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## **An optimised milk testing protocol to ensure accurate enumeration of viable *Mycobacterium avium* subsp. *paratuberculosis* by the PMS-phage assay**

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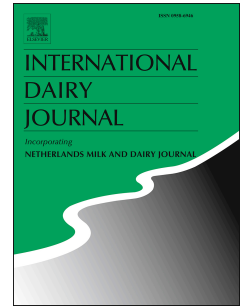
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# Accepted Manuscript

An optimised milk testing protocol to ensure accurate enumeration of viable *Mycobacterium avium* subsp. *paratuberculosis* by the PMS-phage assay

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1 **An optimised milk testing protocol to ensure accurate enumeration of viable**  
2 ***Mycobacterium avium* subsp. *paratuberculosis* by the PMS-phage assay**

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23

24 **Abstract**

25

26       There is interest in determining levels of *Mycobacterium avium* subsp.  
27 *paratuberculosis* (MAP) contamination in milk. The optimal sample preparation for  
28 raw cows' milk to ensure accurate enumeration of viable MAP by the peptide-  
29 mediated magnetic separation (PMS)-phage assay was determined. Results  
30 indicated that milk samples should be refrigerated at 4 °C after collection and MAP  
31 testing should commence within 24 h, or samples can be frozen at -70 °C for up to  
32 one month without loss of MAP viability. Use of Bronopol is not advised as MAP  
33 viability is affected. The vast majority (> 95%) of MAP in raw milk sedimented to the  
34 pellet upon centrifugation at 2500 × g for 15 min, so this milk fraction should be  
35 tested. De-clumping of MAP cells was most effectively achieved by ultrasonication of  
36 the resuspended milk pellet on ice in a sonicator bath at 37 kHz for 4 min in 'Pulse'  
37 mode.

38

39

40

## 41 1. Introduction

42

43 Due to its possible association with various human conditions, such as  
44 Crohn's disease and ulcerative colitis (Pierce, 2010) and Type 1 diabetes (Rosu et  
45 al., 2009), the presence of viable *Mycobacterium avium* subsp. *paratuberculosis*  
46 (MAP) cells in milk and dairy products is viewed as a potential food safety issue.  
47 MAP is the causative agent of Johne's disease, a contagious, chronic and  
48 sometimes fatal infection that primarily affects the small intestine of domestic  
49 ruminants such as cattle, sheep and goats (Stabel, 1998). Dairy cattle infected by  
50 MAP can shed live organisms in both milk and faeces, so potentially bulk milk could  
51 be contaminated either directly or indirectly by this bacterium. Whilst uncertainty  
52 remains about the public health/food safety significance of MAP, it would be prudent  
53 to monitor milk for presence of MAP to assess levels of contamination and hence  
54 potential for human exposure. The general desire is that our food should come from  
55 disease-free (including Johne's disease-free) animals (Regulation (EC) No  
56 853/2004; European Parliament, 2004).

57 There is interest amongst dairy producers and processors to identify a rapid  
58 method that could be adopted to detect the presence of viable MAP in raw milk or  
59 dairy products. Whilst a number of conventional and quantitative PCR methods, able  
60 to detect and estimate numbers of MAP in milk have been developed in recent years  
61 (Timms, Gehringer, Mitchel, Daskalopoulos, & Neilan, 2011), the problem remains of  
62 differentiating whether a PCR positive result is due to DNA from viable or dead cells  
63 (Hanna, Connor, & Wang, 2005). More recently, use of a phage-based test  
64 (*FASTPlaqueTB*<sup>TM</sup> assay, Biotec Laboratories Limited, Ipswich), as a quicker  
65 alternative to culture, for rapid detection of viable MAP was described (Stanley et al.,

2007); its application to test naturally infected milk and milk products was subsequently reported (Botsaris, Liapi, Kakogiannis, Dodd, & Rees, 2013; Botsaris et al., 2010). This phage amplification method exploits the ability of a mycobacteriophage to replicate and amplify only within viable mycobacterial cells. The number of viable cells can be quickly estimated within 24-48 h based on the count of plaques (lysed areas) produced when infected cells burst in a lawn of *Mycobacterium smegmatis* (indicator) cells in an agar plate. We have previously optimised the original *FASTPlaqueTB*<sup>TM</sup> test conditions to maximise detection sensitivity and achieve accurate enumeration (Foddai, Elliott, & Grant, 2009). A magnetic separation step using paramagnetic beads coated with two MAP-specific peptide binders (aMp3 and aMptD; Stratmann, Dohmann, Heinzmann, & Gerlach, 2006; Stratmann, Strommenger, Stevenson, & Gerlach, 2002) was introduced before the phage assay to add specificity for MAP (Foddai, Elliott, & Grant, 2010b), since the phage involved has a broader host range (Rybniker, Kramme, & Small, 2006). The peptide-mediated magnetic separation (PMS) both captures and concentrates MAP cells, separating them from non-target microorganisms and other possible inhibitors, thereby increasing the specificity and sensitivity of the subsequent phage assay.

