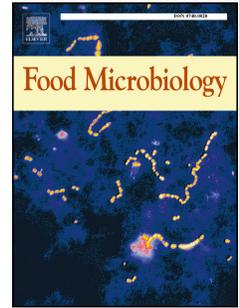


Accepted Manuscript

Survival of *Mycobacterium avium* subspecies *paratuberculosis* in retail pasteurised milk

Zara E. Gerrard, Benjamin M.C. Swift, George Botsaris, Ross S. Davidson, Michael R. Hutchings, Jonathon N. Huxley, Catherine E.D. Rees



PII: S0740-0020(17)30936-X

DOI: [10.1016/j.fm.2018.03.004](https://doi.org/10.1016/j.fm.2018.03.004)

Reference: YFMIC 2975

To appear in: *Food Microbiology*

Received Date: 26 September 2017

Revised Date: 28 February 2018

Accepted Date: 8 March 2018

Please cite this article as: Gerrard, Z.E., Swift, B.M.C., Botsaris, G., Davidson, R.S., Hutchings, M.R., Huxley, J.N., Rees, C.E.D., Survival of *Mycobacterium avium* subspecies *paratuberculosis* in retail pasteurised milk, *Food Microbiology* (2018), doi: 10.1016/j.fm.2018.03.004.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Survival of *Mycobacterium avium* subspecies *paratuberculosis* in retail**
2 **pasteurised milk**

3 Zara E. Gerrard ^{a,c} , Benjamin M. C. Swift^{b*}, George Botsaris^d, Ross S. Davidson^c, Michael R.
4 Hutchings^c, Jonathon N. Huxley^a, Catherine E.D. Rees^a

5 ^aUniversity of Nottingham, Sutton Bonington Campus, College Road, Leicestershire, LE12 5RD

6 ^bRoyal Veterinary College, Hawkshead Campus, Hertfordshire, AL9 7TA

7 ^cSRUC, West Mains Road, Edinburgh EH9 3JG

8 ^dDepartment of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of
9 Technology, Limassol, Cyprus

10

11 *Corresponding author

12 Email: bswift@rvc.ac.uk

13 **Abstract**

14 A survey of retail purchased semi-skimmed pasteurised milk ($n=368$) for *Mycobacterium avium*
15 subspecies *paratuberculosis* (MAP) was conducted between May 2014 and June 2015 across the
16 midlands of England using the Phage-PCR assay. Overall, 10.3 % of the total samples collected
17 contained viable MAP cells, confirming that pasteurisation is not capable of fully eliminating human
18 exposure to viable MAP through milk. Comparison of the results gained using the Phage-PCR assay
19 with the results of surveys using either culture or direct PCR suggest that the phage-PCR assay is able
20 to detect lower numbers of cells, resulting in an increase in the number of MAP-positive samples
21 detected. Comparison of viable count and levels of MAP detected in bulk milk samples suggest that
22 MAP is not primarily introduced into the milk by faecal contamination but rather are shed directly
23 into the milk within the udder. In addition results detected an asymmetric distribution of MAP exists
24 in the milk matrix prior to somatic cell lysis, indicating that the bacterial cells in naturally
25 contaminated milk are clustered together and may primarily be located within somatic cells. These
26 latter two results lead to the hypothesis that intracellular MAP within the somatic cells may be
27 protected against heat inactivation during pasteurisation, accounting for the presence of low levels
28 of MAP detected in retail milk.

29

30 **Keywords**

31 *Mycobacterium avium* subsp. *paratuberculosis*; *Johne's disease*; *human exposure*; *phage-PCR assay*;
32 *pasteurised milk*

33 1.0 Introduction

34 *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease
35 (JD), a chronic wasting disease of cattle and other ruminants, characterised by a reduction in milk
36 yield, severe diarrhoea, weight loss and intermandibular oedema (commonly referred to as bottle
37 jaw). In addition to causing a significant health impact on the national herd, this disease has a
38 significant economic impact on the dairy industry as a whole. MAP is an extremely slow growing
39 bacterium, taking up to 8-12 weeks to grow in liquid culture media, which makes detection by
40 traditional culture methods problematic (Sweeney et al., 2012). MAP is also implicated in the
41 development of Crohn's disease (CD), an inflammatory bowel condition of humans with similar
42 aetiology to JD (Bull et al., 2003; Feller et al., 2007; Rhodes et al., 2014), and although a causal
43 relationship has not been fully established, it has been recommended that limiting human exposure
44 would be sensible on a precautionary principle (NACMCF, 2010).

45 Cattle infected with MAP can shed substantial levels of the bacteria into their milk (up to 540 cfu ml⁻¹;
46 Slana et al., 2008), faeces and semen (Antognoli et al., 2008), with milk being highlighted as a key
47 transmission vehicle for human exposure to MAP, making the efficiency of pasteurisation an
48 important factor in controlling transmission (Stabel and Lambertz, 2004). Although many studies
49 have shown that the conditions used for pasteurisation are sufficient to inactivate MAP, some
50 studies have shown that both low-temperature long-time (LTLT) and high-temperature short-time
51 (HTST) pasteurisation did not totally inactivate MAP when present in milk at levels greater than 1 x
52 10⁴ cfu ml⁻¹ (Chiodini and Hermon-Taylor, 1993; Grant et al., 1996). This finding led to dairy
53 processing centres to adjust the holding time of HTST pasteurisation from 15 s (the legal minimum in
54 the UK), to 25 s to increase the likelihood of totally inactivating MAP. Despite this, MAP has been
55 reported to survive the pasteurisation process and has been detected by culture in a number of
56 surveys of retail pasteurised milk in different parts of the world with a prevalence ranging between

57 1.7 to 6.7 % of samples tested (Ayele et al., 2005; Grant et al., 2002a; Grant et al., 2002b; Paolicchi et
58 al., 2012).

