Introduction

This document clearly explains the experimental studies and results reached therein to demonstrate that Listex™ P100 has no technical effect in the final product. Subchapter 1 contains a detailed analysis of the experimental data in a variety of RTE foodstuffs which demonstrate, among other things:

- Although the magnitude of the initial effectiveness of bacteriophages may differ in various foodstuffs because of the different surface structure of the foodstuffs, experimental studies in a broad range of foodstuffs consistently demonstrate that the phages lose their function shortly after application (within 24 hours);

- The presence of other antimicrobials in foodstuffs has no influence on the period of activity of phages;

Subchapter 2 discusses the primary mechanism by which phages quickly lose their function in foodstuffs (adsorption to the matrix). Subchapter 3 sets forth the factors that lead to structural degradation of phages in foodstuffs some time after the initial adsorption steps. Based on this information, EBI respectfully submits that Listex™ P100 is a processing aid.

1. Experimental Data

Except where mentioned otherwise, all the experimental data presented here is derived from the same study, performed at the Swiss Federal Institute of Technology, within the framework of a Ph. D. thesis. All experiments were performed in triplicate with duplicate sampling, but the error bars are left out for ease of interpretation. All the food products were store-bought in these particular experiments with different formulations. However, formulation does not affect adsorption kinetics of phages, and thus the time frame of functionality. Also formulation does not affect the stability of the phages within the time frame of functionality, as will be demonstrated with experimental data.
Two phages, A511 and P100 were used in this study. They are two closely related phages of the myoviridae family with significant genetic sequence identity and similarly broad host-ranges. They are almost identical in size and the adsorption kinetics of the structural proteins are identical. Several experiments show that their efficacy in food experiments is virtually identical (see Annex 1b).

The experimental data clearly show that after a significant initial reduction, regular growth of the remaining *Listeria* resumes within 6-24 hours after application in all foodstuffs.

It will of course take a certain amount of time (lag-phase) before the bacterial population (which has been reduced because of the initial killing effect of the phages, soon after the phages application) has reached its original number (the number prior to the reduction caused by the phages). As bacterial growth subsequently resumes with the same speed as the growth in the untreated control (seen in the graph as lines with an identical slope), it can be concluded that no residual function of the phages remains.

It is possible to make hypothetical growth curves that plot the growth after such a reduction. This is possible because the growth speed of bacteria under the given conditions can be calculated by analyzing the growth curve of the untreated control.

Exponential growth of bacterial cultures follows the following equation:

$$N = (N_0)\, e^{kt}$$

In a semi-logarithmic graph k is represented by the slope of growth

$$k = \frac{\text{actual rise}}{\text{actual run}} = \frac{\ln Y_2 - \ln Y_1}{t2 - t1}$$

Values Y_2 and Y_1 are established by taking any two y-values of the slope at two time-points t_2 and t_1

The doubling time of the bacterial population is therefore:

$$t_d = \frac{\ln 2}{k}$$

Fig. 1 shows the effect of phage treatment of minced meat. In addition a hypothetical growth curve is included in the graph. This hypothetical curve shows the development of a bacterial population with identical growth characteristics as the untreated control but which starts with cell numbers identical to those of the treated samples following the initial reduction. In other words this curve represents the growth of the population if it had been reduced to that level by a treatment known to have no residual effect such as heat treatment or irradiation. It is obvious from this graph that growth of bacteria that have survived initial treatment is not impeded by the presence of the phages.