In light of the possibility of the PMS-phage assay being adopted by the dairy industry for milk testing, certain aspects of the milk testing protocol being applied at Queen's University Belfast were investigated to ensure that milk sample storage and preparation before testing were optimal. The following were examined: a) effect of milk storage conditions prior to commencement of testing on the viability of MAP cells, and identification of the best milk storage conditions to be applied when immediate analysis of milk samples is not possible; b) the partitioning of MAP to milk

91 pellet, cream and whey fractions after centrifugation, to identify which milk fraction  
92 should be tested to most accurately determine MAP contamination levels; and c)  
93 potential to introduce a de-clumping step before PMS to maximise accuracy of MAP  
94 counts obtained with the phage assay. The optimised phage assay was previously  
95 demonstrated to be a useful research tool for studies involving milk spiked with pure  
96 cultures of MAP (Foddai, Elliott, & Grant, 2010a), as it provides quantitative results  
97 for viable cells within 24 h. Therefore, the optimised phage assay was principally  
98 used throughout this study, although conventional culture on solid agar and qPCR  
99 were employed in some experiments to supplement or confirm phage assay results.

100

## 101 **2. Materials and methods**

102

### 103 *2.1. Bacterial strains*

104

105 Four MAP strains, including two type strains (ATCC 19698 and NCTC 8578)  
106 and two milk isolates (796PSS and 806R; Grant, Ball, & Rowe, 2002), were grown in  
107 a shaker incubator for 4–6 weeks at 37 °C to stationary phase in Middlebrook 7H9  
108 broth containing 10% OADC supplement (both from Difco) and 2 µg mL<sup>-1</sup> mycobactin  
109 J (Synbiotics Europe SAS, Lyon, France). Cultures on solid agar for comparison of  
110 colony counts (cfu mL<sup>-1</sup>) with plaque counts (pfu mL<sup>-1</sup>) achieved by phage assay  
111 were obtained by spreading appropriate dilutions of *Mycobacterium avium* subsp.  
112 *paratuberculosis* onto Herrold's egg yolk medium (comprised of 9 g bacteriological  
113 peptone, 4.5 g sodium chloride, 15.3 g bacteriological agar, 2.7 g beef extract, 4.1 g  
114 sodium pyruvate, 27 mL glycerol, 5 mL 2% malachite green, 100 mL sterile egg yolk,  
115 2 mg L<sup>-1</sup> mycobactin J) and no antibiotics (HEYM). Agar plates were wrapped in

116 Duraseal laboratory sealing film (Diversified Biotech, MA, USA) and incubated at 37  
117 °C for 4–6 weeks before counting colonies. *Mycobacterium smegmatis* mc<sup>2</sup> 155, to  
118 be used as fast-growing sensor cells for the phage assay (see below) was cultured  
119 for 2–3 days at 37 °C to stationary phase in the same 7H9 medium without the  
120 addition of mycobactin J.

121

## 122 2.2. Optimised phage amplification assay

123

124 The optimised phage assay was carried out as previously described by  
125 Foddai et al. (2009). Briefly, after a previous overnight incubation of samples at 37  
126 °C in 1 mL of 7H9 medium containing 2 mM CaCl<sub>2</sub>, 100 µL D29 mycobacteriophage  
127 (10<sup>8</sup> pfu mL<sup>-1</sup>) was added to samples before incubation at 37 °C for 2 h during which,  
128 particularly post-PMS samples, were shaken periodically every 20–30 min to keep  
129 uniform resuspension of paramagnetic beads. Virucide (100 mM ferrous ammonium  
130 sulphate (FAS, Sigma), 100 µL) was then added, samples were mixed thoroughly by  
131 accurate inverting and rolling (10 min at room temperature) in order to inactivate all  
132 exogenous seed phage before addition of 5 mL 7H9 medium containing 2 mM CaCl<sub>2</sub>.  
133 The samples were returned to the incubator at 37 °C for a further 90 min before  
134 being plated onto agar plates prepared with 1 mL *Mycobacterium smegmatis* mc<sup>2</sup>  
135 155 (10<sup>8</sup> cfu mL<sup>-1</sup>) and tempered 7H9 agar. Plaques were counted the following day  
136 after incubation of plates at 37 °C overnight.

137

## 138 2.3. Peptide magnetic separation

139



140 PMS was performed on 1 mL of sample using 5  $\mu$ L biotinylated-aMp3 peptide-  
141 and 5  $\mu$ L biotinylated-aMptD peptide-coated MyOne™ Tosylactivated Dynabeads®  
142 (Life Technologies), prepared in-house as described previously (Foddai et al.,  
143 2010b). Magnetic separation was carried out using the Dynal BeadRetriever (Life  
144 Technologies) as described previously (Foddai et al., 2010b). Magnetic capture was  
145 carried out for 30 min at room temperature under continuous mixing, followed by two  
146 washes in 1 mL Phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-  
147 T20, Sigma), and final resuspension of the beads in 1 mL 7H9 broth containing 10%  
148 OADC and 2 mM  $\text{CaCl}_2$ . For experiments involving PMS of spiked raw milk samples,  
149 7H9 medium was also supplemented with NOA Antimicrobial Supplement (Product  
150 code 3A201N-300, Abtek Biological Ltd, Liverpool UK; final concentrations per  $\text{mL}^{-1}$   
151 of broth: Nystatin 50 IU, Oxacillin 2  $\mu$ g, Aztreonam 30  $\mu$ g). NOA inhibited growth of  
152 any milk bacteria adhering to the paramagnetic beads after PMS without impacting  
153 viability of the MAP cells, or affecting subsequent phage assay.

