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Executive Summary

Campylobacter is a key food-safety pathogen and bio-control using bacteriophages can be an option, since such products are already in use for other food safety pathogens. The present study is based on the outcomes of a previously carried out Rural Industries Research and Development Corporation funded study that resulted in the isolation of bacteriophages from a range of Queensland poultry farms. Thus there is potential to use these bacteriophages in future studies targeting *Campylobacter* bio-control in Australia.

This is the first such study and the main focus of the present study was to find a cost effective and easy option to assess both bacteriophages and *Campylobacter* to support future phage therapy studies. More specifically, an insect model can fill a niche between the required laboratory and subsequent chicken trials. Carrying out extensive chicken trials can be challenging due to the cost, work involved and other ethical requirements.

The present study validated the use of the larvae of the wax moth, *G. mellonella* for the purpose. Insect models have been developed for a range of infective agents, but have received little attention to date as a means of screening for therapeutic approaches destined for either human or animal studies. Additionally, the use of insect models enables the use of many greater experimental units for multi-factorial studies, allowing the design of statistically robust experiments. Thus the *G. mellonella* insect model can be an intermediary option with possibilities to reduce extensive chicken trials and contribute to more effective laboratory screening.

The key outcomes of the study are as follows:

- The conditions required for maintaining a robust laboratory colony of *G. mellonella* which routinely delivered the final instar larvae for experimentation was established and is described under the “Methods” section.

- Methodologies for working with the larvae including a micro-injection technique were validated. The conditions that did not impact on larval stress (or death) when using the various techniques (including microbiology media or reagents) required for working with either *Campylobacter* or bacteriophages were also validated. This included the ability to successfully inject the larvae with high numbers of *Campylobacter* and bacteriophages without detriment to the larvae (over time). The developed (and validated) methodologies are described in “Methods” section.

- Prior to commencement of the therapy studies, both *Campylobacter* and phage numbers injected and recovered was estimated over a series of experiments to demonstrate the sound working of the validated insect model. With bacteriophage, a decrease in counts ranging from 0.63 - 2.24 log PFU/ml, with an average decrease of 1.21 log PFU/ml was observed at 0.5h. Similarly with *Campylobacter* there was a decrease of 0.86 - 2.42 log CFU/ml with an average reduction of -1.49 log CFU/ml over an hour. Irrespective of these reductions, the doses achieved are sufficient to undertake therapy studies. These natural reductions can be accommodated by using two controls, i.e. an un-inoculated insect control and an injected insect control, during therapy studies.

- Finally, the validated insect model was used to demonstrate its use for both “Active” and “Passive” phage therapy. During active therapy, a standard high dose of *Campylobacter* was subjected to varying doses of bacteriophage, thus assessing the potential for the bacteriophages to increase in number during treatment. This is an advantage during phage therapy. With the *Campylobacter* – bacteriophage combination used in the present study, there were increases (i.e. log 0.4, log 0.5, log

0.76, log 0.86, log 0.87, log 1.10 and log 2.38 respectively) across the series of experiments undertaken. Thus we were able to demonstrate an outcome of active therapy with the model.

- In contrast, passive therapy depended on the addition of a high dose of bacteriophage and was demonstrated for a range of *Campylobacter* concentrations. During passive therapy, the *Campylobacter* and bacteriophage used demonstrated a log reduction in *Campylobacter* at 24h (i.e. control log 8.64 and test log 5.26; control log 4.15 and test log 3.28). *Campylobacter* reduction was also observed during active therapy at 24h (i.e. control log 9.47 and test log 8.51 and control log 9.51 and test log 5.15). Interestingly, the latter occurred at a phage concentration of 10^3 PFU/ml. During passive therapy, the phage levels across both test and control remained at 10^7PFU/ml and were stable in the *G. mellonella* model.

All the above outcomes demonstrate that the current validated insect model is a valuable tool and has a definitive role in future studies relevant to phage therapy. More specifically, the current model will enable *in vivo* screening by assessing both active and passive phage therapy based on either individual phages or phage cocktails developed, following their screening against a range of target *Campylobacter* isolates. The data generated from this study also has use with modelling applications (i.e. by using *Campylobacter* and bacteriophage numbers generated) as a predictive tool for phage therapy applications. Thus the present study has validated the *G. mellonella* model and demonstrated the options that could be targeted for phage therapy studies in an efficient, rapid and cost effective manner.

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Introduction

There is interest in using bacteriophages against food-borne pathogens either at the pre- or post-harvest stages to support the reduction of foodborne pathogens along the food chain. Experimental studies have demonstrated that a three log reduction could be achieved for *Campylobacter* with the use of bacteriophages (Orquera *et al.* 2015) and is a promising outcome for food-safety. Bacteriophage therapy has been suggested as an option for *Campylobacter* control in poultry (Loc Carrillo *et al.* 2005). Overseas studies (Atterbury *et al.* 2005) have demonstrated a significant ($P < 0.001$) reduction in *C. jejuni* counts in the presence of bacteriophages (mean log 5.1 CFU/g) from broiler chicken ceaca selected from 90 United Kingdom flocks ($n = 205$) when compared to the counts with *Campylobacter* alone (mean log 6.9 CFU/g). The potential to use bacteriophage therapy has been comprehensively reviewed (Connerton *et al.* 2011) with studies addressing the various aspects of the relationship between bacteriophages and commercial broilers (Connerton *et al.* 2004; Kittler *et al.* 2014).

More specifically, experimental studies using inoculated phage to *C. jejuni*-colonized birds have demonstrated a reduction in *Campylobacter* counts between 0.5 and 5 log CFU/g of caecal contents compared to untreated controls (Carrillo *et al.* 2005). The reductions were dependent on the phage-*Campylobacter* combination, the dose of phage applied, and the time elapsed after administration (Carrillo *et al.* 2005). These studies were directly done using chickens and thus the use of a chicken model is required to trial various phage combinations, dosing and timing of doses for studies leading to phage therapy. Thus, addressing all these variables can be a costly and time consuming exercise. The use of an insect model can potentially play an intermediary role in supporting the necessary chicken or farm based trials in a more simple and cost effective manner.

Insect models have been developed for a range of infective agents, but have received little attention to date as a means of screening for therapeutic approaches destined for either human or animal studies. Insects, similarly to animals, mount humoral and cellular responses to infection. Additionally, the use of insect models enables the use of many greater experimental units and the design of statistically robust studies. There is emerging evidence that the use of insect models can provide a quick and effective means for addressing the problems of cost, animal ethics and the need for specialised facilities that are typically associated with *in vivo* trials for novel agents, such as bacteriophages in animals. Examples of such insect models used in studies with bacteriophages in particular, include *Pseudomonas aeruginosa* with *Drosphila melanogaster* (Heo *et al.* 2009) clinical (human) isolates of *Burkholderia cepacia* in *Galleria mellonella* (Seed and Dennis 2009) and *Staphylococcus aureus* in *Bombyx mori* (Takemura-Uchiyama *et al.* 2013).

The *G. mellonella* insect model has been used against food-borne pathogens such as *Campylobacter* and *Listeria monocytogenes* strains (Schrama *et al.* 2013). A recent study (Champion *et al.* 2010) validated the *G. mellonella* insect model for the identification of novel virulence genes in *Campylobacter*. The authors commented that the insect model is easy to adopt and far cheaper than alternative models, such as the ferret diarrheal model and the colostrum-deprived piglet model. *G. mellonella* was chosen to be used in the current study due to its sterile haemocoel area (with sterile haemolymph) between the gut and the external surface of the insect providing an aseptic environment to assess *Campylobacter* – bacteriophage reactions. In addition to providing an aseptic platform, *G. mellonella* enables the assessment of such interactions at 37^{o}C, the optimum temperature for *Campylobacter*, a temperature suitable for most other human pathogens (Schrama *et al.* 2013).

The use of *G. mellonella* (larvae) is suitable for the adoption of precision based micro-injection techniques enabling the delivery of precision doses of either organism. Further, the model will allow cost-effective screening of bacteriophage pools for their efficacy to reduce

Campylobacter in a living organism. Such a model has the previously noted advantages over animal systems and would provide an intermediary stage of assessment prior to undertaking chicken trials. The present study was undertaken to validate such an insect model for this purpose, and will facilitate the screening of large numbers of bacteriophages and target bacterial strains, also enabling the design of multifactorial approaches that would be difficult to accommodate with animal studies.

A key requirement to the success of any model organism requires the standardisation of the on-going propagation and maintenance conditions to produce organisms of uniformity across generations (Cook and McArthur 2013). The Integrated Parasite Management team at the Ecosciences Precinct, at Boggo Road have long term experience in culturing and experimentation with a range of insect species and also maintain a colony of *G. mellonella* (James *et al.* 2010). Insect colonies maintained by this group are regularly used for screening potential chemical and biological control agents in insects such as *Lucilia cuprina* and *Chrysomya* spp. All such insect studies have also adopted precision micro-injection technique -based model systems for detecting agents such as ciquatoxin, a toxic contaminant of reef fish (Stewart 2011). Cook and McArthur (2013) have reviewed the conditions for *G. mellonella* studies and the temperatures and survival periods for a range of organisms including food-safety pathogens. Studies reviewed by Cook and McArthur (2013) have used *G. mellonella* with various pathogens at temperatures ranging from 25 - 37^{o}C (Table 1). The larvae were also able to survive for up to four days enabling the inclusion of a "time factor" to the various studies, Table 1 (Cook and McArthur 2013).

Table 1: Conditions adopted for *G. mellonella* model studies after (Cook and McArthur 2013)

Pathogen	Temperature	Duration
Bacillus cereus	-	*Oral inoculation, 24h Oral inoculation, 48h
Listeria monocytogenes	30°C 37°C	7d Injection into hind right pro-leg, 5 d
Staphylococcus aureus	30°C and 37°C 25°C, 30°C, and 37°C	*150h 120h
Pseudomonas aeruginosa	25°C 37°C	* 60h 4d

*injection - hindmost left proleg at fifth or sixth instar

The present study was designed to support future studies that may target the use of bacteriophage for bio-control of *Campylobacter* (or other food-safety pathogens). A previous RIRDC study (on *Campylobacter* dynamics) resulted in a collection of bacteriophages from a range of litter practices across different farms in Queensland and representative bacteriophages were stored.

The objectives of the study were addressed in the following manner:

1. The conditions for maintaining a laboratory colony of *G. mellonella* was established and is described under the "Methods" section

2. Methodologies for the successful exposure (and survival over time) of the larvae to high numbers of *Campylobacter* and bacteriophages were developed. This included microbiology methodology described in the "Methods"

3. *Campylobacter* and phage survival was initially validated individually within the larvae, followed by the “therapy studies” (using the validated insect model) and is described in the “Results” section

4. Other options for the *G. mellonella* model (including the therapy studies) are discussed

Objectives

- To validate the *G. mellonella* insect model to assess *Campylobacter* – bacteriophage dynamics and choose the most effective phages or phage combinations for testing in naturally infected chickens

- To use the insect model as a stage between *in-vitro* and *in-vivo* studies to better target *in-vivo* studies using *Campylobacter* bacteriophages in chickens

Methodology

G. mellonella laboratory colony for experimental studies

Larval feed formulation

The final instar larvae are required for this study. Experimentation was carried out towards maintaining a robust "*Galleria* colony" to enable the supply of larvae of this size for routine laboratory work. This included formulating the feed to ensure that an optimum diet was used to support the necessary growth conditions plus ensuring a disease-free status. The colony can be susceptible to fungal infections. Figure 1 illustrates the larvae used for trials.

Figure 1: *G. mellonella* used for trials

Table 2 presents the components used in the diet which was put together after trialling different ratios of commonly used feed components. The food components tested are illustrated in Figure 2 as diet 1 and diet 2.

Table 2: *G. mellonella* diet

Glycerol	140g
Honey	140g
Water	24g
Farex (baby food)	300g
Yeast	20g

Preparation of feed

Weigh out glycerol, honey and water into a bowl, microwave for 1 minute and leave on bench to cool. Weigh out the dry ingredients, and combine with wet ingredients when at room temperature. Combine with dry ingredients and refrigerate at 4^{o}C.

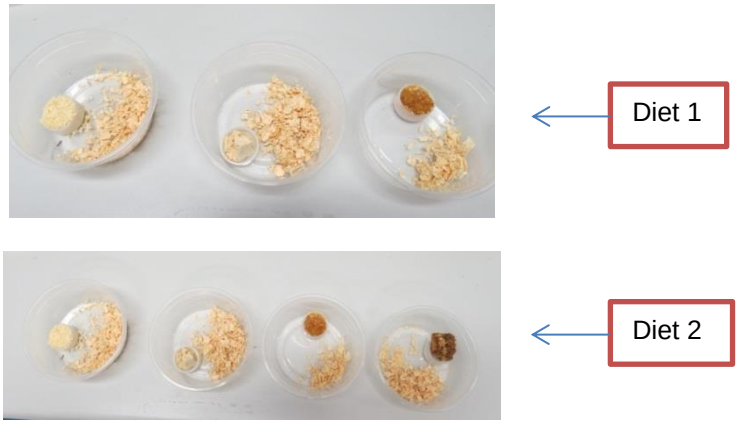


Figure 2: *G. mellonella* diets 1 and 2

Culturing *G. mellonella*

Figure 3, illustrates both the breeding moths and the eggs. When the moths emerge, they are transferred into a new jar containing approximately 4cm of fresh diet. They can be sedated using CO_2 and 10 males and 10 females each are put into the jar. The sexes can be differentiated by the male being lighter in colour and slightly larger than the female. They are left for one week to lay eggs and then gassed (with CO_2). The adults are then transferred into a plastic container and put into the freezer (as they are no longer needed). A fine layer of brown which is visible on the surface of the diet and are the eggs. This stage of the lifecycle is completed in the controlled environment room (CER) B3.C.615 basement level at the Ecosciences Precinct.

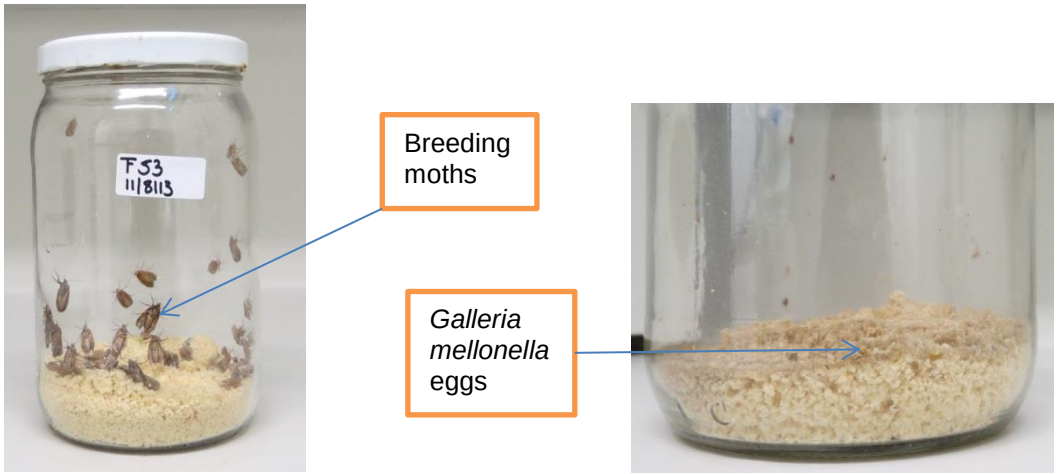


Figure 3: *G.* mellonella breeders and eggs

Maintenance of colony and preparation of insects for injection

The colony is stored in an incubator located in the laboratory and is kept at 26^{o}C; the generation of the insects and date of culturing is written on the jar. The condition of the diet is watched carefully as colonies as susceptible to fungal infection. If the fungus is visible in the colony (Figure 4), the larva are immediately removed and placed into a new jar and the old jar is disinfected. The jars are monitored for signs of fungal infections and diet is topped up when needed.

