Promotion of competitive exclusion by 'good' bacteria against *Salmonella*

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L.L. Duffy and E. Fox

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Promotion of competitive exclusion by 'good' bacteria against *Salmonella*

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Researcher/Author Contact Details
Name: Lesley Duffy
Address: CSIRO Agriculture and Food
39 Kessels Road, Coopers Plains, 4108
Phone: 07 3214 2055
Email: Lesley.Duffy@csiro.au

In submitting this report, the researcher has agreed to Australian Eggs Limited publishing this material in its edited form.

Australian Eggs Limited Contact Details:

Australian Eggs Limited
A.B.N: 6610 2859 585
Suite 6.02, Level 6, 132 Arthur St
North Sydney NSW 2060

Phone: 02 9409 6999
Email: research@australianeggs.org
Website: www.australianeggs.org.au

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Foreword

This project was conducted to improve the biocontrol of *Salmonella* in egg production through the use of 'good' bacteria presenting an approach which will be favourably viewed by consumers. Control of undesirable bacteria in food production systems typically involves application of chemical sanitisers or an antimicrobial processing control such as heat or pressure treatment. Bacteria constantly compete for space and nutrients in localised environments. As a result of this competitive pressure, bacteria have developed mechanisms to inhibit other bacterial species and thus give themselves a competitive edge in colonising their environmental niches. This project therefore proposed to isolate naturally occurring non-pathogenic bacteria from the layer farm environment and assess them for the ability to inhibit *Salmonella* growth via agar overlay assays. The ability of selected candidates to competitively exclude *Salmonella* was assayed by examining competitive exclusion dynamics on stainless steel via the use of pre-formed biofilms.

This project was funded from industry revenue which is matched by funds provided by the Australian Government and by the Commonwealth Scientific and Industrial Research Organisation.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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About the Authors

Dr Edward Fox was the initial Principal Investigator until September 2018. Dr Fox was a Research Scientist and member of the CSIRO Food Safety and Stability Group. He has been the PI on a number of projects in the five years to the start of this project totalling more than $2 million. He has published over 20 peer reviewed journal articles and five book chapters in the five years to the start of this project. Dr Edward Fox currently holds the position of Senior Lecturer, Department of Applied Sciences at Northumbria University, Newcastle, UK.

Dr Lesley Duffy is a Research Microbiologist and member of the CSIRO Food safety and Stability Group. Lesley took the role of PI of this project in October 2018. Lesley has been the PI on a number of poultry based projects that investigated *Campylobacter* and *Salmonella*. Lesley is familiar with *Salmonella* through her poultry projects and has experience in assessing the effectiveness of alternative antimicrobials such as nanoparticles and the use of attachment assays.

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Abbreviations

BC	Biocontrol
CE	Competitive Exclusion
CFU	Colony Forming Unit
MRSA	De Man Regosa Sharpe agar
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar
TSA	Tryptone Soya Agar
XLD	Xylose Lysine Deoxycholate agar

Executive Summary

This project aimed to determine if biocontrol of *Salmonella* in egg production through the use of 'good' bacteria could be achieved hence presenting an approach which will be favourably viewed by consumers.

Potential control organisms (n=225) were first isolated from the layer farm environment by demonstration of inhibition to a single *Salmonella* Typhimurium ATCC 14028 strain. A subset (n=20) of these strains, chosen to represent the various genus isolated, were further characterised for inhibition of the growth of strains four other *Salmonella* serovars; *S*. Kiambu, *S*. Agona, *S*. Montevideo and *S*. Typhimurium PT 135a, all previously isolated from within the poultry industry. Inhibition of *Salmonella* growth by the agar overlay method was low with only small zones of clearing noted. A total of 13 isolates demonstrated inhibition across all serovars tested.

The selected isolates were also assessed for the competitive exclusion of *Salmonella* attachment to stainless steel coupons by the use of pre-formed biofilms. Total exclusion of at least one strain of *Salmonella* from attachment to stainless steel was achieved by five isolates with a further four demonstrating high levels of competitive exclusion. By using a cocktail of isolates there is good potential to develop an application that can exclude numerous *Salmonella* serovars from attaching to abiotic surfaces that may precede the development of a biofilm.