154

#### 155 2.4. Investigation of use of ultrasound sonication to achieve MAP de-clumping

156

157 The ultrasonic cleaning unit employed in this set of experiments was the  
158 Ultrasonic PH 30 (Fisher Scientific Ltd), which has two ultrasonic frequencies (37  
159 kHz and 80 kHz) and three operation modes (Sweep, Pulse, Degas). Initially, 2 mL  
160 aliquots of broth suspensions containing approximately  $10^5$ – $10^6$  MAP pfu  $\text{mL}^{-1}$  were  
161 processed through different ultrasonication treatments applied at ambient  
162 temperature (20–21 °C), using the three operation modes at 37 and 80 kHz, for  
163 increasing time periods (0 to 5 min). Conventional de-clumping method consisting of  
164 vortexing samples with five sterile 3 mm glass beads for 2 min, routinely used at

165 Queen's University Belfast (Rowe, Grant, Dundee, & Ball, 2000) to disperse clumps  
166 of MAP cells, was included as the control treatment for each experiment. Once the  
167 most promising ultrasonication setting had been established, the effect of  
168 ultrasonication treatment of samples on ice (suggested by Schleig et al., 2005),  
169 rather than directly in water bath at ambient temperature, was also evaluated. The  
170 effect of each de-clumping treatment was evaluated by comparing pfu, or cfu, counts  
171 obtained before and after treatment.

172 To investigate the potential for MAP cells to re-clump quickly in samples not  
173 immediately processed after ultrasonication, 2 mL broth and whole UHT milk  
174 suspensions of four MAP strains containing approximately  $10^5$ – $10^6$  cells mL<sup>-1</sup> were  
175 de-clumped by the optimised ultrasonication treatment (identified during course of  
176 experiments outlined above), and then incubated for 0 min, 15 min, 30 min, 1 h and  
177 overnight (approximately 18 h) in a shaker incubator (100 rpm) at 37 °C before being  
178 processed in duplicate through the phage assay. If pfu counts recorded after each  
179 incubation time decreased then this was taken as evidence of re-clumping.

180

### 181 2.5. *Assessment of the fractionation of MAP cells in spiked milk samples upon* 182 *centrifugation*

183

184 Raw bulk tank milk from herds without a previous history of Johne's disease  
185 or ultra-high temperature (UHT) processed milk purchased from a local supermarket  
186 was used for these experiments. Milk samples (10 mL raw and 50 mL UHT) were  
187 inoculated with broth suspensions of MAP to give an initial concentration of  
188 approximately  $10^6$  pfu mL<sup>-1</sup>. Samples were centrifuged for 15 min at 2,500 × g in an  
189 Eppendorf centrifuge (model 5702) at room temperature to separate three milk

190 fractions (cream, whey, pellet). After determining the weight of each milk fraction,  
191 samples were de-clumped by optimised ultrasonication and processed immediately  
192 through the PMS-phage assay. For the cream and pellet fractions, which had been  
193 resuspended in 1 mL PBS-T20, the number of MAP cells was calculated as pfu  
194 counts per entire fraction. For the whey fraction the number of cells was determined  
195 by processing 1 mL of whey through the PMS-phage assay and then correcting the  
196 pfu count to take into account the total volume of the original whey fraction. The  
197 experiment was carried out in duplicate for each bacterial strain considered.

198 Quantitative PCR targeting both IS900 and f57 specific target-genes of MAP,  
199 used either alone (direct qPCR) or combined with prior PMS (PMS-qPCR), was  
200 carried out in parallel with the PMS-phage assay to verify the results obtained. DNA  
201 for samples processed through direct qPCR was extracted from each milk fraction as  
202 described by Hanifian, Khani, Barzegari, and Shayegh (2013). For samples to be  
203 processed through qPCR after PMS, bead samples were resuspended in a final  
204 volume of 50  $\mu$ L RNase/DNase free water and DNA was released from captured  
205 cells by heating samples at 95  $^{\circ}$ C for 25 min. A 2.5  $\mu$ L aliquot of the supernatant,  
206 after centrifugation to sediment beads, was used for each qPCR reaction.