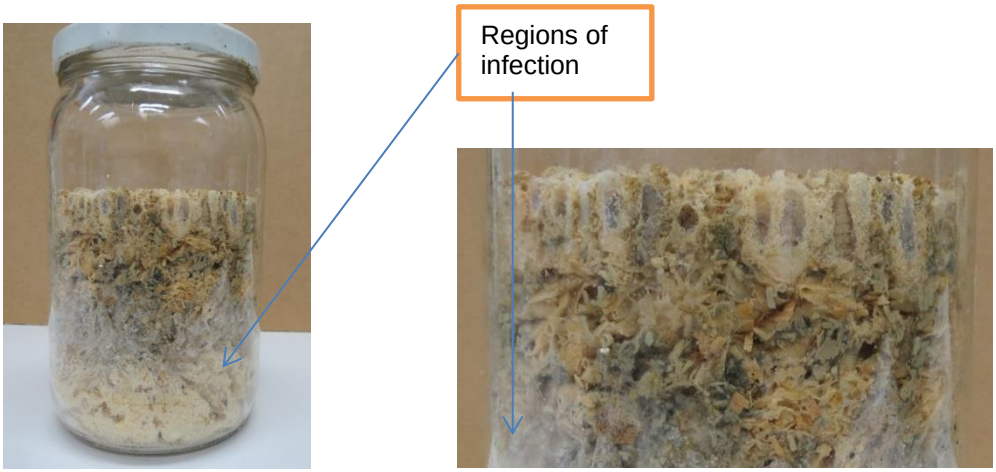


Figure 4: Fungal infection in *G. mellonella* colonies

The first instar larvae of *G. mellonella* are quite difficult to see when the eggs have hatched. At this stage more diet is added along with approximately 8 - 10cm of wood shavings. Figure 5 illustrates the colony of *G. mellonella* in a jar.

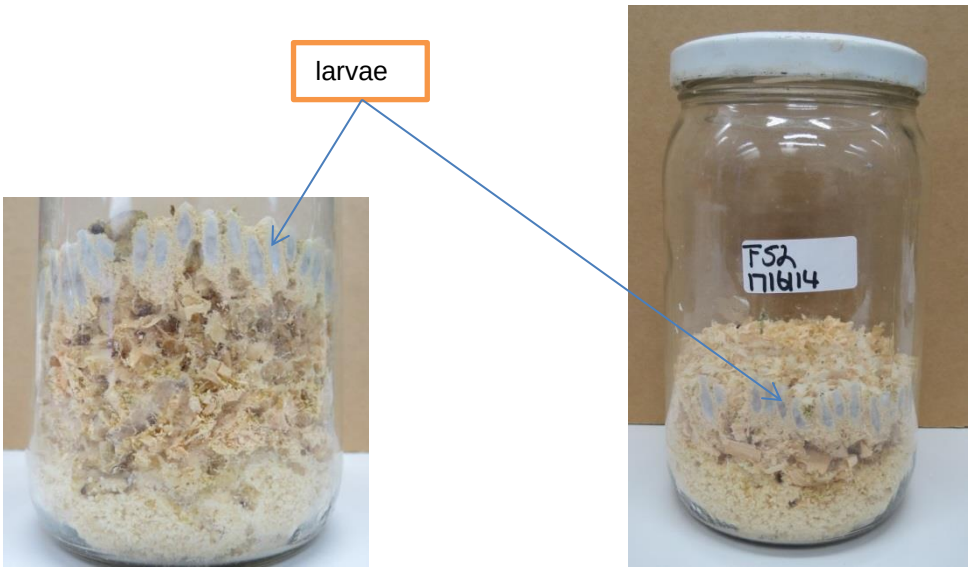


Figure 5: *G. mellonella* colony in a jar

The day before the injection process, *G. mellonella* are prepared to ensure they are the correct weight, length and colour (i.e. weight 330mg and length 2.5cm at the final instar). It is vital that the same instar larvae are used across the trial to aid the comparison of results. The required numbers of larvae are transferred into a clean jar containing approximately 220g of fresh diet. Figure 6, illustrates *G. mellonella* final instar larvae with their diet. They are allowed to feed for 24 to 48h. In order to not stress the insects, 50 to 80 individuals are placed into each jar. When selecting the insects they need to be at least 2cm long especially if they are needed within 24h, smaller individuals require more time to develop and are not removed.

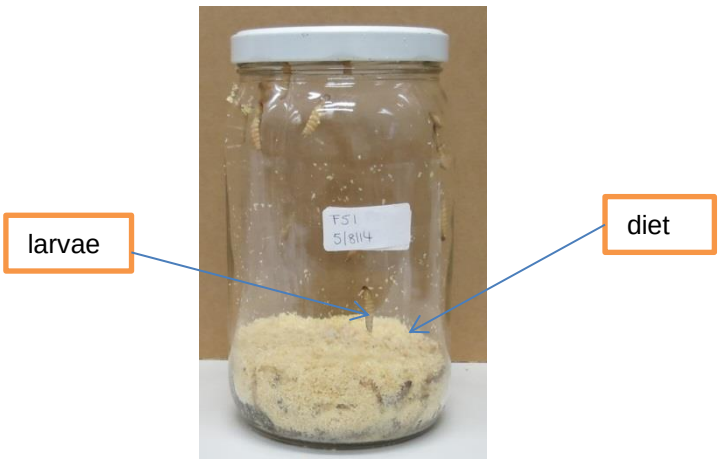


Figure 6: Final instar larvae in diet

Following the establishment of the methodology to maintain an insect colony, the larvae were tested for their survival when subjected to the various aseptic techniques, including the validation of the relevant procedures (such as micro-injection) that needed to be adopted during the study. Table 3 describes the sequence of the work undertaken in order to achieve these outcomes.

For the purpose of the current study, a representative *Campylobacter jejuni* isolate (NC3195) and bacteriophage (phage 181) from our collection was used to both validate and demonstrate the use of the *G. mellonella* insect model.

Table 3: Micro-injection and the use of aseptic techniques

Date	Activity	Details	Note
Part 1			
14/07/2014	**Insect experiments – sterility**.	Testing dis-infection efficacy of insects using 70% alcohol on different sites of the insect	
		Testing sterility of haemocoel after injection	
		Micro-injection of green dye	
		Micro-injection of PBS	
		Micro-injection of SM buffer	
		Micro-injection of Tap water sterile + green dye	
		Micro-injection of 20 µL (10 µl PBS + 10 µL Tap water)	
18/07/2014		Micro-injection of *Campylobacter* 10^8, 10^7, 10^6, 10^5, 10^4, 10^3	
10/2014		Micro-injection of Phage 10^7, 10^6,10^5, 10^4, 10^3	
12/2014		Continuation	

"Validation of the *G. mellonella* laboratory model"

The success of the *G. mellonella* model is based on the organisms' survival when subjected to the various experimental conditions adopted for *Campylobacter,* such as the ability to survive at 37°C and sensitivity to aseptic micro-injection techniques. The techniques adopted should not trigger either stress or mortality when precision injection (and removal) of 10µl volumes using a fine sterile needle via the fourth pro-leg is used. The methodologies for these factors were developed via a series of experiments and are listed below:

- Experiment 1: - *G. mellonella* survival at 37°C
- Experiment 2: - Sterilisation options for larvae
- Experiment 3: - Development of a score sheet to assess survival and melanosis
- Experiment 4: - "Draining" and plating insect haemolymph
- Experiment 5: - "Pipetting" and plating insect haemolymph
- Experiment 6: - Dye, as tracer for injected material
- Experiment 7: - Developing the precise micro-injection technique

Experiment 1*: G. mellonella* survival at 37°C
The temperature to be adopted across the study is 37°C which is the optimum temperature for the growth of *Campylobacter*. The insect was incubated at 37°C initially without and then with injected *Campylobacter* or bacteriophage.

Larvae (final instar) were introduced into petri dishes and incubated at 37°C and tested at 2h, 24h and 48h. The petri dish was secured with rubber bands to prevent the escape of the insect but in a manner that there was sufficient air for survival. Figure 7, illustrates that *G. mellonella* survival at 37°C when tested at 2h, 24h and 48h. The insect was still viable with no signs of melanosis (stress). These conditions would be adopted for incubating the inoculated insect during the trial.

Figure 7 Survival of *G. mellonella* at 37°C (without bacteria or bacteriophage)

Figure 8 illustrates the survival of *G. mellonella* after different concentrations of *Campylobacter* (10 - 10^8 CFU/ml) and phage were separately injected into the haemocoel.

Figure 8: Survival of *G. mellonella* with different *Campylobacter* dilutions

The insect larvae survived when tested on introduction of either bacteria or bacteriophage.

Experiment 2 – Sterilisation options for larvae

The micro-injection (and extraction) of *Campylobacter* and phage would be carried out under aseptic conditions. The agent used for sterilisation (70% alcohol) can impact on the delicate surface of the larvae. The best disinfection technique was selected based on optimum survival time with least melanosis (an indication of stress). Several options were trialled to

ensure minimum stress (no melanosis) to larvae or their natural survival ability under laboratory conditions.

Before injection, *G. mellonella* were removed and placed into a metal bowl which was chilled on ice ensuring all debris was removed. The required number was then placed in a petri dish (on ice) in preparation for the injection. The options for sterilising were tested by using 70% alcohol on different sites of the insect as listed.

1) Spray insect with 70% alcohol very lightly
2) Swab all underside of insect with a sterile swab 70% alcohol
3) Swab only the left foreleg (point of injection) with 70% alcohol
4) Submerge entire insect in 70% alcohol
5) Control - no application

The insect was Incubated at 37ºC and the survival and melanosis was documented using the developed score sheet (next experiment).

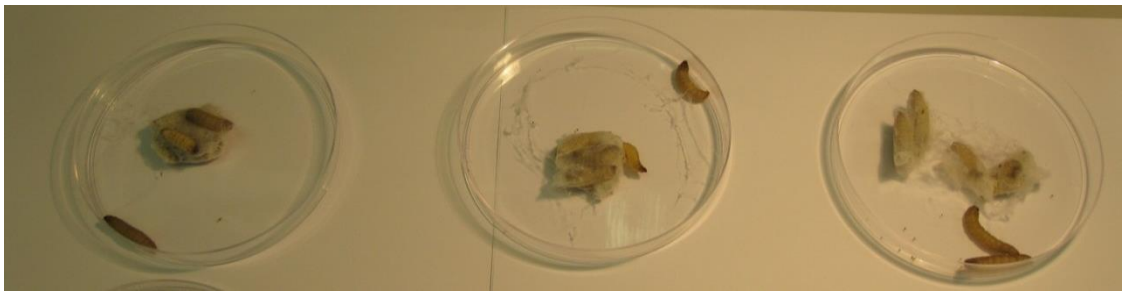
Figure 9 illustrates *G. mellonella* during the various sterilisation options tested noting for a normal insect, insect death, melanosis and the weaving of cacoons leading to a dormant stage of the insect. Following the observations, it was decided that the best option was to swab the front pro-legs (left and right) for minimum impact on death and melanosis (stress). Melanosis is a criterion that will be scored following *Campylobacter* injection over time and should not be triggered as a result of the "surface sterilisation" techniques adopted.

G. mellonella sprayed with 70% alcohol

G. mellonella Control (spinning of cacoons)

G. mellonella dipped in 70% alcohol melanosis clearly visible

G. mellonella Swabbed whole underside with 70% alcohol (spinning of cacoons)



G. mellonella Swabbed on injection point with 70% alcohol (spinning of cacoons)

Figure 9: Survival of *G. mellonella* during various sterilising options

Experiment 3 - Development of a score sheet to assess survival and melanosis

Melanosis can also be an indicator of "insect stress" in the presence of both *Campylobacter* (and or phage) impacting on future experiments. Thus a score sheet was developed to enable grade the levels of insect stress during the various experiments undertaken, as part of the *Campylobacter* – bacteriophage validation studies using the *G. mellonella* model.

Figure 10 lists the criteria for the various stages of melanosis and this score sheet developed based on the different conditions exhibited by the insect. The melanosis "score sheet" shows the grades or stages of melanosis developed. This score sheet will be used across the study to assess the degree of stress following the various *Campylobacter* – bacteriophage interactions.

