Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Inactivation of *Mycobacterium avium* ssp. *paratuberculosis* in milk by UV treatment

J. Donaghy[1], M. Keyser[2], J. Johnston[1], F.P. Cilliers[3], P.A. Gouws[4] and M.T. Rowe[1]

1 Agriculture, Food and Environmental Science Division (Food Microbiology Branch), Agri-Food and Biosciences Institute, Belfast, Northern Ireland
2 Cape Peninsula University of Technology, Bellville, South Africa
3 University of Stellenbosch, Stellenbosch, South Africa
4 Food Microbiology Research Group, Department of Biotechnology, University of the Western Cape, Bellville, South Africa

Keywords
milk, *Mycobacterium avium* ssp. *paratuberculosis* (Map), UV radiation.

Correspondence
Michael T. Rowe, Agriculture, Food and Environmental Science Division (Food Microbiology Branch), Agri-Food and Biosciences Institute, Newforge Lane, Belfast BT9 5PX, Northern Ireland.
E-mail: michael.rowe@afbini.gov.uk

2009/0063: received 12 January 2009, revised 24 March 2009 and accepted 3 April 2009

doi:10.1111/j.1472-765X.2009.02644.x

Abstract

Aims: To determine the effect of UV radiation on the viability of two strains of *Mycobacterium avium* ssp. *paratuberculosis* (Map) inoculated into milk.
Methods and Results: *Mycobacterium avium* ssp. *paratuberculosis* in a ultra heat treated milk matrix was subjected to increasing doses of UV-C radiation from 0 to 1836 mJ ml^{-1} using a pilot-scale UV reactor (20 l capacity). Survival of Map was monitored by culture on Herrold's egg yolk medium, Middlebrook 7H10 medium and the *FASTPlaque*TB™ phage assay. Differences in sensitivity to UV treatment were observed between strains, however, at 1000 mJ ml^{-1} a Map kill rate of 0·1–0·6 log_{10} was achieved regardless of strain used or method employed to enumerate Map. Although the inactivation trend was similar on the culture and phage assay, the former gave a consistently higher viable count.
Conclusions: The use of UV radiation alone does not represent an alternative to current pasteurization regimes for a large reduction in viable Map in milk.
Significance and Impact of the Study: To the authors' knowledge the work here represents the first pilot-scale UV treatment process used to assess UV efficacy to inactivate Map in milk. The results are similar to those obtained with a laboratory-scale process indicating the difficulties associated with UV treatment of an opaque liquid and the recalcitrance of Map towards inimical treatments.

Introduction

Mycobacterium avium ssp. *paratuberculosis* (Map) is the known cause of Johne's disease which affects both wild and domestic ruminants (Beard *et al.* 2001), particularly dairy cattle (Clarke 1997). The organism has also been implicated as a cause of Crohn's disease in humans that manifests as a chronic enteritis which is incurable, although sufferers can experience periods of remission (Behr and Kapur 2008). *Myco. avium* ssp. *paratuberculosis* is excreted directly into the milk and faeces of infected animals in low and very high numbers respectively (Cocito *et al.* 1994). In the latter case even with good hygienic practices some faeces inevitably finds its way into milk. Milk and milk products must therefore be considered possible routes of exposure to the public from this organism.

The possible presence of Map in raw milk is compounded by the fact that there is evidence the organism may, under certain conditions, survive the milk pasteurization process (Grant 2005). Therefore, in the absence of a treatment guaranteed to kill any Map present, the dairy industry is investigating other processes, either to replace or as an adjunct to pasteurization, that are demonstrably lethal for Map. Such a process would, of necessity, as well as being effective need to be economic, continuous rather than batch, not involve addition or removal of chemical agents to retain the natural image of the product and produce minimal organoleptic changes. Examples of such processes that have been investigated to date are use of high pressures (Donaghy *et al.* 2007) microfiltration and bactofugation (Grant *et al.* 2005). These have shown varying degrees of success but to the authors' knowledge have

not been widely adopted by the dairy processing industry for the purpose of dealing with the Map issue.

