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A "proof of concept" study to control Campylobacter using bacteriophages
Sub-Project No. 3.1.6

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Executive Summary

Campylobacter is a key food-safety pathogen and bio-control using bacteriophages may be an option. The current 1.5 year short study, is the first Australian study to demonstrate the ability of phage to reduce *Campylobacter* levels (via the use of an appropriate cocktail of bacteriophages) in the caeca of birds' on-farm and under commercial farming conditions. This was demonstrated via the Farm R study with the reduction in *Campylobacter* levels in the caeca of the treated birds compared with the controls (at the farm), being statistically significant ($P < 0.05$). The Farm D did not meet trial conditions due to phage presence in the control chickens. Further work is required to understand sustaining this reduction through transport stress under commercial conditions. The absence of phage following phage therapy (or any residual treatments other than those of normal food origin) is an important aspect for "consumer acceptance" of phage therapy and receiving regulatory approval for use in food. The treated carcasses from both farms (and controls) showed an absence of phage (residue) on the carcass was also a positive aspect of this study. This was a short proof of concept study from which an extensive range of knowledge and biological resources have been gained.

Prior to farm trials, initial screening of 35 phages was done in Australia following screening at the University of Nottingham during a visit to the UK laboratory. The screening was done using the "UK *Campylobacter* screening panel" that consisted of difficult to lyse *Campylobacter* isolates. This allowed us to narrow down and add additional phages to the screening effort. Following this, extensive screening was carried out, i.e. a total of 128 phages and 486 *Campylobacter* isolates were screened via multiple combinations to narrow down the choice to a 19 member cocktail panel to be used for farm trials. The phages were narrowed down based on their lytic profiles against farm *Campylobacter.*

Phages survived well in tap water and delivery of the phage was via oral gavage for each test chicken. Three sets of farm pre-screenings were carried, during the initial screening one farm (Farm P), though sensitive to cocktail candidates was rejected, as it did not meet trial conditions set for the "proof of concept study" (due to phage presence in the birds). Irrespective of this phage presence, the *Campylobacter* isolates still remained sensitive to selected cocktail candidates. This was encouraging, as the cocktail phages did not show resistance to the *Campylobacter* population that was already infected with resident phage.

Farm R farm was selected for the first farm trial that occurred in November 2016 as it met all trial conditions (during pre-screening). Four phages (PH 265, PH 323, PH 377, and PH 431) showed lysis potential of the *Campylobacter* isolates across all the sheds tested but more specifically in shed 7, which was selected for the trial. The *Campylobacter* levels in the caeca (on farm) for treatment and control were significantly different ($P < 0.05$) and ranged from log 5.18 – log 6.25 CFU/g (with one exception) for treatment compared to the control (log 6.05 – log 8.15 CFU/g). In summary, the reduction in *Campylobacter* levels (on farm) ranged from 1 – 3 log reductions across the treatment chickens when comparing the lowest treatment count (log 5.18 CFU/g) with the highest count (log 8.16 CFU/g) for control chickens. In contrast, whilst difference between treated and control were not statistically significant at the plant (caeca), the *Campylobacter* levels in six treatment chickens ranged from log 5.14 – 6.27 CFU/g, with only one control chicken in this range (log 5.68 CFU/g)

Farm D was selected for the second trial that occurred in December 2016. During pre-screening against the 19 panel cocktail two cocktail candidates (PH 677, PH 722) were able to lyse all the selected isolates across all four sheds at Farm D with wide coverage across the farm, one shed was selected but had a lower than optimum *Campylobacter* count for caeca. Unlike Farm R trial, this farm trial failed to meet the trial conditions during the farm trial, due to the presence of phage across all controls (farm and plant). The *Campylobacter* counts in the caeca on-farm (and plant) for both treatment and control were not significantly different, possibly due to the interference of resident phage (in the controls). A fact of interest is the that the spread of counts for *Campylobacter* for the controls (and treatment) ranged from a minimum of log 5.15 CFU/g to a maximum of log 6.58 CFU/g which was lower than at time of pre-screening performed a week

earlier (maximum count log 7.8 CFU/g). The lower than expected *Campylobacter* levels also could have played a contributory role. The *Campylobacter* levels for carcasses in Farm D, treated and control were not significantly different, though six carcasses were below detection limit of <6000 organisms per carcass compared to four from control.

Continued sensitivity to *Campylobacter* isolates (from farm and plant birds) was demonstrated when the isolates were recovered post phage treatment and tested against the phages (PH 677 and PH 722) used in that trial. This is a positive outcome, where concern for phage resistance had been considered. This aspect is also addressed the way chickens are treated i.e. 24h before pick-up (as in the current study). There was no residual phage detected on carcasses from both farm trials. This is an important requirement for consumer acceptance and registration issues of phage products destined for use in food processing. These aspects make phage therapy (for control of *Campylobacter*) promising. The data generated from this study can contribute to both outcomes and knowledge for the regulators in Australia to further support the registration and acceptance of such products in the future adding to the already available background information.

This was a short proof of concept study and extensive knowledge has been gained within this 1.5 year study which has been challenging due to the time constraints as a result of the both the scientific and practical (commercial farm trials) requirements of the study. This study has however addressed the “proof of concept” by demonstrating the ability to use phages from commercial farm environments to reduce *Campylobacter* numbers in the caeca of the bird on-farm. Whilst the milestones of this study have been successfully addressed, the work on *Campylobacter* phages is not complete and the reason for the new RIRDC study (currently 6 months into the study). In the interest of the Australian poultry industry the outcomes from this study will continue to be addressed and are progressing under the new RIRDC study.

Table of Contents

Introduction 6

Methodology 9

Laboratory studies – phage isolation and selection of cocktail candidates 9

STEP 1: Isolation of phages from stored material 9

STEP 2: Selection of phages (stage 1) from stored collection 9

STEP 3: Selection of (35) phages (stage 1) for lytic profile with farm *Campylobacter* isolates 10

STEP 4: Lytic profile of selected (35) phages assessed at the University of Nottingham 10

STEP 5: Selection of (45) phages (stage 2) and *Campylobacter* isolates from stored collection to create a screening panel) 10

STEP 6: Selection of (32) phages (stage 3) from stored collection 10

STEP 7: Selection of phages (stage 4) from stored collection 11

STEP 8: Selection of *Campylobacter* (host) screening panel based on *flaA*-SVR grouping 11

STEP 9: Screening of 19 phage candidates with 39 *Campylobacter* isolate screening panel 11

Farm trials 12

Selection of farms and conditions during the trial 12

Establish the suitability of water delivery of the selected phage or phages 18

Microbiological sampling (*Campylobacter* and phages) 18

Testing of *Campylobacter* isolates for continued sensitivity 17

Statistical Analysis 19

Results 20

Isolation of phages from stored material 20

Screening for cocktail candidates 20

Stage 1 (a): Lytic profile of a select group of 35 phages using farm isolates from two phage dominant farms (coded GILSH and OMA) 20

Stage 1 (b): Lytic profile of a select group of 35 phages assessed at the University of Nottingham 20

Stage 2: Screening of a select group of 45 phages against a select group of 214 *Campylobacter* isolates 21

Stage 3: Screening of 32 phages with 139 *Campylobacter* isolates to source additional cocktail candidates 21

Stage 4: Screening of 15 phages with 133 *Campylobacter* isolates to source additional cocktail candidates 22

Selection of final cocktail candidates 23

Establish the suitability of water delivery for selected phages 23

Pre-screening for farm trials 24

Pre-screening -1 24

Pre-screening - 2 26

Pre-screening - 3 27

On-farm trials 28

Farm trial 1 - *Campylobacter* levels in caeca and ileum from farm, caeca and ileum following transport and carcasses from the plant – Farm R 28

Farm trial 2 - *Campylobacter* levels in caeca and ileum from farm, caeca and ileum and carcasses from the plant – farm D 30

Continued sensitivity to isolates to cocktail before and after phage treatment 34

Discussion of Results 35

First Australian study, via a “proof of concept” demonstrate the potential to use phages to control *Campylobacter* levels on-farm 35

Cocktail development using phages sourced from commercial farms 35

Reduction in *Campylobacter* levels in the caeca of the bird 35

Campylobacter and phage interactions – Farm P 35
Farm D farm trial and continued sensitivity – Farm D *Campylobacter* isolates 36
Carcass rinses phage presence and *Campylobacter* levels. 36
Delivery options 36
Resistance as a result of phage therapy 36
Regulation for the use of *Campylobacter* phage products in Australia 37
Future work 37
Plain English Compendium Summary 44

Introduction

It has been estimated that *Campylobacter* causes 223,000 cases of gastroenteritis a year in Australia with >50,000 of these being associated with chicken meat (Stafford et al. 2008). Risk assessment studies predict that a reduction of *Campylobacter* levels on chicken meat can contribute to significantly less human illness (Rosenquist et al. 2006). European studies indicate that on-farm interventions can be very effective, i.e. a 2.0 log reduction in faecal *Campylobacter* counts will reduce human infections by 75%. Similarly, a 1.0 log reduction in faecal count supported by a 1.0 log reduction in contamination of the exterior of chickens, during processing would result in a 90% reduction of human infections (Havelaar et al. 2007). Hence, the development and validation of on-farm control options for reducing *Campylobacter* levels by 1.0 to 2.0 logs can realistically result in a lowering of human infections from 50,000 to 5,000 case per year. The use of bacteriophages is one such possible option, to control *Campylobacter* numbers on-farm.

Overseas models have suggested that phage treatment has the greatest potential of all known/potential methods to reduce *Campylobacter* levels in the live chicken (Havelaar et al. 2007). Studies on flock (n=205) from the United Kingdom (UK), have shown that there is potential for a significant reduction ($P < 0.001$) of *Campylobacter jejuni* counts in broiler chicken caeca in the presence of *Campylobacter*-specific bacteriophages, with mean counts of $\log_{10}$ 5.1 CFU/g Vs 6.9 CFU/g (Atterbury et al. 2005). Phages can infect their hosts via a lytic infection, during which time, the infecting phage increases in numbers producing phage virions and killing (or lysing) the infected bacterium (Abedon 2012). This is an on-going process which can be exploited for the reduction of the pathogen. Bacteriophages have been shown to be naturally present in poultry environments in the UK along with their host *Campylobacter* (Connerton et al. 2004). Similarly *Campylobacter* bacteriophages are naturally present in caeca, litter, soil (in Australian farms) and on carcasses (from the processing plant) (Chinivasagam et al. 2015).

Experimental studies have demonstrated the reduction of “introduced” *Campylobacter* on both skin (Goode et al. 2003) and caeca (El-Shibiny et al. 2009). The application of bacteriophages to the surface of chicken skin has also been shown to reduce *C jejuni* (Atterbury et al. 2003a). Connerton *et al.* (2011) in an extensive review on *Campylobacter* bacteriophages have highlighted the potential for using bacteriophages either on-farm or on food (via bio-sanitisation) to reduce *Campylobacter* in poultry or the processed product. Bio-control has been exploited for controlling the numbers of food-borne pathogens such as *Salmonella, E. coli* 0157:H7 and *Listeria monocytogenes* (and now available as commercial applications) (Goodridge and Bisha 2011) though to date, none are available for *Campylobacter.*

A range of phage based products are available against food-safety pathogens. The availability of such products has demonstrated economic viability of phage based options with no limitations in uptake, mainly for food-safety organisms such as *Listeria*, *Salmonella* and *Escherichia coli* 0157:(H7). Commercial phage products are already marketed against these organisms by various companies in Europe and USA. These phage type products are largely marketed as “processing aids”. Listed below are some examples of products from selected companies.

Listeria - Listex P100. 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 to be used in Australia (http://www.foodproductiondaily.com/Safety-Regulation/Listeria-killing-phage-product-gets-FSANZ-approval)

Listsheild (targets *Listeria monocytogenes*), Ecosheild (targets *E. coli* 0157) and Salmofresh (targets *Salmonella*) are all products registered for use by “Intralytix (http://www.intralytix.com/index.php?page=prod) see listsheild, Salmosheild and Ecosheild (*E. coli* 0157) for the same purpose.

Salmonellex (targets *Salmonella*) is approved for use by the FDA (Food and Drug Administration, USA) and USDA (United States Food and Drug Administration) as a GRAS (generally recognised

as safe), and produced by Micreos. This company is going further to seek approval by the Organic Material Review Institute (OMRI) to enable the product to be used in organic products

These products exemplify the fact that international companies are actively developing and marketing phage based products. Market activity clearly indicates there is uptake and active use of these products due to their nature and ability to control food-safety pathogens. Phage based applications also have the additional advantage of being able to target either the pre or post-harvest stage thus offering a flexibility in application and cost structures.

The successful use of phage products is demonstrated by both their availability and approvals granted for use at agricultural or food processing stages for relevant pathogens present in crops or foods. Experimental studies have been carried out to assess bio-control at a 'pre-harvest stage' for ruminants (*E. coli* 0157), pigs (*Salmonella*) and poultry (*Campylobacter* and *Salmonella*). Irrespective of this research, there is still a need for regulatory approval for phage based products to be used on 'live animals' (Goodridge and Bisha 2011). However, the USDA has "supported" the use of *E. coli* 0157 and *Salmonella* based "hide sprays" on cattle prior to slaughter (Goodridge and Bisha 2011). Bacteriophage preparations are also being used on 'post-harvest' product with FDA and USDA approval against *Listeria* on meat and cheese (Goodridge and Bisha 2011). Overall, the proposed study is backed by both on-going research overseas and positive regulatory acceptance of bacteriophage based bio-control of food-borne pathogens.