59 The detection of MAP has been historically difficult due to limitations in standard culture and PCR-
60 based detection methods. Milk culture requires chemical decontamination to eliminate fast growing
61 bacteria, but this also causes a 1-2 log₁₀ drop in viable MAP cells, which in turn can decrease the
62 chance of successful culture (Dundee et al., 2001). For pasteurised dairy products, PCR-based
63 detection is less useful since it will detect any MAP DNA present in the sample, whether this is from
64 viable bacteria or from cells that have been inactivated by the heat treatment. The bacteriophage
65 amplification assay coupled with PCR (phage-PCR) has been developed to rapidly and sensitively
66 detect viable MAP as an alternative to culture-based methods (Swift and Rees, 2013). This assay uses
67 Mycobacteriophage D29 to infect any mycobacteria present in a milk sample, and any viable
68 mycobacteria will be indicated by amplification of the bacteriophage within MAP cells and their
69 subsequent lysis to release progeny phage and MAP DNA. PCR can then be used to confirm the
70 identity of the mycobacteria detected (Stanley et al., 2007). The phage assay is faster than culture as
71 results can be gained within 48 h, as well as being more sensitive than culture as there is no need for
72 chemical decontamination. The phage assay has been successfully used to detect viable MAP in raw
73 milk (Botsaris et al., 2010; Stanley et al., 2007), powdered infant formula (Botsaris et al., 2016),
74 cheese (Botsaris et al., 2010) and blood of infected animals (Swift et al., 2013). In this study we used
75 the phage-PCR assay to rapidly estimate the prevalence of MAP in retail pasteurised milk. In addition
76 experiments were carried out to try and establish how MAP enters the milk and where the bacteria
77 are located in naturally contaminated milk, and the pattern of results led to a hypotheses about how
78 MAP may be able to survive pasteurisation.

79

80 **2.0 Material and Methods**

81 **2.1 Collection of pasteurised milk samples**

82 Three-hundred and eighty-six semi-skimmed milk (1.7 % fat) samples were collected at four time
83 points (May 2014, November 2014, January 2015, and June 2015) using volunteers from the
84 University of Nottingham Sutton Bonington campus; this type of milk was chosen as it was the most
85 popular purchased by the volunteers providing the samples. The milk had been purchased from
86 either retail suppliers or doorstep providers primarily within three UK counties (Nottinghamshire,
87 Derbyshire, and Leicestershire). Volunteers were provided with a sterile 50 ml tube and detailed
88 instructions on how to take the sample, including specifying that it must be taken from a previously
89 unopened carton (carton size not specified) that was shaken to uniformly mix the contents before
90 samples were taken and that the 50 ml sample should be delivered to the laboratory within two
91 days of purchase. Details of the date of pasteurisation and retail supplier were also recorded, but
92 are not discussed here.

93 **2.2 Total viable count**

94 The total viable count (TVC) was performed using milk count agar according to Botsaris et al. (2013).
95 Serial dilutions of the milk sample were prepared and samples (100 μ l) plated on plate count agar
96 before incubating aerobically at 30 °C for 3 d prior to enumeration of colonies and calculation of cfu
97 ml^{-1} .

98 **2.3 Phage-PCR assay**

99 Briefly, 25 or 50 ml of milk was centrifuged at 2,500 $\times g$ for 15 min to separate the pellet, milk and
100 cream layers. The upper two layers were removed, and the pellet resuspended in 3 ml Modified
101 Middlebrook 7H9 media plus (MP; Middlebrook 7H9 broth supplemented with 10 % OADC (oleic
102 acid, bovine albumin, dextrose and catalase; Becton Dickinson, UK), NOA (nystatin, oxacillin and
103 aztreonam Mole et al., 2007), and 2 mM CaCl_2) and centrifuged at 2,500 $\times g$ for 10 min. The resulting
104 pellet was finally resuspended in 1 ml of MP and mycobacteriophage D29 (100 μ l of 10^9 pfu ml^{-1}

105 phage suspension) added to the sample. After incubation at 37 °C for 1 h a virucide (100 µl of 10
106 mM ferrous ammonium sulphate) was added and the sample incubated for 6 min at room
107 temperature to destroy any exogenous phage. To inactivate the virucide 5 ml of MP was added, and
108 the sample finally plated with *M. smegmatis* (1 ml of 10⁸ cfu ml⁻¹ culture grown in MP) and 6 ml of
109 Middlebrook 7H10 agar. Plates were then incubated for 24 h and plaques enumerated. DNA was
110 extracted from plaque agar using Zymoclean Gel DNA Recovery kit (Zymo Research) and the
111 presence of MAP DNA detected by amplification of MAP-specific IS900 region using a nested-PCR
112 (Bull et al., 2003) with particular care being taken during sample preparation and handling to ensure
113 that cross-contamination did not occur. As controls, plaques generated from the detection of *M.*
114 *smegmatis* (non-MAP DNA control) and sterile water (no template control) were included for all sets
115 of PCR reactions. Genomic MAP (K10) DNA was used as an additional positive control. Only samples
116 that produced a positive IS900 plaque-PCR result were classified as containing viable MAP cells.

117

118 **2.4 Investigation of location of MAP cells in milk**

119 One litre of raw milk was taken and split into twenty 50 ml samples and ten of these were tested
120 using the phage assay (Section 2.3) for the presence of mycobacteria (termed 'whole' samples). Prior
121 to conducting the phage assay pellet is washed with MP which lyses any somatic cell present and
122 releases intracellular mycobacteria (Swift et al., 2013, Donnellan et al., 2017). The other ten samples
123 were prepared for the phage assay in the same way, but after resuspending in MP each of these
124 samples were further split into two 25 ml portions (termed 'paired' samples) and each was tested
125 separately using the phage assay (Section 2.3).

126 **2.5 Statistical Analysis**

127 Pfu and cfu counts were compared using Pearson correlation and MAP status (positive or negative)
128 with total viable count (TVC) by performing a t-test in IBM SPSS Statistics 22 (SPSS Inc., Chicago,

129 USA). Further analysis of the correlation between the pfu counts in the paired split bulk tank milk
130 samples was conducted in Microsoft Excel 2010. Details of the statistical methods used to determine
131 the complete spatial randomness (CSR) of cells in bulk tank milk are provided in the text.

132

133 **3.0 Results and Discussions**

134 ***3.1 Detection of MAP in pasteurised milk***

135 The phage-PCR assay detected viable MAP in 10.3 % (37/368) of the pasteurised milk samples
136 collected as defined by the presence of plaques which gave a positive IS900-PCR result (see Figs. 1 &
137 2). Figure 1 shows the number of MAP-positive samples recorded at each of the four sampling time
138 points (approximately 90 samples at each time point). The results show that although the overall
139 average percentage of MAP-positive samples detected was 10.3 %, there was a large variation in the
140 number of positive samples detected at each time point (range 1-27 %), but there was no seasonal
141 pattern. This is consistent with the results of Grant et al. (2002) who detected variable levels of MAP
142 in retail pasteurised milk using a direct PCR-method (average 10 %, range 0-27 %; Grant et al.,
143 2002a).

