ACM/486

ADVISORY COMMITTEE ON THE MICROBIOLOGICAL SAFETY OF FOOD

Heat resistance of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk at pasteurisation temperatures

1. The attached paper reports on Food Standards Agency (previously MAFF)-funded work carried out within the Department of Food Science (Food Microbiology) at Queen's University, Belfast on the heat resistance of MAP.[1]

2. The paper will be presented by Dr Irene Grant (QUB).

Secretariat
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[1] In previous ACMSF papers, MAP has been referred to as *Mycobacterium paratuberculosis* (MPTB).

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Background

1. Since mid 1993 the Ministry of Agriculture, Fisheries and Food, and latterly the Food Standards Agency, has been funding a *Mycobacterium avium* subsp. *paratuberculosis* (MAP) research programme within the Department of Food Science (Food Microbiology) at Queen's University Belfast. The primary objective of this research has been to establish whether current milk pasteurisation conditions would inactivate MAP if present in raw milk.

2. Pasteurisation was introduced into the UK dairy industry as a public health measure around 1920. It was designed to kill the most heat-resistant, non-spore-forming, pathogenic bacteria likely to be present in milk, namely *Mycobacterium tuberculosis*, the cause of TB, and *Coxiella burnetti*, the cause of Q fever. The destruction of these organisms is achieved by either of the following time/temperature regimes: 63°C for 30 min (holder pasteurisation) or 72°C for 15 sec (high-temperature, short-time (HTST) pasteurisation). Holder and HTST pasteurisation conditions were simulated in the laboratory by a test-tube method and by use of Franklin HTST heat exchanger units, respectively. Temperature profiles of the milk during these heat treatments were obtained and found to closely resemble published temperature profiles for commercial pasteurisation processes. The only difference between laboratory pasteurisation and commercial pasteurisation was that the milk was static in both laboratory systems during the holding period of pasteurisation rather than in turbulent flow.

Laboratory pasteurisation studies

3. Raw milk spiked with high numbers of MAP (10^4 and 10^7 CFU/ml) was pasteurised by both holder and HTST methods. Ten strains of MAP (three type strains

and seven field isolates from around the world) were tested on three separate occasions by each of the heating methods (a total of 120 heat treatments). The number of MAP surviving each heat treatment was determined by culture on Herrold's egg yolk medium with mycobactin J (HEYM). Additional holding times at 63°C were included so that the thermal inactivation kinetics of the organism could be determined. Surviving MAP were isolated after holder and HTST pasteurisation from milk samples spiked with both 10^4 and 10^7 CFU/ml before heat treatment. In contrast, when *M. bovis* (10^6 CFU/ml) was spiked into raw milk and subjected to HTST pasteurisation no viable cells were recovered, i.e. HTST pasteurisation effectively inactivated *M. bovis* but not MAP.

4. Subsequently, the HTST pasteurisation experiments were repeated using the same 10 strains of MAP added to raw milk at lower levels (10^3, 10^2 and 10 CFU/ml and 10 CFU/50 ml) totalling a further 120 heat treatments. Surviving MAP were isolated from pasteurised milk samples originally inoculated with 10^3 and 10^2 CFU/ml. However, survival of MAP was never observed when raw milk was spiked with $\leq$ 10 CFU/ml before pasteurisation (which would equate to 5000 CFU per 50 ml of milk). The combined results of the high and low inoculum level pasteurisation experiments showed that MAP may survive current HTST pasteurisation (i.e. 72°C for 15 s) if present in milk at levels $\geq$ 100 CFU/ml.

Examination of the thermal inactivation kinetics of MAP

5. The thermal inactivation curve for MAP heated in milk at holder pasteurisation temperature (63°C) was found to be non-linear and exhibited "tailing". In the early stages of heating (0-10 min) rapid cell inactivation occurred but in the latter stages of heating low numbers of MAP survived for extended periods (10-30 min). In contrast, *M. bovis* was shown to exhibit linear thermal inactivation kinetics when heated in milk at 63°C. This finding prompted further investigations to provide an explanation for the shape of the thermal death curve.

6. A number of possible explanations for "tailing" were investigated and results indicated that the "tailing" phenomenon was not simply an artefact of the heating

method, nor due to some effect of the milk constituents, nor due to a heat-resistant sub-population. Rather it was postulated that the natural tendency of MAP to aggregate into clumps due to the hydrophobic nature of their cell wall was in some way responsible for the observed heat resistance. By means of a viability stain, combining tetrazolium reduction and auramine O acid-fast staining, developed for the purpose of assessing cell viability and clumping during heating, viable MAP cells were only observed within clumps of predominantly heat-killed cells at heating times corresponding to the "tail" region. Furthermore, clumped MAP cells have been shown to be twice as heat resistant as declumped (single) MAP cells, and macrophage-engulfed MAP cells were more heat resistant than freely suspended cells.