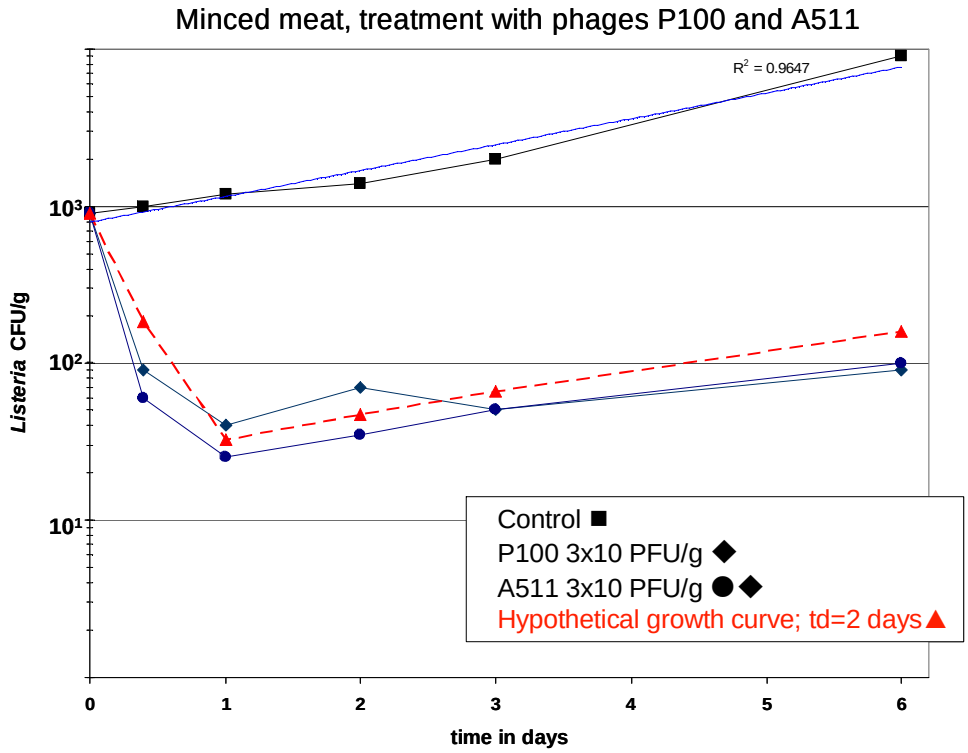
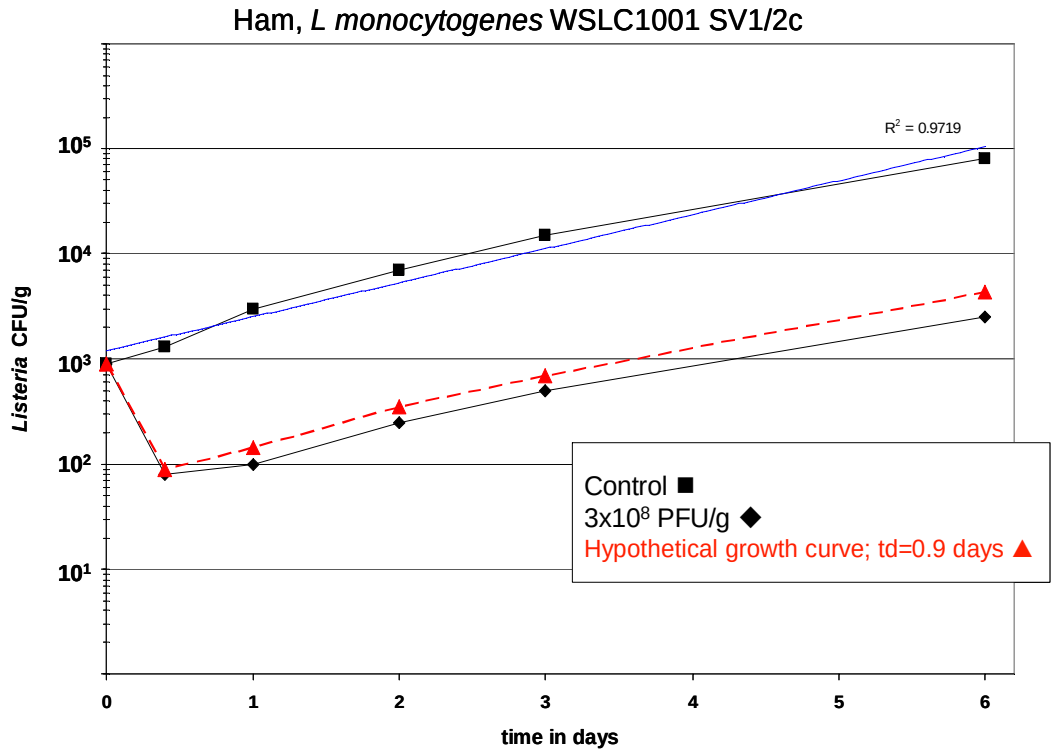