144 Since each plaque arising from the phage-PCR assay represents detection of one viable MAP cell,
145 counting the plaques allows enumeration of MAP as well as absolute detection. Figure 2 shows the
146 distribution of plaque numbers detected in MAP-positive samples collected throughout the survey.
147 Only 1.1 % of these contained more than 10 detectable MAP cells per 50 ml (range 10-32). It is
148 known that chemical decontamination methods reduce the number of MAP cells detectable by
149 culture by least 1 \log_{10} giving a low limit of detection (Bradner et al., 2013). Therefore it is unlikely
150 that samples that contained less than 10 MAP cells per ml would have given a culture-positive result
151 in previous raw milk studies. On this basis, given that other published surveys of retail pasteurised

152 milk generally report levels of detection in the 1-2 % range (Grant et al., 2002a), our results are in
153 line with previous culture-based studies.

154 Direct PCR detection of MAP in milk is believed to be more sensitive than culture, but even then
155 reported limits of detection are relatively high, ranging from 15-50 cfu per 50 ml (Gao et al., 2007) to
156 10^5 cfu ml⁻¹ (Grant et al., 2000). However methods have been reported for direct PCR detection of
157 MAP in faeces with a sensitivity as low as 2.4 cfu per g of sample (Logar et al., 2012) and therefore
158 detection of very low numbers of cells is possible from complex samples. In this study, 3.5 % of
159 MAP-positive samples (1.1 % >10 plus 2.4 % 3-9) contained more than 2 cells and therefore would
160 theoretically be detectable by direct PCR. Previous published surveys of retail pasteurised milk
161 samples by direct PCR have reported a prevalence of MAP detection of between 7 and 12 % (Grant
162 et al., 2002b), thus our results are within the range predicted by the published literature. However it
163 must be noted that direct PCR-methods used in these other surveys do not discriminate between
164 viable cells and cells that have been inactivated by pasteurisation, and therefore the MAP-positive
165 samples would also include samples where DNA from inactivated cells were detected, perhaps
166 explaining the high prevalence reported in some of these direct PCR studies.

167 The largest percentage of samples in our dataset (6.8 %) contained 1-2 detectable MAP cells. Given
168 that these samples contained fewer cells than the reported limit of detection of either culture or
169 PCR-based detection, this result suggests that the level of MAP present would not be detected by
170 either of these methods and that the phage-PCR assay is a more sensitive technique. This is
171 consistent with our previous findings when low levels of MAP were detected in blood samples from
172 infected animals that could not be detected by either culture or using a commercial direct PCR kit
173 (Swift et al., 2013). Similarly in two recent surveys of the prevalence of viable MAP cells in
174 powdered milk, the phage-based methods always detected more positive samples than either
175 culture or direct PCR (Botsaris et al., 2016; Grant et al. 2017).

176 Commercial pasteurisation has been shown by many surveys of retail milk to not be sufficient to
177 inactivate all MAP cells present in raw milk. Although determinations of D-values indicated MAP may
178 survive HTST pasteurisation when the initial organism concentration is greater than 1×10^4 cells ml^{-1}
179 (Grant et al., 1996), many other studies have shown that the cells are not intrinsically resistant to
180 heat and pasteurisation should result in a seven-fold reduction in viable MAP (Rademaker et al.,
181 2007). However Grant et al. (1996) has previously reported that after an initial rapid drop in viable
182 cell number, a 'long tail' in inactivation curves is seen whereby low numbers of viable MAP cells in a
183 sample survive, even after heating for an extended period of time. This type of kinetics indicates
184 that there is a heat-resistant sub-population, and would be consistent with our observation that the
185 largest number of samples contained only 1-2 detectable MAP cells. One possible explanation for
186 this phenomenon could be if the MAP cells were internalised within somatic cells which provided
187 some protection against heat inactivation.

188 ***3.2 Evidence that MAP in milk is primarily intracellular***

189 ***3.2.1 Comparison of TVC and Phage-PCR***

190 It has been well established that low level faecal contamination of milk can occur during routine
191 milking practises (Vissers et al., 2007), therefore providing a route of entry for MAP into raw milk,
192 and it would be expected that these cells would be released into the milk along with other enteric
193 bacteria. Since we have previously shown that MAP present in the circulating blood is located
194 within white blood cells (Swift et al., 2013), MAP cells entering the milk from the udder may be
195 expected to be intracellular, either within infected somatic cells or macrophages. Total viable count
196 is a common method used to determine the level of faecal contamination and therefore hygienic
197 status in milk. To provide evidence that the MAP cells being detected in the somatic cell pellet
198 represented cells being shed directly into the milk rather than being introduced by faecal
199 contamination, a comparison was made of the TVC of bulk tank milk samples and levels of MAP
200 detected using the phage-PCR assay. The samples were collected from 225 separate farms as

201 previously described by Botsaris et al. (2013). Since the phage D29 has a very broad host range the
202 phage assay results (plaque number) report on the total number of viable mycobacteria present in a
203 sample. Figure 3a shows a comparison of the TVC results for all samples that were found to contain
204 mycobacteria due to the formation of plaques ($n = 218$). These samples were then further stratified
205 in to MAP-positive and MAP-negative as determined by the detection or non-detection of the *IS900*
206 genetic element by PCR in the DNA extracted from plaques, respectively. The number of plaques
207 detected for the samples in these two groups were then compared with the TVC results for each of
208 the samples (Fig. 3b). As there was no significant difference in the mean TVC value of the MAP-
209 positive samples and those that contained mycobacteria but with no MAP detected (approx. 1×10^4
210 cfu ml^{-1} ; $p = 0.276$), this suggests that there is no relationship between the likelihood that MAP will
211 be detected and the level of faecal contamination. Therefore the presence of MAP does not appear
212 to correlate with the hygienic status of the milk, suggesting that faecal contamination is not the main
213 route of entry of the MAP into the bulk milk. Similarly when TVC and the plaque number results of
214 the individual MAP-positive samples are compared, there is no correlation ($p = 0.270$) between the
215 number of plaques detected in a MAP-positive sample and the TVC (Fig. 3b) again indicating that a
216 high plaque count, which is associated with a high probability that MAP will be present in a sample
217 (Botsaris et al., 2013), is not due to faecal contamination. These results are in agreement with a
218 previous study that showed that there was no correlation between the total bacteriological count,
219 somatic cell count and the detection of *IS900* sequences by PCR in a survey of bulk tank milk (Corti
220 and Stephan, 2002) and therefore faecal contamination alone cannot account for the presence of
221 MAP in raw milk and rather suggests that these cells are being directly shed into the milk.

222 ***3.2.2 Evidence for asymmetric distribution of MAP in raw milk due to intracellular location***

223 If MAP is not present in the milk due to faecal contamination, it must have entered the milk by a
224 different route. As an intracellular pathogen, one such route is via infected somatic cells which are
225 shed into the milk, and in this case it is predicted that the cells would not be free in the milk matrix,

226 but would be clustered together inside the infected cells, since intracellular MAP have been shown
227 to located together inside vacuoles (Bannantine and Stabel, 2002).