The present study has an on-farm focus and is targeting the development of an on-farm option to control *Campylobacter* numbers in the caeca of chickens. This study is a "proof of concept" study to initially demonstrate that bacteriophages sourced from Australian farms can have an impact on *Campylobacter* numbers in the caeca of the farmed bird, when used as an on-farm treatment. The study is being carried out in collaboration with the industry (who assisted with the farm trials) and with the University of Nottingham, (UK) who have a long history in working with *Campylobacter* phages. This short 1.5 year project was carried out in two stages:

(a) Laboratory work (to select the best phage candidates to be used for on-farm trials)
(b) Farm trials (undertaken during commercial farming)

Objectives

The hypothesis underpinning this work is that an appropriate cocktail of phages delivered via drinking water prior to pick will lower the levels of *Campylobacter* in the caeca of treated chickens and further that the treatment will reduce the final load of *Campylobacter* on the carcass of the treated chickens.

The aims of the work are as follows:

- Perform the necessary laboratory studies to establish a collection of characterised bacteriophages of known capacity to kill *Campylobacter*
- Establish the suitability of water delivery of the selected phage or phages
- Perform two farm trials that will evaluate the performance of the phage/phages administered in reducing the levels of *Campylobacter* in the chicken caecum (Just prior to pick up to help assess phage treatment efficacy and the impact of transport stress) and at two points in the processing plant i.e. at the point of caeca removal and final product which is destined for the market to assess what is being delivered to the consumer

Methodology

The methodology in this section is presented as follows:

1. Laboratory studies targeted at phage cocktail development
2. Farm studies to assess cocktail application (on farmed birds)

The overall study was based on an existing collection of 600 phages (and characterised *Campylobacter* isolates) obtained during the sampling of 17 farms (which also resulted in 24 independent farm sampling) that occurred from 2012 – 2013 (Chinivasagam et al. 2015). These farms adopted a range of litter practices. The nature of the extended study contributed to a diverse collection of *Campylobacter* isolates and phages which formed the basis of the current study.

Laboratory studies – phage isolation and selection of cocktail candidates

The selection of the phages for use in the final cocktail was carried out in a sequential manner as listed as a series of steps below.

STEP 1: Isolation of phages from stored material

Stored caeca filtrates (from the 2012 stored collection) were used for the isolation of phages to increase phage diversity via the "enrichment technique" (using farm based *Campylobacter* hosts) (Chinivasagam et al. 2015).

Table 1 lists the origin of these caeca filtrates (farms) and the *Campylobacter* isolates that were used for enrichment. The stored caeca (at -80° C) were enriched with *Campylobacter jejuni* PT14 (NCTC 12662) and 13 farm *Campylobacter* hosts NC 3198……..(Table 1)

Table 1 Origin of caeca samples (C1 + C2) and *C. jejuni* farm hosts (NC numbered) used for enrichment

Source - 2012	samples	Farm *Campylobacter* hosts
Farm GILSH	A = C1+C2	
	B = C3+C4	
Farm M	A = C1+C2	
	B = C3+C4	
Farm OMA	A = C1+C2	NC3198, NC3210,
	B = C3+C4	NC3219, NC3223,
Farm T	A = C1+C2	NC3280, NC3301,
		NC3340, NC3381,
		NC3527, NC3653,
		NC3661, NC3795,
		NC3945, (farm hosts)
	B = C3+C4	
Farm C	A = C1+C2	PT14 (international host)
	B = C3+C4	
Farm B	A = C1+C2	
	B = C3+C4	

The methodology for phage isolation was based on (Atterbury et al. 2003b) and (Connerton and Timms 12) with some modifications adopted at the enrichment stage (Chinivasagam et al. 2015). The phages were isolated using the international and commonly used host strain *Campylobacter jejuni* PT14 (NCTC 12662) and a selection of farm *Campylobacter* isolates from a previous study (Chinivasagam et al. 2015).

STEP 2: Selection of phages (stage 1) from stored collection

A total of another 35 phages were selected from our stored collection. These phages were previously isolated via two enrichment approaches developed for our previous study. These phages came from a range of farms, sample types and were isolated using either farm hosts or the

universal host *C. jejuni* PT14 (NCTC 12662). The phages were specially selected to ensure phage diversity and the details of the source of the phages are listed in Table 2.

Table 2 Farm[1], sample source[2], method of isolation[3] and *Campylobacter* isolates[4] used to isolate the 35 phages[5] as described in step 2

Phage #	source		Phage #	source
PH181	Bar-C-enr		PH633	Frib-C-sme
PH194	Bar-S-enr		PH674	Mar-C-3195
PH199	Mar-C-enr		PH675	Mar-S-3195
PH215	Tur-C-enr		PH676	Mar-L1-3195
PH216	Tur-C-enr		PH677	pig-PT14
PH229	Tur-S-enr		PH678	pig-PT14
PH232	Tur-S-enr		PH712	Mar-C-3295
PH248	Gilsn-C-enr		PH713	Mar-L-3295
PH253	Gilsn-L-enr		PH714	Mar-S-3295
PH388	Mar-L-enr		PH715	Mar-S-3199
PH415	Qlan_C		PH716	Mar-L-3199
PH420	Qlan-C-enr		PH717	Tur-C-3322
PH437	Gilsn-L-enr		PH718	Tur-L-3322
PH470	Qlan-CR		PH719	Tur-L-3322
PH530	Duke-S-enr		PH720	pig-PT14-enr
PH611	Irv-C		PH721	pig-PT14-enr
PH613	Irv-C		PH722	pig-PT14-enr
PH627	Irv-L-sme			

[1]Bar, Mar, Tur, Gilsn, Qlan, Duke, Irv, Frib, Tur,
[2] (C – caeca; S- carcass, L – litter
[3]enrichment (enr) or "SM enrichment" (sme)
[4] *Campylobacter jejuni* PT14, 3195, 3295, 3199, 3322
[5]PH = phage number

STEP 3: Selection of (35) phages (stage 1) for lytic profile with farm *Campylobacter* isolates

The *Campylobacter* isolates from two phage dominant farms - GILSH and OMA (from our previous study) were used to carry out lytic profiles as in (Connerton and Timms 2012) and (Atterbury et al. 2003b). The 35 phages candidates has been previously described (Table 2).

STEP 4: Lytic profile of selected (35) phages assessed at the University of Nottingham

The same 35 phages (Table 2) were taken to the University of Nottingham by Ms Wiyada Estella (Research Scientist) as a part of our collaborative work in the UK. During this visit these phages were screened against the UK phage screening panel of difficult to lyse *Campylobacter* isolates to help narrow down the selection.

STEP 5: Selection of (45) phages (stage 2) and *Campylobacter* isolates from stored collection to create a screening panel)

Based on the outcomes of steps 3 and 4, there was a need to screen more phages. A further 45 phages were selected from our collection. There was also a need to develop a "*Campylobacter* screening panel" to help with more efficient cocktail candidate selection. For this purpose, we sourced 214 *Campylobacter* isolates from our stored collection. The aim was to narrow this set of *Campylobacter* isolates down to a suitable number, to enable routine screening.

STEP 6: Selection of (32) phages (stage 3) from stored collection

Another set of 32 phages and 139 *Campylobacter* isolates were sourced from our stored collection to further enhance screening.

STEP 7: Selection of phages (stage 4) from stored collection
During stage 4, a set of 16 phages and 133 *Campylobacter* isolates were sourced from our stored collection for further screening.

STEP 8: Selection of *Campylobacter* (host) screening panel based on *flaA*-SVR grouping
A *Campylobacter* screening panel consisting of 39 isolates was selected from the stored collection. The selection was based on the origin of the various isolates (farms), their *flaA*-SVR grouping (Table 3) and their lytic profile across the phages screened. This approach was adopted simply as a means to aid isolate selection from our large number of stored *Campylobacter* isolates. The panel was used to screen the final set of selected phages (i.e.19 candidates).

Table 3 Source (farm), isolate number and species identity of *Campylobacter* (host) screening panel of 39 isolates

Farm	NC #	Strain	*flaA*-SVR grouping	Farm	NC #	Strain	*flaA*-SVR grouping
AKS_12	**3165**	*C. jejuni*	4	Page	**3439**	*C. jejuni*	10
	3179	*C. jejuni*	4		**3449**	*C. jejuni*	10
	3167	*C. jejuni*	4		**3461**	*C. jejuni*	7
	3182	*C. jejuni*	4		**3468**	*C. jejuni*	10
CWT	**3209**	*C. jejuni*	7	Peter13	**3628**	*C. coli*	10
	3210	*C. jejuni*	10		**3632**	*C. jejuni*	7
	3217	*C. jejuni*	8		**3645**	*C. jejuni*	12
	3223	*C. jejuni*	4		**3653**	*C. jejuni*	8
	3234	*C. jejuni*	4	AKS_13	**3677**	*C. jejuni*	7
Barrett	**3247**	*C. jejuni*	10		**3678**	*C. jejuni*	7
	3250	*C. jejuni*	2		**3679**	*C. jejuni*	10
	3252	*C. jejuni*	4	Qlan	**3742**	*C. jejuni*	10
	3270	*C. jejuni*	3		**3766**	*C. coli*	10
	3282	*C. jejuni*	3		**3770**	*C. coli*	10
Peter	**3385**	*C. jejuni*	10		**3771**	*C. jejuni*	10
	3388	*C. jejuni*	10	Strn13	**3841**	*C. coli*	14
	3395	*C. coli*	_		**3843**	*C. jejuni*	10
	3418	*C. jejuni*	10		**3844**	*C. coli*	14
					3854	*C. jejuni*	14
					3872	*C. jejuni*	6

STEP 9: Screening of 19 phage candidates with 39 *Campylobacter* isolate screening panel
Finally, the *Campylobacter* screening panel was used to screen the 19 phage cocktail candidates to finalise selection.

Farm trials

Two commercial farms were selected for the farm trials based on a select set of conditions.

Conditions for selection of the two trial farms/sheds were as follows;
One week before pick-up (and at the time of pre-screening) the birds:

- present a high *Campylobacter* count (caeca) to enable the demonstration of log reductions (this is not unusual for *Campylobacter* levels in caeca, close to final pick-up)
- present a phage negative status (caeca)
- the *Campylobacter* isolates (caeca) demonstrate the potential to be lysed by one or more of the selected cocktail candidates

Selection of farms and conditions during the trial
The farms sourced were selected based on the commercial pick-up schedule of the collaborating company and to enable us to match our sampling schedule with the various commercial pick-up schedules already in place. This trial was carried out under the commercial conditions operated by the company, with the exception of the trial birds were treated within 24 hours of pick-up

Pre-screening – one week before pick-up
The required Animal Ethics Approval was sought prior to screening. Three chickens were randomly picked from 2 -4 sheds of two to three farms. The caeca were removed on farm and transported chilled to the laboratory. On arrival, the caecal samples were analysed for both phage and *Campylobacter* levels as previously described (Chinivasagam et al. 2016). Ten well separated *Campylobacter* colonies were randomly picked across the three samples. Their lysis profiles were then assessed against the cocktail candidates. The presence/absence of phages was also analysed (Chinivasagam et al. 2015).

Farm trial design
The optimum number of chickens to be used for the study was selected in collaboration with the biometrician, and the required Animal Ethics approval for the farm trial obtained from the DAF Animal Ethics Committee. A total of 60 chickens were used for the study (i.e. 30 for test and 30 for control). The trial design is illustrated in Figure 1.

Two farms were selected:

(1) Farm R – the shed set up is illustrated in Figure 2
(2) Farm D - the shed set up is illustrated in Figure 3

Briefly, two sets of 30 chickens were segregated from the original trial birds that remained in the shed prior to final pick-up. The birds were segregated using wire mesh such that they still had access to feed and water lines accessed by the rest of the birds. All conditions remained un-altered during the trial, with the exception of the delivery of either tap water or phage cocktail to the trial birds (all times are presented in Tables 8 and 9).

The control chickens received 3ml of sterile tap water and test birds received 3ml of the phage cocktail containing 10^7PFU/ml that was administered via oral gavage. Other than this there were no other differences between the both the trial and the rest of the birds in the shed.