207 Quantitative PCR targeting both IS900 and f57 was performed as described  
208 previously by Donaghy, Johnson, and Rowe (2010). An internal amplification control,  
209 labelled with VIC at the 5'-end (EXO IPC - Exogenous Internal Positive Control  
210 Reagent) purchased from Applied Biosystems was included for each qPCR reaction.  
211 Comparison of EXO-IPC  $C_T$  values obtained in negative test control (NTC) wells and  
212 unknown samples was undertaken to assess the possible presence of PCR  
213 inhibitors in samples tested. Higher EXO IPC  $C_T$  values in unknown samples relative  
214 to NTC wells were taken as indicative of PCR inhibition. Each qPCR reaction was

215 performed in a final volume of 25  $\mu\text{L}$  including: TaqMan Universal 2X PCR master  
216 mix (Applied Biosystems), EXO IPC 10x mix and EXO IPC 10x DNA (Applied  
217 Biosystems), 10  $\mu\text{M}$  of each forward and reverse primer and 5  $\mu\text{M}$  of specific probe  
218 (Donaghy et al., 2010), and 2.5  $\mu\text{L}$  template DNA. A calibration curve with DNA  
219 released from known amount of MAP ( $10\text{-}10^5$  pfu  $\text{mL}^{-1}$ ) suspended in 7H9 broth was  
220 included with each qPCR run to allow quantification of numbers detected. qPCR  
221 reactions were performed using an Eco<sup>TM</sup> Real-Time PCR system (Illumina, Inc) with  
222 the following thermal cycling conditions: stage 1: 50  $^{\circ}\text{C}$  for 2 min; stage 2: 95  $^{\circ}\text{C}$  for  
223 10 min and stage 3 (40 cycles): 95  $^{\circ}\text{C}$  for 15 s and 60  $^{\circ}\text{C}$  for 1 min.

224

225 *2.6. Assessment of the effect of refrigeration, freezing, and use of preservative on*  
226 *numbers of viable MAP in milk*

227

228 Whole UHT milk samples spiked with moderate ( $\sim 10^4$  cfu  $50\text{ mL}^{-1}$ ) and low  
229 levels ( $\sim 10^2$  cfu  $50\text{ mL}^{-1}$ ) of the four strains were processed through the PMS-phage  
230 assay applied after centrifugation at  $2,500 \times g$ , resuspension of milk pellet fraction in  
231 1 mL PBS-T20 and de-clumping by optimised ultrasonication. Samples were divided  
232 between three storage categories: 1) fresh - samples processed immediately or after  
233 overnight at 4  $^{\circ}\text{C}$ ; 2) frozen - samples tested after 1 week, 2 weeks and 1 month at -  
234 70  $^{\circ}\text{C}$ ; and 3) chemically preserved - milk samples treated with a milk preservative [1  
235 mL of a solution containing 1% Bronopol (Sigma-Aldrich, UK) added to 50 mL spiked  
236 milk, (i.e., final concentration 10 mg  $50\text{ mL}^{-1}$  milk)] and tested after 1 and 2 weeks  
237 storage at 4  $^{\circ}\text{C}$ . Duplicate milk samples were tested for each strain under all  
238 experimental conditions. Counts (pfu  $50\text{ mL}^{-1}$ ) were compared for each storage  
239 condition relative to the starting bacterial spiking level; a decrease in pfu count was

240 considered to be an indication of loss of viability by a proportion of MAP cells  
241 present.

242

## 243 2.7. *Statistical analysis of results*

244

245 The statistical significance of the increase or reduction in plaque (pfu) and  
246 colony (cfu) counts observed for each treatment applied to broth and milk samples  
247 inoculated with viable MAP was assessed by a paired *t* test (Instat 3; GraphPad, La  
248 Jolla, CA); differences with  $P < 0.05$  were considered significant. Correlation  
249 between numbers of MAP detected through PMS-phage assay and qPCR was  
250 assessed using linear regression analysis (Microsoft Excel).

251

## 252 3. **Results**

253

### 254 3.1. *Use of ultrasonication to disperse MAP clumps*

255

256 To determine the optimal ultrasonication treatment to disperse clumps of MAP  
257 cells various treatments were studied. Of the different sonication settings applied in a  
258 sonicator bath at room temperature, the greatest degree of de-clumping was  
259 consistently achieved in samples processed through ultrasonication using Pulse  
260 mode; mean  $\log_{10}$  increase in pfu counts for MAP strains ATCC 19698 and NCTC  
261 8578 were  $0.17 \pm 0.02$  and  $0.23 \pm 0.08$  ( $P < 0.01$ ), respectively (Fig. 1A).

262 Corresponding increases observed for samples de-clumped by vortexing samples  
263 with glass beads were  $0.35 \pm 0.06$  and  $0.44 \pm 0.04$ , respectively ( $P < 0.001$ ). Generally  
264 similar trends in terms of effects of each de-clumping treatment were observed with

265 cfu counts on HEYM (Fig. 1B), although  $\log_{10}$  increases tended not to be as large as  
266 observed with pfu mL<sup>-1</sup> results.