Potential biocontrol isolates were whole genome sequenced to identify the organisms and assess the carriage of antimicrobial resistance genes and potential virulence genes. Two *Aerococcus* and a *Lactobacillus* isolate did not have any antimicrobial resistance genes or recognised virulence genes. All of the *Enterococcus* isolates contained at least one acquired antibiotic resistance gene and most also have point mutations that produce ampicillin resistance. Several of the acquired antibiotic resistance genes, across the isolates, are for medically important antibiotics such as tetracycline, vancomycin, and erythromycin.

Overall the results confirm that naturally occurring microorganisms that are inhibitory to *Salmonella* can be readily isolated from the layer farm environment. A limited number of these demonstrate complete exclusion of *Salmonella* attachment to stainless steel coupons when used in pre-formed biofilms. To enable this work to move along a pathway to commercialisation further work to assess the suitability of these and other isolates for use along the egg production chain will need to be conducted.

Overall Conclusions

The approach used to isolate potential biocontrol organisms has demonstrated that bacteria capable of inhibiting *Salmonella* can be readily isolated from the layer farm environment. Total exclusion of at least one strain of *Salmonella* from attachment to stainless steel was achieved by five isolates with a further four demonstrating high levels of competitive exclusion using pre-formed biofilms on stainless steel. By using a cocktail of isolates there is good potential to develop an application that can exclude numerous *Salmonella* serovars from attaching to abiotic surfaces that may precede the development of a biofilm.

There are a number of isolates with potential to act as biocontrol agents that contain some antimicrobial resistance genes or potential virulence genes which may be of concern for the egg industry. Not all initially isolated organisms were further characterised for their competitive exclusion ability and these represent an accessible but untapped resource of potential biocontrol agents.

1 Introduction

Pathogenic bacteria may contaminate food production supply chains through a plethora of different contamination vectors, including the associated environment, animals or pests, and human interactions. Although control measures can be implemented to reduce this burden of contamination, sporadic contamination can nevertheless occur. Bacterial pathogens may colonise different parts of the production chain, cross-contaminate final food products, and ultimately be consumed by humans and cause clinical illness. Each food production chain has associated bacterial pathogens of particular significance, with *Salmonella* species being one such key contaminant associated with egg production.

Control of undesirable bacteria in food production systems typically involves application of chemical sanitisers or an antimicrobial processing control such as heat or pressure treatment. These approaches may have associated problems such as altering the sensory profile of foods or being negatively perceived by consumers or retail.

Probiotic bacteria, or 'good' bacteria, are naturally occurring bacteria that have traits which have a positive impact on human health. This is often due to their capacity to target and destroy harmful bacteria, or 'bad' bacteria. Biocontrol can be defined as the reduction or suppression of an undesirable organism by one or more other microorganisms. 'Good' bacteria capable of targeting specific 'bad' bacteria present a relatively new biocontrol strategy, which has key advantages in that it is seen as a natural process. The use of these bacteria in a food production environment, means 'good' bacteria are viewed favourably by consumers and retail, and minimising the need to use chemical sanitisers is seen as a priority for both consumers and retail.

While the application of bacteria antagonistic to *Salmonella* has often taken the approach of feeding to hens as a probiotic, this study will focus on environmental application (although this does not preclude the use of strains isolated in this study to be administered in feed as a probiotic).

1.1 Project description

This research will benefit egg producers by helping to minimise the occurrence of *Salmonella* on eggs and in the associated production environment. Food Standards Australia New Zealand recorded 14 recalls of egg and egg products between 1 January 2009 and 31 December 2018, 6 of which were due to *Salmonella* contamination (FSANZ, 2019).