Colour chart for melanosis score for *G. mellonella*

Sterility
Test conditions: Date Prepared: 16/07/2014
Insect stage: *Galleria mellonella weight: ~330mg length: ~*
Percentage alcohol: 70%
Test period: 1hour and 24 hours observations **Conditions:** Incubated at 37°C

					Colour				
Treatment	Number of *G. mellonella* added	# Dead	# Alive	**# Morbid**	**1**	**2**	3	4	5
Control Nil	10	0	10	0	10				
Control Nil	10	0	10	0	8	2			
Control Nil	10	0	10	0	8	2			
Spray	10	0	10	0	7	3			
Spray	10	8	0	2		2	6		2
Spray	10	10	0	0		4		6	
Dip 5 sec	10	6	0	4		4	2		4
Dip 5 sec	10	10	0	0		3	3	2	2
Dip 5 sec	10	6	0	4		4	1	2	3
Swab all prolegs	10	0	10	0	7	3			
Swab all prolegs	10	0	10	0	8	2			
Swab all prolegs	10	0	10	0	8	2			
Swab front prolegs left & right	10	0	10	0	8	2			
Swab front prolegs left & right	10	0	10	0	10				
Swab front prolegs left & right	10	0	10	0	9				

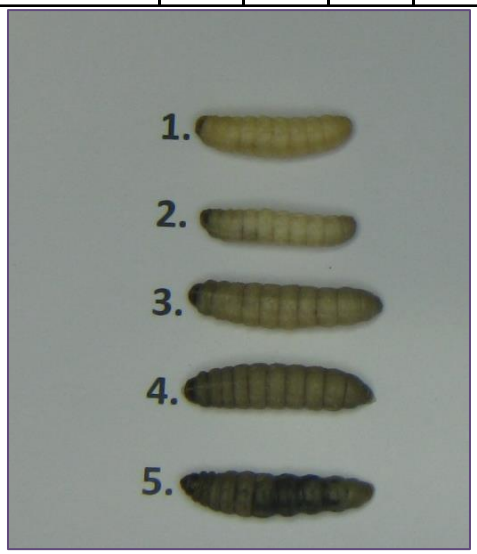


Figure 10: *G. mellonella* score sheet - survival and melanosis

Experiment 4: "Draining" and plating insect haemolymph

Aseptic removal of insect haemolymph – via draining the cut insect

1) Sterilise insect platform on injection unit with 70% alcohol, place disinfected insect (as above)
2) Use sterile needle and inject 10µl *Campylobacter* into insect last left foreleg
3) Cut the bottom 2cm of insect to drain haemolymph
4) "directly deposit" the drained haemolymph from the cut insect on to a horse blood plate (HBA) and use a sterile swab to spread
5) Incubate as usual and check for sterility (or growth)
6) Check for sterility by assessing growth on the plate following incubation at 37^{o}C

Figure 11 illustrates the haemolymph deposited directly onto HBA; some deposits are free from contamination while others have numerous colonies. Direct deposition is thus not suitable and pipetting the haemolymph directly from the insect can be a much better option. The disadvantages of this technique are as follows:

1) Difficult to estimate amount of haemolymph being extracted
2) Contamination through the epidermis of the insect touching the plate
3) Contamination through the gut or the silk gland touching the plate

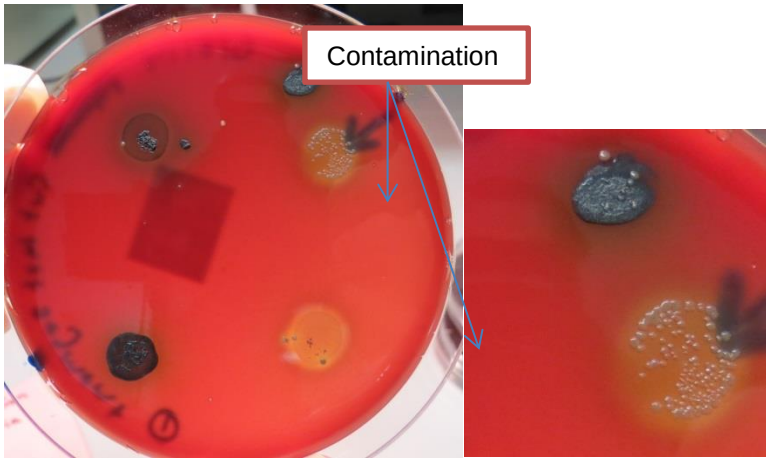


Figure 11: Blood agar plate with directly deposited haemolymph

Experiment 5: Pipetting and plating insect haemolymph
Aseptic removal of insect haemolymph – via pipetting from the cut insect.

Listed is the methodology adopted for this purpose

1) One hour after *G. mellonella* has been injected the haemolymph is removed
2) The insects are sedated on ice until no movement is visible, any movement can lead to contamination via the haemolymph touching the epidermis of the insect.
3) The anal proleg of the insect is swabbed with 70% alcohol, once dry the proleg is removed using sterile scissors.
4) The haemolymph is removed via pipetting from the insect and plated or stored on ice to prevent coagulation

Figure 12 illustrates the minimal amount of freshly removed (via pipette) haemolymph from a single larva. On removal, there is no melanosis which only tends to occur with time and thus the need to chill the removed haemolymph without delay.

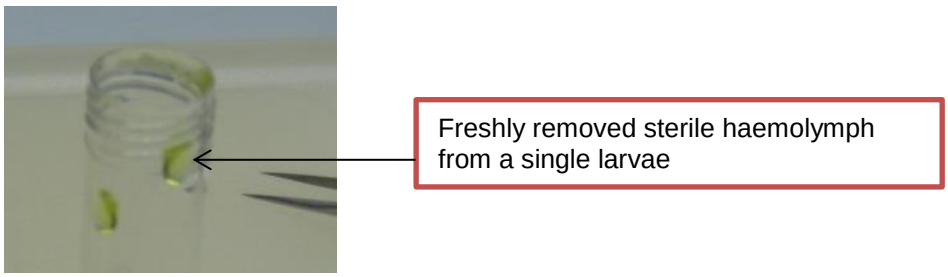


Figure 12 – haemolymph from a single larva

Haemolymph removed with a sterile pipette from the insect is plated (also spread using the same tip) onto HBA shows no contamination at point of plating (Figure 13a), but hardened with melanosis after 24h at 37°C (Figure 13b). Thus the haemolymph will need to be diluted very quickly on removal from larvae and kept chilled, prior to deposition on agar. Further, the

bacterial or phage numbers will have to be enumerated from a diluted haemolymph sample that can be pipetted.

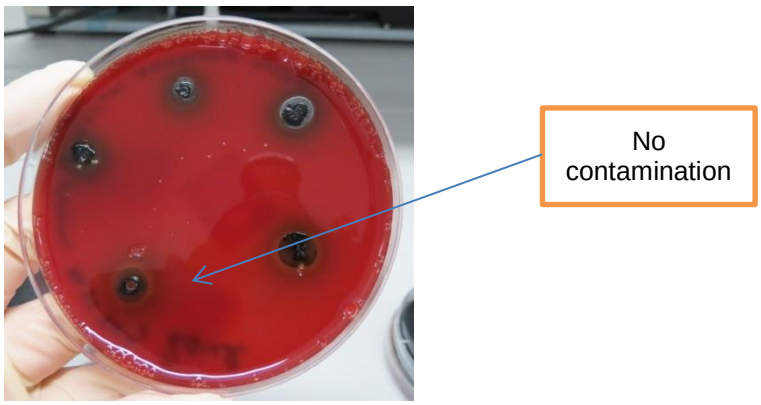


Figure 13a: Plated haemolymph (with no contamination at point of transfer)

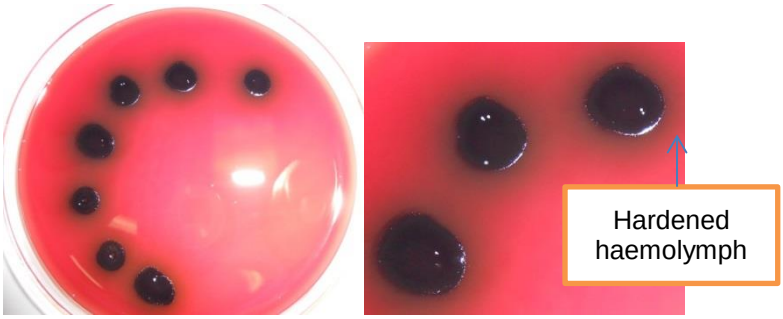


Figure 13b: Plated haemolymph (hardened with melanosis after 24h incubation at 37ºC)

Sufficient volume of haemolymph can also be removed from the larvae by removal with a pipette tip. This enables the possibility to enumerate the organisms (i.e. CFU/ml for *Campylobacter* or PFU/ml for bacteriophages) from a larger sample volume. Due to the minimisation of contamination via this technique there is no need to use antibiotics with the plating media used for enumeration which is either HBA or CCDA (without antibiotics). The non-use of antibiotics when working with bacteriophages and *Campylobacter* is an advantage.

Some of the other advantages of removing haemolymph via pipetting are:

1) minimum contamination and is a much cleaner process
2) ability to accurately measure extracted haemolymph
3) ability to extract from the centre of drop of haemolymph, thus minimising the risk of further contamination This technique will be adopted for all future experiments.

Experiment 6: Dye, as tracer for injected material
The day before injecting, final instar *G. mellonella* (Figure 14) are removed from the colony and put into a separate jar containing only diet. They are left overnight to allow the insects to feed and minimise stress so no melanosis is present and they are cream in colour to enable the easy visualisation of the dye.

The “micro-injector” was used for more precision dose delivery (10µl) with the use of food dye to ensure uniform spread of inoculant through the haemolymph. Queens green food dye

is mixed with sterile water at the dilution of 1ml in 25ml. This is then autoclaved at 121^{o}C for 15 min, all equipment is sterilised with 70% alcohol. *G. mellonella* are sedated and sterilised via swabbing the right proleg and 10µl is injected into the insect, which is allowed to rest for one hour before the haemolymph is removed.

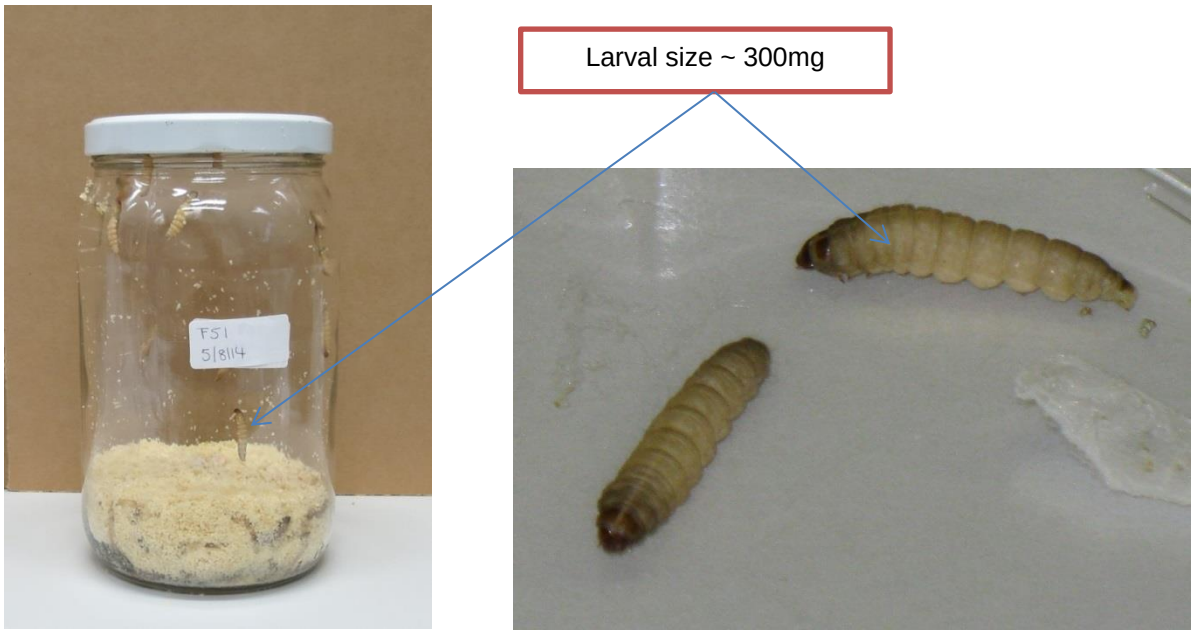


Figure 14: Mature larvae

The methodology that was developed is listed as follows:

1. *G. mellonella* are then removed from diet, weighed, i.e. average weight 220 - 330mg; length 2 - 2.5cm; (Figure 14) and sedated by putting them on ice.
2. The Insect platform and the micro injector are sterilised with 70% alcohol.
3. Individual *G. mellonella* are washed in sterile distilled water and placed onto filter paper in a petri dish sitting on top of ice.
4. The injection site (the right foreleg) is swabbed with 70% alcohol which is then left to dry.
5. Once dry, *G. mellonella* is positioned on the injecting platform which is then moved so the needle can enter the insects' right foreleg.
6. Once the needle has entered the insect, 10µl of fluid is injected into the haemolymph.

Experiment 7: Developing the precise micro-injection technique
A special micro-injector (Figure 16) was used to inject the required low volume of doses of bacteria or phage with precision right into the sterile haemolymph, but not penetrate the gut which can result in contamination. This stage required a lot of practice both to ensure precision and safety when dealing with working with an organism such as *Campylobacter*.

The adopted steps are illustrated in Figures 15, 16 and 17.

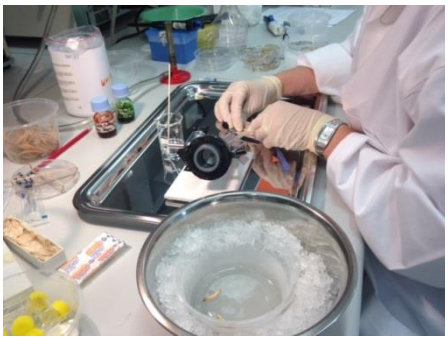
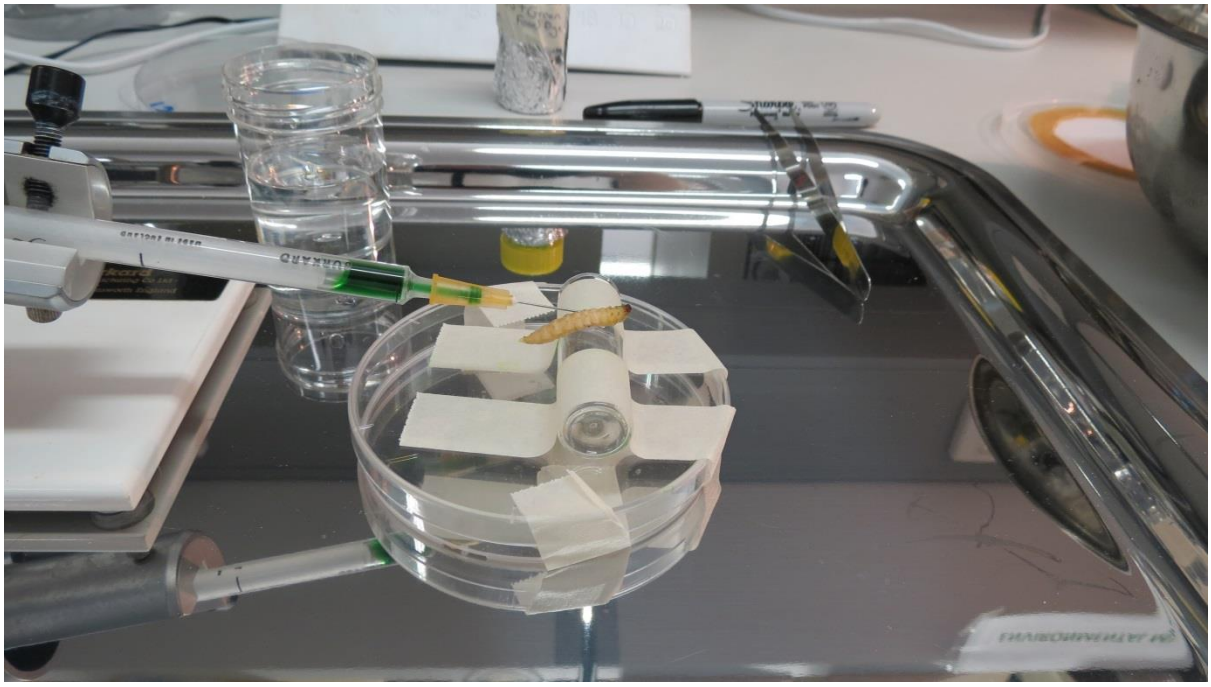


Figure 15: Set- up for injection and haemolymph removal from larvae under aseptic conditions



Correct placement of the needle can be observed by the colour change of the insect