267       Significantly greater de-clumping was achieved when ultrasonication was  
268 applied to samples on ice in the sonicator bath ( $P < 0.001$ ), rather than directly in the  
269 water bath at ambient temperature (Fig. 2A). This was the case for all strains with  
270 the exception of MAP strain 806R which showed considerable variability in counts  
271 (large error bar) when vortexed with glass beads. Mean  $\log_{10}$  increase in pfu counts  
272 observed for two type strains (ATCC 19698 and NCTC 8578) and two milk isolates  
273 (796PSS and 806R) were  $0.90 \pm 0.08$ ,  $0.87 \pm 0.14$ ,  $0.36 \pm 0.03$  and  $0.51 \pm 0.08$ ,  
274 respectively, with overall mean of  $0.66 \pm 0.08$ . The corresponding mean  $\log_{10}$  increase  
275 in pfu counts observed for samples de-clumped by vortexing with glass beads was  
276 lower for each MAP strain ( $0.60 \pm 0.10$ ,  $0.44 \pm 0.04$ ,  $0.20 \pm 0.02$  and  $0.37 \pm 0.30$ ,  
277 respectively, with overall mean  $\log_{10}$  increase in pfu of  $0.40 \pm 0.12$ ,  $P < 0.01$ ). No  
278 significant increase in pfu counts was observed for samples subjected to extended  
279 incubation on ice for 5 or 10 min before the ultrasonication treatment ( $P > 0.05$  in  
280 both cases). Generally, similar trends in terms of effects of each de-clumping  
281 treatment were observed with cfu counts on HEYM (Fig. 2B), although  $\log_{10}$   
282 increases tended not to be as large as observed with pfu mL<sup>-1</sup> results.

283       The possibility of MAP cells re-clumping quickly after sonication treatment  
284 was estimated by comparing counts recorded from samples enumerated at various  
285 times after de-clumping. Mean pfu counts recorded for spiked milk samples  
286 immediately after de-clumping and after each incubation time did not vary  
287 significantly ( $P = 0.96$ ), suggesting that no re-clumping occurred (Fig. 3A). Similar  
288 results were obtained for MAP broth cultures processed through the phage assay  
289 started after 0 min, 15 min, 30 min and 1 h ( $P = 0.06$ , Fig. 3B). However, a significant

290 increase in pfu mL<sup>-1</sup> counts (~ 2 log<sub>10</sub>) was consistently recorded for MAP broth  
291 cultures incubated overnight in 7H9-OADC-2 mM CaCl<sub>2</sub> at 37 °C before the phage  
292 assay commenced (Fig. 3B). This substantial increase in pfu mL<sup>-1</sup> was not  
293 unexpected, as it is consistent with our previous finding (Foddai et al., 2009) that  
294 there is enhanced phage infection of MAP cells and increased pfu counts when they  
295 have been incubated overnight in the presence of 2 mM CaCl<sub>2</sub>.

296

### 297 3.2. Fractionation of MAP cells within milk upon centrifugation

298

299 Upon centrifugation of 10 or 50 mL volumes of MAP spiked milk at 2500 × g  
300 for 15 min, the vast majority of the MAP cells in both raw and UHT homogenised milk  
301 were consistently recovered from the milk pellet. Mean log<sub>10</sub> pfu observed from both  
302 homogenised and non-homogenised milk inoculated with various MAP strains was  
303 6.28±0.25 and 6.38±0.06, respectively, corresponding to approximately  
304 81.53±5.76% and 96.7±1.7%, respectively, of the initial inocula. The percentage of  
305 bacterial cells recovered from the milk whey fraction ranged from 11.4 to 25.6%  
306 (overall mean 18.4±5.8%) for UHT homogenised milk and 0.4 to 2.9% (mean  
307 1.6±0.21%) for raw milk. The percentage of MAP recovered from the cream was  
308 lower than expected, on the basis of previous studies, in both cases; overall mean  
309 observed was 1.6±0.21% for whole raw and 0.06±0.02% for UHT homogenised milk.  
310 No significant difference in fractionation between MAP strains employed to spike raw  
311 and UHT milk was observed (*P* > 0.05).

312 Numbers of MAP in each milk fraction estimated via direct-qPCR were more  
313 variable than PMS-qPCR, but similar trends in numbers of MAP cells per fraction  
314 were generally observed. Lack of sensitivity was observed when qPCR was applied

315 alone on DNA extracted from each raw milk fraction after centrifugation;  $C_T$  values  
316 obtained from EXO IPC assay from samples were generally higher than those  
317 obtained from negative test control (NTC) samples indicating the presence of PCR  
318 inhibitors. Numbers of MAP estimated through IS900 and f57 direct-qPCR were  
319 consistently lower (by 1–2  $\log_{10}$ ) than corresponding values achieved through PMS-  
320 phage assay and PMS-qPCR ( $P < 0.05$  and  $P < 0.01$ , respectively). Best correlation  
321 was observed between qPCR and phage assay results ( $r^2 = 0.8568$  for IS900 qPCR  
322 and  $r^2 = 0.7631$  for f57 qPCR) when PMS was applied before qPCR for both types of  
323 spiked milk samples (Fig. 4A,B). No significant variance of EXO-IPC  $C_T$  values for  
324 all post-PMS DNA samples relative to NTC wells was observed, indicating apparent  
325 absence of PCR inhibitors in these samples.