228 To provide evidence that the MAP being detected in raw milk were primarily intracellular, raw bulk
229 tank milk was obtained which was likely to contain viable MAP cells from a farm with a known
230 Johne's disease problem. To investigate the spatial distribution of MAP, the variation in number of
231 mycobacteria present in individual samples taken from bulk tank milk was investigated. To do this
232 ten 50 ml samples were taken from one litre of raw milk and were tested using the standard phage
233 assay method for the presence of mycobacteria (termed 'whole' samples). To determine if variability
234 in the number of Mycobacteria detected was due an asymmetric distribution of cells in the milk
235 rather than the sampling method, another ten samples were prepared for the phage assay, but after
236 the pellet had been washed with MP which lyses any somatic cells and releases the mycobacteria
237 into the media, each of these samples was then further split into two portions (termed 'paired'
238 samples) and each of these was tested separately using the phage assay (see Fig. 4A for
239 experimental plan). The results in Figure 4B shows that for each of the two groups of samples
240 ('whole' or 'paired'), the median number of mycobacteria detected per 50 ml was the same (50 vs
241 49) and the range of the plaque numbers detected within each group of samples was also very
242 similar (range for 'whole' samples = 16-145 per 50 ml; range for paired samples = 13-131 per 50 ml).
243 These results showed that processing the samples in two different ways did not affect the overall
244 number of mycobacteria detected per 500 ml but that there was quite a large range in the number
245 of mycobacteria detected per 50 ml sample, typical of the pattern seen when low numbers of cells
246 are asymmetrically distributed in a sample.

247 When the numbers of mycobacteria detected in the paired samples is examined, it can be seen that
248 although there is still variation in the number of mycobacterial detected in individual 50 ml samples,
249 there is a good correlation between the number of mycobacteria detected for each of the pairs
250 when individual 50 ml samples were further split into two samples ($r^2 = 0.79$; Fig. 4C), showing that

251 the method reproducibly detects mycobacteria in samples predicted to contain the same number of
252 free Mycobacteria cells.

253 It is not possible to carry out the same simple correlation analysis for the number of mycobacteria
254 detected in the replicate 'whole' samples as there is no non-arbitrary method that can be used to
255 pair them. Hence two statistical analyses were performed to examine a null hypothesis of complete
256 spatial randomness (CSR) of cell distribution within the bulk milk when samples are split into sub-
257 samples by these two different methods. The first analysis examined the number of bacteria present
258 in each of the 50 ml samples, and the data set used consisted of ten 'whole' samples, and (by
259 summing the counts in each of the two paired samples) ten 'paired samples', giving 20 samples in
260 total. To analyse the initial allocation into 50 ml sub-samples, a Pearson Chi-squared test was used,
261 in which there are 19 d.f. and the predicted number of cells in each sample would be 59.4 (i.e. the
262 total number of cells detected divided by 20). This test essentially assumes that the counts in each
263 subsample are drawn from a Poisson distribution with mean equal to 59.4. The result of this analysis
264 was that this null hypothesis of CSR across the subsamples was strongly rejected ($p < 0.001$) and
265 suggests that the distribution of mycobacteria present in the bulk tank milk is uneven so that when
266 an individual 50 ml sample is taken there is likely to be a significant variation in the numbers of
267 mycobacteria detected.

268 The second analysis used a permutation test to compare the total number of mycobacteria detected
269 in the 'paired' samples with that expected from a random (binomial) allocation. Expressing each of
270 the 10 allocations as a proportion $p_i = N_{1i} / N_{Ti}$ of the total $N_{Ti} = N_{1i} + N_{2i}$ of the two halves for that
271 sample, 10,000 sets of equivalent proportions were generated from a binomial distribution. Each of
272 these sets takes the form $p_i^* = r_i / N_{Ti}$ where $r_i \sim B(N_{Ti}, 0.5)$ for $i = 1, \dots, 10$, thus being a random allocation
273 of the same total count as the corresponding sample. For each of these sets, a summary statistic - in
274 this case the variance of these proportions - is calculated. The position (order statistic) of the
275 variance of the proportions from the data within this list of 10,000 variances from the binomial

276 allocations, gives the p-value. As a two-tailed test is to be used, the smaller of the two order
277 statistics are taken and doubled to obtain the final p-value, which was $p=0.625$. Thus for the 'paired'
278 samples the null hypothesis of randomness cannot be rejected, since the evidence of randomness
279 seen in the initial analysis is no longer present after this lysis step has been carried out to release the
280 mycobacteria into the media.

281 The difference in the treatment of the 'whole' and 'paired' samples was rather than resuspending
282 the pellet in 1 ml and independently testing each 50 ml 'whole' sample, the pellet from the 'paired'
283 samples was resuspended into 2 ml of media which lyses the somatic cells releasing the
284 Mycobacteria into the media before further splitting into two samples (see Fig. 4A). The overall
285 plaque number detected per 50 ml sample was not affected by the change in sample preparation
286 method (Fig. 4B), this pattern of results could be best explained if the mycobacteria in the original
287 milk were not free in the milk matrix but were located inside somatic cells, explaining the
288 heterogeneous distribution of MAP cells in the 50 ml milk samples. This hypothesis is consistent
289 with the fact that Patel et al. (2006) have shown that bovine mammary epithelial cells can contain up
290 to 10^4 MAP cells after infection, and MAP are known to be able to replicate within macrophage
291 (Arsenault et al., 2014), so it is also likely that MAP-infected somatic cells will not contain only a
292 single bacterium.

293 The results of the survey carried out in this study, and the results of other published surveys of retail
294 pasteurised milk, clearly show that MAP in retail milk is able to survive pasteurisation. Laboratory
295 studies of heat inactivation of MAP milk have consistently shown that HTST pasteurisation
296 conditions are effective at reducing the levels of viable MAP by 3-6 \log_{10} (McDonald et al., 2005).
297 Since levels of MAP previously reported in milk from infected cows are generally not reported to be
298 more than 10^2 cfu ml⁻¹ MAP (Slana et al., 2008; Sweeney et al., 1992), pasteurisation should achieve
299 complete inactivation of MAP. However these heat inactivation studies used laboratory grown
300 bacteria that were artificially added to milk. Our results suggest that MAP in raw milk is primarily

301 located inside somatic cells which are made up of polymorphonuclear leukocytes, macrophages,
302 lymphocytes and a small number of mammary epithelial cells (Boutinaud and Jammes, 2002).
303 Despite the fact that it has never been demonstrated that MAP cells found intracellularly are
304 resistance to heat, it has previously been shown that MAP surviving inside amoeba are
305 approximately 2-fold more resistant to chlorine disinfection (Whan et al., 2006) and this in part is due
306 to the physical protection provided by the large mass of the host cell surrounding the bacteria
307 (amoeba = 15-35 μm). Although further experiments are required to confirm this hypothesis, it is
308 therefore possible that the survival of low levels of MAP during pasteurisation of naturally infected
309 milk could be as a result of their intracellular location, with the somatic cell providing sufficient
310 protection to prevent complete inactivation of the bacteria by heat treatment. If some of these
311 infected cells do contain high levels of MAP cells (Patel et al., 2006), this would increase the
312 likelihood that the low numbers of survivors detected after pasteurisation represent a residual
313 population of cells that have been physically protected from the heat treatment, especially as it has
314 been previously reported that high numbers of MAP cells can confer heat resistance during
315 pasteurisation (Klijn et al., 2001).

