32.39
G1.140		58.43	65.48
G24.130		65.02	54.39

Bovis_Type	Media	7H11++ PANTA	7H11++ PCAT
AF2122		40.35	41.36
G1.140		62.47	61.45
G24.130		58.48	60.93

Cheese_Type	Media	7H11++ PANTA	7H11++ PCAT
Caerphilly		56.52	58.65
Cheddar		51.00	50.51

	Cheese_Type	Caerphilly		Cheddar	
Bovis_Type	Media	7H11++ PANTA	7H11++ PCAT	7H11++ PANTA	7H11++ PCAT
AF2122		49.12	49.51	31.57	33.21
G1.140		56.41	60.45	68.52	62.44
G24.130		64.04	66.00	52.92	55.87

Appendix 5

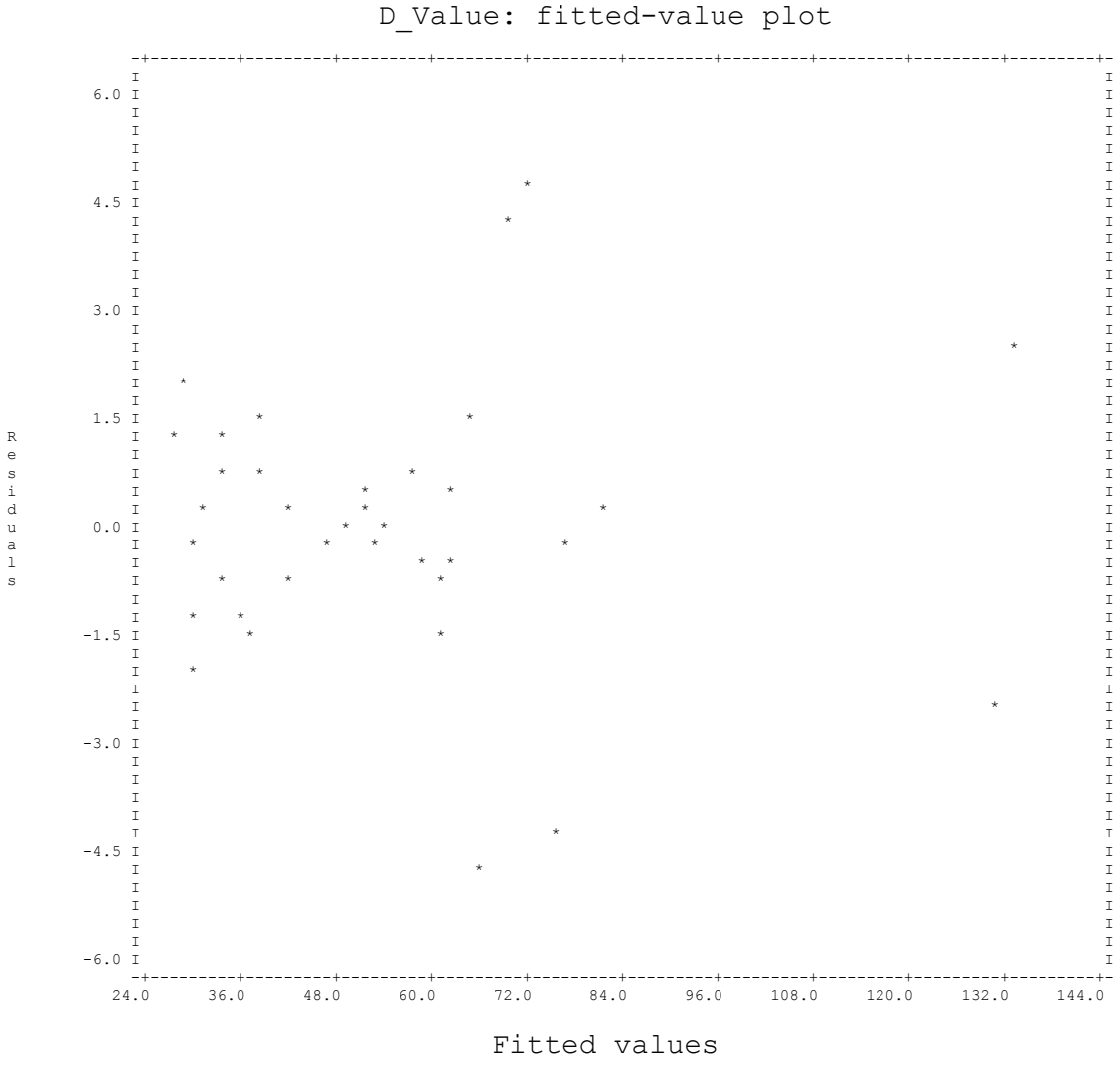
Standard errors of differences of means

Table	Bovis_Type	Cheese_Type	Media	Bovis_Type Cheese_Type	Bovis_Type Media	Cheese_Type Media	Bovis_Type Cheese_Type Media
rep.	12	18	18	6	6	9	3
s.e.d.	15.162	12.379	1.073	21.442	15.219	12.426	21.522
d.f.	12	12	12	12	12.18	12.18	12.18
Except when comparing means with the same level(s) of							
Bovis_Type					1.859		
d.f.					12		
Cheese_Type						1.518	
d.f.						12	
Bovis_Type.Cheese_Type							2.629
d.f.							12

Least significant differences of means (5% level)

Table	Bovis_Type	Cheese_Type	Media	Bovis_Type Cheese_Type	Bovis_Type Media	Cheese_Type Media	Bovis_Type Cheese_Type Media
rep.	12	18	18	6	6	9	3
l.s.d.	33.035	26.973	2.339	46.718	33.104	27.029	46.816
d.f.	12	12	12	12	12.18	12.18	12.18
Except when comparing means with the same level(s) of							
Bovis_Type					4.051		
d.f.					12		
Cheese_Type						3.307	
d.f.						12	
Bovis_Type.Cheese_Type							5.729
d.f.							12

Appendix 5


D_Value: fitted-value plot
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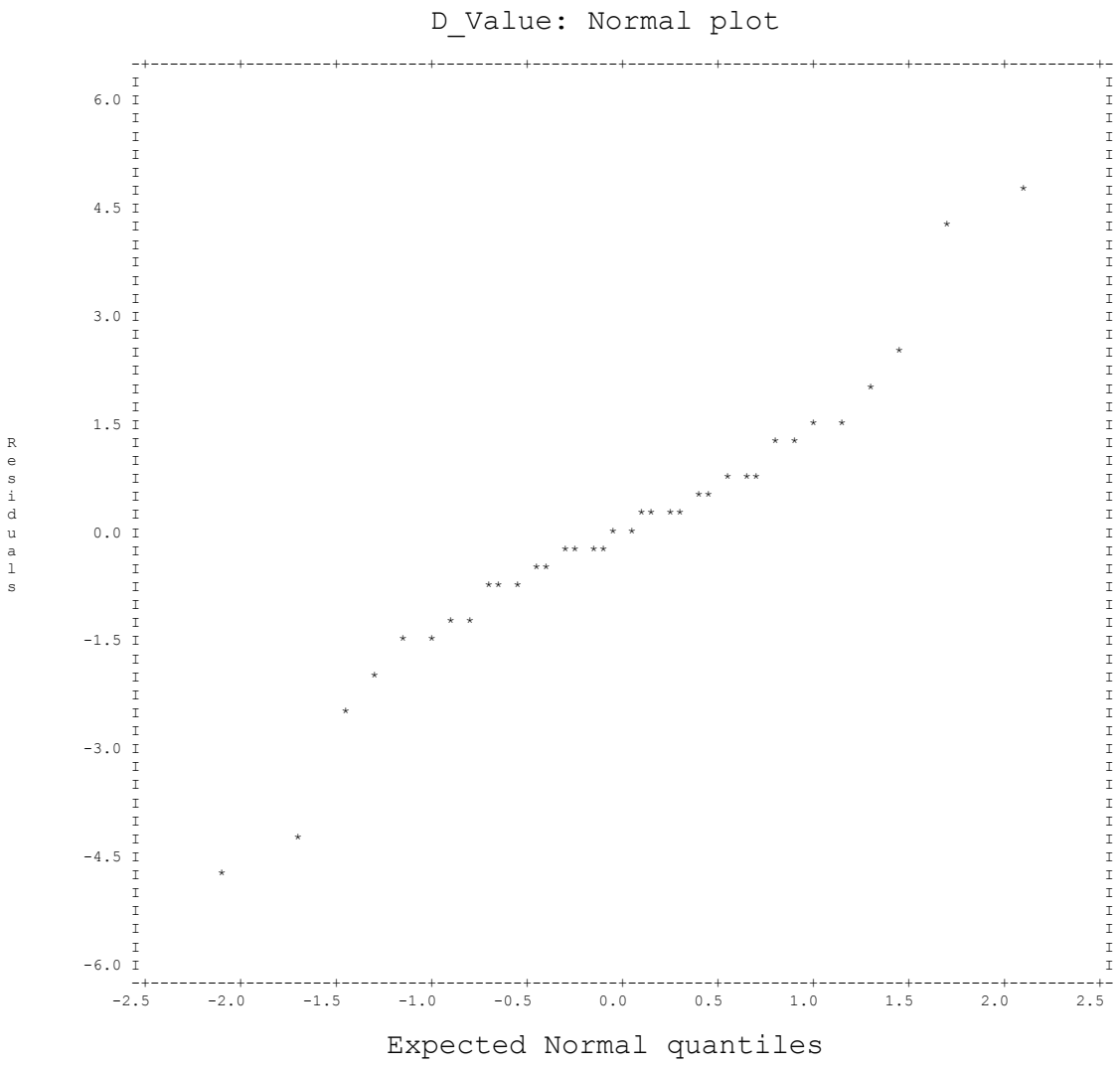
4.5
3.0
1.5
0.0
-1.5
-3.0
-4.5
-6.0
24.0
36.0
48.0
60.0
72.0
84.0
96.0
108.0
120.0
132.0
144.0
Fitted values