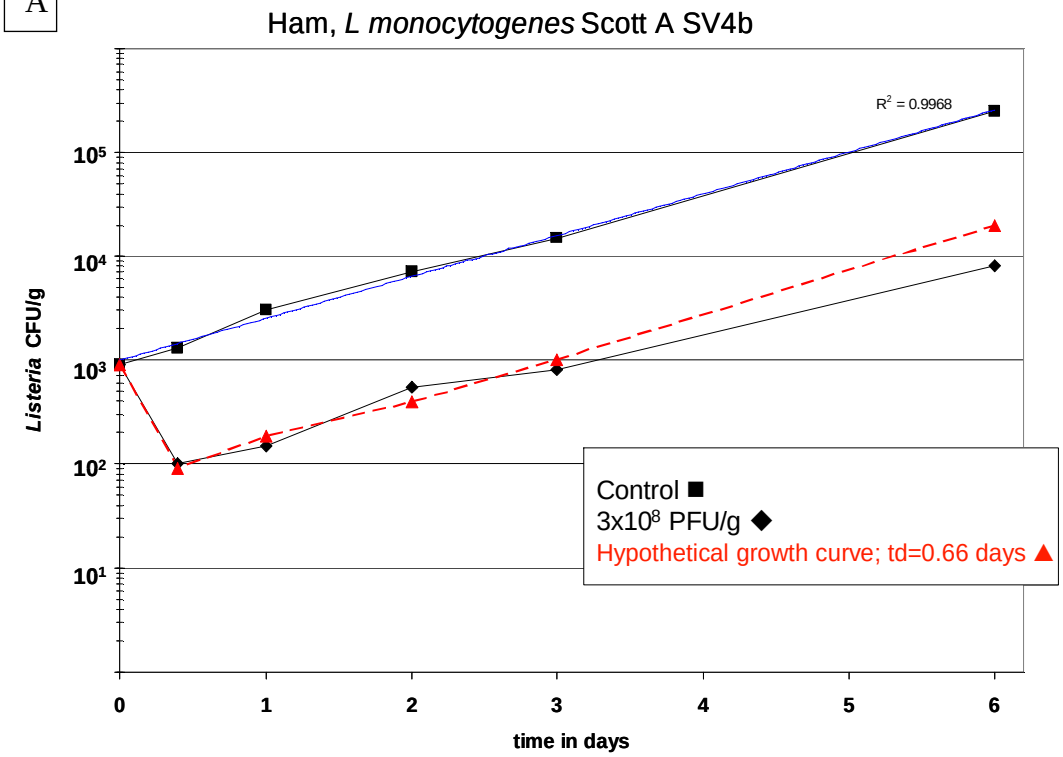
Fig. 1: Effect of phage treatment on listerial growth with phages P100 and A511 in minced meat at 6ºC. The red line shows hypothetical growth after a 1.5 $\log_{10}$ reduction and a doubling time as observed in the untreated control.

Fig. 2 and 3 show data for similar phage treatment of ham and cooked turkey breast. A large part of the data was recently published (Guenther *et al.*, 2008). Hypothetical growth curves following an initial reduction have also been included into the graphs showing the effect of phage treatment on ham and turkey breast artificially contaminated with high numbers of two different *L. monocytogenes* strains ScottA (outbreak strain SV4b) and WSLC1001 (food isolate SV1/2c) in figures 2 and 3. Doubling times of the bacterial population were calculated based on the growth rate of the untreated controls under the experimental conditions. In all cases resumption and rate of growth in the actual experiment follow the hypothetical growth curves.

The remaining cells after initial reduction grow almost exactly as those in the untreated control. The highest reduction is obtained after approximately 6-24 hours and after that no remaining residual function of the phages can be observed in the experimental foodstuffs.



A



B

2: Effect of phage treatment on listerial growth of strain WSLC1001 (A) and strain Scott A (B) on ham at The red lines show hypothetical growth after a 1log10 reduction and a doubling time as observed in the untreated controls.