316

317 **4. Conclusion**

318 The possible association between MAP and Crohn's disease has been discussed for many years
319 (Feller et al., 2007; Rhodes et al., 2014), with pasteurised milk thought to be a key vehicle of
320 transmission to humans. One of the advantages of using phage to detect bacteria is that the
321 bacterial cell must be viable before detection can occur and therefore, unlike PCR-based methods,
322 the phage-based method differentiates between live and dead cells (Stanley et al., 2007; Swift et al.,
323 2014). This makes the method very useful for studying pasteurised products which could contain
324 both viable and inactivated MAP cells. In this study the phage-PCR assay was shown to be able to
325 detect low levels of viable MAP in pasteurised milk with 10 % of the samples found to contain viable

326 MAP. The prevalence of MAP was higher than that reported in a number of other published studies
327 that used culture to detect MAP (1.7 to 6.7%) but this does not suggest that our samples had a
328 higher prevalence rate, just that the phage-PCR assay is more sensitive and therefore increases the
329 frequency with which samples containing very low numbers of MAP cells give a positive result. The
330 specificity of the assay is demonstrated by the end-point PCR, which has been previously
331 demonstrated as being specific for MAP (Bull et al., 2003). Given the rapidity of this method, there
332 is now potential to perform a larger trial to ascertain ability of this method to monitor the efficacy of
333 milk pasteurisation processes on a larger scale. There is a clear need for further research to be
334 carried out to fully understand this hypothesised mechanism of survival and the fact that
335 mycobacteria that reside inside eukaryotic cells can be protected from external sources of stress.
336 Here we have demonstrated the ability to rapidly detect and enumerate MAP using the phage assay
337 providing a useful tool to allow such studies to be completed without the need for prolonged
338 incubation of cultures required when using traditional culture methods.

339

340 **Acknowledgements**

341 Z.E.G was funded by a joint studentship between the University of Nottingham and SRUC. B.M.C.S
342 was funding by a University of Nottingham Hermes Innovation. G.B was sponsored by an EU
343 research scholarship. Thanks is extended to staff and students who supplied samples of retail
344 pasteurised milk samples for the study, and to a local farm for supplying the bulk tank milk. SRUC
345 receives support from the Scottish Government

346

347 **References**

348 Antognoli, M.C., Garry, F.B., Hirst, H.L., Lombard, J.E., Dennis, M.M., Gould, D.H., Salman, M.D.,
349 2008. Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection

350 in dairy cattle and its association with antemortem test results. *Veterinary Microbiology* 127, 300-
351 308.

352

353 Arsenault, R.J., Maattanen, P., Daigle, J., Potter, A., Griebel, P., Napper, S., 2014. From mouth to
354 macrophage: mechanisms of innate immune subversion by *Mycobacterium avium* subsp
355 *paratuberculosis*. *Veterinary Research* 45.

356

357 Ayele, W.Y., Svastova, P., Roubal, P., Bartos, M., Pavlik, I., 2005. *Mycobacterium avium* subspecies
358 *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech
359 Republic. *Applied and Environmental Microbiology*. 71, 1210-1214.

360

361 Bannantine, J.P., Stabel, J.R., 2002. Killing of *Mycobacterium avium* subspecies *paratuberculosis*
362 within macrophages. *BMC Microbiology* 2.

363

364 Botsaris, G., Liapi, M., Kakogiannis, C., Dodd, C.E.R., Rees, C.E.D., 2013. Detection of *Mycobacterium*
365 *avium* subsp *paratuberculosis* in bulk tank milk by combined phage-PCR assay: Evidence that plaque
366 number is a good predictor of MAP. *International Journal of Food Microbiology*. 164, 76-80.

367

368 Botsaris, G., Slana, I., Liapi, M., Dodd, C., Economides, C., Rees, C., Pavlik, I., 2010. Rapid detection
369 methods for viable *Mycobacterium avium* subspecies *paratuberculosis* in milk and cheese.
370 *International Journal of Food Microbiology*. 141, S87-S90.

371

372 Botsaris, G., Swift, B.M., Slana, I., Liapi, M., Christodoulou, M., Hatzitofi, M., Christodoulou, V., Rees,
373 C.E., 2016. Detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in powdered
374 infant formula by phage-PCR and confirmed by culture. *Internatinoal Journal of Food Microbiology*.
375 216, 91-94.

376

377 Boutinaud, M., Jammes, H., 2002. Potential uses of milk epithelial cells: a review. *Reproduction*
378 *Nutrition Development*. 42, 133-147.

379

380 Bradner, L., Robbe-Austerman, S., Beitz, D.C., Stabel, J.R., 2013. Optimization of Hexadecylpyridinium
381 Chloride Decontamination for Culture of *Mycobacterium avium* subsp. *paratuberculosis* from Milk.
382 *Journal of Clinical Microbiology*. 51, 2804-2804.

383

384 Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R.,
385 Hermon-Taylor, J., 2003. Detection and verification of *Mycobacterium avium* subsp *paratuberculosis*
386 in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease.
387 *Journal of Clinical Microbiology*. 41, 2915-2923.

388

389 Chiodini, R.J., Hermon-Taylor, J., 1993. The Thermal-Resistance of *Mycobacterium-Paratuberculosis*
390 in Raw-Milk under Conditions Simulating Pasteurization. *Journal of Veterinary Diagnostic*
391 *Investigation*. 5, 629-631.

392

393 Corti, S., Stephan, R., 2002. Detection of *Mycobacterium avium* subspecies *paratuberculosis* specific
394 IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout
395 Switzerland. *BMC Microbiology* 2.