7. These findings provide circumstantial evidence that the non-linear thermal inactivation kinetics exhibited by MAP in the laboratory studies are due to the existence of cells as tight clumps. MAP cells are likely to occur as clumps in naturally infected milk. Within the udder of an infected cow the MAP cells are likely to be present within macrophage, essentially clumps of cells, and MAP cells arising from faecal contamination of raw milk are also likely to exist as clumps of cells. Therefore, MAP cells could be present as clumps in naturally infected raw milk prior to commercial pasteurisation. If high enough numbers of clumped cells were present in naturally infected raw milk current HTST pasteurisation conditions (72°C for 15 s) may not effectively inactivate MAP.

Investigation of other factors influencing the heat sensitivity of MAP

8. Freezing and thawing of *MAP* cells prior to heating - Freezing can affect MAP cell viability. Studies carried out at QUB have shown that when MAP inoculum is frozen (-80^{o}C for between two days and six months), thawed and then used to spike milk for thermal inactivation studies the MAP cells are much less heat resistant than cells which have never been frozen. It is possible that the actual process of freezing and subsequent thawing of MAP cells causes sub-lethal injury making the cells more sensitive to the lethal effects of heat.

9. Rate of heating of *MAP* cells to pasteurisation temperature - Studies have shown that MAP cells exhibit greater heat resistance when cells were added to pre-heated milk (i.e. heated instantaneously) than when cells were added to cold milk and gradually heated to 63^{o}C. It is possible that during gradual heating a portion of the MAP cells are injured and hence more easily inactivated during the holding period. Alternatively, MAP cells heated instantaneously may be producing heat shock proteins and this may have contributed to the apparent increased heat resistance observed.

10. Effect of heating menstruum (Raw v UHT whole v UHT skim milk) –Studies were carried out to determine the legitimacy of performing thermal inactivation studies on MAP using UHT skim or UHT whole milk as the heating menstruum rather than raw milk with a background microflora. The thermal inactivation kinetics of MAP cells in UHT whole milk were shown to be comparable to those obtained using raw milk and so it was concluded that use of UHT whole milk in pasteurisation studies was unlikely to affect the heat sensitivity data obtained for MAP. However, MAP cells were inactivated more quickly in UHT skim milk than in whole milk, and therefore erroneous data on the heat sensitivity of MAP would be obtained if skim milk were to be substituted for whole milk in pasteurisation studies.

11. Source of MAP inoculum (solid or liquid medium) –Studies showed that MAP cells obtained from broth culture were consistently more heat resistant than cells obtained from slope culture. When stained and viewed under the microscope inoculum obtained from broth cultures exhibits a greater degree of clumping (larger clumps and a greater number of clumps). We speculate that this may explain the greater heat resistance observed.

Effect of higher pasteurisation temperatures and longer holding times at 72°C

12. In an effort to identify a time/temperature combination that would ensure the complete inactivation of high numbers (10^6 CFU/ml) of MAP spiked into raw milk, further experiments were carried out to investigate the effect of higher pasteurisation temperatures (75, 78, 80, 85 and 90°C for 15 s) and longer holding times at 72°C (20 and 25 s) on the destruction of MAP. Three of the most heat resistant MAP strains identified during previous studies (B2, DVL 943 and NCTC 8578) were subjected to each of the above time/temperature combinations. Survival of viable MAP after HTST pasteurisation was found to be sporadic, but not impossible, at temperatures up to 90°C.

13. A longer holding period at 72°C proved to be more effective in inactivating MAP than a higher pasteurisation temperature. Of the three strains studied, only B2 was isolated from milk heated at 72°C for 20 s and none of the strains was isolated from milk heated at 72°C for 25 s. These findings suggest that the duration of heating is more important for the inactivation of MAP in milk than the intensity of heating. It must be noted that these experiments were carried out using milk spiked with laboratory-grown MAP cells that may not behave in the same way as MAP cells present in milk as a result of natural infection. Also the pasteurised milk was tested within a couple of hours of leaving the pasteuriser and the largest volume of milk tested was only 10 ml. These factors may have influenced the recovery of heat-injured MAP cells from the laboratory-pasteurised milk.

Methodology for isolation of MAP

14. In addition to the work on heat resistance, much work was carried out to develop a test for isolating MAP from milk. This led to a method which, in contrast to methods used by other researchers around the world, did not apply chemical decontamination to milk samples directly after heat treatment, did not include antibiotics in the culture media

and used as large a volume of pasteurised milk as possible in order to increase the chances of isolating any surviving MAP present. These actions were taken as it was recognised from the outset that MAP cells surviving heat treatments could be sub-lethally heat injured. Therefore, they should not be subjected to any additional stresses (such as chemical treatment or antibiotics) that may render the potentially viable cells non-recoverable.