One additional process that has received some attention is the use of UV radiation. This has been widely used for the disinfection of air, surfaces, water and fruit juices (Bintsis *et al.* 2000; Guerrero-Beltran and Barbosa-Canovas 2004). Disinfection using UV radiation is a process that can be simple and effective at low cost in comparison to other treatment methods (Bachmann 1975), lethal to most types of micro-organisms (Bintsis *et al.* 2000), does not generate chemical residues or change the sensory characteristics of the final product (Guerrero-Beltran and Barbosa-Canovas 2004) and be applied as a continuous process.

UV radiation treatments have been applied to milk, indeed Matak *et al.* (2005) reported a >5 $\log_{10}$ reduction in viable bacterial numbers in goat's milk after a cumulative UV dose of 15·8 ± 1·6 mJ cm^{-2}. Reinemann *et al.* (2006) found a 3 $\log_{10}$ reduction in total viable bacterial count in raw cow's milk using a treatment of 15 kJ l^{-1}. In more relevant work Altic *et al.* (2007), using a dose of 1000 mJ ml^{-1}, found a reduction in viable Map of 2·5–3·3 and 0·5–1·0 $\log_{10}$ when Middlebrook 7H9 broth and milk were the suspending menstrua respectively. It is interesting to note that in the work of Altic *et al.* (2007) in addition to conventional culture a commercially available phage amplification assay, *FASTPlaque*TB was used to give a more rapid enumeration of viable Map cells. In addition, with Altic *et al.* (2007) the laboratory apparatus incorporated static mixers which caused turbulent flow thereby increasing the volume of milk exposed to the UV source. Indeed designers of UV treatment devices for opaque fluids have adopted a wide variety of approaches to achieve the necessary turbulent flow. This must be seen in the context that not only does the effectiveness of UV penetration of the product depend on light source, product composition, flow profile but also on the geometric configuration of the equipment (Guerrero-Beltran and Barbosa-Canovas 2004). Therefore, even if all the other factors are the same, the resultant lethality of the process will be different if the geometric configuration is changed. It should be recognized that although milk and Map were the suspending mentruum and target organism respectively in both the work of Altic *et al.* (2007) and that reported here the method and extent of turbulent flow generation and the scale of milk processed were significantly different in both systems used.

In the work reported here Ultra Heat Treated (UHT) whole milk was spiked with Map and subjected to UV-C radiation using a pilot plant scale UV-C reactor which incorporates a mechanism to create turbulent flow conditions. Surviving Map cells were enumerated using the *FASTPlaque*TB assay in conjunction with viable plate counts.

Materials and methods

Map strains

Two Map strains were tested: a type strain ATCC 43012 (obtained from the National Collection of Type Cultures, London, UK) and strain 806R, originally isolated from raw cows' milk (Grant *et al.* 2002; kindly provided by Dr. I. Grant, School of Agriculture, Food and Land Use, Queens University of Belfast, Belfast, N. Ireland, UK).

Preparation of inoculum for UV treatment

Each strain was grown on Herrold's egg yolk medium (HEYM) containing 2 μg of mycobactin J ml^{-1} (Synbiotics Europe SAS, Lyon, France) (6 weeks, 37°C). A single colony of each strain was transferred into Middlebrook 7H9 broth medium containing 10% v/v Middlebrook OADC (oleic acid-albumin-dextrose-catalase; Becton Dickinson UK Ltd, Oxford, UK), 0·5% v/v Tween 80 (Sigma, Dorset, UK), and 0·0002% w/v mycobactin J. Culture broths (200 ml), for each strain, were incubated (with agitation, 100 rev min^{-1}) at 37°C for 8–10 weeks. Cells were harvested by centrifugation (4000 ***g***, 20 min) and re-suspended in 20 ml phosphate buffered saline (PBS; Oxoid, Basingstoke, UK). Cell suspensions were agitated with a Vortex Genie 2 (Scientific Industries Inc., Bohemia, NY, USA) for 3 min to disperse large clumps of Map. Triplicate experiments were performed for each strain.