396

397 Donnellan, S., Stone, V., Johnston, H., Giardiello, M., Owen, A., Rannard, S., Aljayyousi, G., Swift, B.,
398 Tran, L., Watkins, C., and Stevenson, K., 2017 Intracellular delivery of nano-formulated
399 antituberculosis drugs enhances bactericidal activity. *Journal of Interdisciplinary Nanomedicine*, 2:
400 146–156.

401

402 Dundee, L., Grant, I.R., Ball, H.J., Rowe, M.T., 2001. Comparative evaluation of four decontamination
403 protocols for the isolation of *Mycobacterium avium* subsp *paratuberculosis* from milk. *Letters in*
404 *Applied Microbiology*. 33, 173-177.

405

406 Feller, M., Huwiler, K., Stephan, R., Altpeter, E., Shang, A., Furrer, H., Pfyffer, G.E., Jemmi, T.,
407 Baumgartner, A., Egger, M., 2007. *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's
408 disease: a systematic review and meta-analysis. *Lancet Infectious Diseases*. 7, 607-613.

409

410 Gao, A., Mutharia, L., Raymond, M., Odumeru, J., 2007. Improved template DNA preparation
411 procedure for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk by PCR. *Journal of*
412 *Microbiological Methods*. 69, 417-420.

413

414 Grant, I.R., Ball, H.J., Neill, S.D., Rowe, M.T., 1996. Inactivation of *Mycobacterium paratuberculosis* in
415 cows' milk at pasteurization temperatures. *Applied and Environmental Microbiology*. 62, 631-636.

416

417 Grant, I.R., Pope, C.M., O'Riordan, L.M., Ball, H.J., Rowe, M.T., 2000. Improved detection of
418 *Mycobacterium avium* subsp. *paratuberculosis* in milk by immunomagnetic PCR. *Veterinary*
419 *Microbiology*. 77, 369-378.

420

421 Grant, I.R., Ball, H.J., Rowe, M.T., 2002a. Incidence of *Mycobacterium paratuberculosis* in bulk raw
422 and commercially pasteurized cows' milk from approved dairy processing establishments in the
423 United Kingdom. *Applied and Environmental Microbiology*. 68, 2428-2435.

424

425 Grant, I.R., Hitchings, E.I., McCartney, A., Ferguson, F., Rowe, M.T., 2002b. Effect of commercial-scale
426 high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in
427 naturally infected cows' milk. *Applied and Environmental Microbiology*. 68, 602-607.

428

429 Grant, I.R., Foddai, A.C.G., Tarrant, J.C., Kunkel, B., Hartmann, F.A., McGuirk, S., Hansen, C., Talaat,
430 A.M., Collins, M.T, 2017. Viable *Mycobacterium avium* ssp. *paratuberculosis* isolated from calf milk
431 replacer. *Journal of Dairy Science*. 100, 9723 - 9735.

432

433 King, C.H., Shotts, E.B., Wooley, R.E., Porter, K.G., 1988. Survival of Coliforms and Bacterial
434 Pathogens within Protozoa during Chlorination. *Applied and Environmental Microbiology*. 54, 3023-
435 3033.

436

437 Klijn, N., Herrewegh, A.A., de Jong, P., 2001. Heat inactivation data for *Mycobacterium avium* subsp.
438 *paratuberculosis*: implication for interpretation. *Journal of Applied Microbiology*. 4, 697-704.

439

440 Logar, K., Kopinc, R., Bandelj, P., Staric, J., Lapanje, A., Ocepek, M., 2012. Evaluation of combined
441 high-efficiency DNA extraction and real-time PCR for detection of *Mycobacterium avium* subsp
442 *paratuberculosis* in subclinically infected dairy cattle: comparison with faecal culture, milk real-time
443 PCR and milk ELISA. *BMC Veterinary Research*. 8.

444

445 McDonald, W.L., O'Riley, K.J., Schroen, C.J., Condron, R.J., 2005. Heat inactivation of *Mycobacterium*
446 *avium* subsp *paratuberculosis* in milk. *Applied and Environmental Microbiology*. 71, 1785-1789.

447

448 Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T., HermonTaylor, J., 1996. IS900 PCR
449 to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cows' milk in
450 England and Wales. *Applied and Environmental Microbiology*. 62, 3446-3452.

451

- 452 Mole, R., Trollip, A., Abrahams, C., Bosman, M., Albert, H., 2007. Improved contamination control for
453 a rapid phage-based rifampicin resistance test for *Mycobacterium tuberculosis*. *Journal of Medical*
454 *Microbiology*. 56, 1334-1339.
- 455
- 456 NATIONAL ADVISORY COMMITTEE ON MICROBIOLOGICAL CRITERIA FOR FOODS (2010) Assessment of Food as
457 a Source of Exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) *Journal of Food Protection*,
458 73, 1357–1397
- 459
- 460 Paolicchi, F., Cirone, K., Morsella, C., Gioffre, A., 2012. First Isolation of *Mycobacterium Avium* Subsp
461 *Paratuberculosis* from Commercial Pasteurized Milk in Argentina. *Brazilian Journal of Microbiology*.
462 43, 1034-1037.
- 463
- 464 Patel, D., Danelishvili, L., Yamazaki, Y., Alonso, M., Paustian, M.L., Bannantine, J.P., Meunier-Goddik,
465 L., Bermudez, L.E., 2006. The ability of *Mycobacterium avium* subsp *paratuberculosis* to enter bovine
466 epithelial cells is influenced by preexposure to a hyperosmolar environment and intracellular
467 passage in bovine mammary epithelial cells. *Infection and Immunity*. 74, 2849-2855.
- 468
- 469 Rademaker, J.L.W., Vissers, M.M.M., Giffel, M.C.T., 2007. Effective heat inactivation of
470 *Mycobacterium avium* subsp *paratuberculosis* in raw milk contaminated with naturally infected
471 feces. *Applied and Environmental Microbiology*. 73, 4185-4190.
- 472
- 473 Rhodes, G., Richardson, H., Hermon-Taylor, J., Weightman, A., Higham, A., Pickup, R., 2014.
474 *Mycobacterium avium* Subspecies *paratuberculosis*: Human Exposure through Environmental and
475 Domestic Aerosols. *Pathogens*. 3, 557-595.
- 476