Appendix 5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Residuals
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4.5
3.0
1.5
0.0
-1.5
-3.0
-4.5
-6.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
Expected Normal quantiles

Analysis for Low Level

Analysis of variance
==========

Variate: D_Value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum					
Cheese_Type	1	243.290	243.290	2.97	0.160
Residual	4	327.328	81.832	21.62	
Block.*Units* stratum					
Media	1	40.837	40.837	10.79	0.030
Cheese_Type.Media	1	1.197	1.197	0.32	0.604
Residual	4	15.140	3.785		
Total	11	627.793			

Tables of means
==========

Variate: D_Value

Grand mean 25.98

Cheese_Type	Caerphilly	Cheddar
	21.48	30.49

Media	7H11++ PANTA	7H11++ PCAT
	24.14	27.83

Cheese_Type	Media	7H11++ PANTA	7H11++ PCAT
Caerphilly		19.95	23.01
Cheddar		28.33	32.65

Standard errors of differences of means

Table	Cheese_Type	Media	Cheese_Type Media
rep.	6	6	3
s.e.d.	5.223	1.123	5.342
d.f.	4	4	4.37
Except when comparing means with the same level(s) of Cheese_Type			1.588
d.f.			4

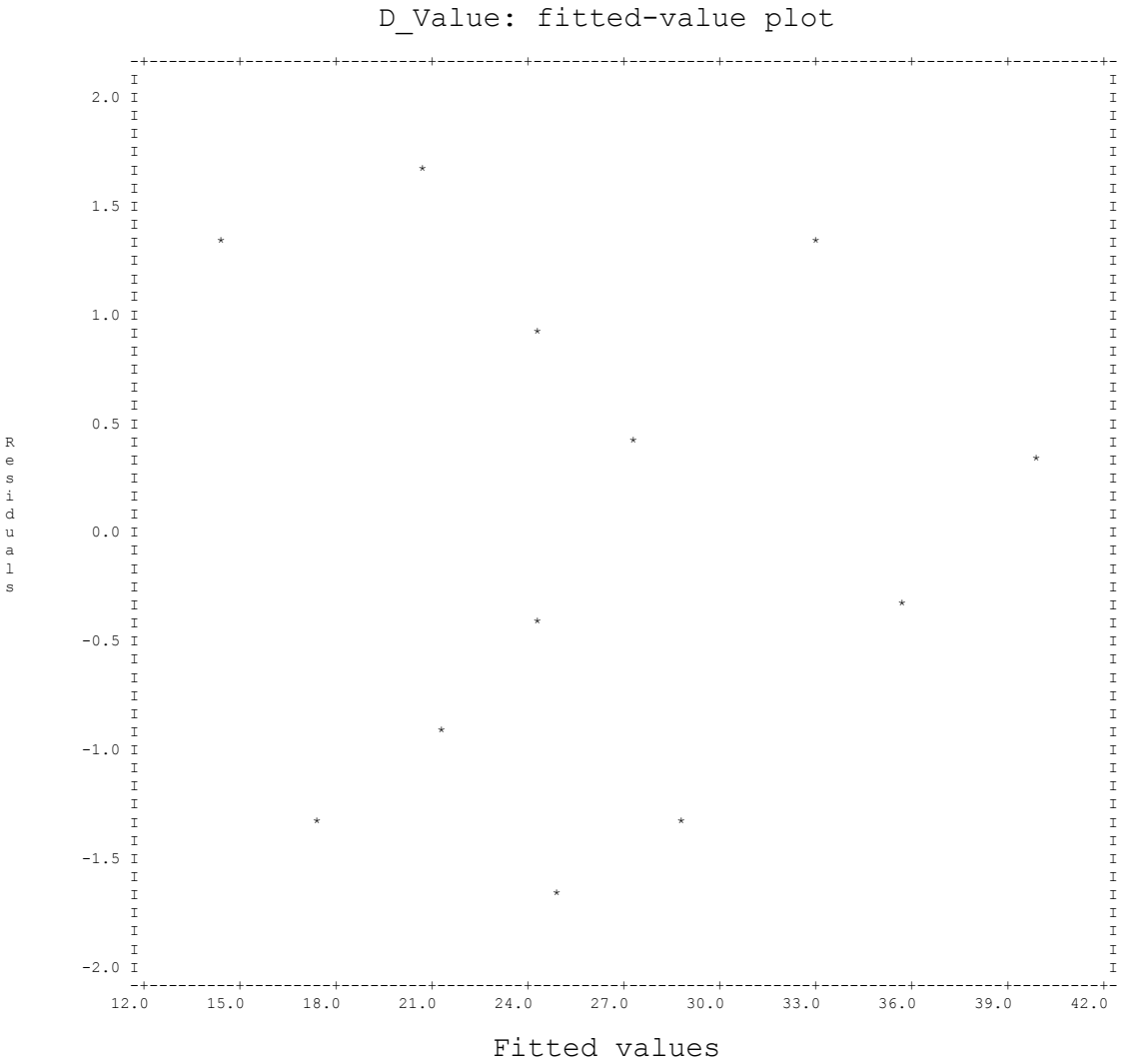
Appendix 5

Least significant differences of means (5% level)

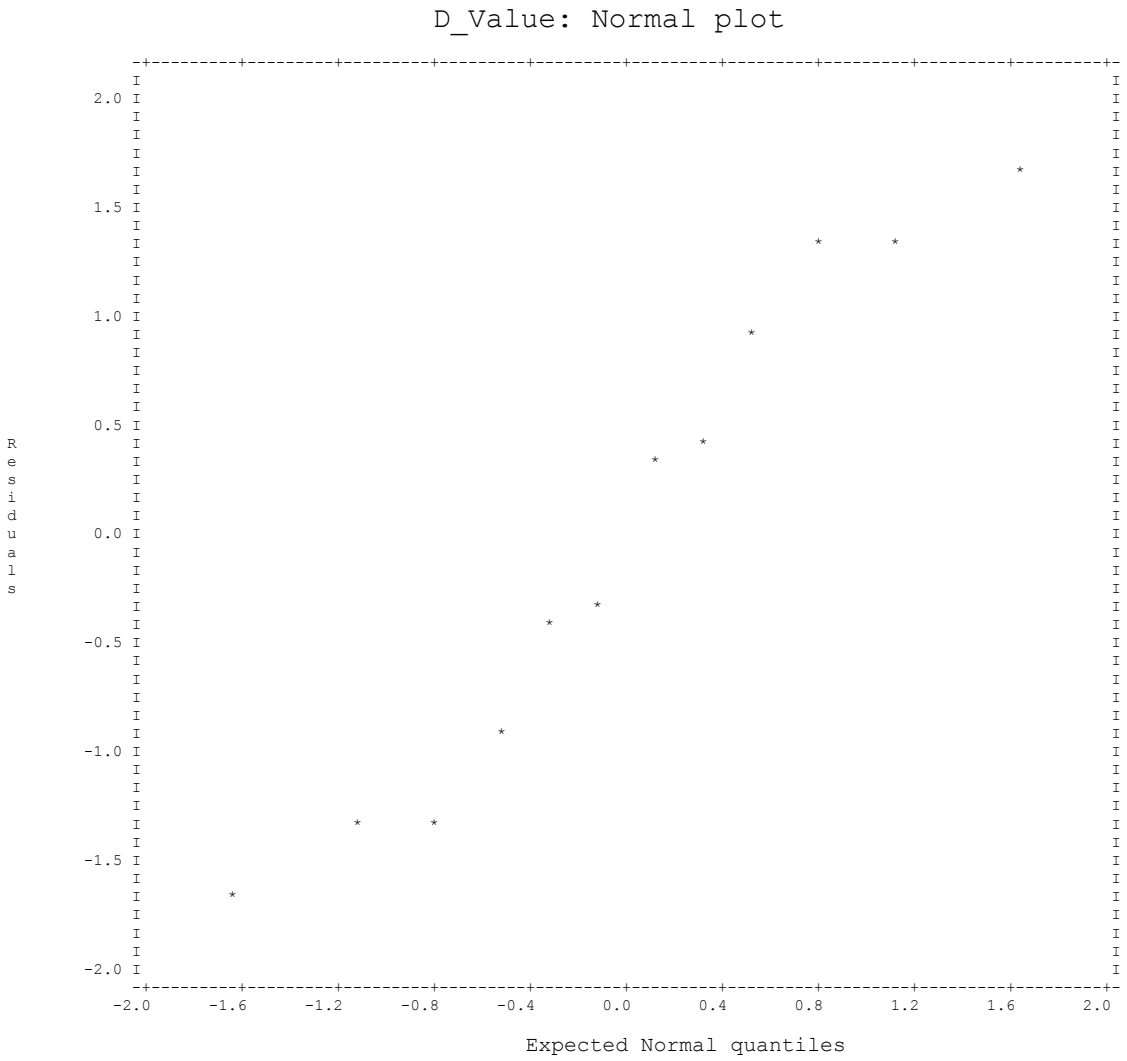
Table	Cheese_Type	Media	Cheese_Type Media
rep.	6	6	3
l.s.d.	14.500	3.118	14.351
d.f.	4	4	4.37

Except when comparing means with the same level(s) of

Cheese_Type	4.410
d.f.	4



Appendix 5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1.0
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0.0
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-1.0
-1.5
-2.0
-2.0
-1.6
-1.2
-0.8
-0.4
0.0
0.4
0.8
1.2
1.6
2.0
Expected Normal quantiles

Analysis for Both Levels

Analysis of variance
==========

Variate: D_Value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum					
Level	1	1326.544	1326.544	8.56	0.019
Cheese_Type	1	94.147	94.147	0.61	0.458
Level.Cheese_Type	1	1008.795	1008.795	6.51	0.034
Residual	8	1239.569	154.946	42.72	
Block.*Units* stratum					
Media	1	33.217	33.217	9.16	0.016
Level.Media	1	10.719	10.719	2.96	0.124
Cheese_Type.Media	1	2.381	2.381	0.66	0.441
Level.Cheese_Type.Media	1	0.000	0.000	0.00	0.998
Residual	8	29.015	3.627		
Total	23	3744.387			

* MESSAGE: the following units have large residuals.