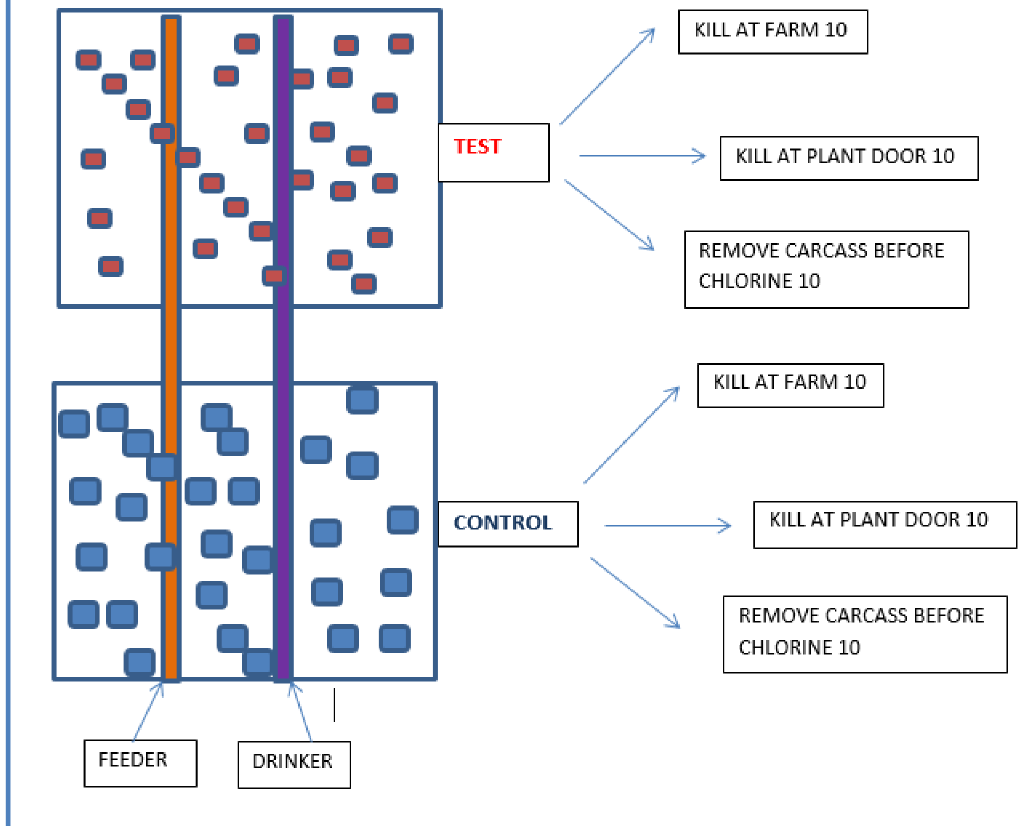


Figure 1 Trial design adopted on-farm

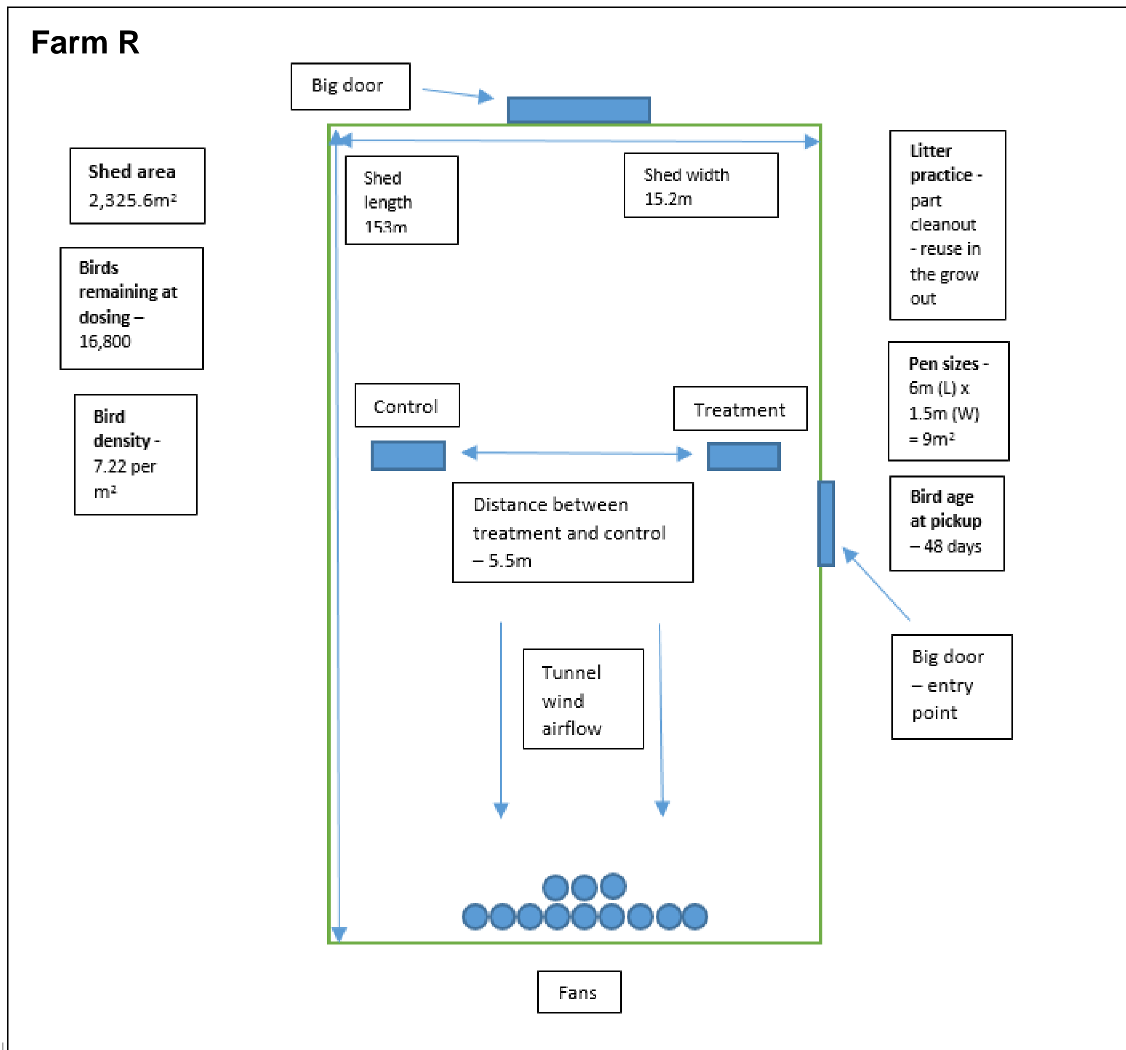


Figure 2 farm R shed layout during the trial

Table 4 Farm R trial –sequence of events

Farm R Shed 7 - Logistics

Monday 7/11/2016

Understand farm layout and logistics
Setup pens for trial and control birds
Understand operating procedures for field sample collection, etc.

Tuesday 8/11/2016

Activity	Time
Select and dose control birds	12:00 PM
Select and dose trial birds	1:00 PM

Wednesday 9/11/2016

Activity	Time
Remove feed from birds - 8 hours feed withdrawal prior to pickup	12:30 AM
Remove water from birds	8:00 AM
Arrive at Farm R for sampling	8:30 AM
Sample at Farm R Shed 7	9:30 AM
Lance take samples to lab	11:30 AM
Travel to processing plant, arrive approximately	2:00 PM
Lairage time then collect sample	2:30 PM
Process birds	3:00 PM
Collect carcases	3:15 PM
Package and organise transport of carcasses to Springwood lab	3:30 PM
Transport carcasses to Springwood lab	3:45 PM

Thursday 10/11/2016

Activity	Time
Carcass rinses at Springwood lab	10:00 AM

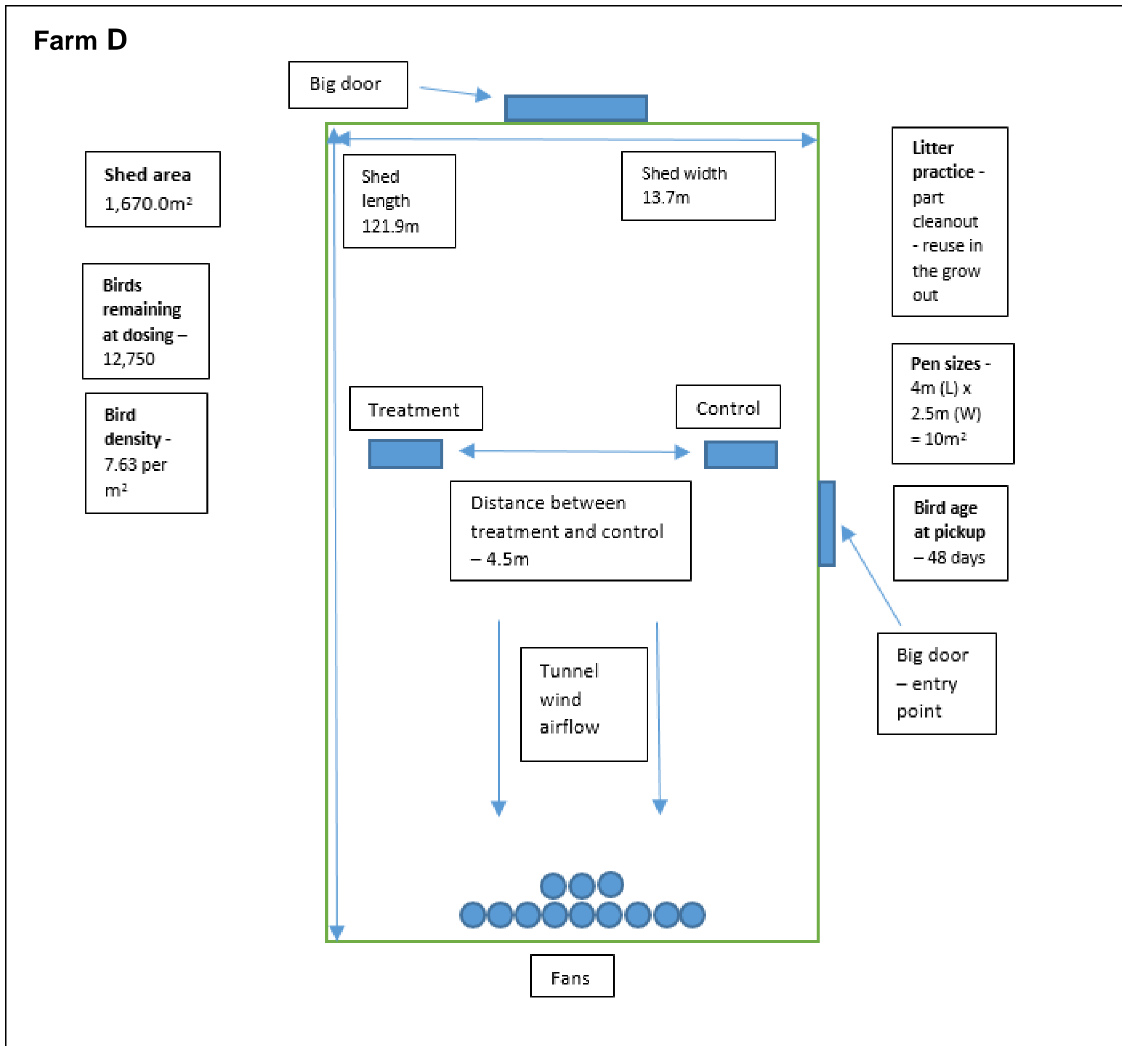


Figure 3 Farm D shed layout during the trial

Table 5 Farm D trial – sequence of events

Farm D Shed 3 - Logistics	
Tuesday 13/12/2016	
Arrive farm D	10:00 AM
Setup pens for trial and control birds	
Setup ready to dose	11:00 AM
Select and dose control birds	12:00 PM
Select and dose trial birds	1:00 PM
Stay Beaudesert Motel	
Wednesday 14/12/2016	
Remove feed from birds - 8 hours feed withdrawal prior to pickup	2:30 AM
Arrive at Farm D and setup for sampling	7:30 AM
Remove water from control bird pen	8:00 AM
Sample Control bird pen at Farm D farm Shed 3	8:30 AM
Remove water from trial bird pen	9:00 AM
Sample Trial bird pen at Farm D farm Shed 3	9:30 AM
Samples taken to lab	10:30 AM
Pickup Shed 3	10:30 AM
Trial and control birds will be placed in a designated marked module, these birds will be removed from the shed at the beginning of the catch and loaded on the first trailer from Shed 3	
Travel to processing plant, arrive approximately	1:00 PM
Lairage time then collect sample	1:30 PM
Process birds	2:00 PM
Collect carcases	2:15 PM
Package and organise transport of carcasses to Springwood lab	2:30 PM
Thursday 15/12/2016	
Carcass rinses at Springwood lab	10:00 AM

Establish the suitability of water for delivery of the selected phage or phages
The suitability of the water as delivery vehicle for the phage cocktail was assessed by;

(a) Estimating the pH of Brisbane tap water
(b) Estimating the survival of phage in tap water

Tap water was sourced from three Brisbane suburbs to assess pH. A random phage was sourced from the cocktail and added tap water to reach a concentration 10^7 PFU/ml. The tap water was stored at room temperature and the phage concentration was assessed on day 1, 2 and 7. The stability of the added phage was assessed in drinking water in this manner.

Microbiological sampling (*Campylobacter* and phages)
Ten chickens (from test and control each) were killed on-farm and both the caeca and the ileum removed and stored chilled until arrival at the laboratory. These samples were tested on the day of arrival.

Following transport, ten chickens (from test and control) entering the process line were removed, once killed and feather plucked. The caeca and the ileum were removed and stored chilled until arrival at the laboratory. These samples were tested on the day of arrival.

Once the trial birds had reached the carcass stage, they (10 test and 10 control) were removed prior to being sent for spin chilling (the next stage), packed ready for transport to the collaborating laboratory for carcass rinses performed the following morning. The chilled rinses were brought to the laboratory for testing the same day.

Campylobacter and phage enumeration

(a) The whole caeca was prepared for *Campylobacter* and phage enumeration as described in Chinivasagam et al. (2016). A one in ten dilution was prepared by using 5 g caeca (at Farm R) for *Campylobacter* and the rest of the sample was adjusted to reach a one in ten dilution for phage. A slight variation was adopted for Farm D, i.e. 1g caeca was prepared as a one in ten dilution for both *Campylobacter* and phage (so as to have an enough for phage enumeration).

(b) All ileum samples obtained at day before pick-up were empty as result of feed withdrawal. Pressure was applied on the empty ileum to get any sample contents. The contents were more representative of mucus and not ileum contents.

(c) In summary, the *Campylobacter* levels presented in this study represent the whole caeca (for caeca) and ileum contents only (for ileum). The levels from these are thus are not comparable.

Campylobacter and phage enumeration was carried out as per Connerton and Timms (2012) and confirmation of *Campylobacter* was carried out as in Chinivasagam et al. (2016). In short, 5g of caeca was macerated with 45 ml of Preston broth (minus antibiotics) for bacteria or 45 ml SM buffer for phage. *Campylobacter* were assessed on the day, whereas phages were shaken overnight prior to filtering and storing filtrates at 4°C ready for enumeration. Caeca and ileum samples were tested in triplicate for the enumeration of the *Campylobacter* and levels and are presented as log CFU/g. Individual samples were tested for phage levels (Chinivasagam et al. 2015) and are presented as PFU/g for caeca and ileum. Carcass rinses were tested the day after (Chinivasagam et al. 2015) and levels were presented as CFU/carcass or PFU/carcass.

MPN – carcass rinses
Carcasses for Farm R were assessed as previously described (Chinivasagam et al. 2015)
A Most Probable Number (MPN) approach was adopted for carcasses adapted from the procedure for litter (Chinivasagam et al. 2016), with the exception that Preston Broth was incubated at 42°C instead of 37°C. The levels are presented as MPN/carcass.