- 477 Slana, I., Kralik, P., Kralova, A., Pavlik, I., 2008. On-farm spread of *Mycobacterium avium* subsp
478 *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and
479 culture examination. *International Journal of Food Microbiology*. 128, 250-257.
- 480
- 481 Stabel, J.R., Lambertz, A., 2004. Efficacy of pasteurization conditions for the inactivation of
482 *Mycobacterium avium subsp paratuberculosis* in milk. *Journal of Food Protection*. 67, 2719-2726.
- 483
- 484 Stanley, E.C., Mole, R.J., Smith, R.J., Glenn, S.M., Barer, M.R., McGowan, M., Rees, C.E.D., 2007.
485 Development of a new, combined rapid method using phage and PCR for detection and
486 identification of viable *Mycobacterium paratuberculosis* bacteria within 48 hours. *Applied and*
487 *Environmental Microbiology*. 73, 1851-1857.
- 488
- 489 Sweeney, R.W., Collins, M.T., Koets, A.P., McGuirk, S.M., Roussel, A.J., 2012. Paratuberculosis
490 (Johne's Disease) in Cattle and Other Susceptible Species. *Journal of Veterinary Internal Medicine*.
491 26, 1239-1250.
- 492
- 493 Sweeney, R.W., Whitlock, R.H., Rosenberger, A.E., 1992. Mycobacterium-Paratuberculosis Cultured
494 from Milk and Supramammary Lymph-Nodes of Infected Asymptomatic Cows. *Journal of Clinical*
495 *Microbiology*. 30, 166-171.
- 496
- 497 Swift, B.M., Denton, E.J., Mahendran, S.A., Huxley, J.N., Rees, C.E., 2013. Development of a rapid
498 phage-based method for the detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in
499 blood within 48 h. *Journal of Microbiological Methods*. 94, 175-179.
- 500
- 501 Swift, B.M., Gerrard, Z.E., Huxley, J.N., Rees, C.E., 2014. Factors Affecting Phage D29 Infection: A Tool
502 to Investigate Different Growth States of Mycobacteria. *PLoS One*. 9, e106690.

503

504 Swift, B.M., Rees, C.E., 2013. Detecting mycobacteria in cattle blood. *Veterinary Record*. 173, 522-

505 523.

506

507 Vissers, M.M.M., Driehuis, F., Te Giffel, M.C., De Jong, P., Lankveld, J.M.G., 2007 Short

508 Communication: Quantification of the transmission of microorganisms to milk via dirt attached to

509 the exterior of teats. *Journal of Dairy Science*. 90, 3579-3582.

510

511 Whan, L., Grant, I.R., Rowe, M.T., 2006. Interaction between *Mycobacterium avium* subsp

512 *paratuberculosis* and environmental protozoa. *BMC Microbiology*. 6.

Figure 1. Seasonality of MAP positive samples isolated from pasteurised milk

Dashed line represents the average percentage prevalence of viable MAP detected in pasteurised milk over the four sampling periods. The graph shows the seasonal variation in the frequency of MAP-positive samples detected using the phage-PCR assay.

ACCEPTED MANUSCRIPT

Figure 2. Distribution of the number of viable MAP samples across the whole survey

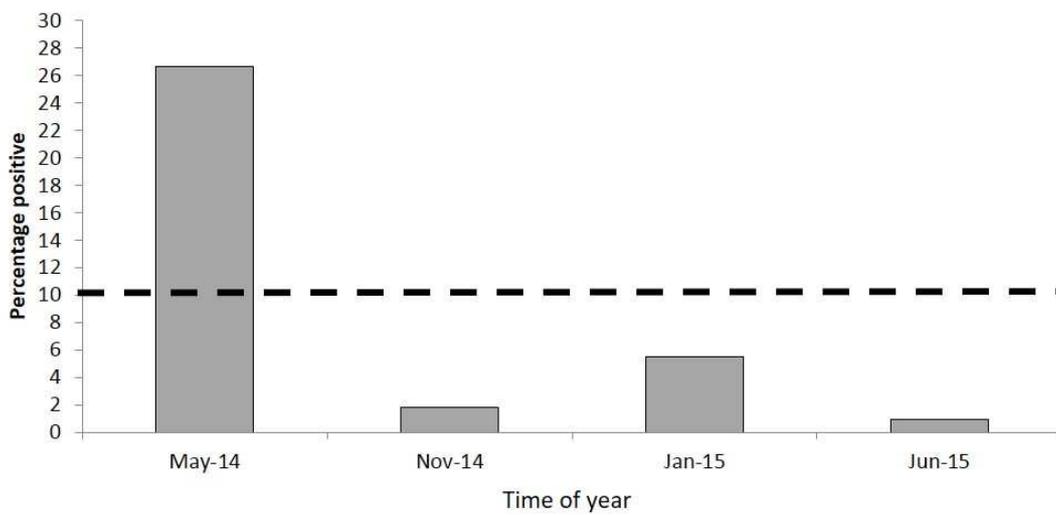
A total of 38 MAP-positive samples were detected, defined as those that produced plaques that were IS900-PCR positive. Data were grouped by plaque number per 50 ml sample and the results for each group reported as percentage of the total sample set (n = 368 semi skimmed (1.7 % fat) milk). The groupings were based on the reported sensitivities of other methods *i.e.* 1-2 cells, no other method reports being able to detect this low level, 3-9 cells and >10 cells, potentially detectable by direct PCR; >10 cells, potentially detectable by culture.

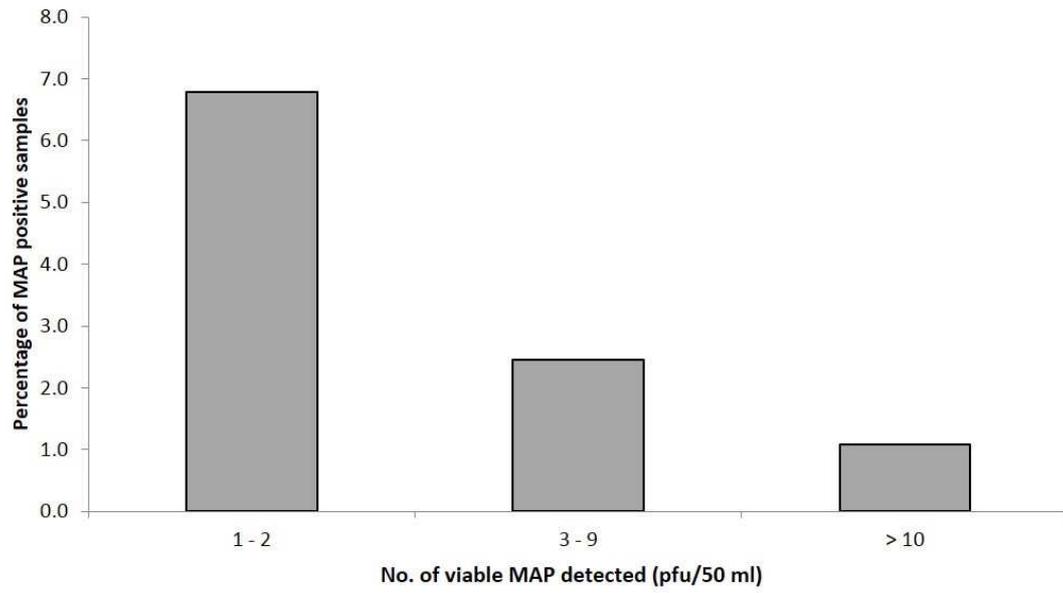
Figure 3. Comparison of MAP status and TVC of bulk milk samples

