FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

Supporting document 2

Technical issues raised in submissions – Application A1045

Bacteriophage Preparation P100 as Processing Aid

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1. Introduction

Submissions on draft variations to the Code for Application A1045 were called for between 16 March and 27 April 2012. Eight submissions were received, of which five supported the approval of P100 for the proposed purpose and three did not provide a position. However seven submissions, which were all government agencies with responsibility for enforcing or assessing compliance with the Code, raised a number of issues. No submitters raised concerns about the safety of adding P100 to treat various solid RTE foods as proposed by the Applicant.

A number of common themes relating to the technical aspects of the Application were identified in the submissions including:

- persistence of bacteriophage on foods
- ongoing functionality
- mode of action
- development of resistance to bacteriophage by the bacteria
- methodology for bacteriophage-treated foods.

The first three points considered together were used to argue that the P100 bacteriophage preparation should be considered a food additive rather than a processing aid. Many submitters referred to two recent scientific opinions from the European Food Safety Authority (EFSA) on the use and mode of action of bacteriophages (EFSA 2009) and the safety and efficacy of Listex^{TM} P100 on raw fish (EFSA 2012).

FSANZ obtained further clarification and information from the Applicant and held additional discussions with the jurisdictional submitters to discuss the various issues raised. FSANZ's responses to these key issues are presented below.

2. Key issues raised

2.1 Persistence of bacteriophage

Many studies for the treatment of foods have found that bacteriophage can persist for days in foods (see Table 1). In most cases the number of bacteriophage are stable, although in some foods the concentration may decrease with time, eg lettuce (Guenther et al. 2009). Reports where the bacteriophage numbers have increased with time are limited. For example, Leverentz et al. (2003) who used cocktails of 14 and 6 distinct lytic bacteriophages specific for *L*. *monocytogenes* found an increase of 1 log over a seven day period, as well as stable levels, on treated honeydew melon.

Bacteriophage numbers will increase on treated food only if there is reproduction inside the bacterial host before lysis. This reproductive behaviour will depend on the mode of action (see section 2.3), bacterial cell concentration and the type of bacteriophage. The application of P100 utilises the 'lysis from without' mechanism by applying large numbers of bacteriophage relative to target bacteria. This causes bacterial cell lysis, without the need for bacteriophage replication (see section 1.1 of SD1 for additional information).

Experimental evidence from challenge studies involving bacteriophage A511, a comparable bacteriophage to P100, in a range of solid and liquid foods (Guenther et al. 2009) showed no increases in bacteriophage concentration (Table 1). For the two liquid foods (chocolate milk and mozzarella brine), where an ongoing technological function was observed, the bacteriophage counts were steady over six days of the study. For solid foods the bacteriophage counts were steady, or decreased when applied to cabbage and lettuce.

Table 1: Summary of bacteriophage persistence from challenge studies

Food type	Bacteriophage	Relative changes in bacteriophage concentration	Length of study (days)	Reference
Cabbage	A511	Decline (1 log)	6	Guenther et al. (2009)
Lettuce	A511	Decline (1 log)	6	Guenther et al. (2009)
Hot dogs	A511	Steady	6	Guenther et al. (2009)
Chocolate milk	A511	Steady	6	Guenther et al. (2009)
Turkey breast	A511	Steady	6	Guenther et al. (2009)
Mozzarella brine	A511	Steady	6	Guenther et al. (2009)
Smoked salmon	A511	Steady	6	Guenther et al. (2009)
Mixed seafood	A511	Steady	6	Guenther et al. (2009)
Brazilian sausage	P100	Steady	10	Rossi et al. (2011)
Smeared cheese	P100	Steady	13	Schellekens et al. (2007)

2.1.2 FSANZ conclusion

Bacteriophages can persist on treated foods for up to 1-2 weeks. Leverentz et al. (2003) provided inconclusive evidence relating to the growth of phages on the surfaces of treated food. The definition of processing aid in Standard 1.3.3 in the Code does not require that the processing aid be absent from the food. Furthermore, presence on the food surface does not automatically mean that a bacteriophage preparation would be considered a food additive. Presence is therefore not a criterion used to make a distinction between phage preparations as processing aids or food additives—rather the period of technological function.