The MPN approach was adopted due to high levels of competing flora on the carcasses that were removed prior to chlorination and spin-chilling to enable assessment of the phage counts, a requirement to ensure no treatment residue on the carcasses. The MPN approach was also developed as the competing flora interfered with the *Campylobacter* counts of carcasses from Farm R

Testing of *Campylobacter* isolates for continued sensitivity
The *Campylobacter* isolates recovered from the chicken that received the treatment were tested against the two phage cocktail candidates used during the farm trial for their lytic profile as in STEP 2. Due to time constraints the isolates from Farm R were not tested and work is in progress via the new RIRDC study. All details will be included at the stage of peer-review publication, which is envisaged soon.

Statistical Analysis
Statistical analysis was carried out as previously described Chinivasagam et al. (2016).

Results

Isolation of phages from stored material

A total of 29 phages (PH723 – PH751) were isolated from stored caeca via the enrichment approach and were included in the screening work. These phages were isolated using farm *Campylobacter* hosts, unlike most of the other phages in our collection which were isolated using *C. jejuni* PT14, a universal host.

Screening for cocktail candidates

The following stages present the *Campylobacter* screening results for cocktail candidate selection. Extensive screening was carried out, i.e. a total of 128 phages and 486 *Campylobacter* isolates were screened via multiple combinations to narrow down to a cocktail as described through the following stages

Stage 1 (a): Lytic profile of a select group of 35 phages using farm isolates from two phage dominant farms (coded GILSH and OMA)

Figure 4, presents a snap-shot of the extensive screening undertaken at the initial stages. During this screening process, *Campylobacter* isolates (Figure 4) were selected from two known phage dominant farms, i.e. GILSH and OMA. The isolates selected represented both *C. jejuni* and *C. coli* species, and were also selected across a range of *flaA*-SVR groupings, to enhance further diversity. Similarly, the phages (Figure 4) selected to test for their profile, were also sourced from a range of farms (Figure 4).

The dark blue marking against the selected phage indicates good lysis potential and thus worthy of inclusion for further screening. Such phages were included in the screening that followed.

Farm · Sample code · *flaA*-SVR

Campylobacter	Farm	Sample code	Phage	flaA-SVR	PH181 Bar-C-en	PH194 Bar-S-en	PH199 Mar-C-en	PH215 Tur-C-en	PH216 Tur-C-en	PH229 Tur-S-en	PH232 Tur-S-en	PH248 Gilsn-C-er	PH253 Gilsn-L-er	PH388 Mar-L-en	PH415 Qlan_C	PH420 Qlan-C-er	PH437 Gilsn-L-er
NC3330	GILSN_12	CC2	C.jejuni	7	-	-	-	+++	+++	-	-	-	-	-	+	++	-
NC3332	GILSN_12	CC2	C.jejuni	7	-	-	-	-	+	-	-	-	-	-	-	-	-
NC3378	OMR_12	No.2	C.jejuni	7	-	-	-	-	-	-	-	-	-	-			-
NC3380	OMR_12	No.4	C.jejuni	7	-	-	-	-	-	-	-	-	-	-			-
NC3527	OMR_13	FRL1	C.jejuni	7	-	-	-	-	-	-	-	-	-	-	++	++	-
NC3543	OMR_13	No.7	C.jejuni	7	-	-	-	-	-	-	-	-	-	-	++	+	-
Continuation……..																	
NC3366	OMR_12	FRC2	C.jejuni	8	-	-	-	-	-	-	-	-	-	-	-	-	-
NC3381	OMR_12	No.5	C.jejuni	8	-	-	-	-	-	-	-	-	-	-			-
NC3325	GILSN_12	CC1	C.jejuni	10	+++	+++	+	-	-	+++	+++	++	+++	-	++	+	+++
NC3329	GILSN_12	CC1	C.jejuni	10	+++	+++	++	-	+	+	+++	+	+	-	+++	+++	+
NC3333	GILSN_12	CC2	C.jejuni	10	-	-	-	-	-	-	-	-	-	-	+	-	-
NC3334	GILSN_12	CC2	C.jejuni	10	+++	++	-	-	-	+	+++	-	-	-	+++	+++	-
NC3340	GILSN_12	CC4	C.jejuni	10	+++	++	-	-	-	+++	+++	+	++	-	+++	+++	++
NC3345	GILSN_12	CL1	C.jejuni	10	+++	++	-	-	-	+	+++	+	+	-	+++	+++	+
Continuation…….																	
NC3377	OMR_12	FRC4	C.jejuni	10	-	-	-	-	-	-	-	-	-	-			-
NC3530	OMR_13	FRL1	C.jejuni	10	-	-	-	-	-	-	-	-	-	-	-	-	-
NC3531	OMR_13	FRL2	C.jejuni	10	-	-	-	-	-	-	-	-	-	+++	-	-	-
NC3548	OMR_13	No.11	C.jejuni	10	++	++	++	-	+	++	++	+	+	-	++	+	-
NC3816	GILSN_13	NO.7	C.jejuni	10	+	++	+	-	-	-	-	-	-	-	-	-	-

Figure 4 A snap shot of screening performed using *Campylobacter* isolates (*C. jejuni* or *C. coli*) using phages isolated across a range of farms

Stage 1 (b): Lytic profile of a select group of 35 phages assessed at the University of Nottingham

The same group of phages were tested against the UK screening panel, which consisted of a set of specially chosen *Campylobacter* isolates (i.e. with hard, medium or easy lysis potential against phage). This panel thus assists in selecting broad spectrum phages.

Based on that overall screening, four phages were identified as potential cocktail candidates. They were PH 232, PH 712, PH 722 and PH 677 (Figure 5).

	For Cocktail																														
	PT2	PT44	OR12	TIVC9	HPC5	OCIIIC2	12662	12668	11168	HPIC1	OKVIIIC	GHIIF	GIIIF2	TIIIC9	TIF2	OKIXC	ESI	CIC4	DVIVC	OCIIC1	OCIF3	OCIIF8	CIIC3	EIF10	GIIC8	DVIVF	EIIIC7	MPVC	TIIIC4	TIVF	**Positive**
PH232	-	+++	-	-	+++	-	+++	-	++	+++	+++	-	-	-	-	-	-	-	-	-	-	+++	-'	-	-	-	-	-	-	-	**7**
PH712	+	+++	-	-	+++	-	+++	-	-	+++	+++	-	+	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	**8**
PH722	?	?	-	-	-	-	+++	+++	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	**4**
PH677	+	-	-	-	-	-	+++	+++	-	-	-	-	+	-	+++	-	-	-	-	-	-	-	+++	-		-	-	-	-	-	**6**

Figure 5 Summary of lytic potential of phages selected form overall screening using the UK *Campylobacter* screening panel

Stage 2: Screening of a select group of 45 phages against a select group of 214 *Campylobacter* isolates

A set of 45 phages were screened against 214 *Campylobacter* isolates that were chosen based on their *flaA*-SVR groupings. Figure 6 illustrates the total number of *Campylobacter* isolates lysed by each of the chosen 45 phages (PH238 – 300). Based on this outcome, phages (PH) 260, 264, 265, 283 and 295 were selected for inclusion as cocktail candidates. The latter selection was done on the following basis:

- Initial selection based on phage which could lyse the highest number of *Campylobacter* isolates
- Comparison of the lysis pattern of each phage across each and every *Campylobacter* isolate, (some phages lyse select *Campylobacter* isolates that the others could not lyse)
- Final selection based on broad spectrum coverage across *Campylobacter* isolates used for the lytic profile and also the potential to lyse a large number of *Campylobacter* isolates
- This approach was adopted along the rest of the stages

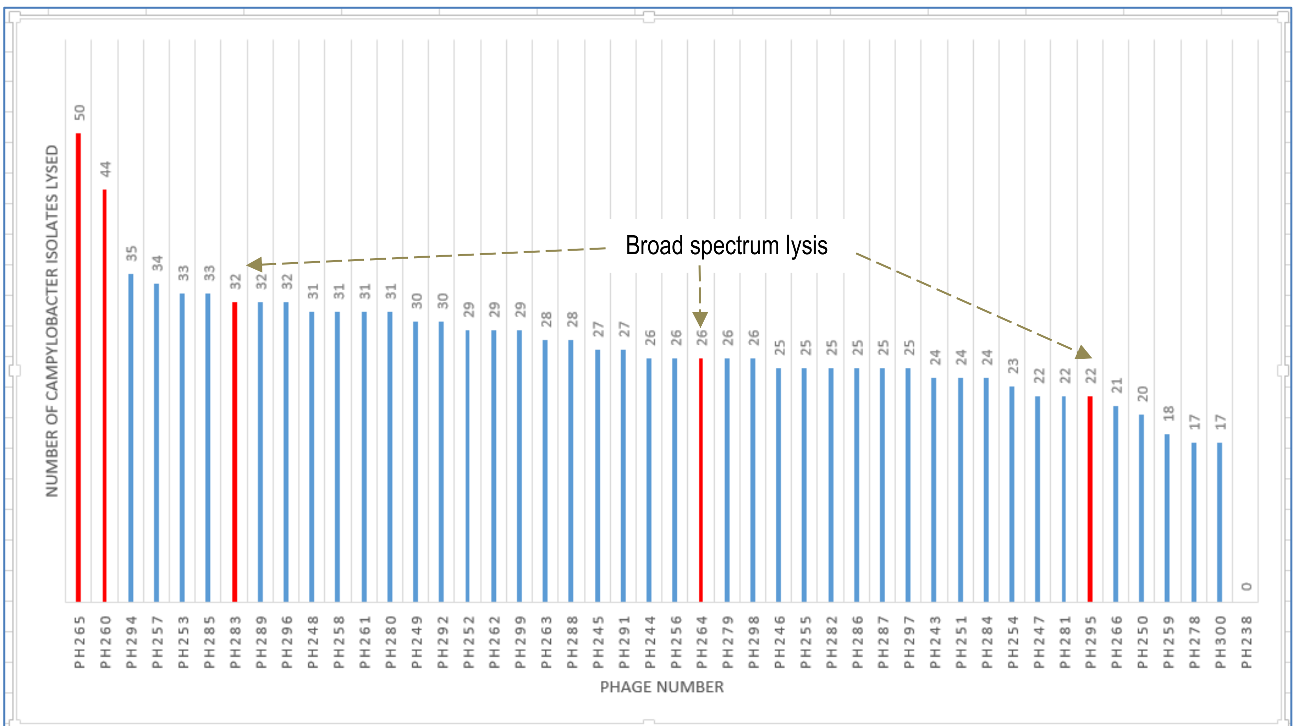


Figure 6 Number of *Campylobacter* isolates lysed by the 45 phages screened (the selected candidates are in red)

Total number of *Campylobacter* isolates tested 214

Stage 3: Screening of 32 phages with 139 *Campylobacter* isolates to source additional cocktail candidates

Additional screening was carried out with a lesser number (139) of isolates previously used against 32 different phages. Figure 7 illustrates the lysis pattern obtained against the various *Campylobacter* isolates.

Selection of phages was not totally based on how many isolates that were lysed but also the phage coverage across those isolates. For example, phage 377 was selected due to its wider coverage of the *Campylobacter* isolates that were also lysed by PH 321, PH 343, PH 354, PH 373, PH 374, PH 375, PH 427 and PH 428 (Figure 7). This approach was routinely adopted across the rest of the screening process that followed.

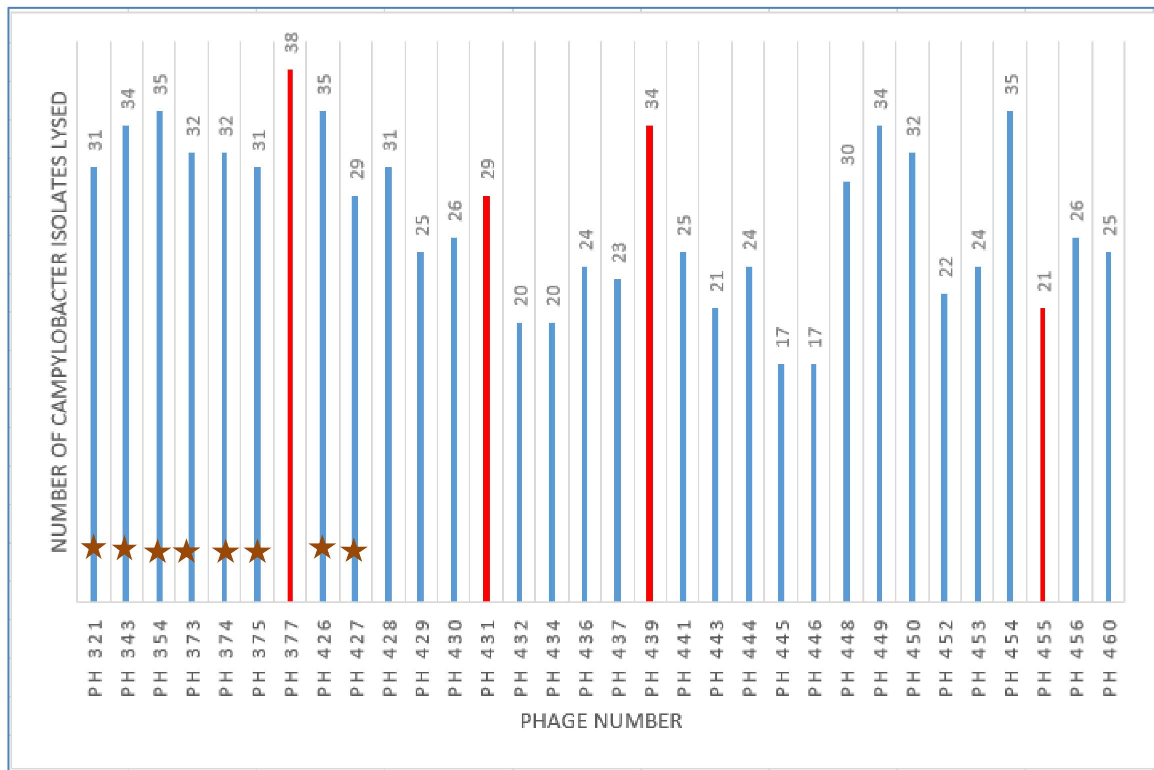


Figure 7 Number of *Campylobacter* isolates lysed by the 32 phages screened (the selected candidates are in red)

Total number of *Campylobacter* isolates tested 139

★ Same isolates lysed by PH 377

Stage 4: Screening of 15 phages with 133 *Campylobacter* isolates to source additional cocktail candidates

As in previous sections, Figure 8 illustrates the phages that were selected. This selection was based not only on those that lysed the highest number of isolates but also had a broader spectrum lysis potential.