Block AF2122 Caerphilly 3 High -15.56 approx. s.e. 7.19

Tables of means
==========

Variate: D_Value

Grand mean 33.42

Level	High	Low	4 Month Mat
	40.85	25.98	

Cheese_Type	Caerphilly	Cheddar
	35.40	31.44

Media	7H11++ PANTA	7H11++ PCAT
	32.24	34.60

Level	Cheese_Type	Caerphilly	Cheddar
High		49.32	32.39
Low		21.48	30.49

Level	Media	7H11++ PANTA	7H11++ PCAT
High		40.35	41.36
Low		24.14	27.83

Cheese_Type	Media	7H11++ PANTA	7H11++ PCAT
Caerphilly		34.54	36.26

Cheddar	29.95	32.93

Appendix 5

Cheese_Type		Caerphilly		Cheddar	
Level	Media	7H11++ PANTA	7H11++ PCAT	7H11++ PANTA	7H11++PCAT
High		49.12	49.51	31.57	33.21
Low		19.95	23.01	28.33	32.65

Standard errors of differences of means

Table	Level	Cheese_Type	Media	Level Cheese_Type	Level Media	Cheese_Type Media	Level Cheese_Type Media	rep.
12	12	12	6	6	6	3		
s.e.d.	5.082	5.082	0.777	7.187	5.141	5.141	7.270	
d.f.	8	8	8	8	8.37	8.37	8.37	

Except when comparing means with the same level(s) of

Level	1.100		
d.f.	8		
Cheese_Type		1.100	
d.f.		8	
Level.Cheese_Type			1.555
d.f.			8

Least significant differences of means (5% level)

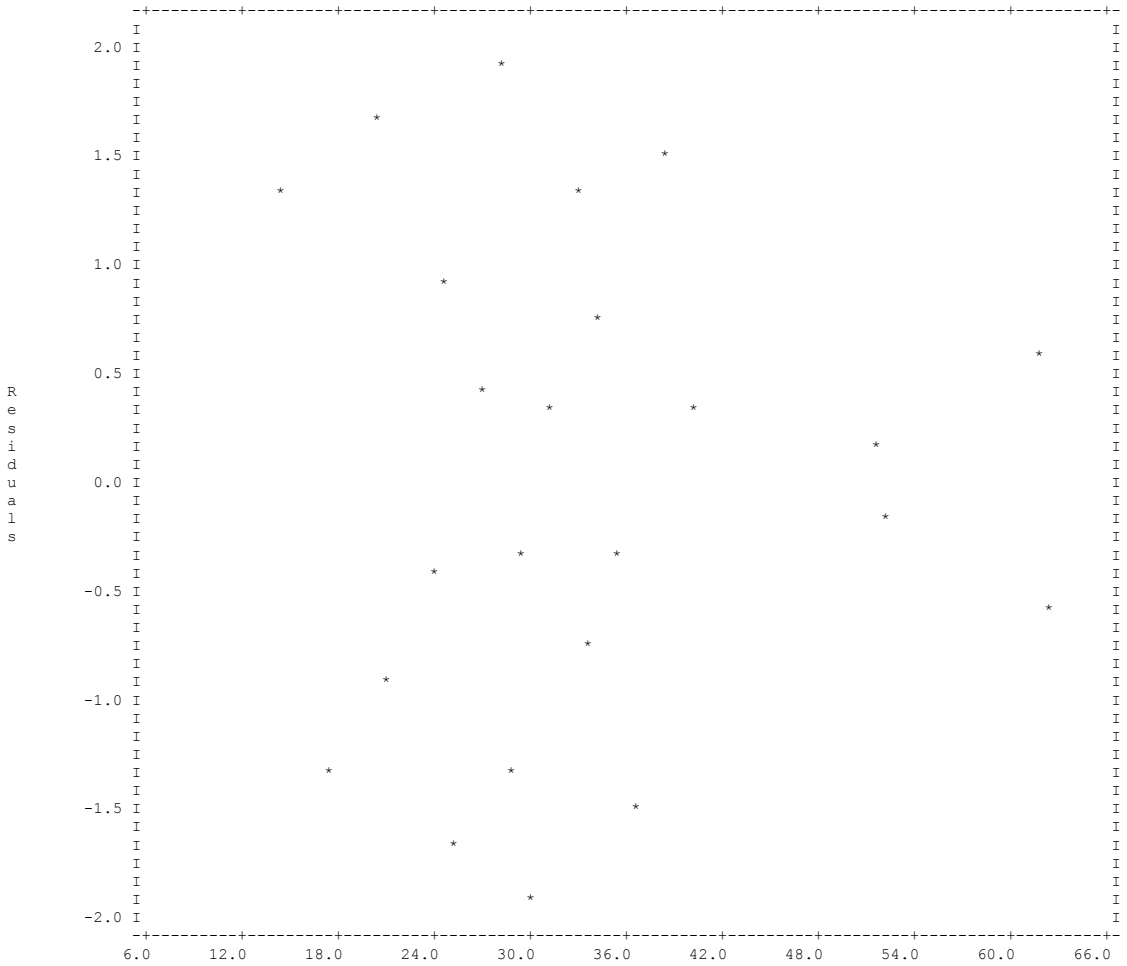
Table	Level	Cheese_Type	Media	Level Cheese_Type	Level Media	Cheese_Type Media	Level Cheese_Type Media	
rep.	12	12	12	6	6	6	3	
l.s.d.	11.719	11.719	1.793	16.573	11.763	11.763	6.636	
d.f.		8	8	8	8	8.37	8.37	
8.37								

Except when comparing means with the same level(s) of

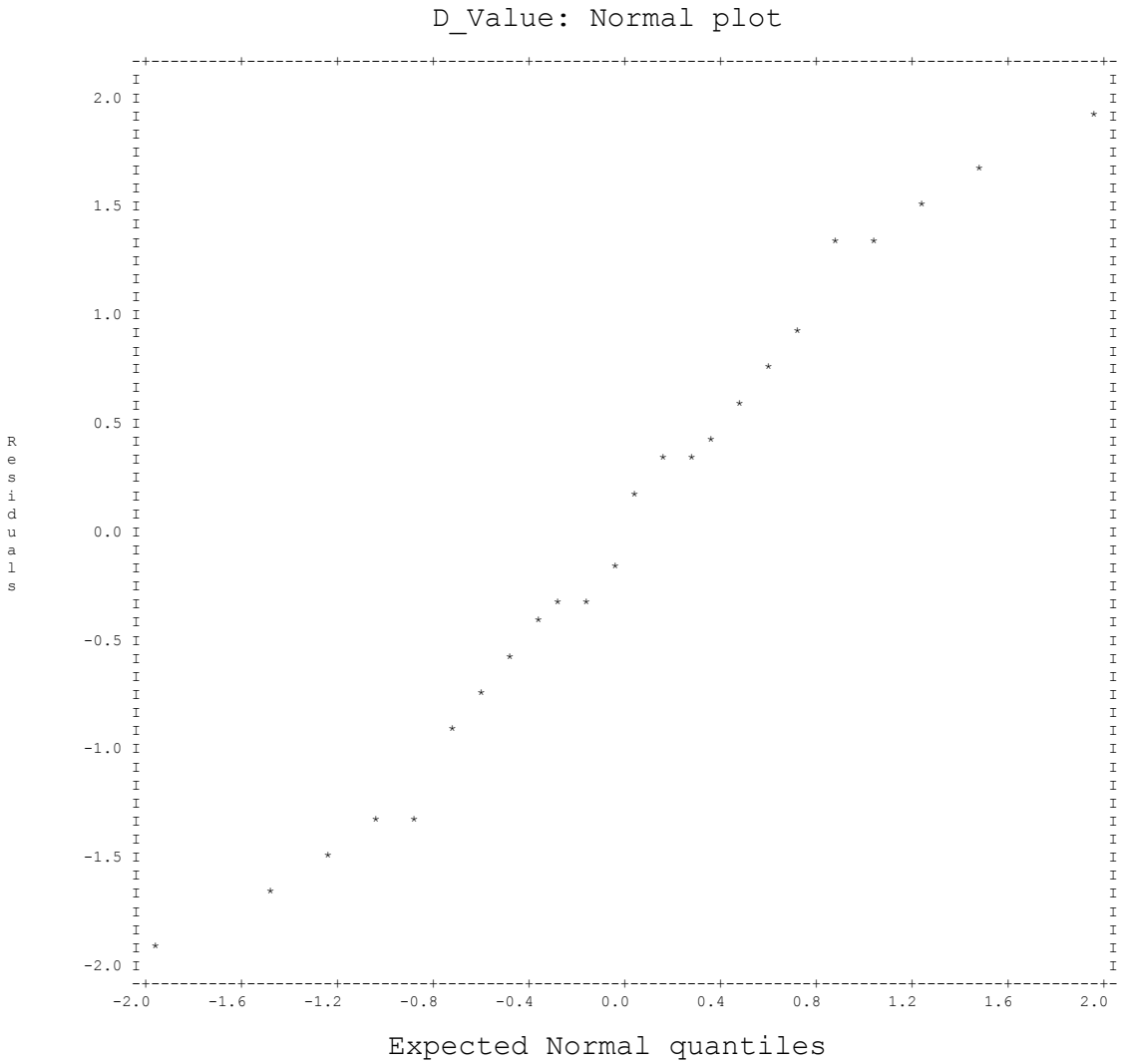
Level	2.535		
d.f.	8		
Cheese_Type		2.535	
d.f.		8	
Level.Cheese_Type			3.586 d.f.
8			

Appendix 5

D_Value: fitted-value plot



Appendix 5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-2.0
-1.6
-1.2
-0.8
-0.4
0.0
0.4
0.8
1.2
1.6
2.0
Expected Normal quantiles

Appendix 5

Analysis for High vs >4mths

Analysis of variance
==========

Variate: D_Value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block1 stratum					
Bovis_Type	2	4205.2	2102.6	0.27	0.774
Residual	6	47167.3	7861.2	525.40	
Block1.Media stratum					
Media	1	58.0	58.0	3.88	0.097
Media.Bovis_Type	2	12.8	6.4	0.43	0.670
Residual	6	89.8	15.0	0.08	
Block1.Media.*Units* stratum					
Level	1	184.7	184.7	0.94	0.352
Media.Level	1	1.5	1.5	0.01	0.932
Bovis_Type.Level	2	967.4	483.7	2.46	0.127
Media.Bovis_Type.Level	2	3.9	2.0	0.01	0.990
Residual	12	2359.0	196.6		
Total	35	55049.7			

* MESSAGE: the following units have large residuals.