Operation of laboratory-scale UV process

The spiked milk was processed using a UV-C reactor manufactured by Surepure Ltd, Milnerton, South Africa. The reactor consisted of a stainless steel inlet and outlet chamber separated by a stainless steel corrugated spiral tube containing a UV germicidal lamp (100 W output, 30 W UV-C output) which is protected by a quartz sleeve (Fig. 1). The liquid flows between the corrugated spiral tube and the quartz sleeve. The tangential inlet of the reactor creates a high velocity and turbulence in the inlet chamber and brings the liquid into contact with the UV radiation. A positive displacement pump is used to transport the liquid from the inlet chamber into the actual reactor, which is the gap between the quartz sleeve and the corrugated spiral tubing, at a minimum flow rate (Fr) of 3800 l h^{-1} with a Reynold's value (Re) in excess of 7000 indicative of turbulent flow. The UV source was used to treat 20 l batches of milk which was circulated at a flow rate of 4000 l h^{-1}. The time for the milk to pass through the system once was 18 s, delivering a UV-C dose of 22·95 J l^{-1} for each pass. The contact time was determined theoretically and assumed that the system was

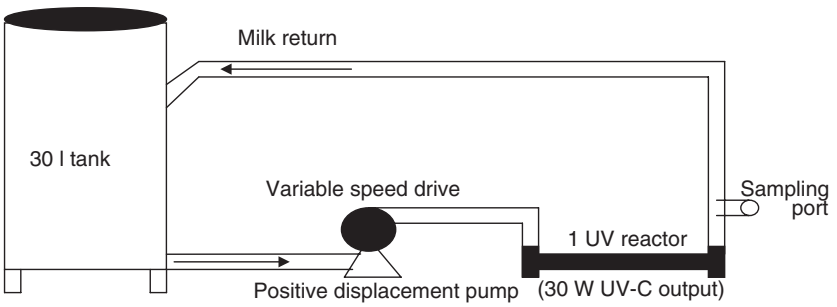

Figure 1 Schematic drawing of the pilot-scale Surepure UV-C treatment system.

in steady state with a uniform product and product flow and that the liquid was nonexpandable and nonvolatile. For health and safety purposes the UV lamp and sampling port of the equipment was located in a Class 1 microbiological safety cabinet during treatments. Between runs the equipment was disinfected and washed using NaOH (1·5% w/v; 10 h treatment) and sterile distilled water respectively.

UV inactivation of Map in milk

Twenty litres of retail UHT whole milk were inoculated with 20 ml of Map suspension in the stainless steel tank of the UV reactor. With the UV lamp switched off, the flow rate was stabilized to 4000 l h^{-1} and after one pass through the system (18 s), the flow rate was reduced and 2×150 ml samples were aseptically collected from the sampling port. (sample OB – no UV treatment before agitation). The flow rate was returned to 4000 l h^{-1} and the milk circulated for a further five passes (1·5 min) without the UV light switched on and milk sampled (sample OA – no UV treatment after agitation). This was to ensure mixing of the Map culture with milk, to create turbulence and de-aggregate Map clumps prior to UV treatment. Two samples (150 ml) were collected at 6, 12, 18 and 24 min (corresponding to 20, 40, 60 and 80 passes respectively) after the UV lamp was switched on and the flow rate was consistent at 4000 l h^{-1}. The UV lamp was turned off and the flow rate reduced to a minimum at each sampling time.

Enumeration of viable Map pre- and post-UV treatment

All microbiological analyses were performed within 2 h of UV treatment. Following UV treatment, samples were vigorously shaken and 1 ml aliquots of treated spiked milks were decimally diluted in maximum recovery diluent (MRD; Oxoid). Where low numbers of Map survivors were expected, cells in a 10 ml aliquot from the treated sample were concentrated by centrifugation (4000 ***g***, 20 min) and re-suspended in 1 ml MRD.