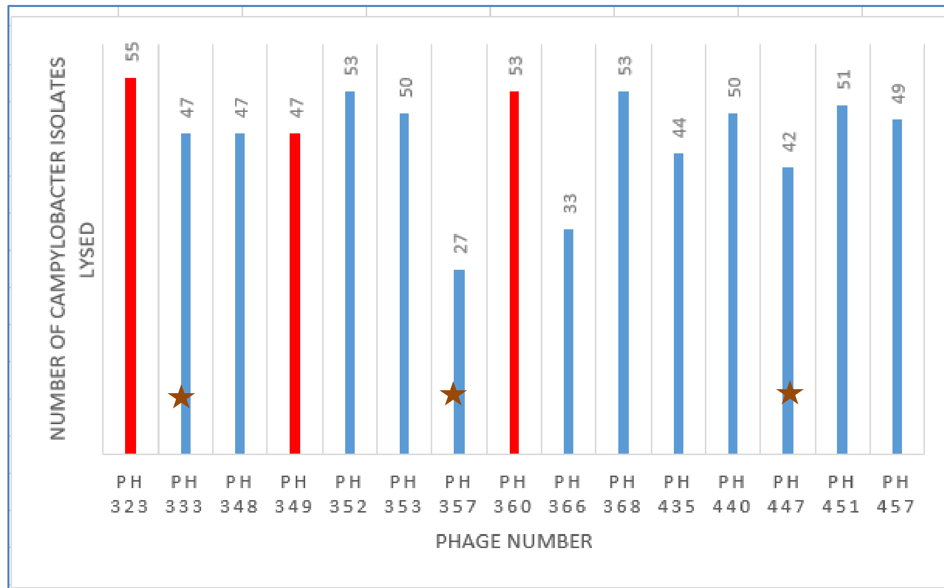


Figure 8 Number of *Campylobacter* isolates lysed by the 15 phages screened (the selected candidates are in red)

★ PH 323 lysed all isolates lysed by PH 333, PH 357 and PH 447

Selection of final cocktail candidates

The cocktail was finally selected and consisted of 12 phages initially tested against the UK host panel. An additional seven phages (PH194, 232, 388, 611, 613, 677 and 722) were added later based on their interesting lytic profile. This set of phages formed the final selection of cocktail candidates that were used for the farm trials.

Table 6 Final cocktail candidates used for farm trials

Phage #	194	232	260	264	265	283	295	323	349	360
Phage #	377	388	431	439	455	611	613	677	722	

Stage 2 selection

Stage 3 selection

UK Selection

Stage 4 selection

Establish the suitability of water delivery for selected phages

The pH of tap water and deionised water (DI) is presented in Table 4 and Table 5 presents the phage counts obtained on days 1, 2 and 7.

Table 7 pH of tap water from Brisbane

Date	Source of water	pH
26/07/16	Tap water Cannon Hill	7.60
26/07/16	Tap water Deagon	7.68
26/07/16	Tap water Dutton Park	7.46
26/07/16	DI water Dutton Park	6.50
04/08/16	Tap water Hamilton	7.45
04/08/16	Tap water Dutton Park	7.45
04/08/16	DI water Dutton Park	7.41

The pH of tap water ranged from 7.45 – 7.68. The phage count intended to be used for the trial is ~ 10^7 PFU/ml. There is no drop in phage titre over the seven day period it was assessed (Table 5). The phage was stable in tap water.

Table 8 Phage survival in tap water over time

Day	PFU/ml
1	2.74 x10^7
1	3.00 x10^7
1	4.12 x10^7
2	3.08 x10^7
2	3.02 x10^7
2	2.90 x10^7
7	6.80 x10^6
7	1.12 x10^7
7	1.04 x10^7

Pre-screening for farm trials

Pre-screening was carried out in September, November and December 2016 (Pre-screenings 1, 2 and 3 respectively). The following section presents these outcomes, which includes assessing *Campylobacter* and phage as well as carrying out lytic profiles of the phage to assess whether the farms/sheds met the required trial conditions.

Pre-screening -1 (September 2016)

Two farms were screened and *Campylobacter* was not detected in the sheds tested on one farm, following first pick-up. This farm did not meet the conditions and was thus rejected. The outcomes for the second farm (Farm P) are presented as follows:

Campylobacter levels in the caeca, ranged from 1.09 x 10^7 - 8.9 x 10^7 CFU/g across sheds 1, 3 and 5. Additionally, shed 3, was phage positive with phage levels in the range of ~ 10^5 PFU/g (caeca). (Table 9).

Table 9 – Pre-screening – *Campylobacter* levels and phage

	Shed 1	Shed 3	Shed 5
	***Campylobacter* levels (CFU/g caeca)**		
Ileum	2.9 x 10^5	2.4 x 10^5	6.7 x 10^6
Caeca	1.09 x 10^7	8.9x 10^7	1.4x 10^7
	Phage levels (PFU/g)		
Ileum	2 x 10^5	5 x 10^5	none(<10^4)
Caeca	none (<10^4)	10^5	none (<10^4)

Further screening was progressed as follows:

(a) A selection of *Campylobacter* isolates from caeca and ileum were screened against the phages isolated from shed 3 and the lytic profile is presented in Figure 9
(b) The same selection of *Campylobacter* isolates from caeca and ileum were screened against the 19 member cocktail and the lytic profile is presented in Figure 10

The screened *Campylobacter* isolates were sensitive to the resident phage (pink and yellow areas, Figure 9) but also showed a better sensitivity to phage cocktail candidates PH265 and PH 377 (and PH 323) in shed 3 (Figure 10). Due to the possibility of the interference of the high level of resident phage with the administered cocktail, it was decided not to progress with the farm trial on Farm P.

Date screened	Farm	NC #	Shed	C1	C3	C5	U1	U3	U5
7/09/2016	Pumicestone	P1	1	1	1	1	1	1	-
7/09/2016	Pumicestone	P2	1	1	1	1	1	1	-
7/09/2016	Pumicestone	P3	1	1	3	2	3	3	1
7/09/2016	Pumicestone	P4	1	-	1	-	1	1	1
7/09/2016	Pumicestone	P5	1	-	1	1	-	1	1
7/09/2016	Pumicestone	P6	1	-	-	-	-	-	-
7/09/2016	Pumicestone	P7	1	1	1	1	1	1	1
7/09/2016	Pumicestone	P8	1	2	2	-	2	2	-
7/09/2016	Pumicestone	P9	3	-	-	-	-	-	-
7/09/2016	Pumicestone	P10	3	-	-	-	-	-	-
7/09/2016	Pumicestone	P11	3	2	2	2	2	2	-
7/09/2016	Pumicestone	P12	3	2	2	-	2	2	-
7/09/2016	Pumicestone	P13	3	-	-	-	-	-	-
7/09/2016	Pumicestone	P14	3	-	-	-	-	-	-
7/09/2016	Pumicestone	P15	3	-	-	-	-	-	-
7/09/2016	Pumicestone	P16	3	2	2	2	2	2	-
7/09/2016	Pumicestone	P17	3	2	2	-	2	2	-
7/09/2016	Pumicestone	P18	3	2	3	-	2	3	-
7/09/2016	Pumicestone	P19	3	2	2	2	2	2	1
7/09/2016	Pumicestone	P20	3	2	2	-	2	2	-
7/09/2016	Pumicestone	P21	3	2	2	2	2	2	2
7/09/2016	Pumicestone	P22	3	1	1	1	1	1	1
7/09/2016	Pumicestone	P23	5	3	3	-	3	3	-
7/09/2016	Pumicestone	P24	5	2	2	2	2	2	2
7/09/2016	Pumicestone	P25	5	2	2	2	2	2	2
7/09/2016	Pumicestone	P26	5	2	2	1	2	2	1
7/09/2016	Pumicestone	P27	5	2	2	1	2	2	1
7/09/2016	Pumicestone	P28	5	?	?	?	?	?	?
7/09/2016	Pumicestone	P29	5	2	3	2	2	2	1
7/09/2016	Pumicestone	P30	5	2	2	-	3	3	-
7/09/2016	Pumicestone	P31	5	2	3	2	2	3	2
7/09/2016	Pumicestone	P32	5	?	?	?	?	?	?

C1= Caeca shed 1
U1= Ileum shed 1
1= lysed but turbid
2= lysed, clearer than 1 but less than 3
3= lysed, clearer than 2

3 = good lysis

Figure 9 Lytic profile of *Campylobacter* isolates from three sheds (Farm P) against resident phage

Date screened	Farm	NC #	Shed	PH194	232	260	264	265	283	295	323	349	360	377	388	431	439	455	611	613	677	722
5/08/2016	Pumicestone	P1	1	-	-	-	-	1	1	-	3	1	2	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P2	1	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P3	1	2	3	3	-	3	3	-	3	3	3	3	3	2	2	3	3	2	2	2
5/08/2016	Pumicestone	P4	1	-	-	-	-	1	1	-	-	1	-	2	3	-	-	-	-	-	-	1
5/08/2016	Pumicestone	P5	1	-	-	-	-	-	1	-	3	-	2	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P6	1	-	-	-	-	-	-	-	3	-	2	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P7	1	-	-	-	-	2	1	-	3	-	2	3	-	-	-	-	-	-	-	2
5/08/2016	Pumicestone	P8	1	-	-	-	-	-	1	-	3	-	2	3	2	-	-	-	2	-	-	2
5/08/2016	Pumicestone	P9	3	3	3	3	-	3	2	1	3	-	3	3	2	-	1	2	3	1	3	3
5/08/2016	Pumicestone	P10	3	-	-	-	-	3	2	-	-	1	3	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P11	3	3	3	3	1	3	2	1	3	3	3	3	3	1	1	3	3	2	-	1
5/08/2016	Pumicestone	P12	3	1	1	1	1	3	2	-	3	-	3	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P13	3	-	-	-	1	3	1	-	3	1	2	3	1	-	-	-	1	-	-	1
5/08/2016	Pumicestone	P14	3	-	-	-	-	3	-	-	3	-	-	3	-	-	-	-	-	-	-	1
5/08/2016	Pumicestone	P15	3	-	-	-	-	3	-	-	3	1	2	3	1	-	-	-	-	-	-	1
5/08/2016	Pumicestone	P16	3	-	-	-	-	3	-	-	3	-	2	3	-	-	-	-	-	-	-	1
5/08/2016	Pumicestone	P17	3	-	-	-	-	3	-	-	3	2	2	-	1	-	-	-	-	-	-	1
5/08/2016	Pumicestone	P18	3	-	-	-	-	3	1	-	3	-	2	3	-	-	-	-	1	-	-	1
5/08/2016	Pumicestone	P19	3	-	1	1	2	3	1	-	3	2	2	3	1	-	-	-	1	-	-	1
5/08/2016	Pumicestone	P20	3	-	1	1	1	3	2	-	3	2	3	3	-	-	-	-	1	-	-	1
5/08/2016	Pumicestone	P21	3	-	-	-	-	3	1	-	-	1	2	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P22	3	-	-	-	-	3	1	-	-	1	2	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P23	5	3	3	3	1	3	3	2	3	2	3	3	2	2	1	3	3	2	2	2
5/08/2016	Pumicestone	P24	5	3	3	3	1	3	3	1	3	2	3	3	3	2	1	3	3	2	2	2
5/08/2016	Pumicestone	P25	5	-	-	-	-	2	1	-	3	-	2	3	-	-	-	-	-	-	-	-
5/08/2016	Pumicestone	P26	5	2	2	2	2	3	2	-	3	2	3	3	2	-	-	1	2	1	-	1
5/08/2016	Pumicestone	P27	5	2	2	2	1	3	2	1	3	2	3	3	2	1	-	2	2	-	-	-
5/08/2016	Pumicestone	P28	5	2	2	2	-	3	2	-	3	2	3	3	2	-	-	-	2	1	-	1
5/08/2016	Pumicestone	P29	5	-	-	-	2	3	1	-	3	-	3	3	1	-	-	-	1	-	-	-
5/08/2016	Pumicestone	P30	5	1	1	1	3	3	-	-	3	-	3	3	-	-	-	1	1	-	-	-
5/08/2016	Pumicestone	P31	5	2	2	2	2	3	2	1	3	2	3	3	2	-	-	1	2	-	-	-
5/08/2016	Pumicestone	P32	5	2	2	2	1	3	3	1	3	2	3	3	2	2	-	-	2	-	-	-

3 = good lysis

Figure 10 Lytic profile of *Campylobacter* isolates against 19 cocktail candidates

Pre-screening – 2

During pre-screening 2, the representative *Campylobacter* isolates from two farms (Farm Re and Farm C) were screened against the 19 panel cocktail. The areas highlighted in green represent a score of 3 and thus good lysis.