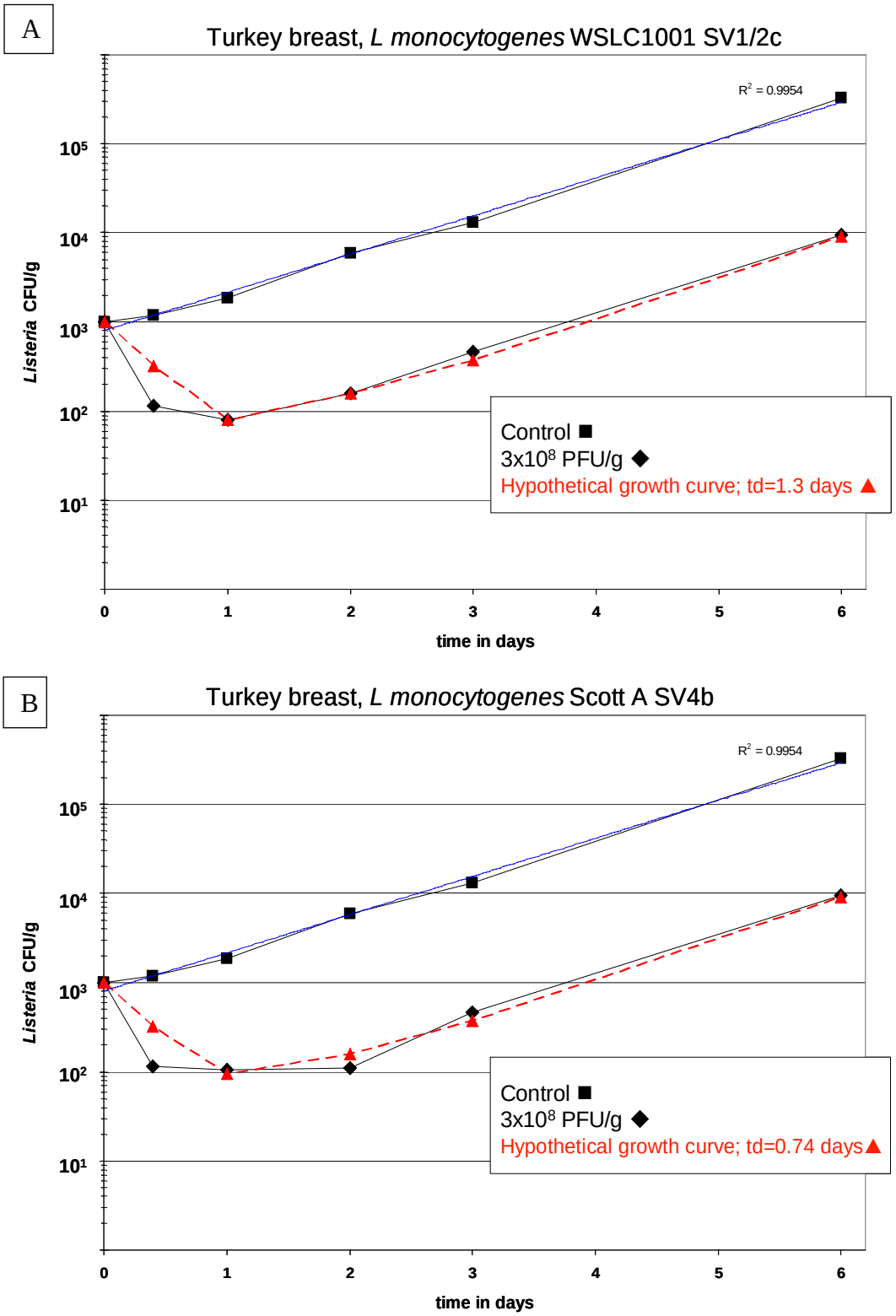


Fig. 3: Effect of phage treatment on listerial growth of strain WSLC1001 (A) and strain Scott A (B) on turkey breast. The red line shows a hypothetical growth after a 1log$_{10}$ reduction and a doubling time as observed in the untreated controls.

Another experiment within the same doctoral thesis framework, using hotdogs, the same contamination levels and the same treatment levels, but a higher incubation temperature, allows us to see how long the phages remain active on hotdogs (no lag-phase). The higher temperature results in an increased growth rate and even though the initial reduction is equally high, resumption of growth can be observed after 6 hours at exactly the same rates as in the untreated control experiment (Fig 4).