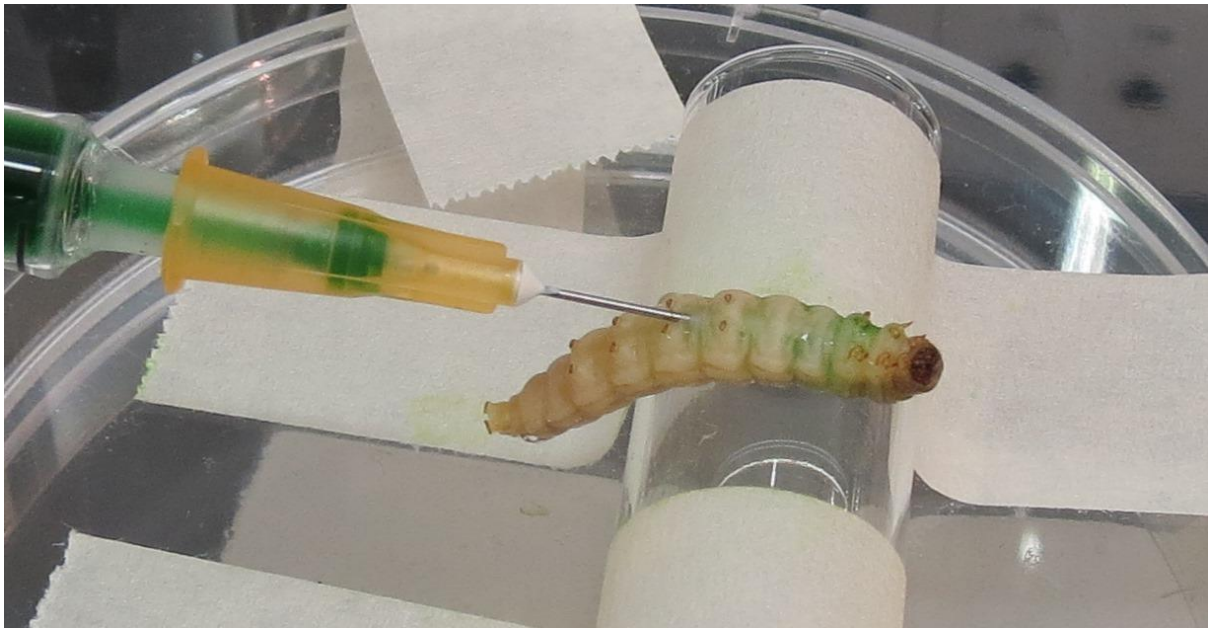


Figure 16: Injection of insect

Once fluid is successfully injected (Figure 17), the insect is carefully removed from the needle and put on filter paper in a petri dish. After approximately 2 to 5 min the insect starts moving or recovering from both the sedation and the injection. The green dye enables the visualisation of the movement of the inoculum within the haemocoel.

Figure 17: Injected and non -injected insects immobilised in ice

The injected larvae wriggle, this movement allows the visualisation of the green dye (or inoculant evenly distributed around the injected larvae. Those that lack even colour distribution are rejected.

Microbiology techniques and *G. mellonella* larvae

This section deals with a series of experiments undertaken to develop the necessary microbiology techniques associated with the micro-injection of the larvae. The larvae need to survive. The various steps undertaken are discussed as a series of experimental studies done in order develop the adopted methodology to facilitate the final outcome of this study, which are the "therapy studies".

The following experiments were undertaken:

Experiment 1: Diluents and *Galleria* survival
Experiment 2: Diluents and green dye on *Galleria* survival
Experiment 3: Enumeration of phage from haemolymph after injection with (10^8, 10^7, 10^6, 10^5, 10^4 PFU/ml) at 0.5, 24 and 48 h
Experiment 4: Enumeration of *Campylobacter* from haemolymph after injection with (10^8, 10^7, 10^6, 10^5, 10^4 CFU/ml) at 0.5, 24 and 48 h

Experiment 1: Diluents and *Galleria* survival
Use of normal diluents or solutions that support the growth of either bacteria or phage can be used as a suitable delivery medium to transfer these organisms into the haemocoel. However, the constituents of those solutions should not impact on the integrity of the "haemolymph micro-environment" that will support the bacteria – phage interactions over time. This study was carried out to validate that the diluents (and volumes) used to inject on the survival of the insect. In order to achieve this, 10 – 20 µl volumes of SM buffer, PBS and

tap water were injected into the insect, and the survival was observed after 24h to see the impact on the insect from the use of these solutions.

Experiment 2: Diluents and green dye on *Galleria* survival
The green food dye enables the assessment of both successful injection and uniform dispersion of the phage inoculum. This provides a better opportunity for inoculated doses of phage to interact with the dispersed *Campylobacter* within the sterile insect haemolymph. The following experiment assesses the use of the green food dye in commonly used phage diluents both with and without the phage, over time (0.5h – 48h). This was carried out twice in Tests 1 and 2.

Survival of *Galleria* with:

(a) 10µl of SM buffer + green dye
(b) 10µl sterile tap water + green dye and
(c) 10µl of sterile tap water + green dye and 10µl PBS+ green dye (20µl total).

Study:

1. Prepare sterile SM buffer + green dye (Dye: SM buffer=1:25).
2. Prepare *Galleria* (weight, sterile surface with 70% Ethanol, put on the filter paper in petri dish, keep on ice etc.)
3. Inject group 1) 10µl SM buffer in to *Galleria*, group 2) 10µl sterile tap water, group 3) 10µl of (b) +10µl PBS.
4. Keep at 37^{o}C for 0.5, 24 and 48h
5. Observe survival, colour and behaviour.

Experiment 3: Enumeration of phage from haemolymph after injection with (10^8, 10^7, 10^6, 10^5, 10^4 PFU/ml) at 0.5, 24 and 48 h

This study involved the enumeration of phage from haemocoel after injection with 10^8, 10^7, 10^6, 10^5, 10^4 PFU/ml at 0.5, 24 and 48h. This was done to assess the impact of these conditions (i.e. phage doses with time) on larval survival prior to testing both together (i.e. *Campylobacter*)

A) Prepare phage dilution with green dye, pipette 100µl of 10^8 PFU/ml phage in to 900µl sterile Tap water + green dye to get the dilution that will inject. For the first lot (10^8) add 10µl green dye in to 240µl phage (1:25).
B) Prepare *Galleria* (weight, sterile surface with 70% Ethanol, put on the filter paper in petri dish, keep on ice etc.)
C) Inject 10µl of phage dilution into *Galleria*
D) Keep at 37^{o}C for 0.5, 24 and 48h.
E) Take haemolymph out after incubation at 0.5, 24 and 48h, pool in microcentrifuge tube, chill on ice
F) Make a 10 fold serial dilution (100µl haemolymph in 900µl sterile tap water).
G) Prepare PT14 host (=McFarland standard No.1) dispense 200µl in to microtube.
H) Mix 100µl of haemolymph dilution with 200µl host, incubate aerobically at 42^{o}C for 30 min
I) Mix with warm overlay agar and pour on top of an agar base plate.
J) For the phage dilution tube that used for injection, make a 10 fold serial dilution (100µl phage in 900µl sterile tap water + green dye).
K) Take 100µl of the each dilution, mix with 200µl host, incubate aerobically at 42^{o}C for 30 min, then mix with warm overlay agar and pour on an agar base plate.
L) Let plate set about 20 min

M) Incubate plate in micro anaerobic condition (in a jar with Campygen) at 42^{o}C for 24h.
N) Count plaque and calculate the number of phage per ml, compare PFU from phage dilution and haemolymph.
O) Repeat A) to N) with **10^{7}, 10^{6}, 10^{5}, 10^{4}** of phage dilution
P) Plating 100µl with overlay agar injected 0 dilution phage with green dye into 5 *Galleria* and pooled the haemolymph

Experiment 4: Enumeration of *Campylobacter* from haemolymph after injection with (10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4} CFU/ml) at 0.5, 24 and 48 h

This study involved the enumeration of *Campylobacter* from haemocoel after injection with 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4} PFU/ml at 0.5, 24 and 48h. This was done to assess the impact of these conditions (i.e. *Campylobacter* doses with time prior to testing both together (i.e. phage) on larval survival.

Select insects as per weight criteria

Insect weight

380mg per insect a total of 7 insects are injected and the best 5 are selected

Insect#	Weight(g)
1.	~ 330
2.	~ 330
3.	~ 330
4.	~ 330
5.	~ 330
6.	~ 330
7.	~ 330

To carry out the phage – bacteria interactions, it is necessary to have a suitable bacterial concentration within the haemolymph of the insect that allows the assessment of *Campylobacter* death (due to bacteriophage). These *Campylobacter* concentrations used should not directly impact on the insect's survival or induce melanosis due to insect stress during the experimentation.

Campylobacter concentrations used to inoculate insect

CFU/1000 µl					
10^{8}	10^{7}	10^{6}	10^{5}	10^{4}	10^{3}
CFU/10µl					
10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10

Media required etc**.**

900µl diluents around 20 (aseptically dispensed and checked for sterility by incubating at 30^{o}C for 24h. or streaking on CCDA and blood agar

5ml nutrient broth to grow an overnight *Campylobacter* isolate

Aseptic handling of larvae

- Select and weigh insects as usually done
- Sterilize larvae with 70v/v alcohol as per your last result outcome i.e. the last pro-leg
- Transfer larvae (10 or a number you choose as appropriate or available) into sterile plastic petri dishes (3 X 4 = 12 or more as available) i.e. 3 or more larvae per dilution per petri dish. Please note we will need slightly more than 100µl haemolymph to be able to serially dilute and count

Preparation of bacterial inoculum

- Grow bacteria in Horse Blood Agar 24h at 37°C. Adjust bacteria to OD590 1.0 in PBS for infections 1X10 ^{8}CFU/ml.
- Dilute as per table for *Campylobacter*
- Store original and all dilutions in fridge until serial dilutions are done
- Confirm concentration of bacteria (infectious doses) by serially plating on HBA and CCDA the appropriate serial dilution (e.g. 1ml in 9ml PBS or Preston with no blood)
- Inject 10µl into the haemolymph via the right foreleg via a Hamilton syringe or miro-injector (Figure 18)
- Leave for one hour for bacteria to move through haemolymph and settle inside
- Record and physical changes based on score card
- Remove haemolymph from 5 insects each and combine (Sample A and Sample B)
- Serially dilute and plate appropriate dilutions using drop plate (as described below)
- Incubate all plates at 37°C for 48h
- Count colonies and calculate numbers of organisms per insect.
- Compare number of bacteria injected Vs what was recovered.
- Repeat to see a pattern of consistency in injected Vs recovered numbers.

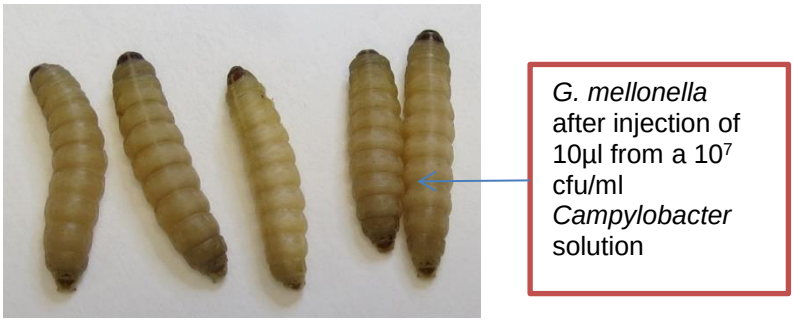


Figure 18 Larvae injected with *Campylobacter*

Drop plate method for testing haemolymph for *Campylobacter*

Materials:
Blood plate
5ml sterile PBS
20 X 90µl sterile diluents aseptically prepared under the laminar flow (after dispensing check 1 or 2 tubes for sterility by swabbing on blood agar and incubating for 24h)

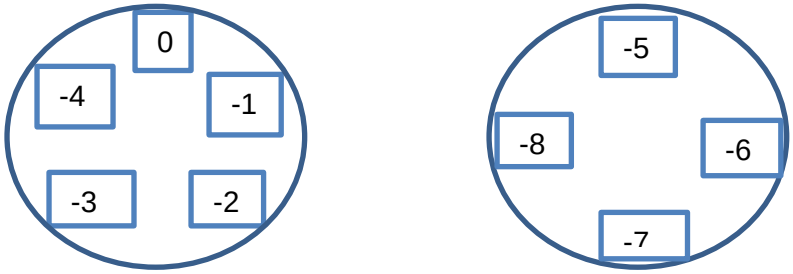
Methodology

1. Prepare overnight culture on blood agar
2. Aseptically prepare are 5ml inoculum of bacteria as follows
   (a) Swab growth from the blood plate
   (b) Mix into a vial of 5ml sterile PBS and adjust bacteria to OD590 (see our unit) ~1X10 ^{8} CFU/ml.
   (c) Do a spread plate 1ml in 9ml serial dilutions (mix well) , keep original dilution in fridge
   (d) Incubate at 37°C for 48h and count

Serial dilution for Sampling *Campylobacter* concentrations in prepared doses used for inoculation of the insect

0 dilution 5 ml tube	-1 Blood plate + CCDA	-2 Blood plate + CCDA	-3 Blood plate + CCDA	-4 Blood plate + CCDA	-5 Blood plate + CCDA	-6 Blood plate + CCDA	-7 Blood plate + CCDA	-8 Blood plate + CCDA
10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1

1) Use original 5ml (tube that OD was done) and do “drop plate” by first doing a 10µl serial dilution in 90µl sterile PBS diluent (mix well) across series
2) Plate using 10µl on both CCDA and Blood Agar
3) Use same table as above to record spots on plates that will represent the different dilutions
4) Incubate at 37°C for 48h and count



Test insects over the required experimental duration. Prior to removal of haemolymph, score insects using score sheet and record.

Score sheet

All alive and green colouration distributed evenly

Insect	**ALIVE**	**DEAD**	**Melanosis Score**			
			1 pink	**2 beige**	**3 brown**	**4 black**
1	YES					
2	YES					
3	YES					
4	YES					
5	YES					
6	YES					
7	YES					

Use of phage and bacteria combinations for therapy experiments
The methodologies developed previously were used for the therapy experiments. The sequence of the therapy experiments undertaken are listed in Table 4.

During the therapy studies some additional methodologies were addressed and are listed below:

1. Modification of presentation of results from “per insect” to “per ml” to facilitate more accurate calculation

2. The use of Chloroform to kill *Campylobacter* on removal of the inoculum from the insect thus ensuring no phage production continued outside insect haemocoel and impact on the calculations.

Turbidity adjustment enables a standard *Campylobacter* inoculum for injection
The calculation of *Campylobacter* count decrease depends on a standard inoculum injected and was carried out as follows for the biolog instrument that was used for the purpose.