Block1 G24.130 Caerphilly 1 85.77 approx. s.e. 36.20

Block1 G24.130 Caerphilly 1 Media 7H11++ PANTA -3.73 approx. s.e. 1.58
Block1 G24.130 Caerphilly 1 Media 7H11++ PCAT 3.73 approx. s.e. 1.58
Block1 G24.130 Caerphilly 1 Media 7H11++ PANTA *units* 1 -17.87 approx. s.e. 8.09
Block1 G24.130 Caerphilly 1 Media 7H11++ PANTA *units* 2 17.87 approx. s.e. 8.09
Block1 G24.130 Caerphilly 1 Media 7H11++ PCAT *units* 1 -20.36 approx. s.e. 8.09
Block1 G24.130 Caerphilly 1 Media 7H11++ PCAT *units* 2 20.36 approx. s.e. 8.09

Appendix 5

Tables of means
==========

Variate: D_Value

Grand mean 59.85

Media	7H11++ PANTA	7H11++ PCAT
	58.58	61.12

Bovis_Type	AF2122	G1.140	G24.130
	48.84	56.18	74.54

Level	High	Low 4 Month Mat
	57.59	62.12

Media	Bovis_Type	AF2122	G1.140	G24.130
7H11++ PANTA		48.41	54.37	72.97
7H11++ PCAT		49.28	57.98	76.11

Media	Level	High	Low 4 Month Mat
7H11++ PANTA		56.52	60.64
7H11++ PCAT		58.65	63.59

Bovis_Type	Level	High	Low 4 Month Mat
AF2122		49.32	48.37
G1.140		58.43	53.92
G24.130		65.02	84.06

Media	Bovis_Type	Level	High	Low 4 Month Mat
7H11++ PANTA	AF2122		49.12	47.69
	G1.140		56.41	52.34
	G24.130		64.04	81.91
7H11++ PCAT	AF2122		49.51	49.05
	G1.140		60.45	55.51
	G24.130		66.00	86.22

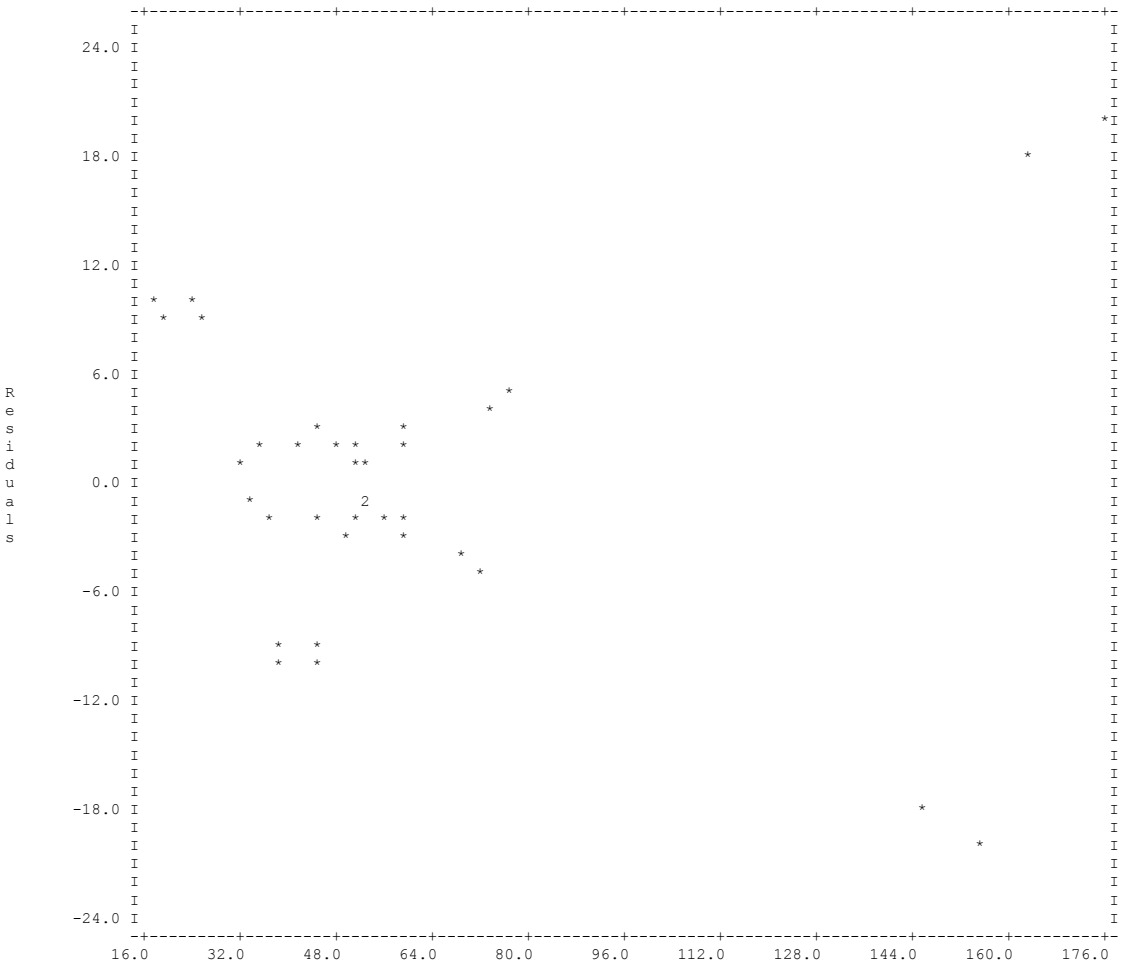
Standard errors of differences of means

Table	Media	Bovis_Type	Level	Media Bovis_Type	Media Level	Bovis_Type Level	Media Bovis_Type Level
rep.	18	12	18	6	9	6	3
s.e.d.	1.289	36.197	4.674	36.231	4.848	36.647	37.124
d.f.	6	6	12	6.02	13.74	6.30	6.63
Except when comparing means with the same level(s) of							
Media					6.610		
d.f.					12		
Bovis_Type				2.233		8.095	8.397
d.f.				6		12	13.74
Media.Bovis_Type							11.448
d.f.							12
Bovis_Type.Level							8.397
d.f.							13.74

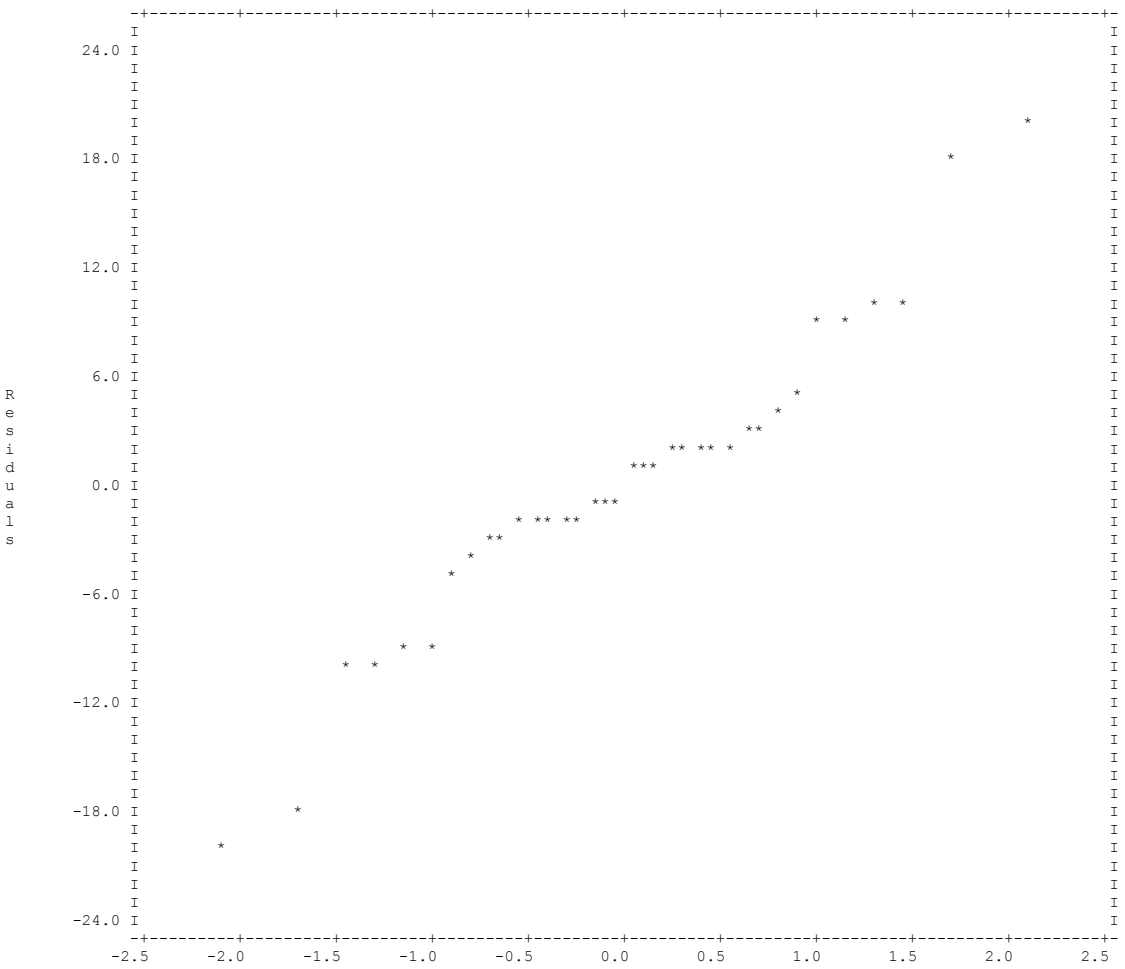
Least significant differences of means (5% level)

Table	Media	Bovis_Type	Level	Media Bovis_Type	Media Level	Bovis_Type Level	Media Bovis_Type Level
rep.	18	12	18	6	9	6	3
l.s.d.	3.155	88.570	10.183	88.573	10.417	88.640	88.786
d.f.	6	6	12	6.02	13.74	6.30	6.63
Except when comparing means with the same level(s) of							
Media					14.401		
d.f.					12		
Bovis_Type				5.465		17.637	18.043
d.f.				6		12	13.74
Media.Bovis_Type							24.943
d.f.							12
Bovis_Type.Level							18.043
d.f.							13.74

D_Value: fitted-value plot



D_Value: Normal plot

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line 81.