Hotdogs *L. monocytogenes* WSLC 1001 SV 1/2c

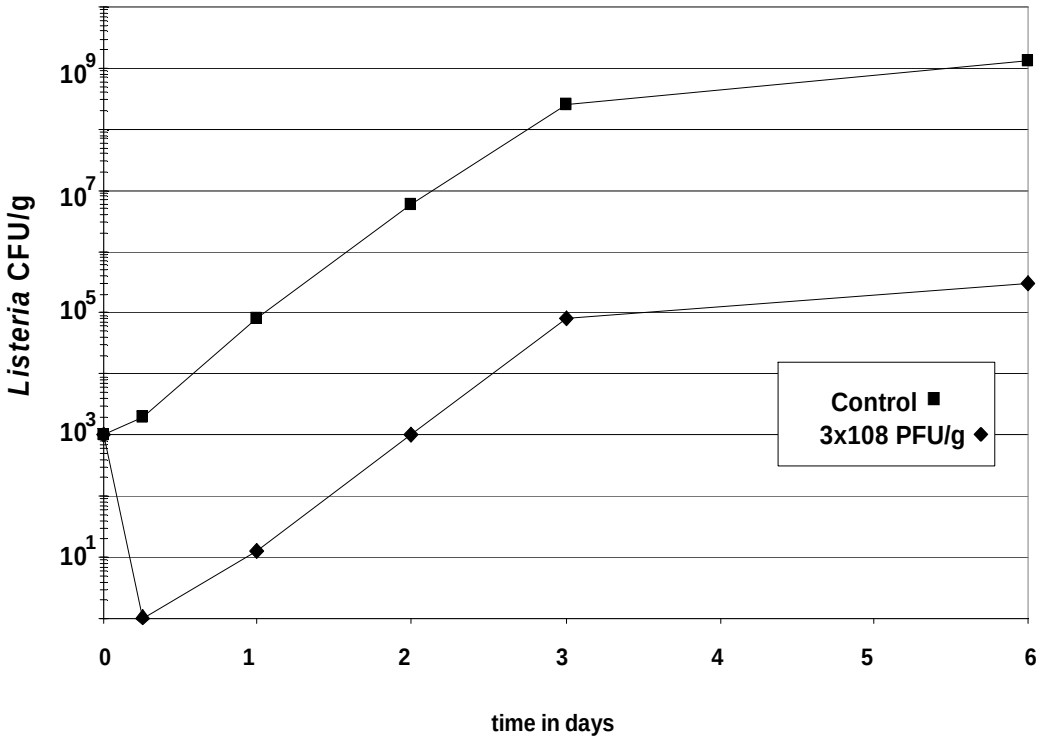


Fig. 4: Effect of the high phage dose on *Listeria*-contaminated hotdogs incubated at a 20ºC. After an initial reduction of approximately $3\log_{10}$, growth resumes after 6 hours at exactly the same rate as in the untreated control experiment.

The major cause for the differences in initial reduction between the foods is the different surface structure of the foodstuffs. On smooth surfaces such as hotdogs and cabbage leaves, there are far fewer recesses where bacteria could find refuge, facilitating the likelihood of phage/host encounters and contributing to higher initial reductions. When there is a higher initial reduction of bacterial numbers, experimental results can make it appear that the phages had a longer kill effect than it actually did. However, as demonstrated in the graphs, even when there is a higher initial reduction in bacteria, resumption of growth always occurs within 24 hours.

In foodstuffs where a relatively high amount of fluid is added or remains after treatment, a high initial killing efficacy can be observed. This is likely caused by high diffusion ability of the bacteriophages in the fluid, allowing the phage to more effectively find their target bacteria. This increased movement does not abolish adsorption in any way.

In order to see whether the presence of other antimicrobials has an influence on the period of activity of the phages, additional experiments were conducted by EBI Food Safety at its own facilities. Cooked ham without added lactate or di-acetate, cooked ham with di-acetate 0.15%

and lactate and di-acetate (1.5% and 0.15% resp.) were used in these experiments to determine if such antimicrobials influence how long the phages remain active. Fig 5 and 6 show that in all cases growth resumes (after an initial reduction) within 24 hours after application, showing that these antimicrobials do not affect the period of phage activity.