Appropriate diluents of all samples were inoculated (200 μl) and spread onto deep (30 ml) plates of each of the following media: Herrold's Egg Yolk Medium (HEYM) which contained (l^{-1}): bacteriological peptone (Difco, Detroit, MI), 9·0 g, sodium chloride 4·5 g, bacteriological agar No. 1 (Oxoid) 15·3 g, beef extract (Sigma) 2·7 g, sodium pyruvate (Sigma) 4·1 g, glycerol (Sigma) 27 ml, 0·01% w/v malachite green and 100 ml sterile egg yolk; Middlebrook 7H10 agar consisting of Middlebrook 7H10 agar base (Becton Dickinson UK Ltd) supplemented with 10% v/v Middlebrook OADC enrichment and 0·5% v/v glycerol. All culture media were supplemented with 2 μg ml^{-1} mycobactin J. Agar plates were sealed with Duraseal™ (Sigma) to minimize the drying of agar during incubation. All media were incubated at 37°C for up to 18 weeks and colonies typical of Map on the agar medium were counted. A representative number of typical and atypical colonies were confirmed by Ziehl-Neelsen acid-fast staining and IS900 PCR (Donaghy *et al.* 2007).

FASTPlaque TB phage assay

The *FASTPlaque* TB assay (Biotec Laboratories Ltd., Ipswich, UK) was originally developed to detect *Mycobacterium tuberculosis* in human sputum samples but has been shown to be suitable for detection of Map. It should be noted that as a phage amplification step, which requires an active metabolism on the part of its host, is integral to the assay it will only detect viable Map cells in this application. The assay consists of sufficient lyophilized Actiphage and sensor cells (*M. smegmatis*), Virusol (viricide), broth, growth supplement, agar and vials sufficient for 50 tests and was used according to the manufacturer's instructions. Essentially duplicate 1-ml aliquots of three dilutions of inoculated milk or UV-treated sample were incubated with Actiphage at 37°C for 1 h, treated with Virusol for 5 min and mixed with rapidly growing sensor cells and the entire sample transferred to an empty Petri dish to which 5 ml of molten (55°C) *FASTPlaque* agar was added. The solidified plates were incubated at 37°C overnight (18 h) before the number of

plaques present (presumed equivalent to the number of viable Map present in the sample) was counted and the result expressed as the number of PFU per ml.

Results

Initial agitation did not result in a significantly ($P < 0{\cdot}001$) higher plaque or plate count. Although the trend in Map inactivation was similar between methods used to enumerate surviving cells, there was a significant difference ($P < 0{\cdot}001$) between the viable count index obtained with the *FASTPlaque* assay and the plate count with the former giving a consistently lower value of approximately 0·5–2·0 log units. Strain ATCC 43012 (called Linda, a clinical isolate from a Crohn's patient) was significantly ($P < 0{\cdot}001$) less resistant to UV radiation than strain 806R (isolated from raw cow's milk in our laboratory). A quadratic relationship between UV-dose and Map survivors was established in this study (Fig. 2) whereby after reaching a defined dose rate the number of survivors decreased more rapidly.

Discussion

In the work reported here UHT whole milk was used as the heating menstruum to eliminate background microflora thus obviating the need to use decontamination procedures or antibiotic cocktail supplements and hence maximize the detection of injured Map cells. It was also noted that in a similar study Altic *et al.* (2007) detected no significant difference ($P < 0{\cdot}258$) on UV inactivation of Map between UHT whole and semi-skim milk. The lack of a significantly higher plaque or plate count after initial agitation indicates that either there were few clumps initially and/or that insufficient shear force was generated to disrupt those present.