Turbidity adjustment = 35% transmittance on Biolog machine

Drop 10 µl on to HBA and CCDA start at
Plate 1 = -1,-2,-3,-4 Plate 2 = -5,-6,-7,-8
The outcomes are presented in Figure 19

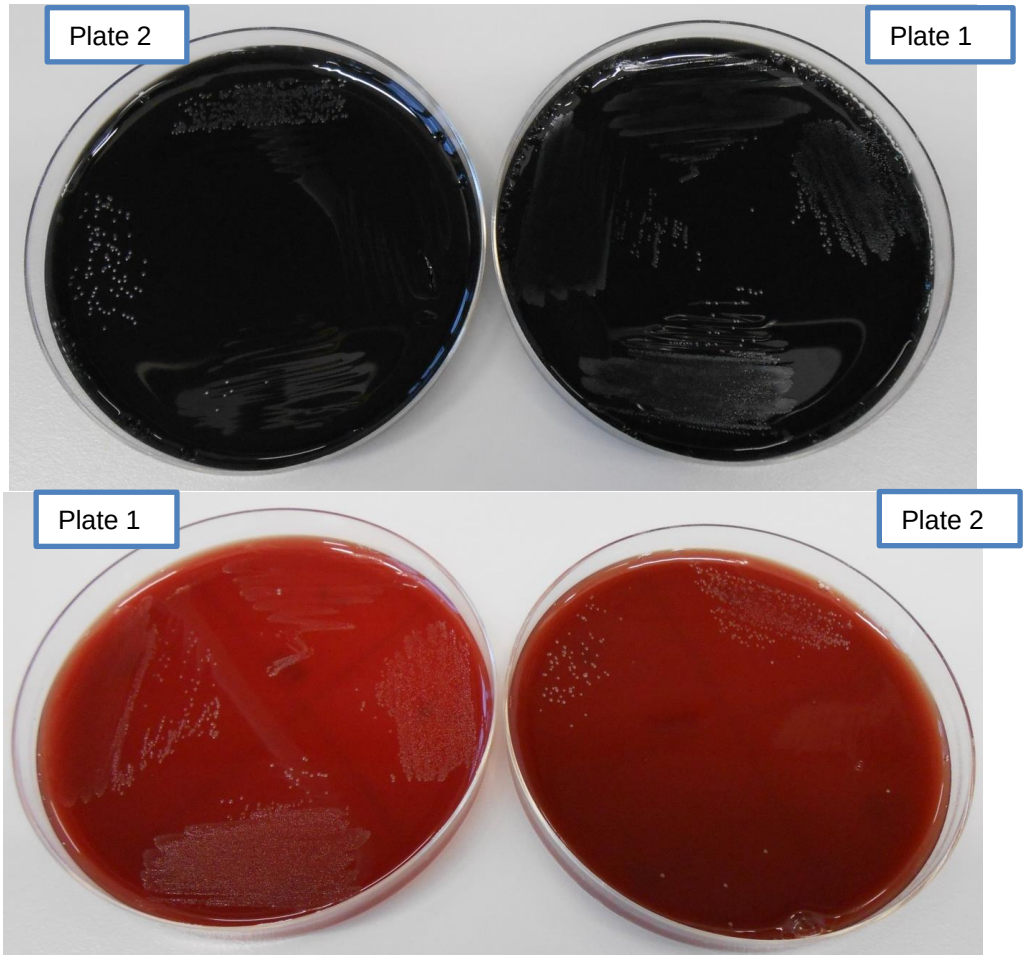


Figure 19: Turbidity adjustments for *Campylobacter* inoculum

Thus it was validated that 0.01ml x 10^7 CFU = 10^9 CFU/ml at the given biolog reading of 35%

Chloroform use and phage enumeration
During phage therapy it is possible that the phage can increase during the lysis (killing *Campylobacter*) process when in the insect. Chloroform was added immediately on removal of the haemocoel to prevent the increase of phage outside the insect. However, prior to undertaking this work, it was necessary to validate that the phage will not be affected by coming into contact with chloroform. Following this, a safe concentration was validated for use during the rest of the study.

The methodology adopted is as follows:

Sample from PH15 high titre phage stock (10^7 PFU/ml).

1. Diluted Phage sample until get 10^3 PFU/ml.
2. Pipette 100µl of 10^3 PFU/ml phage in to 500µl microcentrifuge tube, 4 tubes.
3. Add 5%, 10% and 20% of chloroform in to each tube and leave 1 tube for untreated.
4. Mix and leave it sit for 5 minutes.
5. Centrifuge, and pipette the top liquid in to a new tube.
6. Leave tubes open to evaporate.
7. Process Phage count on overlay agar with PT14 host.
8. Read the results, analyse.

The therapy studies were designed to demonstrate the potential to compare doses of either *Campylobacter* or bacteriophage. This was undertaken via using both "Active" (high doses of both *Campylobacter* and bacteriophage) and "Passive" (High dose of *Campylobacter* and lowering doses of bacteriophage). The sequence of such therapy experiments are listed in Table 4.

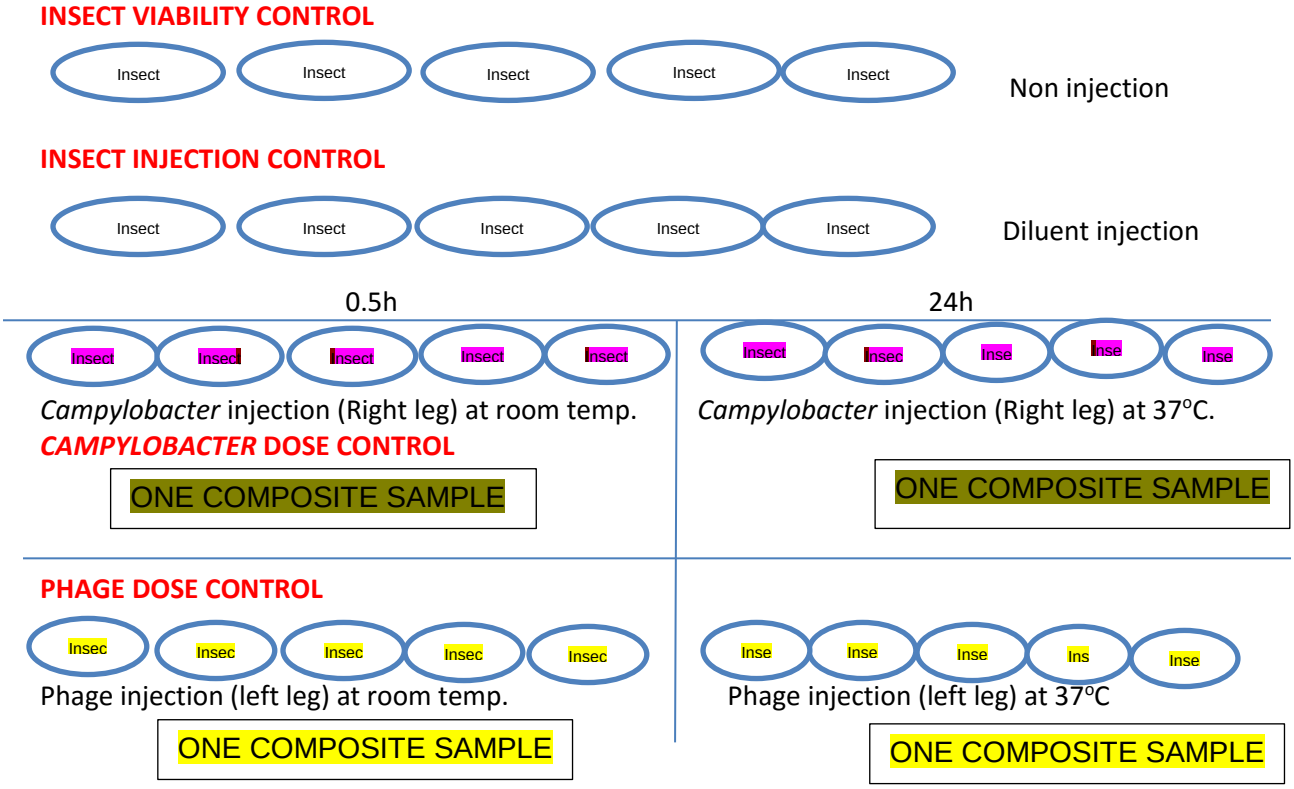
Protocol adopted for "phage therapy" experiments

Active : High *Campylobacter* (10^7 CFU) Versus decreasing phage(10^7, 10^5,10^3 PFU)

Passive : High Phage (10^7 PFU) Versus decreasing *Campylobacter* (10^7, 10^5,10^3 CFU)

Control Group

1. Viability Control: Non injection
2. Injection Control: Inject 10 µl PBS (+green dye) + 10µL dH2O (+ green dye)
3. *Campylobacter* Control: Inject 10µl of *Campylobacter* (=10 insects, 5 for 0.5h, 5 for 24h.)
4. Phage Control: Inject 10µl of Phage (=10 insects, 5 for 0.5h, 5 for 24h.)



A) Active

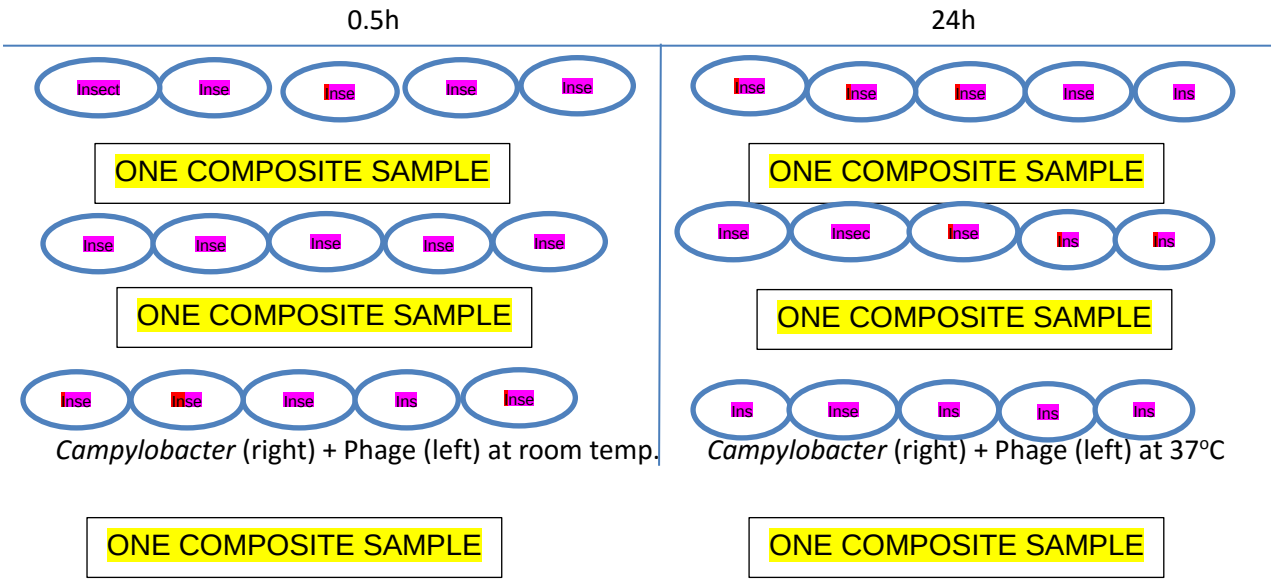
Experiment 1: *Campylobacter* 10^7 CFU + Phage 10^7 PFU

Prepare *Campylobacter* inoculum of 10^9 CFU/mL from grown overnight culture on HBA with BHI by comparing turbidity with biolog = 35 % transmittance (validated).

Inject 10µl of 10^9 CFU/ml of *Campylobacter* (= 10^7 CFU) into the right front pro leg and 10µl of 10^7 PFU/ml of phage (the highest of our phage stock that we have) plus green dye into the left front pro leg of gallerias and then take the haemolymph out at after let remain.

1) 0.5 hour at room temperature.
2) 24 hours at 37°C.

TEST SAMPLES (*Campylobacter* phage) reaction = *Campylobacter* reduction



Measure volume of haemolymph, divide up into 2 parts for *Campylobacter* and phage count. Make a 10 fold serial dilution and plate on agar plate for:

- *Campylobacter*: 10µl in to 90µL of PBS and pipette 10µL drop and spread on HBA and CCDA (4 segments per plate)
- Phage count: the first dilution(10^{-1}) pipette 10µl into 90µl and the next dilution pipette 100µl in to 900µl of sterile dH2O + green dye(10^{-2}, 10^{-3} , 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilution). Take 100µl from each dilution then process per ***phage count method***.

Experiment 2: *Campylobacter* 10^7 CFU + Phage 10^5 PFU

Prepare *Campylobacter* inoculum of 10^9 CFU/ml from grown overnight culture on HBA with BHI by compare turbidity with biology = 35 % transmittance (validated).

Inject 10µl of 10^9 CFU/ml of *Campylobacter* (=10^7 CFU) into the right front pro leg and 10µl of 10^7 PFU/ml of phage (=10^5 PFU/ml) plus green dye into the left front pro leg of *Galleria* and then process same as experiment 1.

Experiment 3: *Campylobacter* 10^7 CFU+ Phage 10^3 PFU

Prepare *Campylobacter* inoculum of 10^9 CFU/ml from grown overnight culture on HBA with BHI by compare turbidity with biology = 35 % transmittance (validated).

Inject 10µl of 10^9 CFU/ml of *Campylobacter* (=10^7 CFU) into the right front pro leg and 10µl of 10^5 PFU/ml of phage (=10^3 PFU/ml) diluted with sterile water plus green dye into the left front pro leg of *Galleria* and then process same as experiment 1.

B) Passive

Experiment 4: Phage 10^7 PFU+ *Campylobacter* 10^7 CFU.
Prepare *Campylobacter* inoculum of 10^9 CFU/ml from grown overnight culture on HBA with BHI by compare turbidity with biology = 35% transmittance (validated).
Inject 10µl of 10^9 CFU/ml of *Campylobacter* (=10^7 CFU) into the right front pro leg and 10µl of 10^7 PFU/ml of phage (the highest we have) plus green dye into the left front pro leg of *Galleria* and then process same as the previous experiment.

Experiment 5: Phage 10^7 PFU+ *Campylobacter* 10^5 CFU.
Prepare *Campylobacter* inoculum of 10^9 CFU/ml from grown overnight culture on HBA with BHI by compare turbidity with biology = 35 % transmittance (validated). Make a serial dilution with sterile water plus green dye (-1,-2 = 10^7 CFU/ml).
Inject 10µl of 10^7 CFU/ml of *Campylobacter* (=10^5 CFU) into the right front pro leg and 10µl of 10^7 PFU/ml of phage (the highest we have) plus green dye into the left front pro leg of Galleria and then process same as the previous experiment.

Experiment 6: Phage 10^7 PFU+ *Campylobacter* 10^3 CFU.
Prepare *Campylobacter* inoculum of 10^9 CFU/ml from grown overnight culture on HBA with BHI by compare turbidity with biolog = 35 % transmittance (validated). Make a serial dilution with sterile water plus green dye (-1,-2,-3,-4 = 10^5 CFU/ml).
Inject 10µl of 10^5 CFU/ml of *Campylobacter* (=10^3 CFU) into the right front pro leg and 10µl of 10^7 PFU/ml of phage (the highest we have) plus green dye into the left front pro leg of *Galleria* and then process same as the previous experiment.

Results

G. mellonella laboratory colony for experimental studies

Successful colony maintenance including the development of optimum feed and disease management was established. This enabled the succession of larvae of the required size and age for routine weekly experimental studies. The colony management protocol established also gave the knowledge (and options) to troubleshoot any problems of insect mortality and the re-establishment of the colony to enable routine work.