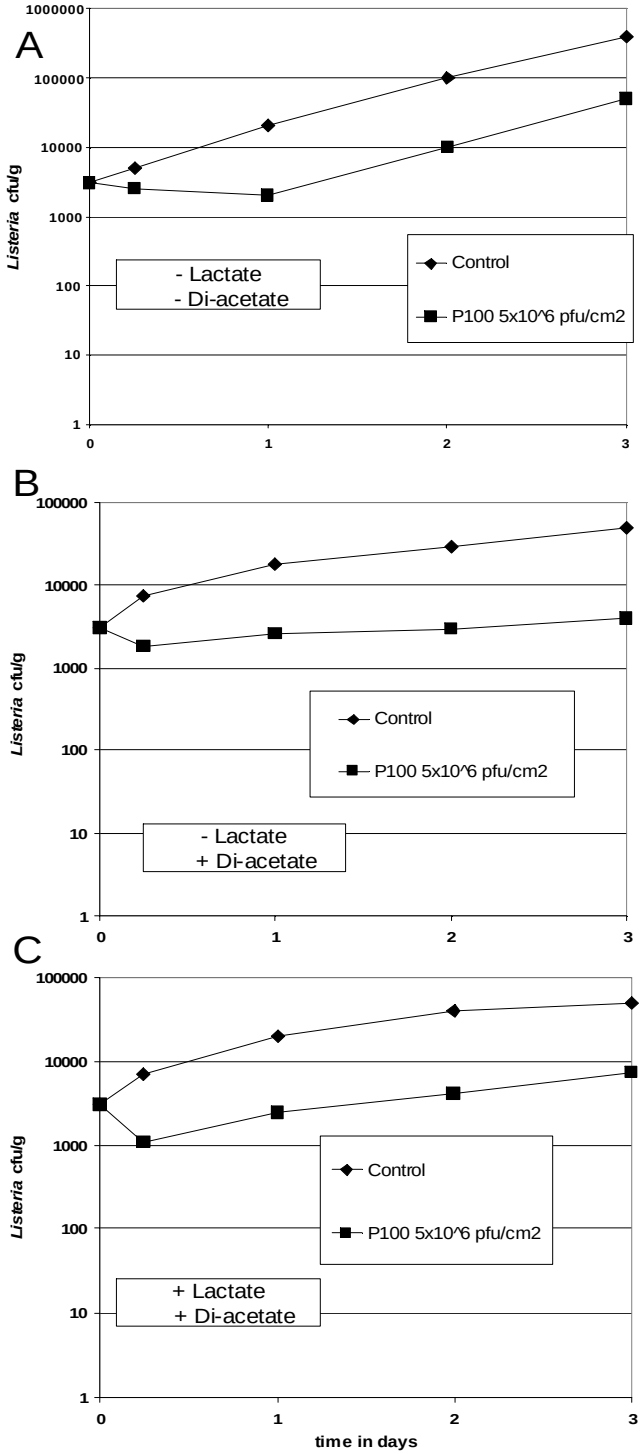
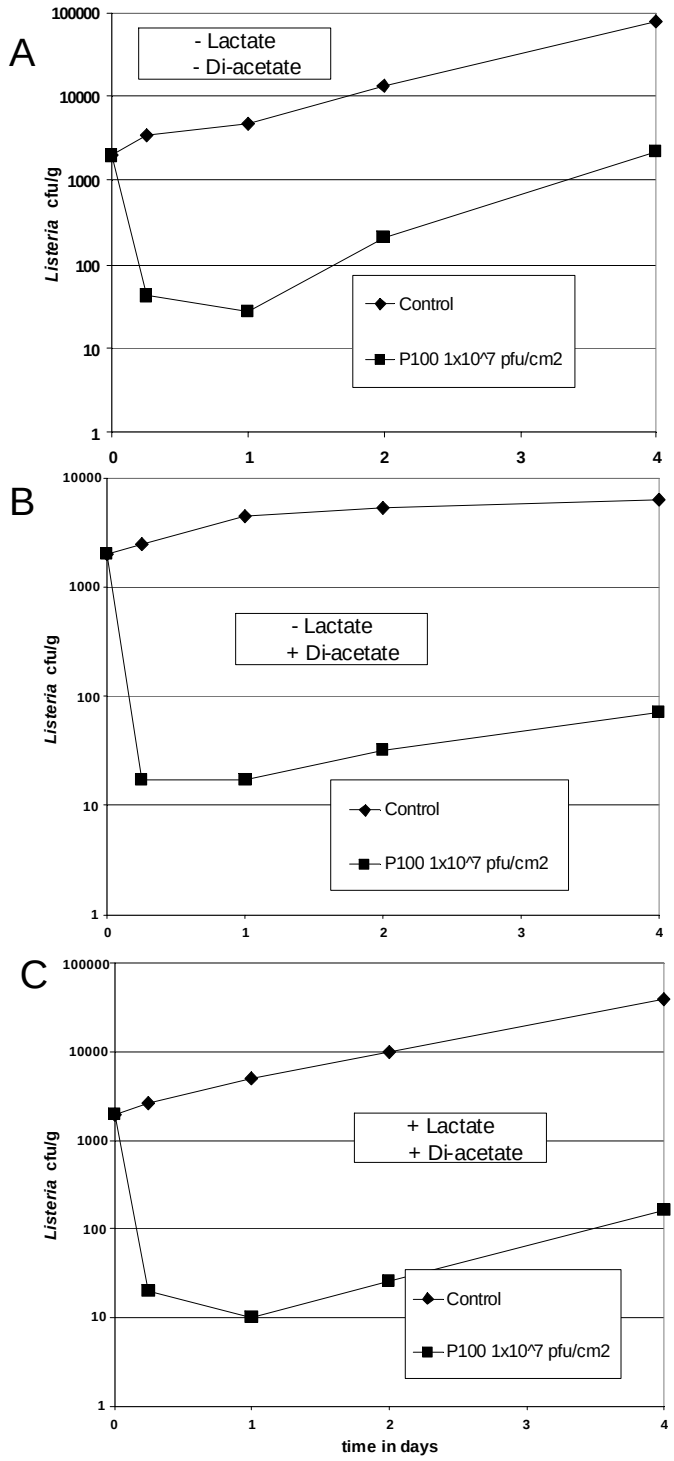


Fig. 5: Effect of bacteriophage P100 at $5x10^6$ pfu/cm^2 on *Listeria* on cooked ham without organic acids (**A**), with di-acetate (**B**) and with both lactate and di-acetate (**C**) over a 3 day period. Although the speed of growth of *Listeria* and total numbers after 4 days varies in the untreated control hams, the effect of phage addition: ~1 $\log_{10}$ reduction and growth resumption after 6-24 hours is identical in all cases.