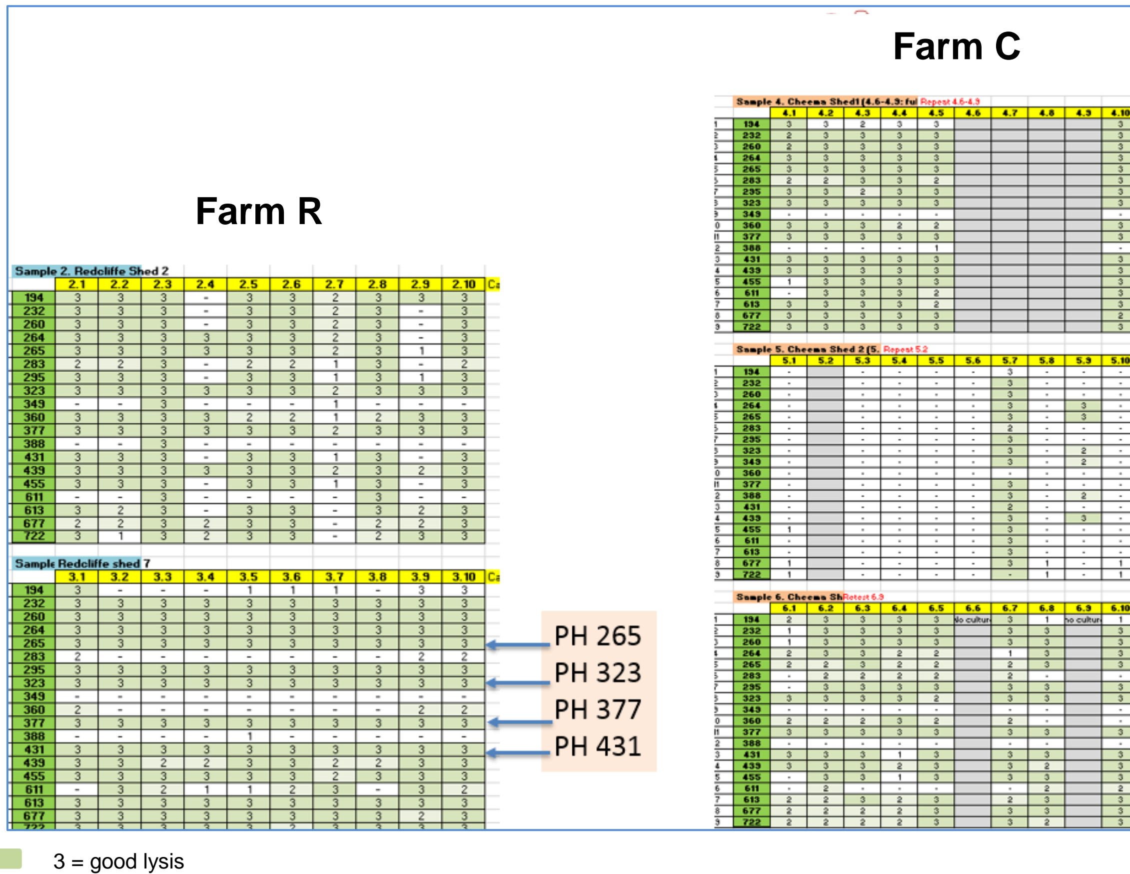


Figure 11. Lytic profile of *Campylobacter* isolates from sheds 2, 7 – Fam R and sheds 4, 5, 6 Farms C

The *Campylobacter* levels across both farms were high and in the range of ~ 10^8CFU/g in caeca. No phages were detected across both farms (Table 10).

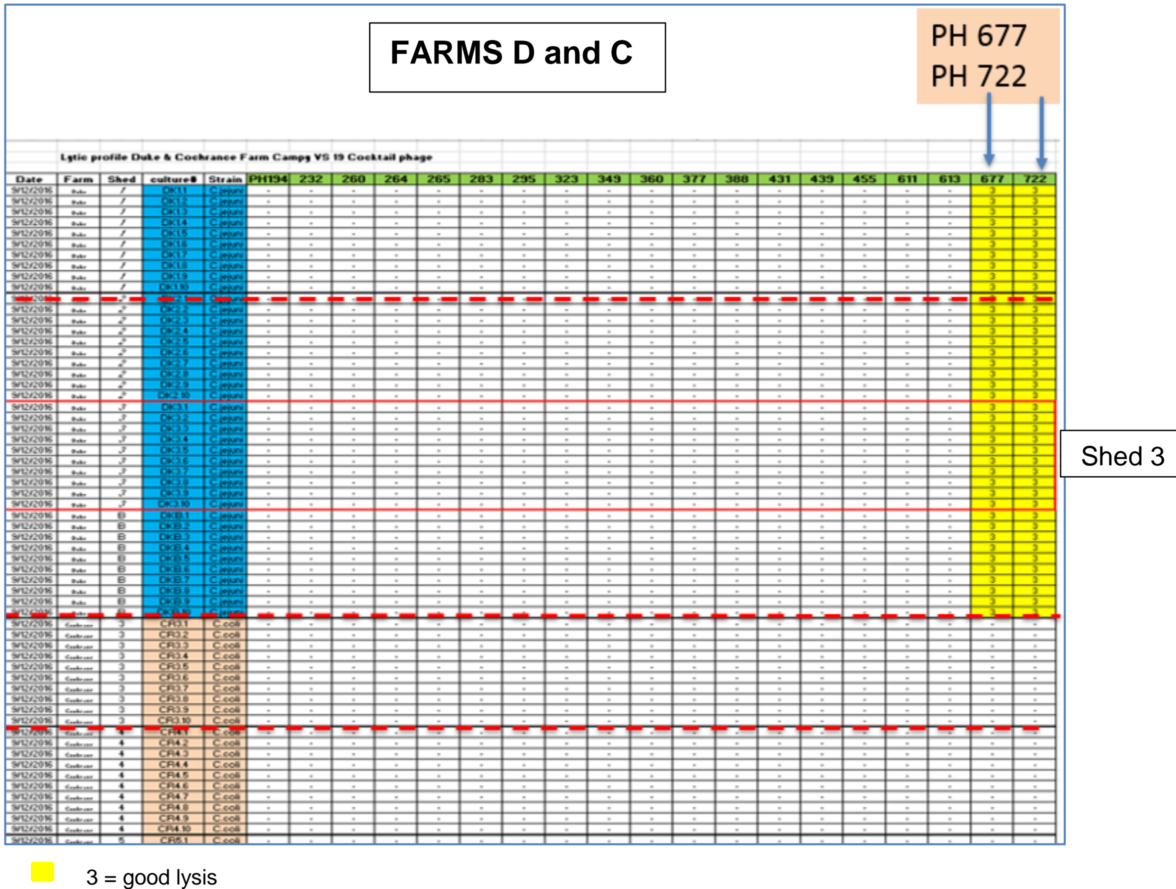
Table 10 *Campylobacter* (CFU/g) and phage (PFU/g) levels – Farms R and C during pre-screening

sample	date	farm	shed	*Campylobacter* CFU/g	Phage PFU/g
1	31/10/16	**R**	1	3.20×10^8	<100
2	31/10/16	**R**	2	1.47×10^8	<100
3	31/10/16	**R**	7	4.10×10^8	<100
4	31/10/16	**C**	1	6.64×10^8	<100
5	31/10/16	**C**	2	3.10×10^8	<100
6	31/10/16	**C**	3	2.73×10^8	<100

Shed 7 (Farm R) had the best coverage from four cocktail candidates (PH 265, PH 323, PH 377, PH 431), was phage negative and had a high *Campylobacter* count. Thus, this shed was selected as the trial shed and the trial progressed.

Pre-screening 3

During pre-screening 3, the representative *Campylobacter* isolates from two farms (Farm D and Farm C) were screened against the 19 panel phage cocktail. In this instance, two cocktail candidates (PH 677, PH 722) were able to lyse all the selected isolates across all four sheds in Farm D with wide coverage across the farm (Figure 12).



3 = good lysis

Figure 12. Lytic profile of *Campylobacter* isolates from sheds 1, 2, 3 and B – Farm D and sheds 3, 4, 5 Farm C

Farm C had the preferred high *Campylobacter* levels but was phage positive and lacked coverage from the cocktail candidates. Irrespective of good cocktail candidate coverage, Farm D farm had lower than usual *Campylobacter* levels. Shed 3, had the highest *Campylobacter* levels (6.9 x 10^7 CFU/g) was phage negative (tested 1 week before pick-up), meeting the trial conditions and progressed to farm trials (Table 11).

Table 11 *Campylobacter* (CFU/g) and phage (PFU/g) levels – Farms D and C farms during pre-screening

farm	shed	*Campylobacter* CFU/g	Phage PFU/g
D	1	1.0 x 10^7	<100
D	2	9.3 x 10^6	<100
D	3	6.9 x 10^7	<100
D	B	1.6 x 10^7	<100
C	3	1.0 x 10^7	<100
C	4	1.2 x 10^9	<100
C	5	1.0 x 10^9	4.6 x 10^3

On-farm trials

The outcomes for the two farm trials are listed below.

Farm trial 1 - *Campylobacter* levels in caeca and ileum from farm, caeca and ileum following transport and carcasses from the plant – Farm R

Table 12 presents the mean *Campylobacter* levels for caeca (farm and plant), ileum (farm and plant) and carcasses and the comparative statistical analysis (i.e. standard errors and *P* values). Figure 13 (a, b, c, d) presents the spread of *Campylobacter* counts across both treatment and control, farm and plant.

Table 12. Farm R – Analysis of *Campylobacter* and phage levels; farm and plant (following transport)

Variable	Stage	Sample	Control*	Treated*	s.e.m.	*P*-level
Campylobacter (log cfu/g)	Farm	Caeca	6.90	5.98	0.256	**0.020**
Campylobacter (log cfu/g)	Farm	Ileum	5.84	5.70	0.248	0.698
Campylobacter (log cfu/g)	Plant	Caeca	7.39	6.76	0.320	0.184
Campylobacter (log cfu/g)	Plant	Ileum	6.96	6.91	0.234	0.889
Phage (log pfu/g)	Farm	Caeca	n.d.	5.37	0.212	
Phage (log pfu/g)	Farm	Ileum	n.d.	3.83	0.977	
Phage (log pfu/g)	Plant	Caeca	n.d.	4.96	0.257	
Phage (log pfu/g)	Plant	Ileum	n.d.	5.12	0.883	
Phage (log pfu/carcass)	Plant	Carcass	n.d.	n.d.		

*means; n.d. = not detected; standard errors (s.e.m.); probability (*P*) levels (bolded where $P < 0.05$).

<u>Phage - caeca farm and plant</u>

Phages were only detected in treatment and not in control indicating that the trial conditions were met, i.e. the trial shed remaining phage negative. The mean phage levels for treatment ranged from log 4.96 (plant) to log 5.37 PFU/g (farm).

The carcasses remained phage free for both treatment and control

<u>*Campylobacter* - caeca farm and plant</u>

The *Campylobacter* levels in the caeca (farm) for treatment and control were significantly different ($P < 0.05$) and ranged from log 5.18 – log 6.25 CFU/g (with one exception) for test compared to the control (log 6.05 – log 8.15 CFU/g), Table 12. In summary the reduction in *Campylobacter* levels (farm) ranged from 1 – 3 log reductions across the treatment chickens when comparing the lowest treatment count (log 5.18 CFU/g) with the highest count (log 8.16 CFU/g) for control chickens (Figure 13a).

Whilst in the processing plant the difference between treated and control were not statistically significant, the *Campylobacter* levels in six treatment chickens ranged from log 5.14 – 6.27 CFU/g, with only one control chicken in this range (log 5.68 CFU/g, Figure 13b).

<u>*Campylobacter* - ileum farm and plant</u>

Unlike caeca, the ileum lacked contents (we were to test the ileum contents). The mucus content tested was more representative of the ileum lining. The *Campylobacter* levels showed no difference between treated and controls, for both farm and plant (Table 12). Though the spread of *Campylobacter* levels were in a lower range (log 4.5 – log 6.5 CFU/g), for farm compared to plant (log 5.5 – log 7.5 CFU/g) across both treated and control chickens (Figure 13 c, d).

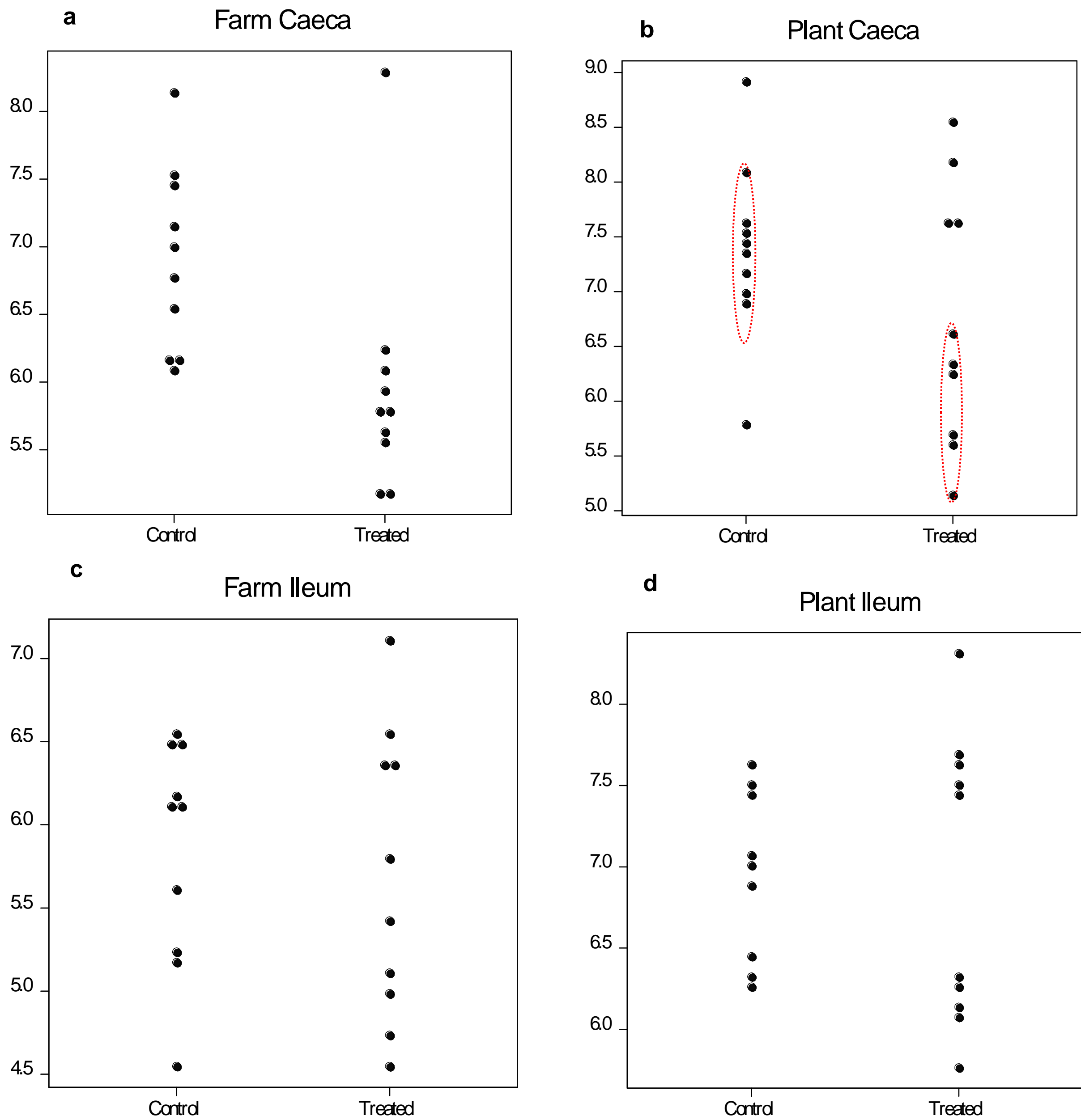


Figure 13 ***Campylobacter*** **levels (CFU/g) in caeca and ileum for treated and control chickens – Farm R**

Farm trial 2 - *Campylobacter* levels in caeca and ileum from farm, caeca and ileum and carcasses from the plant – Farm D

Table 13 presents the mean *Campylobacter* levels for caeca (farm and plant), ileum (farm and plant) and carcasses along with the comparative statistical analysis (i.e. standard errors and P values). Figure 14 (a, b, c, d) presents the spread of phage levels across both treatment and control, farm and plant. Figure 15 (a, b, c, d) presents the spread of *Campylobacter* levels across both treatment and control, farm and plant.