"Validation of the *G. mellonella* laboratory model"

The *G. mellonella* larvae survived for up to 48h at 37^{o}C, thus enabling experimentation to be carried out at temperatures supportive for *Campylobacter*. This was validated prior to the introduction of both organisms allowing larvae to be subjected to normal laboratory incubation conditions in a petri dish at 37^{o}C. The protocol for surface sterilisation using of 70% alcohol at the point of injection was established (i.e. foreleg) following comparison of all other possible options. This option was selected based on least stress and also to facilitate aseptic inoculation. A *G. mellonella* score chart was created to score the insect for stress during surface sterilisation, injection (with *Campylobacter* or bacteriophage), and incubation with these organisms over time. Stress or death indicates that the insect has been affected and that any outcomes at the time are not valid with the need repetition.

Following the establishment of the protocols for the normal laboratory handling of the larvae, both *Campylobacter* and bacteriophage protocols, were validated. Pipetting removal of haemolymph was selected over draining, thus enabling the least contamination during the removal of haemolymph from an inoculated larvae. The use of a food based green tracer dye concentrations was established and sterile dye was incorporated with the injections. The dye, in conjunction with either organism inoculants, allowed visualisation of the movement of the small (10µl) volumes of inoculum within the larvae, validating a successful injection each time and visualisation of the uniform mixing within the haemolymph. The natural healthy larval contractions after injections contribute to the mixing and if this did not happen the larvae were rejected as being unsuitable. Both a Hamilton syringe and a micro-injector were compared. Finally, a precise micro-injection technique that aseptically delivered accurate 10µl volumes with minimum stress was developed. The micro-injector platform was easy to use for the purpose of sequentially injecting larvae plus was easy to decontaminate. The validation of all the above techniques ensured the repeatability of the precision micro-injecting technique (*Campylobacter* and bacteriophages) to a large number of larvae that were required for the study. All these steps were successfully validated.

Recovery of injected bacteriophages from the larval haemolymph

This stage of the study focused on the impact of the adopted microbiology protocols for both *Campylobacter* and bacteriophages on the larvae, including the use of suitable diluents. Figure 20 illustrates *G. mellonella* larval morphology after being injected with different diluents. Excepting for PBS (shriveled larvae) the other diluents were not detrimental to the larvae.

Control – un-injected Larvae at 144h	20µl sterile PBS – Larvae at 144h (for *Campylobacter*)	10µl sterile tap water – Larvae at 144h (for *Campylobacter*)	10µl sterile SM buffer – Larvae at 144h (for phage)

Figure 20: Survival of insect injected with different diluents

Tables 4a and b present the score sheet assessment of 10 larvae following the injection of the three common diluents sterile SM buffer (a phage diluent), PBS (a bacterial diluent) and tap water during two separate studies. The survival of the 10 larvae recorded at 0.5, 24 and 48h showed no detrimental effects in the presence of SM buffer and tap water. Tap water has the least impact on the integrity of the insect haemolymph up to 48h. Tap water (and green food dye) was selected for all future studies and would be an advantage with future chicken studies.

Table 4(a): *G. mellonella* survival in the presence of different diluents – Test 1

	10µl SM buffer	**10µl sterile tap water**	**10µl sterile tap water + 10µL PBS (20µl total)**	**Control No injection**
0.5h	All recovered	All recovered	2 recovered one is a bit slower and another has trouble righting itself	All recovered
24h	All active bright green frass in dish green colour of epidermis faded	All active bright green frass in dish green colour of epidermis faded	Slow individual dead half body is black epidermis still quite green alive individuals active	All active
48h	All active hardly any green colour in epidermis look similar to control	All active hardly any green colour in epidermis look similar to control	Another individual dead surviving individuals epidermis still quite green but are quite active had healthy looking	All active

Table 4(b): *G. mellonella* survival in the presence of different diluents – Test 2

	10µl SM buffer	10µl sterile tap water	10µl sterile tap water + 10µL PBS (20µl total)	Control
0.5h	All recovered	All recovered	2 recovered one is a bit slower and another has trouble righting itself	All recovered
24h	All active green in epidermis faded, bright green in frass	All active green in epidermis faded, bright green in frass	All active noticeable green coloration in epidermis present, also producing bright green frass	All active
48h	All active green in epidermis faded almost similar colour as the controls	All active green in epidermis faded almost similar colour as the controls	All active green in epidermis faded a little more but still very noticeable	All active

The integrity attributed to the injection process to the larvae continued to be assessed. More specifically, the repeatability of the injected *Campylobacter* and bacteriophage numbers within the haemolymph were assessed. This was done using the relevant laboratory protocols, initially looking at the survival of the larvae with high dose injection. Table 5 presents the survival of *G. mellonella* after the injection of both 10µl and 100µl of 10^8 PFU/ml phage in to 900µl tap water,+ green dye and in the presence of phage, there was no detrimental effect. Tap water was chosen as the diluent (with green food dye) and was tested in the presence of the following phage concentration (10µl in 900µl and 100µl in 900µl) and observed at 0.5, 24 and 48h. All larvae were found well and active under these conditions up 48h, Table 5. Thus tap water, green food dye and bacteriophage at high concentrations had no detrimental effect on the larvae.

Table 5: Survival of *Galleria* after phage injection at different concentrations

	10µl and 100µl of 10^8 PFU/ml phage in to 900µl tap water+ green dye
0.5 h	Recovered well quite active green colouration evenly distributed over epidermis, some staining of the filter paper where the dilution has leaked from the injection point a bit
24 h	All active green still visible in epidermis although not as dark, no sign of melanosis
48 h	All active not much green seen in the epidermis

The survival of the bacteriophages was compared across the diluents. Table 6 presents the outcome of the plating options compared for phage, where drop plate (the ability to use low 10µl volumes) versus pour plate (the use of 100µl volumes), the commonly adopted routine technique was compared. Higher counts ~ 10^7 PFU.ml can be achieved with the use of bacteriophage in tap water and dye with the pour plate technique. These were set as the routinely used diluent and standard plating technique for enumerating bacteriophages from insect haemolymph.

Table 6: Bacteriophage counts with the use of different diluents both "drop" and "pour plate"

Method	Drop plate	Pour plate	Drop plate	Pour plate
Date	23.09.14		24.09.14	
	PFU/ml			
SM buffer	$2.0X10^6$	$1.3X10^7$	$1.0x10^4$	$1.4X10^7$
SM+Dye	$1.0X10^6$	$1.3X10^7$	$1.1X10^6$	$1.7X10^7$
Tap Water	$3.0X10^6$	$1.0X10^5$	$1.0x10^4$	$6.6X10^6$
TW+Dye	$2.0X10^6$	$1.7X10^7$	$4.8X10^5$	$1.6X10^7$

Enumeration of phage from haemolymph after injection with 10^8, 10^7, 10^6, 10^5, 10^4 PFU/ml at 0.5, 24 and 48h
Figure 21 illustrates the bacteriophage plaques (areas of clearing on a lawn of *Campylobacter* host). These bacteriophages are on the surface of NZCYM agar.

Figure 21: bacteriophages on NZCYM agar

Appropriate dilutions are counted following which the total count is presented as PFU/ml. Tables 7 presents the bacteriophage counts (PFU/ml) following the injection of 10^8, 10^7, 10^6, 10^5, 10^4 PFU/ml of bacteriophage concentrations into *G. mellonella* larvae and assessed at 0.5h and 24 – 48h (incubated at 37°C). The bacteriophage counts in the original injection solution ranged from a minimum of 2.50×10^6 PFU/ml to a maximum of 1.91×10^9 PFU/ml and when recovered from the haemolymph ranged from a minimum of 3.30×10^4 PFU/ml to a maximum of $2.48x10^8$ PFU/ml (depended on the original concentration at injection). There is a 1 – 2 log reduction (only 10µl is injected) once in the haemocoel of the insect. The achieved concentration within the larvae at 0.5h is sufficient for the therapy studies. At 24h there was not much "natural reduction" in counts in the recovered haemolymph from the incubated insect (minimum of 2.40×10^4 PFU/ml to a maximum of 2.07×10^7 PFU/ml). After 48h the counts ranged from a minimum of 5.90×10^4 PFU/ml to a maximum of 1.25×10^7 PFU/ml with minimal drop in the injected bacteriophage counts in the incubated larvae.

Table 7: Bacteriophage counts (PFU/ml) from insect haemolymph

Dates	15.10.14	22.10.14	29.10.14	05.11.14	13.11.14	19.11.14	10.12.14
	log PFU/ml						
Dilution prior inoculation	2.50 x 10^6	1.91 x 10^9	2.68x 10^9	3.60 x 10^6	5.80 x 10^6	5.80 x 10^6	ND
Haemolymph 0.5 h	5.40 x 10^5	1.38 x 10^7	2.48x 10^8	8.50 x 10^5	3.30 x 10^4	5.90 x 10^5	1.70 x 10^6
Haemolymph 24 h	1.75 x 10^5	ND	2.07 x 10^7	3.20 x 10^5	ND	1.90 x 10^6	2.40 x 10^4
Haemolymph 48 h	6.30 x 10^4	1.25 x 10^7	ND	ND	ND	5.90 x 10^4	ND

Table 8 provides a summary (log PFU/ml) of the above count and, from an overall perspective the insect haemolymph supports sufficiently high counts in the incubated live insect at 48h with the opportunity for designing longer experiments if required.

Table 8: A summary of bacteriophage counts across time

Original Dilution prepared	0.5h	24h	48h
	Plated from haemolymph of injected larvae		
	log PFU/ml		
6.40	5.73	5.24	4.80
9.28	7.60	ND	7.10
9.43	8.39	7.32	ND
6.56	5.93	5.51	ND
6.76	4.52	ND	ND
6.76	5.77	6.28	4.77

Further analyses of these bacteriophage counts (Table 9), show a decrease in counts ranging from 0.63 - 2.24 log PFU/ml, with an average decrease of 1.21 log PFU/ml at 0.5h. This outcome was considered suitable for the therapy studies that will be undertaken.

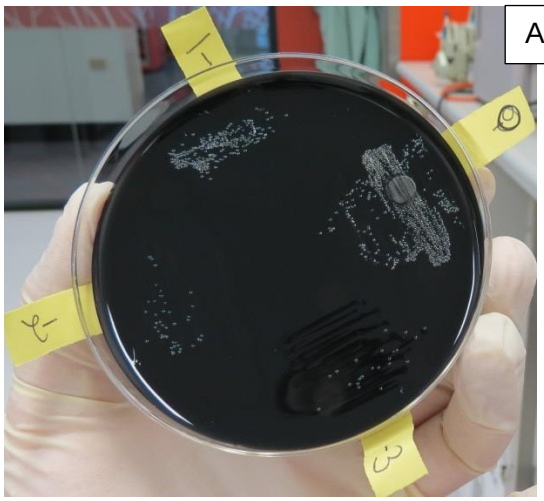
Table 9: Average decrease in bacteriophage counts

0.5h	24h	48h
-0.67	-1.15	-1.60
-1.68		-2.18
-1.03	-2.11	
-0.63	-1.05	
-2.24		
-0.99	-0.48	-1.99

Recovery of injected *Campylobacter* from the larval haemolymph

Figure 22 illustrates the successful use of the drop plate technique to enumerate *Campylobacter* with either blood agar or CCDA. The drop plate (10µl) technique had comparable counts to spread plate (100µl). More insects were used to reach the volumes required spread plating (combine haemolymph). The successful validation of the drop plate technique (plating 10µl volumes) enabled the testing of individual insect/larval (units) during therapy studies (if required). Tap water with green food dye with *Campylobacter* was chosen as the diluent as was for bacteriophage (Table 9). Thus tap water can be used for the

introduction of both organisms. Further blood agar (no antibiotics) presented no contamination and comparable counts to CCDA (with antibiotics), giving the advantage of using an antibiotic free media more suitable for the phage therapy studies.



A

Drop plate method used on CCDA agar to assess *Campylobacter* doses in original solution (used for injection) validated

B

Drop plate method used on HBA to assess *Campylobacter* levels in haemocoel validated (black areas are melanosis from concentrated haemocoel, which disappears with serial dilution

Figure 22: Drop plate method for enumeration of *Campylobacter* from Haemolymph

Repeatability of injection and recovery of *Campylobacter* from haemolymph

Table 10 presents the *Campylobacter* counts over a series of repeated experiments to validate the *Campylobacter* doses injected (starter) Vs those that were recovered from haemolymph. Initial *Campylobacter* doses from ~10^5 to 10^6 CFU per ml were prepared and represent the "starter". Five insects were combined to obtain the necessary inoculum for the preparation of the serial dilutions. The final concentration in these studies was then calculated as counts per insect. (This approach was changed during the therapy studies to calculating CFU/ml in order to achieve further accuracy).

Table 10: *Campylobacter* levels in both dose used (starter CFU/ml) and recovered (per insect CFU/ml) for doses injected (~10^7 – 10^8 CFU/ml) and assessed after 1h.

		Campylobacter CFU/ml		
			Haemolymph CFU/ml	
		Starter	5 insects	1 insect
	Date injected			
-1	18/09/2014 a	$7.60x10^6$	$1.46x10^6$	$2.92x10^5$
	18/09/140b	$7.60x10^6$	$1.65x10^6$	$3.30x10^5$
	26/11/14a	$4.70c10^7$	$6.10x10^6$	$1.22x10^6$
	26/11/14b	$4.70x10^7$	$2.91x10^6$	$5.82x10^5$
-2	17/09/14a	$7.60x10^6$	$4.90x10^5$	$9.80x10^4$
	18/09/2014	$1.80x10^6$	$4.30x10^5$	$8.60x10^4$
	4/11/14a	$1.70x10^6$	$1.02x10^6$	$2.04x10^5$
	4/11/14b	$1.70x10^6$	$1.17x10^6$	$2.34x10^5$
	5/11/2014	$2.60x10^6$	$3.20x10^5$	$6.40x10^4$
	11/11/14a	$1.57x10^6$	$1.15x10^5$	$2.30x10^4$
	18/11/14a	$9.60x10^6$	$1.38x10^6$	$2.76x10^5$
	18/11/14b	$9.60x10^6$	$1.07x10^6$	$2.14\ x10^4$
-3	1	$6.90\ x10^5$	$1.30\ x10^4$	$2.60\ x10^3$
	2	$8.70x10^4$	$5.40\ x10^4$	$1.08\ x10^4$
	3	$2.60\ x10^5$	$9.70\ x10^4$	$1.94\ x10^4$

Table 11 presents the log reduction for counts in Table 10. The reduction ranged from a decrease of 0.86 - 2.42 log CFU/ml with an average reduction of -1.49 log CFU/ml after an hour. Irrespective of this the doses achieved are sufficient to undertake therapy studies. This natural reduction can be accommodated by using two controls, i.e. an un-inoculated insect control and a *Campylobacter* injected insect control.

Table 11: Log *Campylobacter* levels in both dose used (starter CFU/ml) and recovered (per insect CFU/ml) for doses injected (~10^7 – 10^8 CFU/ml) and assessed after 1h.