Fig. 6: Effect of bacteriophage P100 at $1x10^7$ pfu/cm^2 on *Listeria* on cooked ham without organic acids (**A**), with di-acetate (**B**) and with both lactate and di-acetate (**C**) over a 4 day period. Although the speed of growth of



Listeria and total numbers after 4 days varies in the untreated control hams, the effect of phage addition: ~2 $\log_{10}$ reduction and growth resumption after 6-24 hours, is identical in all cases.

Since these organic acids may affect the long term structural integrity of phages, a phage recovery experiment on the three different pieces of ham was also performed.
In all cases the percentage of phages that was recoverable within the period where the phages show activity, was identical (Fig 7). If these substances have an influence on phages' structural stability at all, this happens beyond the point in time where residual activity is observed. Recoverability does not mean that the phages still have their function: they are not able to infect bacterial cells after 24 hours, as shown before.

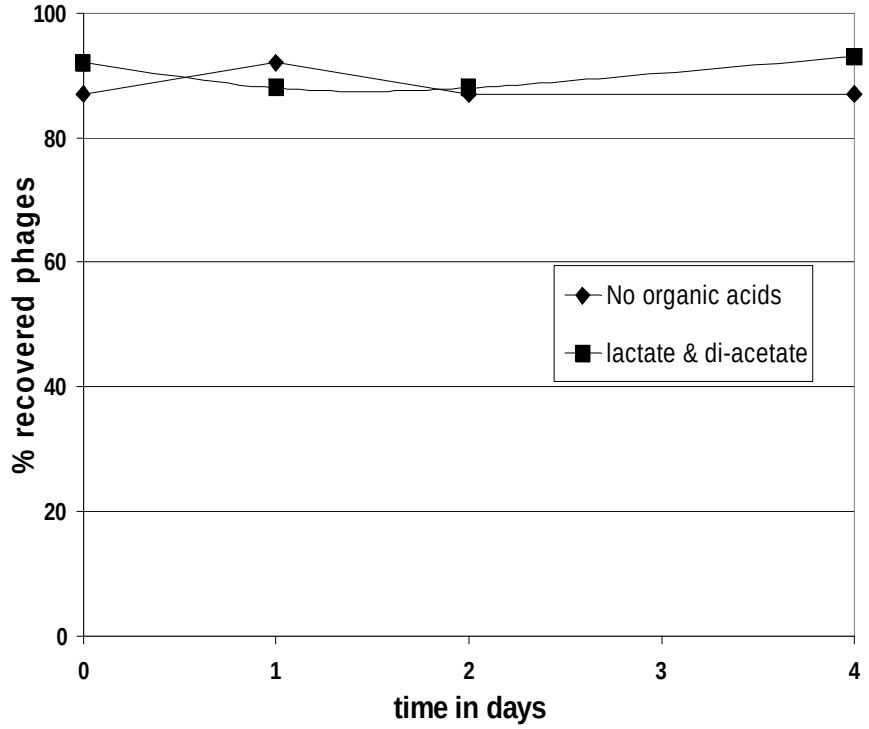


Fig. 7: Recovery of phages from ham (Experiment = Fig 6) over a 4-day period in the presence and absence of organic acids. Roughly 90% of the phages could be recovered from both hams at all times during the 4-day experimental period, showing that the organic acids have no effect on the structural stability of the phages during this period.

In sum, detailed analysis of experimental data shows that in all the tested foodstuffs resumption and rate of growth in the actual experiment follow the hypothetical growth curves. The remaining cells, after initial reduction, grow almost exactly as those in the untreated control. The highest reduction is obtained after approximately 24 hours and after that no remaining residual activity can be observed.

2. Phage adsorption

The food experimental data shows clearly that a rapid loss of phage function can be observed within 24 hours of application. At the same time in these experiments most of the applied phages can still be recovered within and even after this period. The rapid loss of function of phages is caused almost entirely by adsorption. It is commonly known that proteins absorb to surfaces. Phages consist of a protein hull containing DNA. Like all proteins, when phages come into contact with a surface, they will bond with that surface. This adsorption has a number of consequences for a bacteriophage and its ability to interact with the host bacterium.

Phages diffuse passively in liquids and the liquid applied during Listex™ P100 application allows a phage to move and "find" its host bacterium. At the same time, however, phages will collide with the food surface or particles. These collisions can result in the formation of bonds between surface and bacteriophages. Primarily such a bond-formation will impede and/or abolish the ability of the phages to move and thus find host bacteria. This inability to further diffuse is a major factor in the rapid loss of function of phages.