2.2 Ongoing functionality

Submitters cited the EFSA opinions (2009 and 2012) of the lack of evidence regarding the possibility of an ongoing technical function following (re)contamination of a bacteriophage treated food.

The Applicant claimed that the growth rate of *L*. *monocytogenes* on the treated and untreated foods were identical subsequent to the application of bacteriophages on the foods (see

Annex 12 of the Application[1]). The reducing efficacy of the bacteriophage treatment can be observed by the reduction in *L*. *monocytogenes* concentration during the first 6-24 hours after application.

The Applicant's hypothesis is that if the growth rate of the bacteriophage-treated samples (commencing from a lower initial bacterial concentration post phage treatment) and untreated samples were identical, then there is no ongoing technological function. This indicates that the bacteriophage is no longer influencing the growth rate of bacteria. The applied bacteriophages become inactive after the initial effective period, enabling the growth rates of any remaining bacteria in the treated sample to 'pick up' and match the growth rates of the bacteria in the untreated control under the same experimental conditions.

FSANZ performed a detailed statistical analysis of challenge study data submitted, and additional published data, to test this hypothesis. The statistical analysis aimed to determine two important criteria for assessing a bacteriophage preparation: (1) efficacy; and (2) ongoing technological function. The majority of data presented in SD1 was taken from Guenther et al. (2009).

For these data, a simple straight line model was determined to be appropriate (Figure 2A in SD1). Efficacy was determined by the difference in the intercepts between the treated and untreated foods. Ongoing technological function was determined by a comparison of the slopes (=growth rates) of the treated and untreated foods. The analysis considered whether the slopes of the treated and untreated foods were statistically equal, and thus leading to the conclusion of no ongoing technological function. Where differences in slope were observed and with the treated food having a lesser slope, this suggested there was ongoing technological function.

FSANZ found slopes (ie growth rates) to be different between solid foods and liquid foods. Specifically in the case of liquid foods, the slopes were negative, indicating a reduction on *L*. *monocytogenes* concentration with time (bacteriophages continued their activity, reducing bacterial growth rates). For the majority of solid foods (12/15) the slopes for treated and untreated foods were found to be the same, supporting the hypothesis that there was no ongoing technological function. For the three solid foods where the slopes were found to be different, the growth rate of bacteriophage treated food was lower, but positive. This suggests that bacterial cells may have recovered and continued to grow, but not at the same rate as the untreated samples (i.e. limited ongoing effect). A full explanation of these anomalous results is contained in section 5.2.2.1 of SD1 but is summarised below.

There were two mixed seafood results analysed, with the second study found to have equal slopes. For the trial that did not produce equal slopes, it was considered that the difference was only small and not considered to be of practical significance.

For the cabbage result, the high efficacy of the phage treatment meant the bacterial concentrations were very low and close to the limit of detection, so such a study was not appropriate to perform detailed statistical analysis. Unfortunately, the second cabbage trial had insufficient data to enable a complete statistical analysis.

[1]Available at http://www.foodstandards.gov.au/foodstandards/applications/applicationa1045bact4797.cfm

However, there were two studies on comparable food matrices (iceberg lettuce) which both produced parallel slopes.

There was one smoked salmon study where the results were unable to be explained. However, there were two other smoked salmon studies that produced the parallel lines. One of these studies used the P100 phage preparation.

A question raised following the original statistical analysis was whether the experimental challenge studies were performed long enough, and whether bacteriophage is only suitable for foods with short shelf lives. To examine this question, additional challenge studies using P100 and other bacteriophages were analysed. A P100 study from Holck and Berg (2009) was found to be suitable for this statistical analysis. In this study, cooked ham was challenged with *L*. *monocytogenes* and then treated singularly or in combination with P100 bacteriophage and a culture of *Lactobacillus sakai* TH1. As the ham was cooked, there were no other lactic acid bacteria present prior to the study which may inhibit the *L*. *monocytogenes*. The study was performed for 28 days at 10°C, which was enough time for the *L*. *monocytogenes* concentration in the untreated samples to reach a maximum concentration of greater than 10^8 cfu/g. The experiment in which P100 was the only treatment was analysed.