GenStat Release 14.2  (PC/Windows 7)              25 October 2012
14:07:52
Copyright 2011, VSN International Ltd.
Registered to: DARDNI

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JUST=L
  19    & 'Cost code:','44187'; FIELD=39; JUST=L
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Received from:                          Michael Rowe
Cost code:                              44187
Statistical Analysis number:            S20120324
Folder:
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Milk Cheese (44187)
Data File(s):                           Michael Rowe 120702 M bovis
High level Stats Data.xlsx
Output File:                            Michael Rowe 120702 M bovis
High level Stats Data.txt
Output files created by Clare Flanagan on: 06/07/12
Title:                                  M Bovis in Raw Milk Cheese
Analysis

  28
  29  IMPORT InFile; SHEET='Cheddar'; ISAVE=Data

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Catalogue of file
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Sheet Title: Sheet1
Description: Data read from Michael Rowe 120702 M bovis High level
Stats Data.xlsx [Cheddar]A2:Q319
Sheet Type: vector
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      1            text     318   Bovis_Type
      2            text     318   Cheese_Ref
      3            text     318   Media
      4            text     318   Cheese_Type
      5         variate     318   Cheese_Rep
      6         variate     318   Day
      7         variate     318   Log_cfu_g_1

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ISAVE=Data1
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Statistical analysis of high- and low-inoculum Cheddar and Caerphilly: Fisher's LSD test.

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  16    PAGE
  17    SKIP [FILE=OUT] 2
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Received from:                          Michael Rowe/Richard Forgrave
Cost code:                              44187
Statistical Analysis number:            S20120592
Folder:
P:/Biometrics/Analysis/AlanG/Alan/Micro/Michael Rowe/M Bovis in Raw
Milk Cheese (44187)
Data File(s): Michael Rowe 121024 M bovis stats data-DMfit.xlsx
Output File:  Michael Rowe 121024 M bovis stats data-DMfit v3.txt
Output files created by Alan Gordon:    27/11/12
Title: M Bovis in Raw Milk Cheese Analysis

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      3            text      36   Cheese_Type
      4        variate       36   Cheese_Rep
      5        variate       36   Slope
      6        variate       36   D_Value

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      3            text      12   C3
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      5         variate      12   C5
      6         variate      12   C6

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Loading Spreadsheet File
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Catalogue of file
C:\USERS\1006315\APPDATA\LOCAL\TEMP\GENSTAT\G142D21E.GWB

Sheet Title: Sheet1
Description: Data read from Michael Rowe 121024 M bovis stats data-
DMfit.xlsx [Hi lev C'philly 4 Month Mat   ]A3:F20
Sheet Type: vector
  Index            Type    Nval   Name
      1            text      18   D_1
      2            text      18   D_2
      3            text      18   D_3
      4         variate      18   D_4
      5         variate      18   D_5
      6         variate      18   D_6

  32  FOR DUM1=Data[]; DUM2=Data1[]; DUM3=Data2[]
  33    APPEND DUM1,DUM2,DUM3
  34  ENDFOR
  35  GROUP [RED=Y] Bovis_Type,Media,Cheese_Type,Cheese_Rep
  36  FACT [LAB=!T(High,Low,'4 Month Mat'); VAL=36(1),12(2),18(3)]
Level
  37  FACPRODUCT !P(Bovis_Type,Cheese_Type,Cheese_Rep,Level);
PROD=Block
  38  & !P(Bovis_Type,Cheese_Type,Cheese_Rep); PROD=Block1
  39
  40  FOR
  41
  42  "  PAGE
 -43    PRIN
Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Value;
DEC=5(0),4,2"
  44
  45    FOR I1=1...4; I2='High','Low','Both','High vs >4mths'
```

```
  46
  47      IF I1.LE.2
  48        CONC [NEW=TIT] OLD='Analysis for ',I2,' Level'
  49        REST
Block,Block1,Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Valu
e; Level.IN.I2
  50        BLOC Block
  51        IF I1.EQ.1
  52          TREA Bovis_Type*Cheese_Type*Media
  53        ELSE
  54          TREA Cheese_Type*Media
  55        ENDIF
  56      ELSIF I1.EQ.3
  57        CONC [NEW=TIT] OLD='Analysis for ',I2,' Levels'
  58        REST
Block,Block1,Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Valu
e;\
  59         (Bovis_Type.IN.'AF2122').AND.(Level.NI.'4 Month Mat')
  60        BLOC Block
  61        TREA Level*Cheese_Type*Media
  62      ELSE
  63        CONC [NEW=TIT] OLD='Analysis for ',I2
  64        REST
Block,Block1,Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D_Valu
e;\
  65         (Cheese_Type.IN.'Caerphilly').AND.(Level.NI.'Low')
  66        BLOC Block1/Media
  67        TREA Bovis_Type*Media*Level
  68      ENDIF
  69
  70      PAGE
  71      PRIN [IPR=*; SQ=Y] TIT
  72      ANOV [FPROB=Y; PSE=DIFF,LSD] D_Value
  73      IF I1.LE.2
  74        IF I1.EQ.1
  75          AMCOMPARISON [PR=LET; METH=FPLSD]
Bovis_Type*Cheese_Type*Media
  76        ELSE
  77          AMCOMPARISON [PR=LET; METH=FPLSD] Cheese_Type*Media
  78        ENDIF
  79      ELSIF I1.EQ.3
  80        AMCOMPARISON [PR=LET; METH=FPLSD] Level*Cheese_Type*Media
  81      ELSE
  82        AMCOMPARISON [PR=LET; METH=FPLSD] Bovis_Type*Media*Level
  83      ENDIF
  84  "    APLOT [GRAPH=L] FIT,NORM"
  85
  86      REST
Block,Block1,Bovis_Type,Media,Cheese_Type,Cheese_Rep,Level,Slope,D
Value
  87      DELE [RED=Y] TIT
  88
  89    ENDFOR
  90
```

Analysis for High Level

Analysis of variance
==========

Variate: D_Value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum					
Bovis_Type	2	3223.02	1611.51	1.17	0.344
Cheese_Type	1	420.30	420.30	0.30	0.591
Bovis_Type.Cheese_Type	2	926.88	463.44	0.34	0.721
Residual	12	16551.18	1379.26	133.02	
Block.*Units* stratum					
Media	1	6.02	6.02	0.58	0.461
Bovis_Type.Media	2	18.32	9.16	0.88	0.439
Cheese_Type.Media	1	15.50	15.50	1.49	0.245
Bovis_Type.Cheese_Type.Media	2	63.39	31.70	3.06	0.085
Residual	12	124.43	10.37		
Total	35	21349.04			

* MESSAGE: the following units have large residuals.

Block G24.130 Caerphilly 1 High 66.65 approx. s.e. 21.44

Block G1.140 Cheddar 1 High *units* 1 4.82 approx. s.e. 1.86
Block G1.140 Cheddar 1 High *units* 2 -4.82 approx. s.e. 1.86
Block G1.140 Cheddar 2 High *units* 1 -4.37 approx. s.e. 1.86
Block G1.140 Cheddar 2 High *units* 2 4.37 approx. s.e. 1.86

Tables of means
==========

Variate: D_Value

Grand mean 54.17

Bovis_Type	AF2122	G1.140	G24.130
	40.85	61.96	59.71

Cheese_Type	Caerphilly	Cheddar
	57.59	50.75

Media	7H11++ PANTA	7H11++ PCAT
	53.76	54.58

Bovis_Type	Cheese_Type	Caerphilly	Cheddar
AF2122		49.32	32.39
G1.140		58.43	65.48
G24.130		65.02	54.39

Bovis_Type	Media 7H11++ PANTA	7H11++ PCAT
AF2122	40.35	41.36
G1.140	62.47	61.45
G24.130	58.48	60.93

Cheese_Type	Media 7H11++ PANTA	7H11++ PCAT
Caerphilly	56.52	58.65
Cheddar	51.00	50.51

Cheese_Type	Caerphilly		Cheddar		Bovis_Type
Media 7H11++ PANTA	7H11++ PCAT	7H11++ PANTA7	H11++ PCAT		
AF2122	49.12	49.51	31.57	33.21	
G1.140	56.41	60.45	68.52	62.44	
G24.130	64.04	66.00	52.92	55.87	

Standard errors of differences of means

Table	Bovis_Type	Cheese_Type	Media	Bovis_Type Cheese_Type	Bovis_Type Media	Cheese_Type Media	Bovis_Type Cheese_Type Media	rep.
12	18	18	6	6	9	3		
s.e.d.	15.162	12.379	1.073	21.442	15.219	12.426	21.522	
d.f.	12	12	12	12	12.18	12.18	12.18	
Except when comparing means with the same level(s) of								
Bovis_Type					1.859			
d.f.					12			
Cheese_Type						1.518		
d.f.						12		
Bovis_Type.Cheese_Type							2.629	
d.f.							12	

Least significant differences of means (5% level)

Table	Bovis_Type	Cheese_Type	Media	Bovis_Type Cheese_Type	Bovis_Type Media	Cheese_Type Media	Bovis_Type Cheese_Type Media
rep.	12	18	18	6	6	9	3
l.s.d.	33.035	26.973	2.339	46.718	33.104	27.029	46.816
d.f.	12	12	12	12	12.18	12.18	12.18
Except when comparing means with the same level(s) of							
Bovis_Type					4.051		
d.f.					12		
Cheese_Type						3.307	
d.f.						12	
Bovis_Type.Cheese_Type							5.729
d.f.							12

```
Fisher's protected least significant difference test
===================================================

Bovis_Type
----------
******** Warning 289, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type is not significant.