326

### 327 3.3. *Effect of various milk storage conditions pre-testing on MAP viability*

328

329 The effect of refrigeration, freezing, and use of preservative on numbers of  
330 viable MAP in milk was evaluated. Counts (pfu) detected from samples processed  
331 immediately or after one overnight at 4 °C were similar ( $P > 0.05$ , Fig. 5A,B),  
332 suggesting that milk analysis can be performed on fresh or overnight refrigerated  
333 milk samples without adverse effect on MAP counts. Minimal reduction in numbers of  
334 MAP detected ( $P > 0.05$ ) was observed from samples refrigerated for 3 days before  
335 milk analysis. Mean  $\log_{10}$  decrease in pfu counts for both moderate-spiked (Fig. 5A)  
336 and low-spiked (Fig. 5B) milk samples was  $0.41 \pm 0.08$  and  $0.15 \pm 0.10$ , respectively.  
337 No adverse effect on pfu counts ( $P > 0.05$ ) was also observed from milk samples  
338 previously frozen at -70 °C for 1 or 2 weeks and 1 month before testing.  $\log_{10}$   
339 decrease in pfu counts ranged from  $0.29 \pm 0.23$  to  $0.52 \pm 0.20 \log_{10}$  for moderate (Fig.



340 5A), and from  $0.01 \pm 0.20$  and  $0.20 \pm 0.24$  for low-spiked level (Fig. 5B), suggesting  
341 minimal impact of frozen storage at  $-70\text{ }^{\circ}\text{C}$  on viability of MAP cells. In contrast,  
342 addition of Bronopol, as a milk preservative, in combination with refrigeration at  $4\text{ }^{\circ}\text{C}$   
343 for 1 and 2 weeks was found to severely impact viability of MAP cells. In the case of  
344 one MAP strain (806R) viability was so adversely affected that no viable MAP were  
345 detected in low spiked milk when testing was preceded by refrigeration for 2 weeks  
346 in the presence of Bronopol (Fig. 5B). Counts (pfu) observed for milk samples  
347 processed after 1 and 2 weeks were consistently 1-2  $\log_{10}$  lower than those  
348 observed from fresh samples (Fig. 5A,B). Mean  $\log_{10}$  decrease in pfu counts  
349 observed in moderate and low-spiked milk samples was  $1.96 \pm 0.38$  and  $1.04 \pm 0.20$   
350 after 1 week ( $P < 0.01$ ) and  $2.25 \pm 0.56$  and  $1.70 \pm 0.40$  after 2 weeks ( $P < 0.001$ ),  
351 respectively.

352

#### 353 4. Discussion

354

355

356 The objective of this study was to develop an optimised protocol for sample  
357 processing and PMS-phage assay testing of raw milk samples to ensure rapid  
358 detection and accurate enumeration of viable MAP, so a number of aspects of the  
359 current milk testing protocol were investigated. The first part of the study focused on  
360 the development of a non-invasive de-clumping method to maximise accuracy of  
361 MAP cells obtained through PMS-phage assay. Due to the hydrophobic nature of its  
362 cell wall, MAP cells tend to aggregate in clumps, the presence of which in test milk  
363 samples can lead to underestimates of MAP numbers by a factor of 100-1000 cells  
364 (Klijn, Herrewegh, & de Jong, 2001; Pearce et al., 2001). Various de-clumping

365 options, including repeated syringing (Keswani & Frank, 1998; Sung & Collins,  
366 1998), vortexing with glass beads (Rowe et al., 2000), and sonication (Chiodini &  
367 Hermon-Taylor, 1993; Stabel, Steadham, & Bolin, 1997) have previously been  
368 applied in the preparation of MAP inocula by other researchers. The method  
369 routinely applied at Queen's University Belfast involves vortexing the MAP culture  
370 with five sterile 3 mm glass beads for 2 min. However, the sterile glass beads would  
371 generally be added to 7H9 broth at the time of preparation, not at the point of de-  
372 clumping, which would be the case in the context of milk testing. Introduction of  
373 glass beads to each test milk sample would be laborious and may increase the risk  
374 of introducing exogenous MAP DNA to the milk sample, if this approach were to be  
375 taken. A similar situation would exist in the case of syringing, the use of which would  
376 also require extra costs for additional sterile consumables. For these reasons, only  
377 treatment of samples in sealed tubes in a sonicator bath would seem to be a realistic  
378 proposition. The impact of different sonicator bath power settings and treatment  
379 temperatures and times on the viability of MAP cells was evaluated. Amongst the  
380 different sonication settings involving low-frequency ultrasound treatment (37 and  
381 80kHz) at ambient temperature, no significant de-clumping was observed. A small  
382 increase in MAP numbers compared to vortexing with glass beads (control  
383 treatment) suggested a minimal effect on disruption of MAP clumps. With the  
384 possible exception of one of the MAP field strains (MAP 806R), ultrasonication at  
385 37kHz on ice achieved significantly greater de-clumping of MAP suspensions than  
386 the control method (vortexing with glass beads). When grown in broth, 806R forms  
387 particularly large aggregates of cells and generally proves difficult to de-clump and  
388 achieve a visually uniform suspension; the large error bars for vortexing of MAP  
389 806R (Fig. 2A) is reflective of this situation. Overall, no adverse effect of de-

390 clumping by ultrasonication at 37 kHz in Pulse mode for 4 min on the viability of MAP  
391 cells was observed.