Table 13. Farm D - Analysis of *Campylobacter* and phage levels; farm, plant (following transport) and carcasses

Variable	Stage	Sample	Control	Treated	s.e.m.	*P*-level
Campylobacter (log cfu/g)	Farm	Caeca	5.65	5.67	0.162	0.923
Campylobacter (log cfu/g)	Farm	Ileum	6.09	5.96	0.133	0.506
Campylobacter (log cfu/g)	Plant	Caeca	5.75	5.84	0.147	0.690
Campylobacter (log cfu/g)	Plant	Ileum	6.21	6.55	0.200	0.240
Campylobacter (log MPN/carcass)	Plant	Carcass	4.91	4.41	0.415	0.409
Phage (log pfu/g)	Farm	Caeca	3.32	3.65	0.201	0.264
Phage (log pfu/g)	Farm	Ileum	2.57	2.52	0.243	0.891
Phage (log pfu/g)	Plant	Caeca	2.86	3.68	0.324	0.087
Phage (log pfu/g)	Plant	Ileum	2.52	3.00	0.228	0.152
Phage (log MPN/carcass)	Plant	Carcass	n.d.	#		

#: Nine values of zero and one of 40.

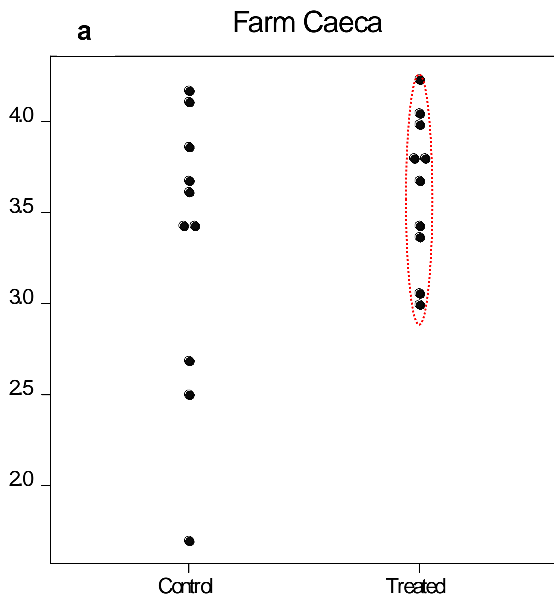
*means; n.d. = not detected; standard errors (s.e.m.); probability (*P*) levels (bolded where $P < 0.05$)

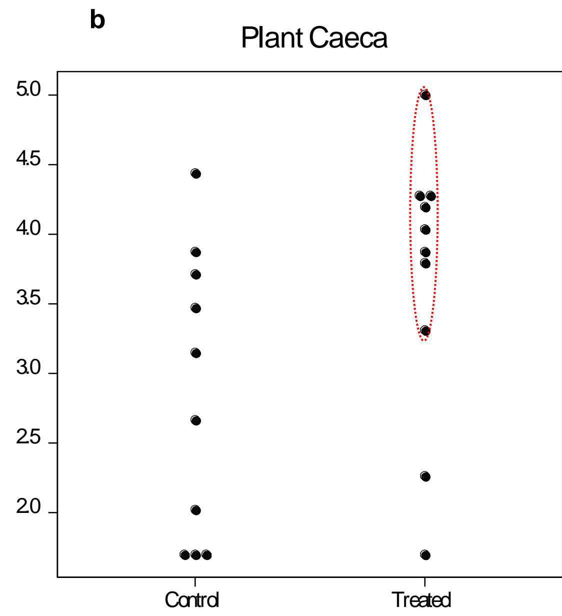
nd not detected

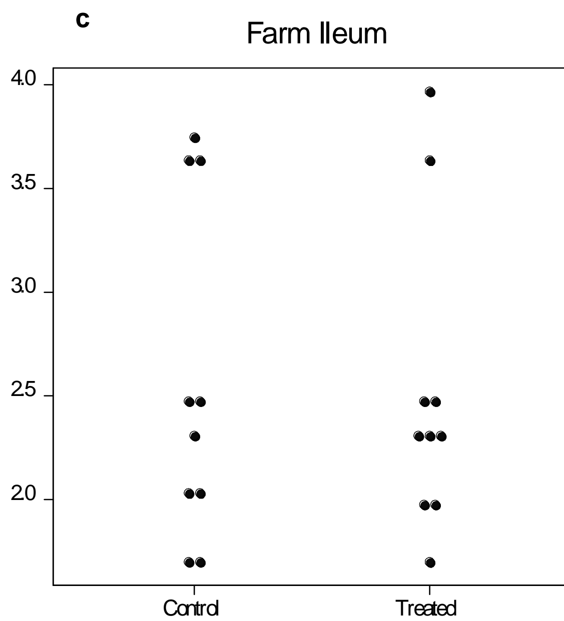
Phage presence and levels – treatment and controls

Unlike Farm R, this farm trial failed to meet the trial conditions at the stage of the farm trial. Phage was detected across all controls (farm and plant) with little variation between the phage levels across treatment and controls (Table 13). Phages were not introduced to controls, thus the phages detected in the controls were native phages. Two types of phage plaque sizes (large and small) were detected from the samples. The smaller phage plaques were present across the treatment and control groups, are thus likely represents the native phage, whereas the larger plaques were only present in the treatment samples and likely represent the introduced cocktail phage (data not presented as work is still in progress under the RIRDC study).

Figure 14 a, b, c, d presents the spread of phage counts across chickens for both farm and plant (treatment and controls). Though the differences were not statistically significant between treatment and control caeca for farm and plant, there was a tendency for higher phage counts in the treated chickens in both farm and plant compared to control with the levels in treatment in the range of log 3.0 – log 5.0 PFU/g (Figure 14 a and b). This range was not too different to the phage levels for treatment of Farm R farm. The ileum did not show much variation in phage levels. In contrast, for the control group the spread for both farm and plant ranged from < log 2.0 – log 4.0 PFU/g.







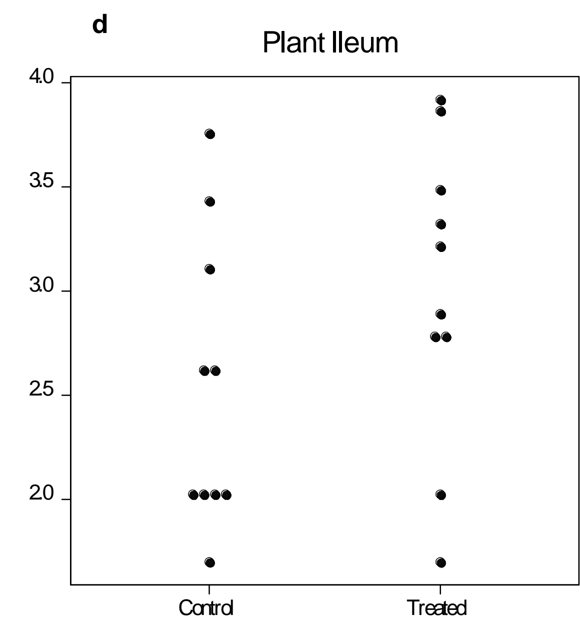


Figure 14 Phage levels (PFU/g) in caeca and ileum for treated and control chickens – Farm D

Caeca farm and plant

The *Campylobacter* counts in the caeca on-farm (and plant) for both treatment and control were not significantly different (Table 13). Another fact of interest is that the *Campylobacter* levels on both treatment and control showed a spread of counts (Figure 14 a, b) from a minimum of log 5.15 CFU/g to a maximum of log 6.58 CFU/g across both treatment and control (lower than at time of screening and one week before pick-up). The maximum count across the three sheds tested during pre-screening was log 7.8 CFU/g or 6.9 X 10^7 CFU/g, Table 8 as previously presented.

Ileum farm and plant

As with the previous farm, unlike caeca, the ileum lacked contents (we were to test the ileum contents). We tested the mucus content, which was more representative of the ileum lining. On Farm D, as with Farm R, the *Campylobacter* levels showed no difference between treated and controls, for both farm and plant. The general spread of *Campylobacter* levels for farm and plant were in the similar range were log 5.5 – log 7.0 CFU/g (plant) and log 5.4 – log 6.6 CFU/g (farm) across both treated and control (Figure 14c and d).

Carcass –*Campylobacter* levels

The *Campylobacter* levels for carcasses from treated and control were not significantly different, though six carcasses were below detection limit of <6000 organisms per carcass compared to four from control Figure 14e).

a Farm Caeca

b Plant Caeca

c Farm Ileum

d Plant Ileum

e Carcass rinse

Figure 15 *Campylobacter* levels (CFU/g) in caeca, ileum and carcasses for treated and control chickens – Farm D

Continued sensitivity to isolates to cocktail before and after phage treatment

Figure 16 shows the lytic profile of *Campylobacter* isolates from the caeca, both before (at the pre-screening stage) and after the introduction of the cocktail from isolates sourced from the farm and the plant. The lytic profile of the *Campylobacter* isolates to PH 677 and PH 722, remains unchanged. This shows the continued sensitivity of the *Campylobacter* isolates following treatment and with no development of resistance.

Campylobacter isolates – **BEFORE** introduction of cocktail

Lytic profile Duke & Cochrance Farm Campy VS 19 Cocktail phage

Date	Farm	Shed	culture#	Strain	PH194	232	260	264	265	283	295	323	349	360	377	388	431	439	455	611	613	677	722
[illegible]	[illegible]	[illegible]	DK2.9	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK2.10	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.1	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.2	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.3	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.4	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.5	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.6	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.7	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.8	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.9	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	J	DK3.10	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
9/12/2016	[illegible]	[illegible]	DK8.1	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3

Campylobacter isolates – **AFTER** introduction of cocktail

Lytic profile Campy from Duke farm trial VS 19 phage cocktail

				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Date	group	Culture		PH194	232	260	264	265	283	295	323	349	360	377	388	431	439	455	611	613	677	722
11/01/2017	Farm test	C1a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C1b	C.jejuni	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	3	3
11/01/2017	Farm test	C2a	C.jejuni	-	-	-	-	-	-	-	2?	-	-	-	-	-	-	-	-	+?	3	3
11/01/2017	Farm test	C2b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C3a	C.jejuni	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	1?	3	3
11/01/2017	Farm test	C3b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C4a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C4b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C5a	C.jejuni	-	-	-	-	-	?	?	?	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C5b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C6a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C6b	C.jejuni	-	-	-	-	-	-	-	-	-	1?	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C7a	C.jejuni	1?	1?	1?	1?	1?	1?	1?	-	1?	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C7b	C.jejuni	1?	1?	1?	1?	1?	1?	1?	-	1?	1?	-	-	1?	-	1?	-	-	3	3
11/01/2017	Farm test	C8a	C.jejuni	1?	-	-	-	1?	-	-	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C8b	C.jejuni	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C9a	C.jejuni	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3
11/01/2017	Farm test	C9b	C.jejuni	1	-	-	1	1	-	-	1	1?	1?	1?	1?	-	-	1?	-	1?	3	3
11/01/2017	Farm test	C10a	C.jejuni	+?	-	-	-	-	1?	1	-	-	-	-	-	-	-	-	-	-	3	3
11/01/2017	Farm test	C10b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C41a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C41b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C42a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C42b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C43a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C43b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C44a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C44b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C45a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C45b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C46a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C46b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C47a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C47b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C48a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C48b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C49a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C49b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	Plant test	C50a	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
12/01/2017	plant test	C50b	C.jejuni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3

Figure 16 Sensitivity of phage cocktail candidates (PH 677 and PH722) to selected *Campylobacter* isolates sourced before during pre-screening and those isolated (farm and plant) after the introduction of the cocktail

Discussion of Results

First Australian study, via a "proof of concept" demonstrate the potential to use phages to control *Campylobacter* levels on-farm

The current 1.5 year short study, is the first Australian study to demonstrate the ability to reduce *Campylobacter* levels (via the use of an appropriate cocktail) in the caeca of birds' on-farm and under commercial farming conditions. This was demonstrated via the Farm R study with the reduction in *Campylobacter* levels in the caeca of the treated birds compared with the controls (at the farm), being statistically significant ($P < 0.5$). The Farm D did not meet trial conditions due to the presence of phage in the control chickens. Further work is required to understand how this reduction can be sustained through transport stress under commercial conditions. Extensive work on *Campylobacter* bacteriophages have been carried out by our UK collaborators and future work will progress via the new RIRDC funded study. For example, this group have sequenced the genome of *Campylobacter* bacteriophages (Brathwaite et al. 2013) and the genome dynamics of *Campylobacter* bacteriophages in the chicken gut has been evaluated (Scott et al. 2007). Such outcomes will help to create a better understanding of *Campylobacter* phages sourced during this study from Australian farm environments.