Cheese_Type
-----------
******** Warning 290, code UF 2, statement 145 in procedure
AMCOMPARISON

The number of MEANS must be greater than 2.

Media
-----
******** Warning 291, code UF 2, statement 145 in procedure
AMCOMPARISON
The number of MEANS must be greater than 2.

Bovis_Type.Cheese_Type
----------------------
******** Warning 292, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type.Cheese_Type is not significant.

Bovis_Type.Media
----------------
******** Warning 293, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type.Media is not significant.

Cheese_Type.Media
-----------------
******** Warning 294, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Cheese_Type.Media is not significant.

Bovis_Type.Cheese_Type.Media
----------------------------
******** Warning 295, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type.Cheese_Type.Media is not significant.
```

Analysis for Low Level

Analysis of variance
==========

Variate: D_Value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum					
Cheese_Type	1	243.290	243.290	2.97	0.160
Residual	4	327.328	81.832	21.62	
Block.*Units* stratum					
Media	1	40.837	40.837	10.79	0.030
Cheese_Type.Media	1	1.197	1.197	0.32	0.604
Residual	4	15.140	3.785		
Total	11	627.793			

Tables of means
==========

Variate: D_Value

Grand mean 25.98

Cheese_Type	Caerphilly	Cheddar
	21.48	30.49

Media	7H11++ PANTA	7H11++ PCAT
	24.14	27.83

Cheese_Type	Media	7H11++ PANTA	7H11++ PCAT
Caerphilly		19.95	23.01
Cheddar		28.33	32.65

Standard errors of differences of means

Table	Cheese_Type	Media	Cheese_Type Media
rep.	6	6	3
s.e.d.	5.223	1.123	5.342
d.f.	4	4	4.37
Except when comparing means with the same level(s) of			
Cheese_Type			1.588
d.f.			4

Appendix 6

```
Least significant differences of means (5% level)
-------------------------------------------------

Table          Cheese_Type       Media Cheese_Type
                                               Media
rep.                     6           6           3
l.s.d.              14.500       3.118      14.351
d.f.                     4           4        4.37
Except when comparing means with the same level(s) of
Cheese_Type                                   4.410
d.f.                                              4


Fisher's protected least significant difference test
====================================================


Cheese_Type
-----------
******** Warning 296, code UF 2, statement 145 in procedure
AMCOMPARISON

The number of MEANS must be greater than 2.

Media
-----
******** Warning 297, code UF 2, statement 145 in procedure
AMCOMPARISON

The number of MEANS must be greater than 2.

Cheese_Type.Media
-----------------
******** Warning 298, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Cheese_Type.Media is not significant.

Analysis for Both Levels
```

Appendix 6

Analysis of variance
==========

Variate: D_Value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum					
Level	1	1326.544	1326.544	8.56	0.019
Cheese_Type	1	94.147	94.147	0.61	0.458
Level.Cheese_Type	1	1008.795	1008.795	6.51	0.034
Residual	8	1239.569	154.946	42.72	
Block.*Units* stratum					
Media	1	33.217	33.217	9.16	0.016
Level.Media	1	10.719	10.719	2.96	0.124
Cheese_Type.Media	1	2.381	2.381	0.66	0.441
Level.Cheese_Type.Media	1	0.000	0.000	0.00	0.998
Residual	8	29.015	3.627		
Total	23	3744.387			

* MESSAGE: the following units have large residuals.

Block AF2122 Caerphilly 3 High -15.56 approx. s.e. 7.19

Tables of means
==========

Variate: D_Value

Grand mean 33.42

Level	High	Low 4 Month Mat
	40.85	25.98

Cheese_Type	Caerphilly	Cheddar
	35.40	31.44

Media	7H11++ PANTA	7H11++ PCAT
	32.24	34.60

Level	Cheese_Type	Caerphilly	Cheddar
High		49.32	32.39
Low		21.48	30.49

Level	Media	7H11++ PANTA	7H11++ PCAT
High		40.35	41.36
Low		24.14	27.83

Cheese_Type	Media	7H11++ PANTA	7H11++ PCAT
Caerphilly		34.54	36.26
Cheddar		29.95	32.93

Cheese_Type		Caerphilly		Cheddar	
Level	Media	7H11++ PANTA	7H11++ PCAT	7H11++ PANTA	7H11++ PCAT
High		49.12	49.51	31.57	33.21
Low		19.95	23.01	28.33	32.65

Standard errors of differences of means

Table	Level	Cheese_Type	Media	Level Cheese_Type	Level Media	Cheese_Type Media	Level Cheese_Type Media
rep.	12	12	12	6	6	6	3
s.e.d.	5.082	5.082	0.777	7.187	5.141	5.141	7.270
d.f.	8	8	8	8	8.37	8.37	8.37
Except when comparing means with the same level(s) of							
Level					1.100		
d.f.					8		
Cheese_Type						1.100	
d.f.						8	
Level.Cheese_Type							1.555
d.f.							8

Least significant differences of means (5% level)

Table	Level	Cheese_Type	Media	Level Cheese_Type	Level Media	Cheese_Type Media	Level Cheese_Type Media
rep.	12	12	12	6	6	6	3
l.s.d.	11.719	11.719	1.793	16.573	11.763	11.763	16.636
d.f.	8	8	8	8	8.37	8.37	8.37
Except when comparing means with the same level(s) of							
Level					2.535		
d.f.					8		
Cheese_Type						2.535	
d.f.						8	
Level.Cheese_Type							3.586
d.f.							8

Fisher's protected least significant difference test

Level

******** Warning 299, code UF 2, statement 145 in procedure AMCOMPARISON

The number of MEANS must be greater than 2.

Cheese_Type

******** Warning 300, code UF 2, statement 145 in procedure AMCOMPARISON

The number of MEANS must be greater than 2.

```
Media
-----
******** Warning 301, code UF 2, statement 145 in procedure
AMCOMPARISON

The number of MEANS must be greater than 2.


Level.Cheese_Type
-----------------
                       Mean
 Low Caerphilly       21.48 a
    Low Cheddar       30.49 a
   High Cheddar       32.39 a
High Caerphilly       49.32 b


Level.Media
-----------
******** Warning 302, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Level.Media is not significant.


Cheese_Type.Media
-----------------
******** Warning 303, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Cheese_Type.Media is not significant.

Level.Cheese_Type.Media
-----------------------
******** Warning 304, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Level.Cheese_Type.Media is not significant.
```

Analysis for High vs >4mths

Analysis of variance
==========

Variate: D_Value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block1 stratum					
Bovis_Type	2	4205.2	2102.6	0.27	0.774
Residual	6	47167.3	7861.2	525.40	
Block1.Media stratum					
Media	1	58.0	58.0	3.88	0.097
Media.Bovis_Type	2	12.8	6.4	0.43	0.670
Residual	6	89.8	15.0	0.08	
Block1.Media.*Units* stratum					
Level	1	184.7	184.7	0.94	0.352
Media.Level	1	1.5	1.5	0.01	0.932
Bovis_Type.Level	2	967.4	483.7	2.46	0.127
Media.Bovis_Type.Level	2	3.9	2.0	0.01	0.990
Residual	12	2359.0	196.6		
Total	35	55049.7			

* MESSAGE: the following units have large residuals.

Block1 G24.130 Caerphilly 1 85.77   approx. s.e. 36.20

Block1 G24.130 Caerphilly 1 Media 7H11++ PANTA -3.73 approx. s.e. 1.58
Block1 G24.130 Caerphilly 1 Media 7H11++ PCAT   3.73 approx. s.e. 1.58
Block1 G24.130 Caerphilly 1 Media 7H11++ PANTA *units* 1   -17.87   approx. s.e. 8.09
Block1 G24.130 Caerphilly 1 Media 7H11++ PANTA *units* 2   17.87   approx. s.e. 8.09
Block1 G24.130 Caerphilly 1 Media 7H11++ PCAT   *units* 1   -20.36   approx. s.e. 8.09
Block1 G24.130 Caerphilly 1 Media 7H11++ PCAT   *units* 2   20.36   approx. s.e. 8.09

Tables of means
==========

Variate: D_Value

Grand mean 59.85

Media	7H11++ PANTA	7H11++ PCAT
	58.58	61.12

Bovis_Type	AF2122	G1.140	G24.130
	48.84	56.18	74.54

Level	High	Low 4 Month Mat
	57.59	62.12

Media	Bovis_Type	AF2122	G1.140	G24.130
7H11++	PANTA	48.41	54.37	72.97
7H11++	PCAT	49.28	57.98	76.11

Media	Level	High	Low 4 Month Mat
7H11++	PANTA	56.52	60.64
7H11++	PCAT	58.65	63.59

Bovis_Type	Level	High	Low 4 Month Mat
AF2122		49.32	48.37
G1.140		58.43	53.92
G24.130		65.02	84.06

Media	Bovis_Type	Level	High	Low 4 Month Mat
7H11++ PANTA	AF2122		49.12	47.69
	G1.140		56.41	52.34
	G24.130		64.04	81.91
7H11++ PCAT	AF2122		49.51	49.05
	G1.140		60.45	55.51
	G24.130		66.00	86.22

Standard errors of differences of means

Table	Media	Bovis_Type	Level	Media Bovis_Type	Media Level	Bovis_Type Level	Media Bovis_Type Level
rep.	18	12	18	6	9	6	3
s.e.d.	1.289	36.197	4.674	36.231	4.848	36.647	37.124
d.f.	6	6	12	6.02	13.74	6.30	6.63
Except when comparing means with the same level(s) of							
Media				6.610			
d.f.				12			
Bovis_Type			2.233		8.095	8.397	
d.f.			6		12	13.74	
Media.Bovis_Type							11.448 d.f. 12
Bovis_Type.Level							8.397
d.f.							13.74

Least significant differences of means (5% level)

Table	Media	Bovis_Type	Level	Media Bovis_Type	Media Level	Bovis_Type Level	Media Bovis_Type Level
rep.	18	12	18	6	9	6	3
l.s.d.	3.155	88.570	10.183	88.573	10.417	88.640	88.786
d.f.	6	6	12	6.02	13.74	6.30	6.63
Except when comparing means with the same level(s) of							
Media					14.401		
d.f.					12		
Bovis_Type				5.465		17.637	18.043
d.f.				6		12	13.74
Media.Bovis_Type							24.943
d.f.							12
Bovis_Type.Level							18.043
d.f.							13.74

```
Fisher's protected least significant difference test
===================================================


Bovis_Type
----------
******** Warning 305, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type is not significant.