		Campylobacter CFU/ml			
			Haemolymph		Log Reduction
		Starter	5 insects	1 insect	
		CFU/ml	CFU/ml		
-1	18/09/14a	6.88	6.16	5.47	-1.42
	18/09/14b	6.88	6.22	5.52	-1.36
	26/11/14a	7.67	6.79	6.09	-1.59
	26/11/14b	7.67	6.46	5.76	-1.91
-2	17/09/14a	6.88	5.69	4.99	-1.89
	18/09/14	6.26	5.63	4.93	-1.32
	4/11/14a	6.23	6.01	5.31	-0.92
	4/11/14b	6.23	6.07	5.37	-0.86
	5/11/14	6.41	5.51	4.81	-1.61
	11/11/14a	6.20	5.06	4.36	-1.83
	18/11/14a	6.98	6.14	5.44	-1.54
	18/11/14b	6.98	6.03	5.33	-1.65
-3	1	5.84	4.11	3.42	-2.42
	2	4.94	4.73	4.03	-0.91
	3	5.41	4.99	4.29	-1.13

Accuracy for calculation, bacteriophage concentration from "per insect" to "per ml"
To increase accuracy, both bacteriophage and *Campylobacter* counts were calculated as "per ml" for the therapy studies (rather than "per insect"). Thus the volume of haemolymph recovered from individual insects was measured. Figure 23 shows a scatter plot of the volumes of haemolymph recovered during the study period. The geometric mean haemolymph volume used for calculations was 26.9 µl.

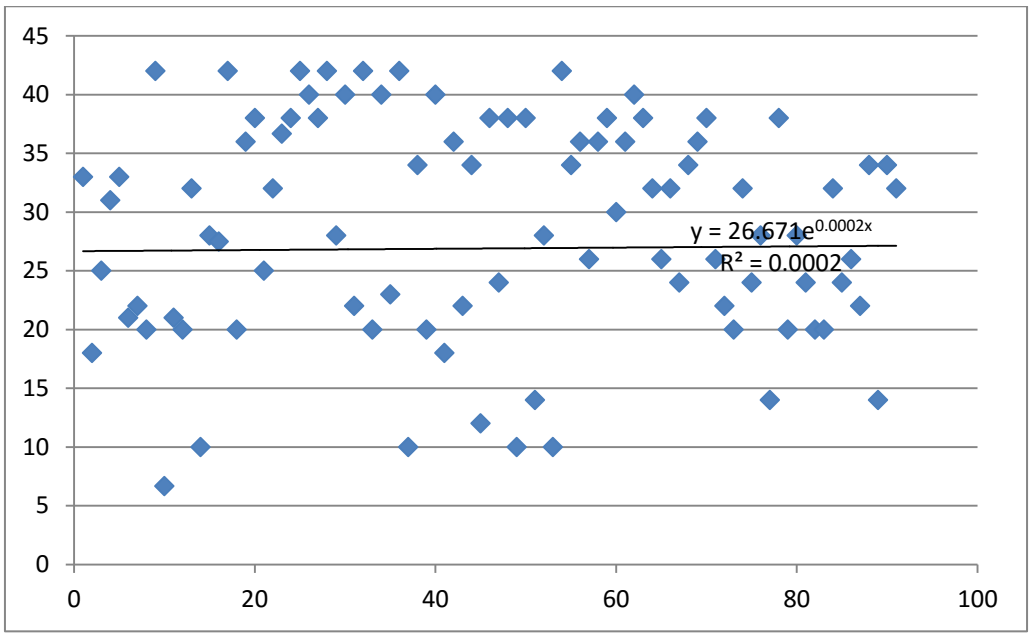


Figure 23: Scatter plot of haemolymph volume per insect

Treatment of haemolymph with chloroform targeting *Campylobacter* death
One of the interesting aspects of phage therapy is the potential for the natural increase of bacteriophages during treatment, a reason for this experiment. Thus the need to kill *Campylobacter* (with chloroform) as soon as the haemolymph is recovered from the larvae using concentrations not detrimental to the bacteriophage. The safe chloroform concentration for bacteriophages was assessed. Table 12 lists the bacteriophage levels following the treatment of haemolymph using 0, 5, 10, and 20% chloroform. The bacteriophage counts were in the same range (~ log 2.70) and, irrespective of the use of the various concentrations of chloroform, the phage counts remained the same, thus 5% Chloroform was used.

Table 12: Bacteriophage counts using different concentrations of chloroform

CHCl3	0%	5%	10%	20%
Test 1	2.95	2.71	2.74	2.77
Test 2	2.83	2.65	2.67	2.66
Test 3	2.88	2.66	2.70	2.72

The use of the validated insect model – phage therapy studies
The insect model was successfully validated following which the therapy experiments were undertaken to demonstrate a possible key use of the insect model. Candidate bacteriophages for inclusion in a cocktail can be selected from laboratory studies based on lytic profiles (i.e. screening a range of bacteriophages with campylobacters). The following demonstrates the use of the model to assess the activity of bacteriophages based on dose

used (or concentration). More specifically, the following demonstrates the testing for both "active and "passive" phage therapy.

"Active phage therapy" represents the use of high *Campylobacter* (10^7CFU) against a range of bacteriophage doses. This approach depends on the bacteriophages introduced actively increasing in the presence of *Campylobacter* and following on to kill more *Campylobacter*. Active phage therapy was tested against a lowering range of bacteriophage doses (we used 10^7, 10^5, 10^3 PFU).

"Passive therapy" represents high bacteriophage (10^7PFU) counts and was tested in the presence of decreasing *Campylobacter* concentrations (10^7, 10^5, 10^3 CFU).

Table 13 presents the sequence of therapy studies undertaken.

Table 13: Sequence of phage therapy experiments

Part 2	Therapy experiment		
09/01/2015	Method Validation	Culture Turbidity adjustment = 35% transmittance on Biolog machine	
15-19/01/2015	Prepare Phage stock PH673	Purify, confluent plate and titration PH 673 (PH181-NC3195)	
14/01/2015	**Therapy Experiment : Active**	Experiment 1 : Active 10^7CFU vs 10^7 PFU	
21/01/2015		Experiment 1.1 : Active 10^7CFU vs 10^7 PFU	
28/01/2015		Experiment 1.2 : Active 10^7CFU vs 10^7 PFU	
04/02/2015		Experiment 1.3 : Active 10^7CFU vs 10^7 PFU	
11/02/2015		Experiment 1.4: Active 10^7CFU vs 10^7 PFU	
18/02/2015		Experiment 1.5: Active 10^7CFU vs 10^7 PFU	
25/02/2015		Experiment 1.6: Active 10^7CFU vs 10^7 PFU	
06/03/2015		Experiment 2: Active 10^7CFU vs 10^5 PFU	*Start Measuring Haemolymph Volume
10/03/2015		Experiment 2.1: Active 10^7CFU vs 10^5 PFU	
11/03/2015	**Method validation**	Chloroform treatment in infected samples	Add $ChCl_3$
19/03/2015		Experiment 2.2: Active 10^7CFU vs 10^5 PFU	
24/03/2015		Experiment 2.3: Active 10^7CFU vs 10^5 PFU	
26/03/2015		Experiment 2.4: Active 10^7CFU vs 10^5 PFU	
01/04/2015		Experiment 2.5: Active 10^7CFU vs 10^5 PFU	
08/04/2015		Experiment 2.6: Active 10^7CFU vs 10^5 PFU	#Start Adding $CHCl_3$
15/04/2015	**Therapy Experiment : Passive**	Experiment 4: Passive 10^7CFU vs 10^7 PFU	
27/04/2015		Experiment 4.1: Passive 10^7CFU vs 10^7 PFU	
06/05/2015		Experiment 5: Passive 10^5CFU vs 10^7 PFU	
29/04/2015		Experiment 5.1: Passive 10^5CFU vs 10^7 PFU	
22/04/2015		Experiment 6: Passive 10^3CFU vs 10^7 PFU	
20/05/2015		Experiment 6.1: Passive 10^3CFU vs 10^7 PFU	
24/06/2015	**Therapy Experiment : Active (catch up)**	Experiment 3: Active 10^7CFU vs 10^3 PFU	
01/07/2015		Experiment 3.1: Active 10^7CFU vs 10^3 PFU	
	Completion of study		

*commenced presenting data as per and not per insect to aid better comparison
#added Chloroform to kill host and contain phage increase after removal from haemolymph from insect

Table 14(a) represents "Active treatment", using *Campylobacter* at 10^7 CFU/ml against bacteriophage concentrations 10^7, 10^5, 10^3 PFU/ml, whereas Table 14(b) presents the relevant phage counts. During active phage therapy, 10^7, 10^5 and 10^3 PFU/ml phages were used against 10^7 CFU/ml *Campylobacter* concentrations. These phage concentrations were maintained across the various tests (Table 14(b). At 0.5h after introduction, the phage levels more or less mirrored the inoculated doses. But at 24h, the test population (of insects) demonstrated a slight increase when compared to the control on seven instances during those series of trials. These increases were log 0.4, log 0.5 log, log 0.76, log 0.86, log 0.87, log 1.10 and log 2.38 respectively across the series of experiments undertaken. These increases for this *Campylobacter* and phage combination indicate that "passive therapy" is occurring with the phage contributing to increase in numbers during therapy in the *G. mellonella* model.

When comparing *Campylobacter* counts, there were two instances when a reduction in *Campylobacter* counts was observed (i.e. control log 9.47 and test log 8.51 and control log 9.51 and test log 5.15, Table 14(a). Interestingly the latter occurred at a phage concentration of 10^3 PFU/ml.

Unlike the phage, there was a drop in *Campylobacter* counts (when comparing both test and control) at 24h of testing. These drop in *Campylobacter* counts were more than what was validated (tested after 1h) for *Campylobacter* at the start of the study (i.e. a reduction of 0.86 - 2.42 log CFU/ml with an average reduction of -1.49 log CFU/ml). Nevertheless, it is still possible to assess phage reduction with the concentrations that prevailed at the time and numbers (or reduction in numbers) are always based on the "controls" at the relevant times.

Table 14: Therapy experiments – Active treatment, using *Campylobacter* at 10^7 CFU/ml against bacteriophage concentrations 10^7 PFU/ml, 10^5 AND 10^3PFU/ml

(a) *Campylobacter* enumeration

ACTIVE *Campylobacter* 10^7 CFU/ml					
		log *Campylobacter* (CFU/ml)			
Date	Phage	0.5 h		24 h	
	PFU	Control	Test	Control	Test
04/02/2015	10^7	8.73	9.96	9.47	8.51
28/01/2015		4.70	8.49	ND	ND
14/01/2015		9.40	8.52	5.58	5.53
21/01/2015		8.88	8.71	4.83	ND
11/02/2015		7.99	9.42	ND	7.30
18/02/2015		8.36	9.40	ND	ND
05/03/2015	10^5	8.42	8.64	4.81	4.88
10/03/2015		9.28	9.36	4.77	5.40
19/03/2015		9.01	9.16	5.00	5.75
24/03/2015		9.32	9.42	ND	ND
1/04/2015		9.31	9.33	4.94	5.62
8/04/2015		9.33	9.24	4.79	6.43
26/06/2015	10^3	8.22	8.66	9.51	5.15
01/07/2015		8.56	8.58	4.90	7.63

(b) Phage enumeration

ACTIVE *Campylobacter* 10^7 CFU/ml					
Date	Phage	log Phage (PFU/ml)			
	PFU	0.5 h		24 h	
		Control	Test	Control	Test
4/02/2015	10^7	7.46	7.28	7.02	7.78
28/01/2015		7.25	7.80	ND	ND
14/01/2015		6.15	6.22	ND	ND
21/01/2015		6.64	6.64	6.18	6.14
11/02/2015		7.37	6.88	6.88	7.38
18/02/2015		7.60	6.58	6.58	7.44
5/03/2015	10^5	5.37	4.91	4.96	5.37
10/03/2015		5.49	5.47	4.73	7.11
19/03/2015		5.57	5.35	ND	5.11
24/03/2015		5.50	5.45	ND	ND
1/04/2015		5.39	5.47	6.26	4.41
8/04/2015		5.31	4.64	5.19	6.29
26/06/2015	10^3	5.05	4.48	4.82	5.69
01/07/2015		5.10	5.04	8.04	6.96

Table 15(a) represents “Passive treatment”, using bacteriophage at 10^7 PFU/ml against *Campylobacter* concentrations 10^7, 10^5,10^3 CFU/ml, whereas Table 15(b) presents the relevant phage counts. During passive treatment, high doses of bacteriophages 10^7 PFU/ml were used right through with *Campylobacter* concentrations ranging from 10^7, 10^5 and 10^3 CFU/ml. Even though in this instance (depends on the *Campylobacter* and phage selected), there were two instances in Table 15 (a), when a log reduction in *Campylobacter* was observed compared to the control at 24h (i.e. control log 8.64 and test log 5.26; control log 4.15 and test log 3.28).

As with active therapy there were two instances with increase in bacteriophage numbers (i.e. control log 7.26 and test log 7.55; control log 6.76 and test log 6.79), Table 15(b), but these increases were low in number and frequency, unlike those observed during active therapy. The key aspect is that during passive therapy, the phage levels across both test and control (and across all *Campylobacter* concentrations) remained equal to the added concentration of 10^7PFU/ml. The phage exhibited stability in concentration in the *G. mellonella* model.

Table 15: Therapy experiments – Passive treatment, using bacteriophage at 10^7 PFU/ml against *Campylobacter* concentrations 10^7 PFU/ml, 10^5 PFU/ml, 10^3 PFU/ml

(a) *Campylobacter* enumeration

PASSIVE Phage10^7 PFU/ml					
Date		log *Campylobacter* CFU/ml			
	Campylobacter	0.5 h		24 h	
	CFU	Control	Test	Control	Test
15/04/2015	10^7	9.24	8.90	8.64	5.26
27/05/2015		8.89	8.77	5.22	5.73
29/04/2015	10^5	6.71	6.91	ND	3.61
6/05/2015		7.11	6.58	4.81	4.19
22/04/2015	10^3	4.51	4.58	4.15	3.28
20/05/2015		4.57	4.55	ND	3.43

(b) Phage enumeration

PASSIVE Phage10^7 PFU/ml					
Date		log Phage PFU/ml			
	Campylobacter	0.5 h		24 h	
	CFU	Control	Test	Control	Test
15/04/2015	10^7	7.67	7.26	6.38	ND
27/05/2015		7.85	7.56	7.26	7.55
29/04/2015	10^5	6.28	7.56	5.79	7.62
6/05/2015		7.68	7.48	6.76	6.79
22/04/2015	10^3	7.04	7.47	ND	6.99
20/05/2015		7.19	7.29	6.78	6.47

Discussion of Results

G. mellonella model - applications

The current study pushed into new territory – the use of insect models to study the interaction of bacteriophages with a food safety organism that is not a pathogen of any host except humans. Further, the study involved the use of a novel insect system – the wax moth larvae (*G. mellonella*) is well suited for this work because of its large size and consequent relative ease of handling. The need to create a better understanding on factors such as the heterogeneity and ecology of both bacteriophages and bacteria, have led to the use of animal models such as mice, poultry and large food animals (Atterbury 2009). However, the study has demonstrated through the validated sequential methodologies developed that *G. mellonella* larvae were able to handle the various techniques required to work with both *Campylobacter* and its bacteriophages with relatively no problems. It was possible to inject high doses and relatively high numbers survived over time, paving way to demonstrate the "phage therapy" studies undertaken. Repeated injections of both *Campylobacter* and bacteriophage with high doses have demonstrated *G. mellonella* to be quite resilient. The concept of using insect models is – to date – rather new, though have been in use for other applications with food-safety pathogens. *G. mellonella* was used to screen diverse *Campylobacter* MLST types to compare their virulence potential (Senior *et al.* 2011). *G. mellonella* larvae were also adapted to study the oxidative and aerobic stress response mechanisms in *Campylobacter,* based on the unique immune responses demonstrated by the insect host (Gundogdu *et al.* 2011). *G. mellonella* model has also been validated for *S*. Typhimurium pathogenicity studies. (Viegas *et al.* 2013). Similarly, Schrama *et al.* (2013) evaluated the effect of the acid and salt adaptation on the virulence potential of *Listeria monocytogenes* strains isolated from cheese and dairy processing environment using the *G. mellonella* model.