As the stationary phase had been reached during the study, a Baranyi equation without a lag time was fitted using the formula obtained from the nlstools library (Baty and Delignette-Muller, 2011) using R (R Development Core Team 2011). Analysis of variance was used to determine the most parsimonious equation: different intercepts, but identical growth rates and maximum concentration. The predictions of the final model are presented below (see Figure 1).

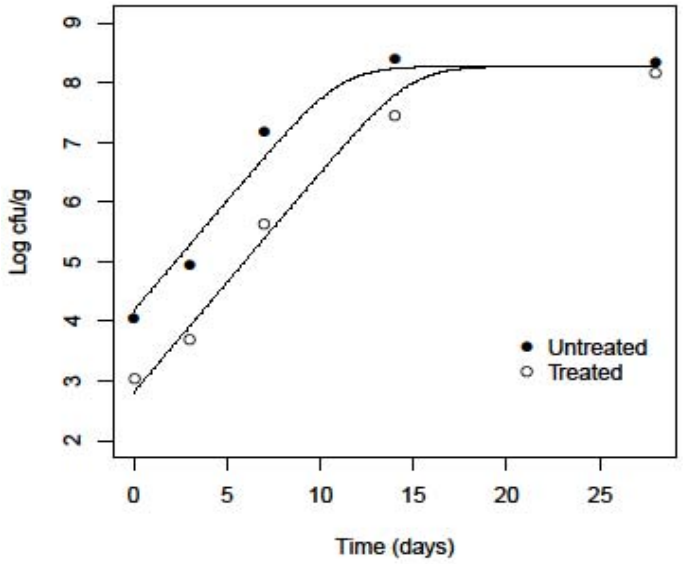

Figure 1: Analysis of treating cooked ham treated with and without P100 for up to 28 days at 10°C (Holck and Berg, 2009)

The finding that the intercepts are different between the treated and untreated foods again highlights the efficacy of P100, in this case 1.4 log difference. The growth rates and maximum concentration were found to be 0.37 $\log_{10}$ day^{-1} and 8.27 $\log_{10}$ cfu/g.

The finding that the maximum concentration was the same for both the treated and untreated foods supports the hypothesis that there is no ongoing technological function for P100, even at very high *L*. *monocytogenes* concentrations in excess of 10^8 cfu/g. It should be noted that the finding of the same maximum concentration for both groups would not be expected for foods which are not sterile, due to the potential inhibitory effects of other microflora.

2.2.1 FSANZ conclusion

For the data of Holck and Berg (2009), using the same considerations as found in SD1, it is concluded that the P100 bacteriophage treatment had an initial effect of 1.4 log reduction in concentration but no ongoing function as indicated by the identical slopes and maximum population. The maximum population of *L*. *monocytogenes* in the treated and untreated foods was $10^{8.2}$ cfu/g. The finding that the maximum population was identical for both the treated and untreated foods provides a new insight into the lack of ongoing function of bacteriophage. The practical outcome of this finding is that *L*. *monocytogenes* concentrations on foods must be controlled to low levels through the use of GMP prior to bacteriophage application.

2.3 Mode of action

A submitter cited Monk et al. (2010) regarding uncertainty in the mode of action of bacteriophages. Monk et al. (2010) discussed the question of whether high enough numbers of bacteriophage can be applied to a food resulting in the 'lysis from without' mechanism (see section 1.1.1 in SD1). In this scenario large number of bacteriophage attach to the bacteria resulting in its destruction without the requirement of bacteriophage replication. Monk et al (2010) conclude that although there is uncertainty regarding the exact mechanism of action bacteriophage are still effective at controlling pathogens in food.

2.3.1 FSANZ conclusion

It is therefore concluded from the mode of action of P100 that it is extremely unlikely that the P100 particles applied to the surface of a solid RTE food would replicate with increased storage time.