Media
-----
******** Warning 306, code UF 2, statement 145 in procedure
AMCOMPARISON

The number of MEANS must be greater than 2.

Level
-----
******** Warning 307, code UF 2, statement 145 in procedure
AMCOMPARISON

The number of MEANS must be greater than 2.

Bovis_Type.Media
----------------
******** Warning 308, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type.Media is not significant.


Bovis_Type.Level
----------------
******** Warning 309, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type.Level is not significant.


Media.Level
-----------
******** Warning 310, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Media.Level is not significant.
```

```
Bovis_Type.Media.Level
----------------------
******** Warning 311, code UF 2, statement 159 in procedure
AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for
Bovis_Type.Media.Level is not significant.

  93  ENDJOB

********* End of job. Current data space: 15 blocks, peak usage 4% at
line 92.

GenStat Release 14.2  (PC/Windows 7)             27 November 2012
15:12:59
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  94  STOP
```

Appendix 7.1

Poster: European Society of Mycobacteriology summer conference ESM 2008.

Investigations into the survival of *Mycobacterium bovis* manufacture and maturation of cheese produced from unpasteurised milk.

Richard Forgrave[1], John Donaghy[2], Michael Rowe[1,2]

[1]School of Biological Sciences, Queen's University Belfast, Newforge Lane, Belfast, N. Ireland, BT9 5PX. [2] Food Microbiology, Agri-Food and Biosciences Institute, Newforge Lane, Belfast, BT9 5PX, N. Ireland.

Purpose of the Study

Epidemiological investigations have attributed recent reports of culture-positive tuberculosis in the US to the consumption of *M bovis* contaminated unpasteurized cheese imports from Mexico[1]. These cases from the US suggest a link between *M bovis* contaminated dairy produce and human infections. The possibility of *M bovis* infection from consumption of UK unpasteurised cheese is unclear and may be compounded by the increasing prevalence of tuberculosis amongst UK cattle and growing demand for artisanal cheese produced from unpasteurised milk.

In order to diminish this risk producers in regions of the UK where the sale of unpasteurised milk products is permitted must operate under additional regulatory criteria. These require that the producers be registered, hold a tuberculosis free status, submit to more regular inspections and label the product with a health warning. Furthermore it has been hypothesised that the cheese manufacturing process and the presence of cheese microflora may reduce the level of *M bovis* contamination should there be a failure in the other regulatory mechanisms. The impact of these additional factors on the survival kinetics of *M bovis* during the manufacture, ripening and storage of unpasteurised milk cheeses is undefined. This investigation seeks to characterise the effect of the cheese making process and unpasteurised milk and cheese microflora on the survival of *M bovis*.

Methodology

Cheese Manufacture: Two hard cheese types have been selected:- Cheddar which has the greatest production volume of UK unpasteurised cheeses and Caerphilly which has a shorter maturation period from 1 to 10 weeks . Laboratory scale cheese production will be conducted according to a protocol described previously[2]. Modifications have been made to the procedure and equipment to minimize the production of aerosols during whey run off (Fig. 1). A high inoculum level will be used to allow investigation of the rate of *M. bovis* decline during cheese maturation and a low level of inoculum should provide a better representation of natural *M. bovis* contamination in milk.

Preliminary Investigation of Mycobacteria Media: The isolation of *M. bovis* from unpasteurised milk and cheeses may be impeded by the overgrowth of indigenous unpasteurised milk microflora. This could potentially obscure positive results or cause an underestimation of *M. bovis* numbers in milk or cheese samples. Preliminary evaluation of Mycobacteria media and antimicrobial supplement combinations was conducted to allow future development of media for isolation of *M. bovis* in the presence of other microorganisms. Prepared samples of direct vat starter culture (CHR Hansen), unpasteurised milk and cheese (Fig. 2) were inoculated onto antibiotic free Lowenstein & Jensen media (BD) and Stonebrinks media[3]. Further samples were inoculated onto oleic acid-albumin agars Middlebrook 7H10 and 7H11 (BD) and Gallaghers media[4]. Samples were tested against antibiotic free agar based media and agar based media containing the antimicrobial combinations described in Table 1. All samples were incubated aerobically at 37° C for 14 days.

Results

Table 1: Antimicrobial combinations used with agar based media.

Antimicrobial Combination	Antimicrobial	Concentration *
PANTA	Polymyxin B	(10)
	Amphotericin B	1
	Nalidixic Acid	4
	Trimethroprim	1
	Azlocillin	1
VAN	Vancomycin	10
	Amphotericin B	10
	Nalidixic Acid	10
PCAT	Polymyxin B	(100)
	Carbenicillin	100
	Amphotericin B	50
	Trimethroprim	20

* All concentrations are in µg ml⁻¹ except those in parentheses which are units ml⁻¹.

Fig. 1 Cheese Vat and containment measures.

- Contaminants observed after only 24 hours incubation for all samples when inoculated onto antibiotic free egg based media.
- On antibiotic free agar based media contaminants were observed after only 24 hours incubation for all samples except direct vat starter culture (0.1% w/v) (no growth after 14 days).
- The growth of indigenous unpasteurised milk and cheese microflora and direct vat starter culture on antimicrobial containing agar-albumin media is described in Table 2.

Table 2: Growth of indigenous unpasteurised milk and cheese microflora and direct vat starter culture on antimicrobial containing agar based media.

	Agar-Albumin Based Media								
	Middlebrook 7H10			Middlebrook 7H11			Gallaghers		
Sample	PANTA	VAN	PCAT	PANTA	VAN	PCAT	PANTA	VAN	PCAT
Unpasteurised Milk	+	+	-	+	+	-	-	+	-
Starter Culture (10 % w/v)	+	+	+	+	+	+	+	+	+
Unpasteurised Cheese 1	+	+	+	+	+	+	+	+	-
Unpasteurised Cheese 2	+	+	+	+	+	-	+	+	-

+ = Growth, - = No Growth.

Fig. 2 Cheese blocks and starter culture.

Conclusions

- Antibiotic free egg based media are insufficiently selective to prevent the growth of direct vat starter culture and indigenous microflora from unpasteurised milk or cheese.
- Addition of antimicrobial compounds to egg based media maybe unviable given the heat treatments and type of matrix involved. It may therefore be preferable to use agar based media.
- Gallaghers PCAT and Middlebrook 7H11 PCAT appear to be sufficiently selective to prevent the growth of indigenous microflora from unpasteurised milk or cheese of most samples tested and these combinations warrant further investigation.
- The efficacy of PANTA media combinations may be improved by increasing the concentration of the antimicrobial supplement.
- It may be appropriate to use specific Mycobacteria media at different points of the manufacture and maturation process as the cheese microflora continues to develop.

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3. Stonebrink. (1958). Acta Tuberculosea Scandinavica 32: 67 – 80.
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This project is funded by the Food Standards Agency.

Appendix 7.2

Poster: European symposium of the International Association of Food Protection IAFP 2009.

A Method for the Laboratory-Scale Manufacture of UK Semi-Hard and Hard Type Cheeses from Milk Contaminated with *Mycobacterium bovis*.

RICHARD FORGRAVE[1], John Donaghy[2], Michael Rowe[1,2]

[1]School of Biological Sciences, Queen's University Belfast, Newforge Lane, Belfast, N. Ireland, BT9 5PX. [2] Food Microbiology, Agri-Food and Biosciences Institute, Newforge Lane, Belfast, BT9 5PX, N. Ireland.

Introduction

Epidemiological investigations have attributed reports of culture-positive tuberculosis in the US to the consumption of *M bovis* contaminated raw milk cheese imports from Mexico[1]. These cases from the US suggest a link between *M bovis* contaminated dairy produce and human infections. Recent reports from the Republic of Ireland have emphasised the risks posed by consumption of raw milk[2]. The possibility of *M bovis* infection from consumption of UK raw milk cheese is unclear and may be compounded by the increasing prevalence of tuberculosis amongst UK cattle and growing demand for artisanal cheese produced from raw milk.

UK producers of raw milk products operate under additional regulatory criteria. Producers must be registered, hold a tuberculosis free status, submit to more regular inspections and label the product with a health warning. It has been hypothesised that the cheese manufacturing process and the presence of cheese microflora may reduce the level of *M bovis* contamination should there be a failure in the other regulatory mechanisms. The impact of these additional factors on the survival kinetics of *M bovis* during the manufacture, ripening and storage of raw milk cheeses is undefined. Two representative cheese types have been selected Caerphilly a semi-hard cheese which has a short maturation period from 1 to 10 weeks and Cheddar a hard cheese which has the greatest production volume of UK raw milk cheeses. This investigation seeks to characterise the effect of the cheese making process and the microflora of raw milk and cheese on the survival of *M bovis*.