392 Milk samples to be tested for MAP generally require initial centrifugation to  
393 reduce large volumes (20–50 mL) to a 1 mL test volume appropriate for PMS. The  
394 PMS-phage assay and qPCR results obtained during the present study indicate that  
395 the milk pellet is the location of maximal numbers of MAP after centrifugation of a  
396 milk sample at  $2,500 \times g$  for 15 min at ambient temperature. Mean percentage  
397 recovery of MAP in the pellet estimated based on pfu counts for spiked homogenised  
398 UHT milk and spiked raw milk were >80% and 95%, respectively. Considerably lower  
399 proportions of MAP cells were generally recovered from the whey (18.4% and 1.6%  
400 for spiked homogenised UHT and raw milk, respectively) and cream (0.06% and  
401 1.6% for spiked homogenised UHT and raw milk, respectively) fractions. Thus, in  
402 the case of raw milk samples negligible numbers of MAP would be lost if the whey  
403 and cream fractions were discarded after centrifugation; the picture for homogenised  
404 milk seems to be rather different. Comparison of results achieved by the two  
405 detection methods showed a better correlation between phage assay and PMS-  
406 qPCR results than direct qPCR results; some evidence of PCR inhibition was  
407 obtained for direct qPCR, but not for PMS-qPCR. PMS was, therefore, confirmed to  
408 be an effective method to achieve selective concentration of target cells plus removal  
409 of PCR inhibitors from test samples. Two previous fractionation studies carried out  
410 on raw milk inoculated with MAP showed that the vast majority of the original  
411 inoculum sediment to the milk pellet after centrifugation (Grant, Ball, & Rowe, 1998;  
412 Van Brandt et al., 2010). Another study (Gao, Odumeru, Raymond, & Mutharia,  
413 2005) reported that MAP cells preferentially partitioned to the cream fraction upon  
414 centrifugation, leading to a recommendation that the pellet and cream fractions

415 should be combined for MAP testing of milk. The centrifugation conditions adopted  
416 by Van Brandt et al. (2010; 974 × *g* for 36 min at 4 °C) and Gao et al. (2005; 3100 ×  
417 *g* for 30 min, temperature not stated) differ from those used by Grant et al. (1998)  
418 and in the current study (2,500 × *g* for 15 min at room temperature). In particular,  
419 temperature of centrifugation, or temperature of milk at time of centrifugation, may  
420 have impacted the differing fractionation profiles observed. When processing raw  
421 milks in our laboratory, we purposely allow milk samples to attain ambient  
422 temperature before starting to test and always centrifuge at ambient temperature  
423 (not at 4 °C) to ensure that sedimentation of MAP cells to the pellet is maximised.

424 Another important consideration for the establishment of an optimised milk  
425 testing protocol was to discover the best milk storage condition to be adopted for  
426 milk samples if milk analysis cannot start immediately upon delivery of samples. Raw  
427 milk samples for MAP testing are usually collected miles away from the testing  
428 laboratory, and refrigeration, use of milk preservatives, or freezing samples are some  
429 of the methods used to preserve milk samples after collection or upon delivery. As  
430 the phage assay exploits the ability of D29 mycobacteriophage to replicate only  
431 within viable mycobacterial cells, maintaining the viability of MAP cells in test  
432 samples is critical. To the best of our knowledge, no information is currently available  
433 in the literature on how storage conditions including refrigeration, freezing, and use  
434 of preservative affect viability of MAP cells in milk. Results of the present study  
435 indicate that processing milk on the same day as collection or after overnight storage  
436 at 4 °C are the ideal conditions to maximise detection and achieve accurate  
437 enumeration of viable MAP cells. If this is not possible, then freezing of milk samples  
438 at -70 °C for up to one month could be alternatively applied with minimal impact on  
439 viable MAP counts. In contrast, the addition of milk preservative (Bronopol) to milk

440 samples which are then refrigerated for 1 or 2 weeks at 4 °C weeks should be  
441 avoided as these conditions significantly affected viability of MAP; this could lead to  
442 underestimation of the number of MAP cells originally present in milk samples, or  
443 false negative results if  $<10^2$  MAP cells are present per 50 mL milk. Our findings are  
444 in general agreement with the literature available on the subject. Use of milk  
445 preservatives, particularly Bronopol, have already been reported to cause bacterial  
446 membrane damage affecting both Gram-negative (*Escherichia coli* and  
447 *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) bacteria  
448 (Shepherd, Waigh, & Gilbert, 1988; Stretton & Manson, 1973). This might explain the  
449 reduction in bacterial count as observed in our study and in other milk surveys  
450 previously carried out (Amores et al., 2010; Pinnow et al., 2001). Freezing of milk  
451 samples at -80 °C without adverse effect on the viability of bacterial cells has  
452 previously been also reported for other pathogenic bacteria, including coagulase-  
453 negative staphylococci and Gram-negative bacilli (Sanchez et al., 2003).