2.4 Bacteriophage-insensitive strains

The issue of the development of resistance of *L*. *monocytogenes* to a bacteriophage should be considered in the context of the reduced effectiveness of the bacteriophage when this does occur. This is over and above the reduced effectiveness due to naturally occurring phage-insensitive strains present in the processing plant. Most bacteriophage are limited to a narrow range of strains of bacteria. The Applicant has provided information that the P100 bacteriophage was effective against 95% of *Listeria* spp. strains tested (section 4.1 of SD1). As it is not effective against all strains of *L*. *monocytogenes* there will be a real possibility that some strains present in a food processing environment will be naturally resistant. P100 is not intended for use as a sole agent to control *L. monocytogenes* in processing facilities.

Following issues raised by submitters, FSANZ sought further information from the Applicant on how they ensure that their P100 preparation does not lose its efficacy in reducing the concentration of *L. monocytogenes* on treated food.

For monitoring purposes, sensitivity tests are conducted on *L. monocytogenes* strains isolated from users' food premises using standardised methods such as efficiency of plating (EOP) tests and pull-down assays.

To date, there has been no evidence of reduced susceptibility of *L. monocytogenes* to P100 found by their long term customers[2]. Any phage-treated product that is not offered for sale needs to be appropriately treated to inactivate phages before reprocessing or removed from the production facility on a regular basis and disposed of with care. Treated product or food ingredients or their packaging material should not re-enter the processing facility unless they are treated to inactivate the phages.

As a result, companies that choose to use the P100 bacteriophage preparation must ensure that GMP and listericidal processes are used to actively control *L*. *monocytogenes* contamination of foods.

2.5 Analytical methodology issues for bacteriophage-treated foods

Submitters have hypothesised that the presence of bacteriophage in foods may influence the detection of any remaining *L*. *monocytogenes* present on a food after treatment. The concern is that if the P100 bacteriophage preparation was approved as a processing aid, and therefore unlabelled, then enforcement authorities would not know that the food had been treated. This was noted as being especially important if compliance agencies were to conduct *L. monocytogenes* analyses on the P100 treated samples. The concern expressed was that residual phage particles that are still active could be extracted from the surface of treated food during homogenisation and inactivate *L. monocytogenes*, so producing artificially low or incorrect measurements of the amounts of *L. monocytogenes* present.

Examination of bacteriophage challenge studies identified a small number that include a centrifugation step to separate the host bacteria from the bacteriophage in the treated food prior to analysis (Bigot et al. 2011; Soni and Nannapaneni 2010). The majority of challenge studies do not include such a step. None of the studies evaluated in the Risk Assessment (SD1) for efficacy and ongoing technological function included the centrifugation step.

The majority of challenge foods analysed statistically in the Risk Assessment (12 out of 15 studies) were found to have equivalent growth rates (parallel lines). As the process of extracting the *L*. *monocytogenes* and bacteriophage occurs in a liquid medium, it would be expected that the higher the bacterial concentration, the greater the potential for bacteria-bacteriophage interactions and reduced counts of bacteria. This was not observed, as the growth rates of treated and untreated foods were the same.

An important reason and explanation for the lack of impact of residual phage on *L. monocytogenes* concentrations relates to the very short time taken to extract the *L. monocytogenes* during the homogenisation step before plating out. This step would usually occurs within 15 minutes, and concluded to be too short a time for phage/*L. monocytogenes* interactions to have an effect on concentrations. Further, the use of a diluent during sample preparation further reduces the bacterial and bacteriophage concentrations so that the chances of the bacteriophage colliding with the bacteria are minimal.

Evidence of the continued detection of *L*. *monocytogenes* in bacteriophage treated foods following enrichment can be found in the study of Schellekens et al. (2007). This study investigated the application of bacteriophage P100 to control *L*. *monocytogenes* on Munster cheese. Schellekens et al. (2007) notes the following observation: “No *Listeria* was detected using the quantitative plate counting method up to 21 days after Packaging DAY (PD + 21). However, the enrichments at PD+21 showed that *Listeria* was still present, although at low numbers”.

[2] The Applicant has patented P100. Therefore consideration has only been given in this assessment to the control practices of the Applicant.

The enrichment method described in the paper, which includes an incubation period of 24h at 37°C, does not include a centrifugation step.

2.5.1 FSANZ conclusion

It is therefore concluded that the treatment of foods with P100 is unlikely to result in false negative *L*. *monocytogenes* results when an enrichment step is included.

3. References

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