Cocktail development using phages sourced from commercial farms

Using both stored bacteriophages and *Campylobacter* isolates from our previous RIRDC study, a collection of suitable phages were assessed via screening, for their lytic potential against *Campylobacter*. The extensive and logical screening approach resulted in a set of 19 phage cocktail candidates, some of which show good lysis potential against *Campylobacter* sourced from the farms/sheds tested. These bacteriophages were originally isolated from commercial farms in Queensland and an additional 29 were obtained (via enrichment) to facilitate phage diversity using Queensland hosts. Bacteriophages isolated from broiler house environments have shown relationships/variations based on successive flocks representing diversity within poultry farming environments, (Connerton et al. 2004). Thus, it was possible to use the phages and *Campylobacter* isolates sourced from commercial farm environments to progress phage therapy studies. Further work on these cocktail candidates is required and is in progress within the new RIRDC study. To date, additional phages have been isolated and re-evaluation of this cocktail will occur.

Reduction in *Campylobacter* levels in the caeca of the bird

At Farm R, following the oral gavage the reductions in *Campylobacter* levels within the bird's caeca ranged from log1.00 – 3.00 CFU/g, within 24h post treatment. Experimental studies in the UK have shown that phage treatment of *C. jejuni*-colonised birds resulted in *Campylobacter* counts decreasing between 0.5 and 5 log CFU/g of ceacal contents, compared to untreated controls over a 5-day treatment period. These reductions were dependent on the phage-*Campylobacter* combinations; doses administered and post treatment time, (Loc Carrillo et al. 2005). Kittler *et al.* (2013), following the phage application against *Campylobacter* in commercial broiler houses have suggested that phages can lead to a reduction of up to log 3.2 CFU in caeca *Campylobacter* loads. Carvalho *et al.* (2010) have reported a 2 log reduction (in faeces) in experimentally colonised 1-week old chicks (with *Campylobacter*) treated with a phage cocktail via the feed in a study that lasted 7 days. The present study directly addressed phage therapy during commercial farming on the farmed bird.

***Campylobacter* and phage interactions– Farm P**

There was a phage presence in Farm P during pre-screening done following first pick-up. Thus irrespective of the high *Campylobacter* numbers ($\log10^9$ CFU/g) in the caeca at the time, there was a possibility of the resident phage contributing to the reduction in *Campylobacter* levels, irrespective of the better lysis potential of the cocktail phage over the resident phage (based on our scoring). We rejected this farm as (a) there was potential for the resident phage to overlap with the cocktail phage; (b) we had to make an assumption that the cocktail phage (introduced at higher numbers) will get to the *Campylobacter* over the possible low numbers of the resident phages. Irrespective of all these factors, it was encouraging to observe that the *Campylobacter* population that was already infected with resident phage did not exhibit resistance to the cocktail phages

applied. This situation suggests that the cocktail phages were appropriate and their surface interactions with host are probably essential for the colonisation of the chickens (personal communication, Professor Ian Connerton).

Farm D trial and continued sensitivity of *Campylobacter* isolates

The Farm D *Campylobacter* levels in the caeca for both treatment and control were lower than expected for both treatment and controls (that did not receive the cocktail). The *Campylobacter* levels in the caeca on-farm were in the range of log 5.0 CFU/g in the control birds. These low *Campylobacter* levels are unusual based on previous work (Chinivasagam et al. 2015) where the *Campylobacter* levels in caeca have ranged from log 8.0 – 9.0 CFU/g (observed during a two year study across 17 farms/ 24 farm samplings).

Such situations could be challenging for "active phage therapy" to be successful, due to the target bacterial densities being below the phage proliferation threshold (personal communication Professor Ian Connerton). The presence of phage in the control flock at the time of the trial also could have been a contributory factor. This situation may have impacted on the study outcomes by (a) reducing the *Campylobacter* count relative to the count at the pre-screening stage; (b) the surviving bacteria may not have been at a level to support active phage proliferation and therefore reduced the impact of the therapeutic phage; (c) the surviving *Campylobacter* may have acquired partial resistance to the resident phage (personal communication Professor Ian Connerton). However, from our post treatment experiments we are now confident that the latter possibility of phage resistance did not occur and may not have impacted on the study. Irrespective of the presence of the resident phage, the farm *Campylobacter* isolates remained sensitive to the virulent treatment phage used to treat the birds during the farm trial. Whilst time constraints prevented us assessing the isolates of Farm R (which will be assessed as part of the new RIRDC study) the fact that Farm D isolates did not show phage resistance to the *Campylobacter* isolates sourced from caeca post phage treatment (at a farm or at plant sampling) is an important finding.

Carcass rinses phage presence and *Campylobacter* levels.

The absence of phage following phage therapy (or any residual treatments other than those of normal food origin) is an important aspect for "consumer acceptance" of phage therapy and receiving regulatory approval for use in food. The treated carcasses from both farms (and controls) showed an absence of phage (residue) on the carcass, when tested prior spin-chilling and chlorination. The carcasses were removed at this stage to gain an understanding without the impact of cool temperatures and chlorine. As a result, the *Campylobacter* counts were higher than normal (Chinivasagam et al. 2015). Note we lost the results for Farm R, as we under-estimated the *Campylobacter* count based on our previous study. Finally, though not statistically significant, there were more carcasses below the detection limit for *Campylobacter* in the treated chickens compared to controls. Overall, the key point to emerge from the carcass work was the absence of residual phage.

Delivery options

Whilst water delivery was adopted as a cheap and easy means on-farm; there potential for future interesting possibilities to become available. Siringan *et al.* (2014) for the first time have demonstrated an interesting relationship between the bacteriophage and *Campylobacter.* The organism, capable of adopting a "Carrier-State-Life- Cycle" where the phage in linked to *Campylobacter* in a non-infective carrier state. This has been observed as a natural association between *Campylobacter* and the phage in biofilms during the above study, which can have practical implications. This aspect has future potential to act as an "expendable vehicle" for phage delivery to pre-colonized chickens and is an interesting new possibility.

Resistance as a result of phage therapy

Whilst there is concern about phage resistance the current study has shown that even on a farm with existing native phages (Farm D) there was no evidence of phage resistance with the Farm D isolates that remained sensitive. In commercial broilers, this is addressed via administering the cocktail 24h post pick-up (as done in the current study) as a means of reducing the contact time

between the phage and bacteria. The selection of appropriate phage and their dose optimization are key elements for the success of phage therapy to reduce campylobacters in broiler chickens". The *Campylobacter* temporarily develops resistance in the presence of the phage, but reverts to a sensitive form in the absence of the phage (Loc Carrillo et al. 2005). The sensitive form is an active coloniser of the chicken gut, thus the trend for *Campylobacter* to revert to a form that allows it to successfully "exploit" this "niche" which is favourable for its on-going survival. Additionally, Loc Carrillo et al. (2005) have shown that "campylobacters resistant to bacteriophage infection were recovered from phage-treated chickens at a frequency of <4%. These resistant types were compromised in their ability to colonize experimental chickens and rapidly reverted to a phage-sensitive phenotype in vivo. Scott *et al.* (2007) have shown that *C. jejuni* populations that survive bacteriophage predation in broiler chicken display genomic re-arrangements resulting in resistance to bacteriophages at the same time being inefficient colonisers of the broiler chicken intestine. When these strains were reintroduced into chickens in the absence of bacteriophage further genomic rearrangements at the same locations resulted in, reversion to bacteriophage sensitivity and colonisation proficiency. Thus the resistance phonotype is of temporary nature with potential to revert to a sensitive phenotype. Thus genomic instability of *C. jejuni* in the avian gut has been adopted as a mechanism to temporarily survive bacteriophage predation and subsequent competition for resources in order to survive local environmental pressures.

Regulation for the use of *Campylobacter* phage products in Australia

The data generated from this study can contribute to both outcomes and knowledge for the regulators in Australia to further support the registration and acceptance of such products in the future when combined with the available background information. This will be unlike the situation in Europe, where the main reason for the limitation for uptake being that of regulation in Europe – in essence, there is no clear path to registration. This is being addressed, but a longer process than elsewhere in the world. However, while there are no phage products marketed in Europe, European companies export to US, (Personal communication, Professor Connerton). From an overall perspective this simple "proof of concept study"

Future work

This was a short proof of concept study from which an extensive range of knowledge and biological resources have been gained. The 1.5 year timeframe has been challenging for both the scientific and practical (commercial farm trials) requirements of the study. This study has however achieved "proof of concept" by demonstrating the ability to use phages from commercial farm environments to reduce *Campylobacter* numbers in the caeca of the bird on-farm. Whilst the milestones of this study have been successfully addressed, the work on *Campylobacter* phages is not complete and the reason for the new RIRDC study (currently 6 months into the study). In the interest of extending the already high food safety standards of the Australian poultry industry the outcomes from this CRC will continue to be addressed and are progressing under the new RIRDC study. .

Implications

This study has shown via a “proof of concept” that

(a) A select cocktail of bacteriophages can be used to target *Campylobacter* in the caeca of broiler chickens on-farm’
(b) *Campylobacter* levels in caeca can be reduced during on-farm treatment
(c) No residual phage were detectable on carcasses for the processing plant, a positive outcome for acceptance of the treatment
(d) Further work is required to understand the impact of *Campylobacter* levels during “transport stress” and the subsequent levels of *Campylobacter* on the carcass following processing.

Recommendations

The outcomes form this study are a short 1.5y proof of concept study. It is recommended that these outcomes be incorporated and research continued in the recently commenced RIRDC study to exploit the full potential of this research.

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Department of Agriculture and Fisheries – Queensland

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	**A "proof of concept" study to control *Campylobacter* using bacteriophages**
Poultry CRC Sub-Project No.:	3.1.6
Researcher:	Dr. Nalini Chinivasagam
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Sub-Project Overview	
Background	*Campylobacter* is a key food-safety pathogen and bio-control using bacteriophages can be an option. The current 1.5 year short study, is the first Australian study to seek and demonstrate the ability of phage to reduce *Campylobacter* levels (via the use of an appropriate cocktail) in the caeca of birds' on-farm and under commercial farming conditions.
Research	The research addressed this study in the following manner (1) Developing a phage cocktail panel using both phages and *Campylobacter* isolates sourced from a previous RIRDC study (from Queensland farms) (2) Addressing the "proof of concept" via carrying out two farm trials under commercial conditions and during commercial farming /processing Outcomes: This was a short proof of concept study from which an extensive range of knowledge and biological resources have been gained. The ability to reduce *Campylobacter* levels in the caeca of the bird was demonstrated in the first farm study with a reduction in *Campylobacter* levels in the caeca of the treated birds compared with the controls (at the farm), that was statistically significant ($P < 0.05$). The second farm did not meet trial conditions (due to phage presence in the control chickens during the trial) leading to the differences between treatment and control, not statistically significant. Further work is required to understand how this reduction can be sustained through commercial transport stress before processing. The absence of phage following phage therapy (or any residual treatments other than those of normal food origin) is an important aspect for "consumer acceptance" of phage therapy and receiving regulatory approval for use in food. The treated carcasses from both farms (and controls) showed an absence of phage (residue) on the carcass, was also a positive aspect of this study. An important finding of this study was the absence of phage resistance. There was continued sensitivity by *Campylobacter* (from isolates on the farm and in the processing plant) recovered post phage treatment and tested against the phages (PH 677 and PH 722) used in that trial. This aspect is also addressed the way chickens are treated i.e. 24h before pick-up (as in the current study). Another key finding was that no residual phage was detected on carcasses from both farm trials. This is an important requirement for consumer acceptance and registration issues of phage products destined for use in food processing. Most importantly this study has identified 19 phages that have the potential to be successful as phage cocktail candidates and will be further evaluated (with the work incorporated into the new RIRDC study). The findings from this study make phage therapy (for control of *Campylobacter*) promising. The data generated from this study can contribute to both outcomes and knowledge for the regulators in Australia

	to further support the registration and acceptance of such products in the future adding to the already available background information. This was a short proof of concept study and extensive knowledge has been gained within this short 1.5 year study which has been challenging due to the time constraints as a result of the both the scientific and practical (commercial farm trials) requirements of the study. This study has however addressed the "proof of concept" by demonstrating the ability to use phages from commercial farm environments to reduce *Campylobacter* numbers in the caeca of the bird on-farm. Whilst the milestones of this study have been successfully addressed, the work on *Campylobacter* phages is not complete and the reason for the new RIRDC study (currently 6 months into the study). In the interest of the Australian poultry industry (and food-safety) the outcomes from this study will continue to be addressed and are progressing under the new RIRDC study.
Sub-Project Progress	Final Report
Implications	This study has shown via a "proof of concept" that (a) A select cocktail of bacteriophages can be used to target *Campylobacter* in the caeca of the bird on-farm' (b) *Campylobacter* levels in caeca can be reduced during on-farm treatment (c) No residual phage is present on carcasses in the processing plant, a positive outcome for acceptance of the treatment Further work is required to understand the impact of *Campylobacter* levels during "transport stress" to the bird and the subsequent levels of *Campylobacter* on the bird.
Publications	We intend to prepare one or two publications as follows: (1) We intend to present a poster at the *Campylobacter* conference in France and will be seeking approval very shortly as the closing date is the 24th February. (2) A peer review publication is being prepared for "Frontiers in Microbiology" and will commence on submission of this report. (3) A possible second publication based on the collaborative work in the UK in PLoS Microbiology.