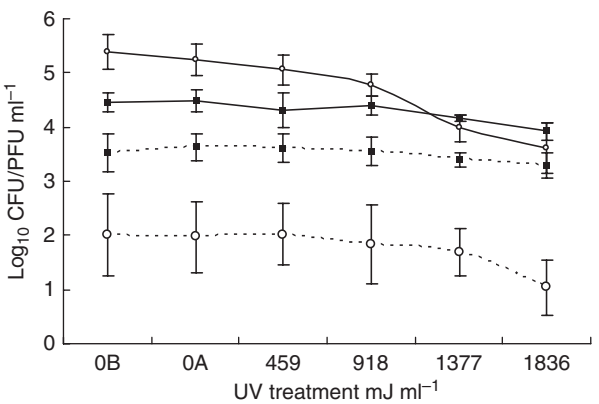

Figure 2 Inactivation of Map strains ATCC 43012 (○) and 806R (■) by UV-radiation measured by CFU (——) and PFU (– – –) count. OA, Zero UV treatment after agitation; OB, Zero UV treatment before agitation.

The consistently lower viable count index values obtained with the *FASTPlaque* assay compared with the plate count was also observed by Altic *et al.* (2007) with Map and by the manufacturer of the *FASTPlaque* assay with *M. tuberculosis*. Three possible explanations for this discrepancy have been advanced by personnel from Biotec Laboratories who developed the *FASTPlaque* assay viz. an intrinsic property of the Map phage relationship, a characteristic of the assay which was originally optimized for *M. smegmatis* or a cell state issue (Andre Trollip and Richard Mole, personal communication to Dr I. Grant, Queen's University of Belfast).

The differences in response to UV radiation between the strains is in contrast to that of Altic *et al.* (2007) who used NCTC 8578, a bovine isolate from faeces, 796PSS, an isolate from pasteurized milk and 806R as used in the work reported here.

The quadratic relationship between UV-dose and Map survivors established in this study (Fig. 2) indicated that at 1000 mJ ml^{-1} the kill rate achieved was in the range 0·1–0·6 $\log_{10}$ depending on strain and method used. Altic *et al.* (2007) reported a kill rate between 0·5 and 1·0 $\log_{10}$ at a similar UV dose albeit using different UV equipment and smaller milk volumes. These authors observed a much greater Map kill when the menstruum used was mycobacterial growth medium compared to semi-skim or whole milk. A similar effect was observed when UV-C treatment was applied to orange juice and fruit nectars compared to a clarified, clear apple juice (Keyser *et al.* 2008).

The two strains used in this study were considerably more resistant to UV compared to *Listeria monocytogenes* since Matak *et al.* (2005) achieved a greater than 5 $\log_{10}$ reduction in viable numbers with a dose of only 15·8 ± 1·6 mJ cm^{-2}. The equipment used differed from that used in this study which make comparisons difficult. However, Reinemann *et al.* (2006) used the same Surepure system as used in this study to determine the UV effect on standard plate counts, thermophiles, psychrotrophs, coliforms and spore-formers in raw cows' milk. The respective $\log_{10}$ reductions at a UV dose rate of 1000 mJ ml^{-1} were: 1·92, 1·42, 2·08, 2·48 and 0·36. Therefore, Map shows similar UV resistance to spore-forming micro-organisms and is much more resistant than the major microflora associated with milk. This greater UV resistance of mycobacteria over other bacterial genera has been observed previously (Collins 1971; Peccia and Hernandez 2004; Shin *et al.* 2008).

It should be recognized that the maximum dose applied in the work reported here (1836 mJ ml^{-1}) exceeds 1000 mJ ml^{-1} which is thought to be the dose limit at

which undesirable organoleptic changes are generated. The conclusion to the work is the same as that of Altic *et al.* (2007) that UV treatment using the process described here is not a viable alternative to current pasteurization regimes for liquid milk which achieve approximately 4 $\log_{10}$ reductions in Map. It would be a logical extension to the current work to determine the efficacy of UV treatment combined with pasteurization with respect to Map. There is also scope to improve the turbulent flow characteristics of the machine and increase the intimate contact of the milk menstruum with the UV radiation. It should however be born in mind that such turbulence may destroy the milk fat globule membrane and increase the lipid substrate surface area to the action of intrinsic and microbial lipases leading to rancidity.

Acknowledgement

The authors wish to thank Dr David Kilpatrick of Biometrics and Information Systems, Agri-Food and Biosciences Institute for the statistical analysis and their interpretation.