Rationale & Objectives

Main considerations when devising the cheese making protocol:- 1. Operations should be possible within the confines of a Class I Microbiological safety cabinet. 2. Operations should be adequate representations of those found in industry. 3. By-products should be contained prior to disinfection and disposal. 4. Equipment should be disinfected prior removal from the cabinet.

Results

1. Milk in cheese vat brought to 32° C before adding Cheese starter & *M. bovis*

2. Ripen for 60 mins before addition of rennet Cease stirring and allow Milk to coagulate for 30 mins before cutting.

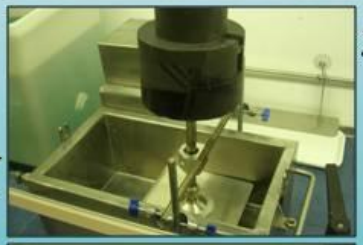
3. Cutting equipment is discarded in the disinfectant tank to the left of the cheese vat

4. Once cut the milk separates into solid curd and liquid whey. Over 1 hr the vat temperature is raised to the scalding temperature of 40° C to increase the expulsion of whey from the curd pieces.

5. After 1 hr at 40° C scalding temperature the whey is ready for draining.

6. Approx. 7 L of *M. bovis* contaminated whey is drained into a sealed carboy filled with disinfectant. The carboy serves as the primary containment for the whey.

7. For Caerphilly the cutting and chedaring of the curd mass is sufficient to produce the desired texture. The quantity of cheddar curd required that apparatus be developed which compensates for the lack of curd mass. Cutting and chedaring of curd whilst increasing the mass applied, simulates the traditional cheddaring process.



8. After completion of the cheddaring process the metal plate can be detached from the rest of the apparatus and transferred to the disinfectant tank.

9. Prior to salting the curd is milled. Caerphilly curd is cut in the small basin using the available utensils. Cheddar requires smaller curd pieces. The curd mass is initially cut in the small basin. Larger curd pieces are milled using the apparatus suspended over the larger basin supported by the cheese vat

10. After salting curd pieces are loaded into the mould wrapped in side cheese cloth. The high pressure required for pressing are produced by an Enerpac ™ pneumatic cylinder and pump set Pressure obtained can be monitored using the pressure gauge. Caerphilly is pressed at 0.2 kg/cm² then 0.8 kg/cm². Cheddar is pressed at 0.7 kg/cm² then 3.2 kg/cm²

11. The cheese is removed from the mould and cheese cloth and the curd/cheese allowed to dry prior to waxing.

12. The cheese is waxed using translucent and coloured wax. The wax is melted over a Bunsen flame in a Pyrex crystallizing basin. The molten wax is then transferred to the safety cabinet where it can be tempered to the desired temperature using a hotplate. The Pyrex crystallizing basin diameter is only slightly larger than that of the cheese allowing the cheese to be submerged in a relatively small volume of molten wax.

Conclusions

- The method allows the investigation of *M. bovis* survival kinetics in raw milk cheeses.
- This small scale procedure and equipment is a good representation of those found in a commercial environment.
- All equipment can be decontaminated by a combination of disinfectant, fumigant and autoclaving.
- Adaptation to the protocol would allow production of a variety of cheese types with Hazard **Group 2&3 pathogens** and therefore facilitate investigation of aspects of food protection relating to cheese.

This project is funded by the Food Standards Agency.

IAFP Fifth European Symposium on Food Safety Berlin 7th – 9th October

Appendix 7.3

Poster: Society for Applied Microbiology summer conference SFAM 2011.

The Inactivation of *Mycobacterium bovis* during the manufacture and maturation of UK raw-milk cheeses.

RICHARD FORGRAVE[1], ANNE FISHER[2], John Donaghy[2], Michael Rowe[1,2]

[1]School of Biological Sciences, Queen's University Belfast, Newforge Lane, Belfast, N. Ireland, BT9 5PX. [2] Food Microbiology, Agri-Food and Biosciences Institute, Newforge Lane, Belfast, BT9 5PX, N. Ireland.

Aims

Culture-positive tuberculosis in the US has been linked to consumption of *M bovis* contaminated raw-milk cheese imports[1]. Further reports have emphasised the risks posed by consumption of raw-milk[2]. The possibility of *M bovis* infection from consumption of UK raw-milk and raw-milk products is unclear at a time when there is increased prevalence of tuberculosis amongst UK cattle and growing demand for artisanal cheese produced from raw-milk.

UK producers of raw-milk products must be registered, hold a tuberculosis (TB) free status, submit to more regular inspections and label the product with a health warning. The survival kinetics of *M bovis* during the manufacture, ripening and storage of raw milk cheeses was undefined. Two cheese types were selected Caerphilly a semi-hard cheese which has a short maturation period from 2 weeks to 4 months and Cheddar which has a longer maturation period of 12 to 24 months. This investigation sought to characterise the effect of the cheesemaking process and cheese maturation on the survival of *M bovis*.

Methods & Results

Traditional cheesemakers produce cheese in vats with a capacity of 1000 litres or more. During this investigation cheese was produced from only 8 litres of raw-milk artificially contaminated (10^3 -10^5 cfu ml^{-1}) with three different *M. bovis* isolates (Table 1). The equipment and the methods used were designed to satisfactorily replicate the larger volume traditional cheesemaking process. Furthermore it was essential that the methods and equipment meet stringent containment level 3 safety regulations. Some of the key equipment and processes are pictured in Fig. 1a – 1d below.

Fig. 1a. Milk in cheese vat brought to 32° C before adding Cheese starter & *M. bovis*.

Fig. 1b. Post renneting, cutting & scalding the vat is raised so contaminated whey can drain into the carboy.

Fig. 1c. Apparatus for cheddar making mimicked the cheddaring of the curd during traditional cheddaring process.

Fig. 1d. A miniature cheese press was produced to achieve high pressure required for pressing of Cheddar & Caerphilly cheese.

***M bovis* contaminated cheeses were initially trialled against Middlebrook 7H11 and Gallagher's media[3] containing antimicrobial supplement PANTA plus (BD, UK) or PCAT[4]. It was observed that although the initial recovery of *M bovis* was similar for both media types after several months maturation the recovery of *M bovis* on Gallagher's media was poor. By omitting malachite green from Gallagher's media to produce a media designated Middlebrook 7H11++ the recovery of *M bovis* was improved (Fig. 2).**

Table 1. *M. bovis* isolates.

M. bovis Isolate	Description
AF2122	Sequenced strain, Prevalent UK mainland particularly Wales and South West England.
G1.140	Most prevalent spoligotype in Northern Ireland.
G24.130	Isolate related to both AF2122 & G1.140. Most prevalent in Republic of Ireland.

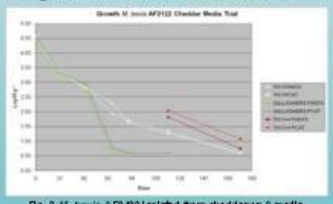


Fig. 2 *M. bovis* AF2122 isolated from cheddar on 6 media type's after 179 Days maturation

Using the three *M bovis* isolates a total of 18 cheeses were produced 3 Cheddar and 3 Caerphilly per isolate. *M bovis* was recovered from milk, whey and cheese samples. Although no inactivation of *M bovis* was observed during cheesemaking an increase in *M bovis* concentration was apparent between the liquid and solid phase for both Cheddar (0.8 $log10\ g^{-1}$) and Caerphilly (0.47 $log10\ g^{-1}$). A proportion of *M bovis* inoculum was removed with the whey on average 11% for Cheddar and 2.7% for Caerphilly

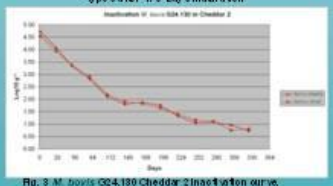


Fig. 3 *M. bovis* G24.130 Cheddar 2 inactivation curve.

Regular sampling and enumeration of *M bovis* during maturation allowed inactivation curves to be plotted for the 18 cheeses. Fig. 3 is representative of the inactivation curves produced. From a trend line fitted to the linear portion of the inactivation curve (Fig. 4) D_{10} values could be calculated (Table 2). In turn Average D_{10} values across all *M bovis* strains for Cheddar and Caerphilly were 48 days and 58 days respectively.

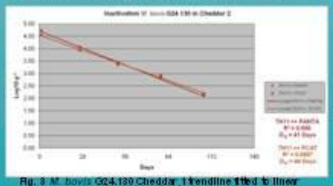


Fig. 4 *M. bovis* G24.130 Cheddar 1 trendline fitted to linear portion of inactivation curve.

Table 2. *M. bovis* D_{10} values for Cheddar and Caerphilly cheese.

	Cheddar D_{10} values			Caerphilly D_{10} values		
M bovis	**Cheddar 1**	**Cheddar 2**	**Cheddar 3**	**Caerphilly 1**	**Caerphilly 2**	**Caerphilly 3**
AF2122	37	33	32	52	62	34
G1.140	51	81	66	79	54	45
G24.130	37	43	47	131	35	27

Conclusions & Significance of study

- The method allows the investigation of *M bovis* survival kinetics in raw-milk cheeses by producing cheeses which are representative of those found in a commercial environment.
- During the extended maturation of cheddar inactivation occurred in two phases an initial linear phase followed by a 'tailing' inactivation effect after 3 – 4 months.
- An average 2.61 $log10\ g^{-1}$ reduction in *M bovis* was observed during a 4-mth ripening period for Caerphilly and a 2.93 $log10\ g^{-1}$ reduction observed during a 6-mth ripening period for Cheddar.
- This study will aid the risk assessment of raw-milk products and particularly where an assessment was needed of maturing cheese which had been produced before a herd lost its TB free status.

This project is funded by the Food Standards Agency.

SFAM Summer Conference Dublin 4th – 7th July