Samples of raw bulk tank milk ($n = 225$) were tested using the phage-PCR assay and the TVC of each sample was separately determined. Samples were classified as MAP-positive or MAP-negative according to the results of the Phage-IS900 PCR assay. Panel A. For each data set, the distribution of TVC values is represented by a Box and Whisker plot. An independent samples t-test revealed no significant difference between the TVC results of the MAP-positive samples and the MAP-negative samples ($p=0.276$). Panel B. MAP-positive samples are shown as open circles and MAP-negative samples are shown as open squares. For each group, the distribution of TVC values is compared with the plaque count for individual samples. For the MAP-positive samples, a Pearson correlation indicated no significant relationship existed between these two variables ($p=0.270$).

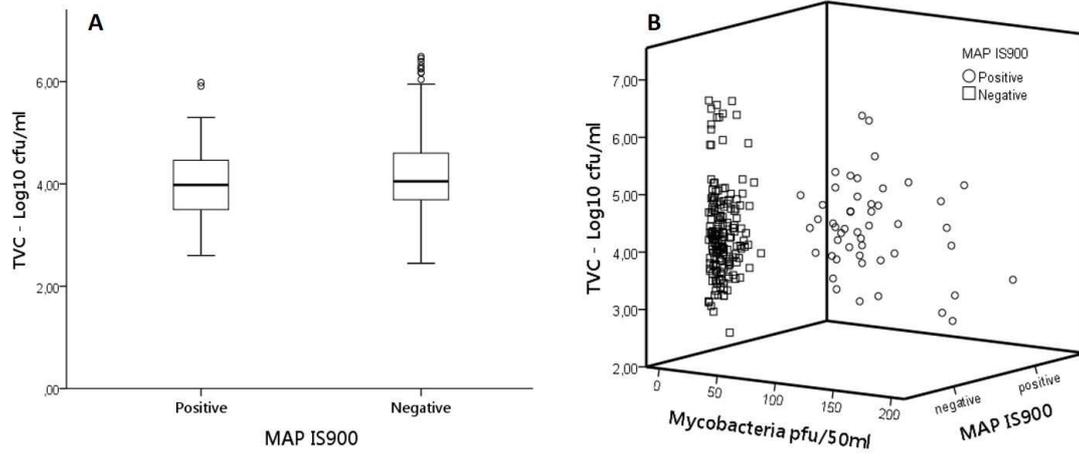
Figure 4. Distribution of viable MAP in whole milk samples with and without somatic cell lysis

Panel A. One litre of raw milk split into twenty 50 ml samples. Ten of these were tested using the phage assay for the presence of mycobacteria ('whole' samples). The other ten samples were centrifuged, and the pellet resuspended in 2 ml MP to lyse the somatic cells. This sample was further split into two 1 ml samples ('paired' samples) before each one was tested separately using the phage assay. Panel B. Box and Whisker plot showing the median number and range of mycobacteria detected for 'whole' (mean 50, range 16 to 145) and 'paired' (mean 49, range 13 to 131) samples for the two different treatments. To normalise the data the results for the two different treatments for the 'paired' samples were multiplied by 2 as the equivalent of 25 ml and not 50 ml had been tested, before determining the median and range of data. Panel C. Plot to determine the correlation between plaque numbers detected in individual pairs of samples. Circles show the pfu results for the paired samples per 25 ml ($r^2 = 0.79$).

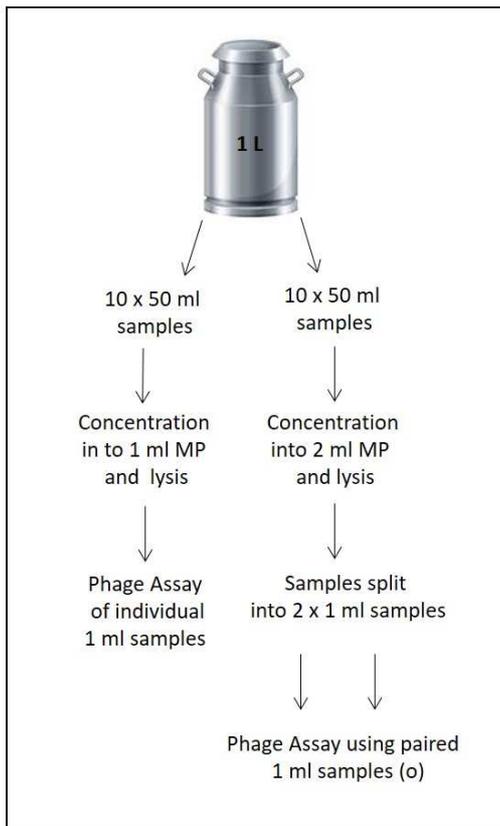




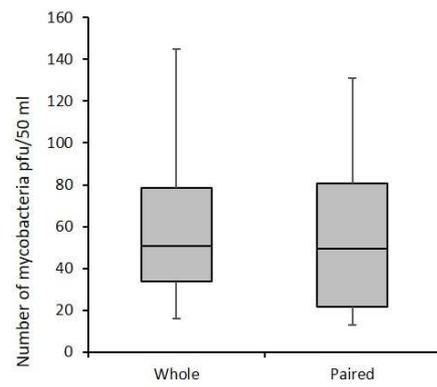
ACCEPTED MANUSCRIPT



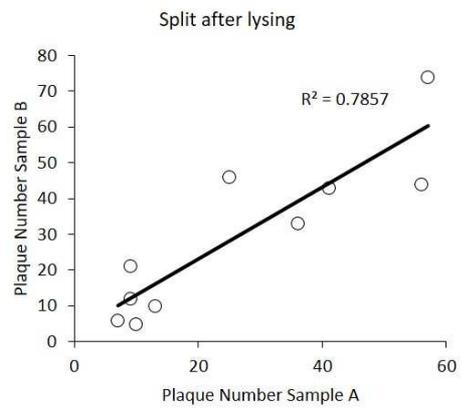
A



B



C



Highlights

- Survey of UK retail pasteurised milk for presence of viable MAP
- Use of phage-PCR to rapidly and sensitively detect viable MAP in milk
- Presence of viable *M. paratuberculosis* observed in 10 % of samples
- Intracellular MAP may aid their survival during pasteurisation