454

## 455 5. Conclusions

456

457 Milk samples should be refrigerated at 4 °C after collection and MAP testing  
458 should commence within 24 h, or, when this is not possible, samples can be frozen  
459 at -70 °C for up to one month without significant loss of MAP viability. The vast  
460 majority (>95%) of MAP in raw milk were found to sediment to the pellet fraction  
461 upon centrifugation at 2500 × g for 15 min, so this milk fraction should be tested. De-  
462 clumping of MAP aggregates before PMS is most effectively achieved by  
463 ultrasonication of the resuspended milk pellet on ice in a sonicator bath at 37 kHz for  
464 4 min in 'Pulse' mode. Our findings regarding optimal milk storage and preparation

465 prior to MAP testing by the PMS-phage assay should theoretically be equally  
466 applicable to milk samples to be cultured; however the effect of chemical  
467 decontaminants on viability of MAP cells that had been previously frozen and thawed  
468 would be an additional consideration.

469

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471

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474

## 475 **References**

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## 1 Figure legends

2

3 **Fig. 1.** Comparison of the de-clumping effects of vortexing with glass beads for 2  
4 min (Control) and three ultrasonication treatments (Pulse Mode at 37 kHz for 2, 3 or  
5 4 min). Middlebrook 7H9 broth cultures of various MAP strains containing  $10^5$ – $10^6$   
6 pfu mL<sup>-1</sup> were placed in a steel basket and processed in a sonicator bath at ambient  
7 temperature (20 °C). MAP was enumerated by (A) phage assay (pfu mL<sup>-1</sup>) and (B)  
8 culture on HEYM (cfu mL<sup>-1</sup>). The larger the increase in log<sub>10</sub> pfu mL<sup>-1</sup> observed, the  
9 greater the de-clumping effect of the treatment. For each MAP strain results are  
10 mean ± standard deviation of two experiments with duplicate samples per  
11 experiment.

12

13 **Fig. 2.** De-clumping effects of ultrasonication (Pulse Mode at 37kHz for 4 min) on ice  
14 compared with vortexing with glass beads for 2 min (Control). Middlebrook 7H9 broth  
15 cultures of four MAP strains containing  $10^5$ – $10^6$  pfu mL<sup>-1</sup> were processed in a  
16 sonicator bath on ice. MAP was enumerated by (A) phage assay (pfu mL<sup>-1</sup>) and (B)  
17 culture on HEYM (cfu mL<sup>-1</sup>). The larger the increase in log<sub>10</sub> pfu or cfu mL<sup>-1</sup>  
18 observed, the greater the de-clumping effect of the treatment. For each MAP strain  
19 results are mean ± standard deviation of two experiments with duplicate samples per  
20 experiment. Bars represent (left to right): vortexing with glass beads for 2 min  
21 (control, ■), ultrasonication on ice at 37 kHz for 4 min (■), incubation for 5 min on  
22 ice before ultrasonication on ice at 37 kHz for 4 min (■), and incubation for 10 min  
23 on ice before ultrasonication on ice at 37 kHz for 4 min (■).

24

25 **Fig. 3.** Investigation of possible re-clumping of MAP cells in (A) spiked milk samples  
26 and (B) Middlebrook 7H9 broth cultures after ultrasonication treatment. Samples  
27 were processed through the phage amplification assay immediately after  
28 ultrasonication (0 min, ■) and at increasing times (15 min, ■; 30 min, ■; 1 h, ■;  
29 overnight at 37 °C, ■) post-ultrasonication. No significant decrease in MAP count,  
30 which would have been indicative of re-clumping, was observed. For each MAP  
31 strain,  $\log_{10}$  pfu mL<sup>-1</sup> results are mean  $\pm$  standard deviation of two experiments with  
32 duplicate samples per experiment.

34 **Fig. 4.** Relationship between MAP counts obtained by PMS-phage assay and  
35 corresponding numbers of MAP estimated by (A) PMS-IS900 qPCR and (B) PMS-  
36 f57 qPCR for cream (white), whey (grey) and pellet (black) fractions after  
37 centrifugation of 10 mL raw milk (circles) and 50 mL homogenised UHT milk  
38 (diamonds) originally spiked with approximately 10<sup>6</sup> pfu MAP mL<sup>-1</sup>. Each data point  
39 represents the mean of two samples.

41 **Fig.5.** Impact of pre-testing storage conditions on numbers of viable MAP in milk  
42 samples spiked with 10<sup>4</sup> pfu 50 mL<sup>-1</sup>. Four different MAP strains were used for  
43 spiking experiments. Milk samples were processed through the PMS-phage assay  
44 immediately after spiking and after different pre-testing storage conditions: (A)  
45 refrigeration at 4 °C, with and without addition of Bronopol preservative, and (B)  
46 freezing at -70 °C. For each MAP strain results are mean  $\pm$  standard deviation of two  
47 experiments with duplicate samples per experiment. Bars represent (left to right):  
48 immediate testing (■), 4 °C overnight (■), 4 °C for 3 days (■), 1 week at 4 °C plus

49 Bronopol (■), 2 weeks at 4 °C plus Bronopol (■), 1 week at -70 °C (■), 2 weeks at -  
50 70 °C (□), and 1 month at -70 °C (▣).

51

52

ACCEPTED MANUSCRIPT