Several interactions likely contribute to strong bonds being immediately formed, with hydrophobic interactions being the most prominent. The side chain of several amino acids (Va, Phe, Ile, Leu) are non-polar and hence interact poorly with polar molecules like water. When non-polar residues are exposed at the surface of two different molecules, it is energetically more favourable for their non-polar surfaces to approach each other closely, displacing the water from between them. The strength of hydrophobic interactions is not appreciably affected by changes in pH or salt concentration, which means that the bond can be broken really only by force.

Ionic interaction bonding also occurs because proteins contain both positively (Lys, Arg, His) and negatively charged amino acids (Asp, Glu). These interact with and bind to other, oppositely charged groups. Shifting the pH results in more negatively charged groups when the pH rises and more positively charged groups when the pH drops. Increasing the ionic strength of the environment decreases ionic interaction between molecules in favour of interaction with free ions.

Formation of hydrogen bonds between phage and food surface may also occur. Hydrogen bonds can form whenever a strongly electronegative atom (e.g., oxygen, nitrogen) approaches a hydrogen atom which is covalently attached to a second strongly electronegative atom. These can be formed in the case of phages between the –C=O group and the H-N- groups of separated peptide bonds in proteins (giving rise to the alpha helix and beta configuration) and between –C=O groups and hydroxyl (H-O-) groups in Ser and Thr residues of proteins and sugars.

Individually these bonds are much weaker than covalent bonds (typically about 20 times), but many of them together can have formidable strength. The first bond to occur brings the phage closer and holds it to the food surface, increasing the likelihood of additional bonds to form. This is the reason why adsorption only becomes stronger over time. Any one bond can be broken with relative ease, but for phages to desorb, all bonds must be broken simultaneously. Bond formation can and does progress to the point where it is in fact virtually irreversible. At this point desorption will be possible only by harsh treatment (for example with chemicals). Before the phage is irriversible bound to the food matrix, phages can be extracted, in the laboratory, from the foodstuff as described below

In order to desorb phages in the lab, the foodstuff is placed in phage stabilization buffer (SM-buffer, pH 7.6) at a ratio of 10:1 buffer to foodstuff (v/w) and 'stomachered' (homogenized) twice, for three minutes each. The pH of SM-buffer is much higher than that of the products Listex™ P100 is used, and this shift may weaken the ionic interactions formed at a lower pH. Monovalent and divalent cations, Na^{+} and Mg^{2+}, in the buffer will shield the negatively charged groups which result from the pH shift. The agitation of the large volume of buffer by the paddles in the stomacher creates sufficient energy to weaken many interactions, including hydrophobic bonds, favouring desorption of phage particles. The recovered phages are no longer active as described above in the experimental data. The protocol for further processing (PCR) can be found in Annex 11.

Conditions that lead to desorption in the laboratory setting are not present in the commercial setting and thus desorption of phages will not occur in production or consumption:

- Purge of solution present in the packeging of the foodstuff will not cause desorption of the phages because no relevant current of force is generated and the pH and ionic strength are the same as the foodstuff.
- Processing steps such as slicing, mincing of grinding would not result in desorption. All of these process steps create new surface areas which would require extremely larger amount of phages in the first place.

It is a fact that fluid aids phage movement (diffusion), but it does not abolish adsorption in any way. This can be seen in the experiments (i.e. purge in hot dogs), where in spite of fluid on the surface, the phages stop functioning after between 6 and 24 hours after application. One of the factors influencing adsorption is the rate of collision with the surface. A heightened mobility of the phages caused by the presence of fluid will also increase the rate of collision with the food surface.

Several detailed information on the subject of protein/surface interactions can be found in publications written by Ruggiero *et al.* (2005) and Kozak *et al.* (2007).

3. Structural phage degradation during further storage of foodstuffs

As discussed above, while phage function will become inactivated within the first 24 hours of application due to absorption, a large part of the phages can be recovered even beyond this time period. However, the number of phages that can be retrieved from a food surface also declines over time.

The inability to be retrieved can be due to permanent adsorption to the surface or to structural decay of the phage particles. The factors contributing to structural degradation are numerous and the different factors may weigh differently in different foodstuffs and may even vary between batches of the same foodstuff. A summary of the various factors leading to structural decay can be found in Annex 9 (Phage inactivation Loessner).

This structural decay and/or irreversible adsorption ensures that the phages cannot be reactivated at later stages regardless of what happens to the foodstuff.