References

Altic, L.C., Rowe, M.T. and Grant, I.R. (2007) UV light inactivation of *Mycobacterium avium* ssp. *paratuberculosis* in milk as assessed by *FASTPlaque*TB phage assay and culture. *Appl Environ Microbiol* **73**, 3728–3733.

Bachmann, R. (1975) Sterilization by intense ultraviolost radiation. *The Brown Boveri Rev* **62**, 206–209.

Beard, P.M., Daniels, M.J., Henderson, D., Piroe, A., Rudge, K., Buxton, D., Rhind, S., Greig, A. *et al.* (2001) Paratuberculosis infection in nonruminant wildlife in Scotland. *J Clin Microbiol* **39**, 1517–1521.

Behr, M.A. and Kapur, V. (2008) The evidence for *Mycobacterium avium* ssp. *paratuberculosis* in Crohn's disease. *Curr Opin Gastroenterol* **24**, 17–21.

Bintsis, T., Litopoulou-Tzanetaki, E. and Robinson, R. (2000) Existing and potential applications of ultraviolet light in the food industry – a critical review. *J Sci Food Agri* **80**, 637–645.

Clarke, E. (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species. *J Comp Pathol* **116**, 217–261.

Cocito, C.P., Gilot, M., Coene, M., De Kesel, P., Poupart, P. and Vannuffel, P. (1994) Paratuberculosis. *Clin Microbiol Rev* **7**, 328–345.

Collins, F.M. (1971) Relative susceptibility of acid-fast and non-acid-fast bacteria to ultraviolet light. *Appl Microbiol* **21**, 411–413.

Donaghy, J.A., Linton, M., Patterson, M.F. and Rowe, M.T. (2007) Effect of high pressure on *Mycobacterium avium* ssp. *paratuberculosis* in milk. *Lett Appl Microbiol* **45**, 154–159.

Grant, I.R. (2005) Zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: the current position. *J Appl Microbiol* **98**, 1282–1293.

Grant, I.R., Hitchings, E.I., McCartney, A., Ferguson, F. and Rowe, M.T. (2002) Effect of commercial-scale HTST pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. *Appl Environ Microbiol* **68**, 602–607.

Grant, I.R., Williams, A.G., Rowe, M.T. and Muir, D.D. (2005) Investigation of the impact of simulated commercial centrifugation and microfiltration on levels of *Mycobacterium avium* ssp. *paratuberculosis* in milk. *Int J Dairy Tech* **58**, 138–142.

Guerrero-Beltran, J.A. and Barbosa-Canovas, G.V. (2004) Review: advantages and limitations on processing foods by UV light. *Food Sci Tech Int* **10**, 137–147.

Keyser, K., Muller, I.A., Cilliers, F.P., Nel, W. and Gouws, P.A. (2008) Ultraviolet radiation as a non-thermal treatment for the inactivation of microorganisms in fruit juice. *Innovative Food Sci Emerg Technol* **9**, 348–354.

Matak, K.E., Churey, J.J., Worobo, R.W., sumner, S.S., Hovingh, E., Hackney, C.R. and Pierson, M.D. (2005) Efficacy of UV light for the reduction of *Listeria monocytogenes* in goat's milk. *J Food Prot* **68**, 2212–2216.

Peccia, J. and Hernandez, M. (2004) UV-induced inactivation rates for airborne *Mycobacterium bovis* BCG. *J Occup Environ Hyg* **1**, 430–435.

Reinemann, D.J., Gouws, P., Cilliers, T., Houck, K. and Bishop, J.R. (2006) *New Methods for UV Treatment of Milk for Improved Food Safety and Product Quality*. American Society of Agricultural and Biological Engineers (ASABE), paper no. 066088. St Joseph, MI: ASABE.

Shin, G.-A., Lee, J.-K., Freeman, R. and Cangelosi, G.A. (2008) Inactivation of Mycobacterium avium complex by UV Irradiation. *Appl Environ Microbiol* **74**, 7067–7069.