Bacteriophages and food-safety applications

There has been resurgence in the research of phage therapy in recent times either from a disease perspective targeting both plants and animals or applications such as food-safety (Hudson *et al.* 2005). More specifically, bio-control options targeting the food-industry have been developed for both food-safety and food-spoilage applications (Greer 2005). Currently there are bacteriophage based products in use by the food industry. Listex P100 (against *Listeria*) is the first bacteriophage product to be permitted to be used in Australasia as a food processing aid and has FSANZ (Food Standards Australia New Zealand) approval. Thus, addressing the potential use of bacteriophages in poultry is a growing area of research. More specifically, there is also the possibility that consumer demands to reduce or eliminate the use of antibiotics and chemical treatments in the poultry industry will contribute to the use of bacteriophages in the future (Cox and Pavic 2009). Bacteriophage based bio-control hold great promise for the various food-safety applications; however, it is essential to understand the conditions that lead to host infection (Denes and Wiedmann 2014) to be able to optimise on the various bio-control applications.

Salmonella and *Campylobacter* are the key food-safety pathogens and still remain a challenge to the food industry. A range of studies addressing the use of bacteriophages in poultry have targeted the key pathogen, *Campylobacter* (Connerton *et al.* 2011). Mathematical modelling predicts that an approximate 2 $\log_{10}$ cfu/g reduction could lead to a 30 fold reduction in the incidences of *Campylobacter* and such reduction has been demonstrated with bacteriophages in chicken experimental studies (Carvalho *et al.* 2010). Studies assessing *Campylobacter* bacteriophages have introduced them via feed into chickens (Carvalho *et al.* 2010). Other studies have looked at the possibility of surface application e.g. *Salmonella* in pig skin (Hooton *et al.* 2011) and *Campylobacter* in chicken skin (Atterbury *et al.* 2003). During the present study we assessed tap water as a possible mode of application based on the validation studies carried out. Irrespective of the routes of delivery, there is a need for assessing other factors, such as cocktails developed and studies

have been done on live chickens (Fischer *et al.* 2013; Kittler *et al.* 2013), all of which can be costly processes. The use of the insect models as adopted in the current study could provide a cost effective means for designing such multifactorial studies, which can be initially carried out prior to final chicken studies. In the current study, the validated *G. mellonella* model was used to demonstrate the applicability of this model to assess dosing via demonstrating the assessment of both "active" and "passive" therapy.

Phage therapy kinetics and the *G. mellonella* model

The kinetics for phage mediated bio-control play a great role in such applications, where a multiplicity of infection (MOI) of 10 is recommended (i.e. at this MOI ~ 1 in 20,000 bacteria will remain unabsorbed by the phage) (Abedon 2009). A successful dose for such applications is suggested to be 10^8 PFU/ml (or greater) for several minutes (or longer) (Abedon 2009). Our studies with the *G. mellonella* model reached these doses for the tested phage, i.e. around log 7.0 on application of a dose of that concentration. The MOI of 10 can be achieved either by "passive" (i.e. the application of continuous or single means of the required dose) or via "active" means (i.e. allowing in situ phage replication to supply the adequate densities) (Abedon 2009). The current study used the *G. mellonella* insect model to demonstrate both "active" and "passive" phage therapy using a single randomly selected *Campylobacter* and phage isolate. With the *Campylobacter* – bacteriophage combination trialled, there were increases (i.e. log 0.4, log, 0.5, log 0.76, log 0.86, log 0.87, log 1.10 and log 2.38 respectively) across the series of experiments undertaken. Thus, we were able to demonstrate an example of "active therapy" using the *Galleria* model. This is a key feature of this validated model.

One of the factors observed during the therapy studies were instances when there was a reduction in the "control" *Campylobacter* counts, between 0.5h and 24h testing. Whilst the exact reason is unknown, it could be due to the natural insect defences in the haemolymph that occurs with time. The cells in the insect haemolymph are capable of phagocytosing or encapsulating microbial invaders within, and other humoral responses such as the production of lysozyme or small antibacterial peptides, can play a contributory role (Ramarao *et al.* 2012). However, the reduction in the control did not impact the study as the controls at the time formed the basis for the initial calculations of the reduction in doses. This phenomenon was not observed during the initial validation studies when concentrations were tested after 1h.

Phage therapy and potential uses of the *G. mellonella* model

The selection of the appropriate bacteriophages and optimisation of both the timing and method of phage delivery are key factors in the successful phage-mediated control of pathogens, such as *Salmonella* in broiler chickens.(Atterbury *et al.* 2007). An apparent minimum host threshold level is needed for phage replication, and sub-optimal performance occurs at temperatures beneath the optimum of the host. (Hudson *et al.* 2006). Wagenaar *et al.* (2005) used a chicken model to evaluate *Campylobacter* phages both for a "preventative" and a "therapeutic" purposes. These studies demonstrated aspects such as the initial reduction in *Campylobacter* counts (during treatment), delay in colonisation followed by reduced colonisation and lastly stabilisation of counts, thus showing the alternating shifts in amplification of bacteria and phages. Thus, the *G. mellonella* model validated during the current study can contribute to the design of multifactorial experiments to assess the range of options as those assessed by Wagenaar *et al.* (2005), to evaluate phage therapy studies.

Carrillo *et al.* (2005) used chicken studies to assess phage treatment of *C. jejuni*-colonised birds and demonstrated a fall in *Campylobacter* counts between 0.5 and 5 log CFU/g of caeca over a 5-day period post administration. These workers also showed that such reductions were dependent on the phage-*Campylobacter* combination, the dose of phage applied, and the time elapsed. Such phage inactivation dynamics plays a key role in establishing the doses delivered, and the developed *G. mellonella* model could be used to

design a range of experimental combinations in a much easier and cost effective manner. Bigwood *et al.* (2009), whilst adopting such approaches, fitted a quadratic polynomial equation to the inactivation data of the varying concentrations of *Salmonella* and *Campylobacter* host cells, and plotted contour maps of inactivation against the phage and host concentrations. These were done *in vitro* and the live insect *in vivo* model can be adopted to address such applications. From totally different perspective studies (Grayson and Molineux 2007) focused on the physical factors, such as hydrodynamic drag at the initiation of infection, related to the injection of phage DNA into the host bacteria. The live *G. mellonella* model described in the present study would be relatively easy to use in such situations, and thus fill a niche between the laboratory experimentation and on-farm chicken studies.

In addition to the therapy studies undertaken, the *G. mellonella* model can also be used for various other applications relevant to phage therapy. These include the potential to assess the emergence of phage resistant bacteria (Orquera *et al.* 2015) plus understanding the ecology of phage interactions with their target bacteria, to further understand their behaviours in the farm environment (Johnson *et al.* 2008). Cairns *et al.* (2009) has shown the importance of using kinetic models: in combination with *in vitro* systems as an essential precursor to building a meaningful picture of the kinetic properties for *in vivo* phage therapy for *Campylobacter*. Thus, the *G. mellonella* model validated during the present study certainly will have other applications relevant to phage therapy other than food-safety. For example, bacteriophages have been used against necrotic enteritis in chickens (Miller *et al.* 2010) and thus could be used for screening of cocktails prior to the complex chicken model studies.

The future of *G. mellonella* model

There could be many future applications for the *G. mellonella* model not only against food-safety organisms but in the various animal (and human) disease applications. Workers (Lynch *et al.* 2010) have used the *G. mellonella* model to demonstrate a proof-of-principle, to show that temperate phages of *Burkholderia cepacia* can be genetically engineered to a lytic form and that these engineered phages are active *in vivo*. There also could be other novel applications where phages may be genetically engineered for the purpose following assessment in live insect models. Some of the benefits of using insects as experimental models to study human pathogens include, the ability to use large numbers, their short life cycle and ease of manipulation, quick infection process and rapid results, fewer ethical concerns when working with pathogens and finally, the similarity between humans and insects in terms of infection and immune responses, i.e. phagocytosis and production of antimicrobial peptides (Abbasifar *et al*. 2014). All these were relevant for the current study when working with the *G. mellonella* insect model.

Finally the validated insect model will have a role in future studies to enable *in vivo* screening prior to chicken trials. This can be achieved by assessing both active and passive phage therapy by either screening individual bacteriophages or phage cocktails. The data generated form this study can also have use with modelling applications (i.e. by using *Campylobacter* and bacteriophage numbers generated) as a predictive tool for phage therapy applications. Thus the present study has validated the *G. mellonella* model and demonstrated the options that could be targeted for phage therapy studies in an efficient, rapid and cost effective manner.

Implications

A cost effective and easy option to assess both bacteriophage and *Campylobacter* to support future phage therapy studies was developed. More specifically, the validated *G. mellonella* insect model can fill a niche between the required laboratory and subsequent chicken trials. Carrying out extensive chicken trials can be challenging due to the cost, work involved and other ethical requirements.

The validated insect model will have a role in future studies to enable *in vivo* screening prior to chicken trials. This can be achieved by assessing both active and passive phage therapy by either screening individual bacteriophages or phage cocktails. The data generated from such a study can also have use with modelling applications (i.e. by using *Campylobacter* and bacteriophage numbers generated), as a predictive tool for phage therapy applications. Thus, the present study has validated the *G. mellonella* model and demonstrated the options that could be targeted for phage therapy studies in an efficient, rapid and cost effective manner.

Recommendations

- It is recommended that the outcomes of this study be used to support further studies on *Campylobacter* bacteriophages

- Explore other options as to how the validated *G. mellonella* model could be used similar to the *Campylobacter* bacteriophage work undertaken in the present study

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Development of an insect model to assess phage/Campylobacter interactions
Poultry CRC Sub-Project No.:	3.1.5
Researcher:	Dr Nalini Chinivasagam
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Sub-Project Overview	The present study is based on the outcomes of a previously carried out Rural Industries Research and Development Corporation funded study that resulted in the isolation of bacteriophages from a range of Queensland poultry farms. These bacteriophages are natural predators of *Campylobacter*. There is potential to use these bacteriophages in future studies targeting *Campylobacter* bio-control in Australia. This is the first such study, and the main focus of the present study was to find a cost effective and easy option to assess bacteriophages-*Campylobacter* interactions to support the required studies. The use of the *G. mellonella* model can be an intermediary option with possibilities to reduce extensive chicken trials and contribute to more effective laboratory screening that is required to support phage therapy studies for *Campylobacter*.
Background	*Campylobacter* is a key food-safety pathogen and bio-control using bacteriophages can be an option, since such products are already in use for other food safety pathogens. This requires extensive laboratory screening of both bacteriophages and bacteriophage cocktails both in the laboratory and carrying out chicken trials to assess the efficacy of such cocktails to kill *Campylobacter*. Chicken trials can be challenging due to the cost, work involved and other ethical requirements. Thus the *G. mellonella* can be an intermediary option with possibilities to reduce extensive chicken trials and contribute to more effective laboratory screening.
Research	• The conditions required for maintaining a robust laboratory colony of *G. mellonella* which routinely delivered the final instar larvae for experimentation was established. • Methodologies for working with the larvae including a micro-injection technique were validated. The conditions that did not impact on larval stress (or death) when using the various techniques (including media or reagents) required for working with either *Campylobacter* or bacteriophages were also validated. This included the ability to successfully inject the larvae with high numbers of *Campylobacter* and bacteriophages without detriment to the larvae (over time).

	• Prior to commencement of the therapy studies, both *Campylobacter* and phage numbers injected and recovered were estimated over a series of experiments to demonstrate the sound working of the validated insect model. With bacteriophage, a decrease in counts ranging from 0.63 - 2.24 log PFU/ml, with an average decrease of 1.21 log PFU/ml was observed over an hour. Similarly with *Campylobacter,* there was a decrease of 0.86 - 2.42 log CFU/ml with an average reduction of -1.49 log CFU/ml over an hour. Irrespective of these reductions the doses achieved are sufficient to undertake therapy studies. These natural reductions can be accommodated by using two controls i.e. an un-inoculated insect control and an injected insect control during therapy studies. • Finally, the validated insect model was used to demonstrate its use for both "Active" and "Passive" phage therapy. During active therapy, a standard high dose of *Campylobacter* was subjected to varying doses of bacteriophage and thus assessing the potential for the bacteriophages to increase in number during treatment. This is an advantage during phage therapy. With the *Campylobacter* – bacteriophage combination used in the present study, there were increases (i.e. log 0.4, log, 0.5 log, log 0.76, log 0.86, log 0.87, log 1.10 and log 2.38 respectively) across the series of experiments undertaken. Thus, we were able to demonstrate an outcome of active therapy with the model. • In contrast, passive therapy depended on the addition of a high dose of bacteriophage and was demonstrated for a range of *Campylobacter* concentrations. During passive therapy, the *Campylobacter* and bacteriophage used demonstrated a log reduction in *Campylobacter* at 24h (i.e. control log 8.64 and test log 5.26; control log 4.15 and test log 3.28). During passive therapy the phage levels across both test and control (remained at 10^7PFU/ml and thus stable in the *G. mellonella* model.
Sub-Project Progress	Completed
Implications	It is possible that the current validated insect model will have a role in future studies to enable *in vivo* screening and assessing both active and passive phage therapy, based on either individual phages or phage cocktails developed following their screening against a range of target *Campylobacter* isolates. The data generated from such a study can also have use with modelling applications (i.e. by using *Campylobacter* and bacteriophage numbers generated) as a predictive tool for phage therapy applications. Thus, the present study has demonstrated the options that could be targeted using the *G. mellonella* insect model for phage therapy studies.

Publications	It is aimed to publish this work in a peer reviewed journal prior to publication of the report. The following abstract is due to be presented at the *Campylobacter* conference (CHRO) to be held in November in New Zealand Development of an insect model to assess phage/*Campylobacter* interactions. K. McGlashan, W. Estella, P. James, I. Connerton, H.N. Chinivasagam