The outcome will also benefit the consumer by reducing the likelihood of consuming *Salmonella* from contaminated eggs, and benefit society as a whole, through improved public health outcome. Recent estimates relating to sources of salmonellosis infections in South Australia from the years 2000 to 2010 associated 37% of sporadic infections and 59% of outbreak infections with contaminated eggs (Glass et al., 2016). Eggs and egg-containing foods were the most frequently identified food vehicle in *Salmonella* outbreaks from 2001 to 2016, with raw egg desserts accounting for the highest number of outbreaks (22 %) followed by egg-based sauce and other egg-based foods with 17 and 10 % of outbreaks respectively (Ford et al., 2018). In 2002 (Yohanes, 2002), each notified salmonellosis case was estimated as costing $1,387. Based on the parameters determined by Glass et al. (2016), this would estimate a burden of approximately $2.8 million to Australia, as a result of salmonellosis infection due to contaminated egg sources.

Reducing recalls will improve environmental sustainability by reducing food loss along with the associated costs of its production.

1.2 Objectives

Australian Eggs project 1FS801CO "Promotion of competitive exclusion by 'good' bacteria against *Salmonella*" commenced on September 26th, 2017.

The aims of this project include:

1. To identify naturally occurring non-pathogenic bacterial isolates ('good' bacteria) which have anti-*Salmonella* properties and are tolerant to Australian climate and farm conditions.

2. To develop a strain cocktail of 'good' bacteria that possess anti-*Salmonella* activity.

3. To examine the feasibility of delivery systems to incorporate the 'good' bacteria cocktail as a low cost control method targeting *Salmonella* in the egg production chain.

4. To generate a bank of strains which can be used for a variety of different application approaches (e.g. environmental application or application as a probiotic in feed).

The primary outcome of this project will be the development of a natural biocontrol agent which can be used by the egg industry to help reduce *Salmonella* contamination of eggs.

2 Isolation of anti-Salmonella organisms

Bacteria constantly compete for space and nutrients in localised environments. As a result of this competitive pressure, bacteria have developed mechanisms to inhibit other bacterial species and thus give themselves a competitive edge in colonising their environmental niches. Non-pathogenic bacteria have been shown to be capable of inhibiting other pathogenic bacteria including foodborne pathogens (Casey et al., 2017; Fox et al., 2014). These bacteria could represent a novel control measures that can be used to reduce food safety risks associated with pathogenic bacteria such as *Salmonella*. Lactic acid bacteria as well as other species, such as *Janthinobacterium* spp., have already been shown to be good candidates for use as biocontrol agents.

2.1 Method

Three sampling rounds were conducted at Victorian-based farms. The first and third sampling was on the same caged egg farm and was carried out on Monday October 16th, 2017 and Monday June 12th, 2018, respectively. The second round of sampling was conducted at a barn egg farm on January 30th, 2018. Samples collected are listed in Table 1 .

Table 1: Sample description from all sampling rounds with sampling point and number of samples.

Sample Point	**Number of Samples**
Sampling no. 1	
Swab – Faecal conveyor belt under cages	14
Swab – Shed floor	4
Feed	3
Faecal material	12
TOTAL	33
Sampling no. 2	
Swab – Faecal conveyor belt under cages	2
Litter material from barn floor	39
Feathers and litter	2
Faecal material	2
TOTAL	45
Sampling no. 3	
Swab – shed floor	10
Feed	2
Faecal material	8
TOTAL	30

Samples were serially diluted and plated on selective and non-selective agar media (i.e. BHIA, brain-heart infusion agar; PCA, plate count agar; and buffered MRSA, buffered De Man, Rogosa, Sharpe agar pH 7.4; Thermo Fisher). Screening was performed using a modified version of Henning et al., (2015) and is fully described in Figure 1. Briefly, serial dilutions were overlay plated on each of the three agar media and incubated overnight at 30 °C, with duplicate plates incubated aerobically or anaerobically.

Plates showing between one and approximately 500 colonies were overlayed with 0.7% agar containing approximately 10^7 CFU/ml of *Salmonella* Typhimurium ATCC 14028. Plates were reincubated overnight at 30 °C under the same atmosphere as the first incubation. Colonies with clearance zones indicating inhibition of the *Salmonella* strain were then purified from the agar plates and rescreened to confirm purity. A screening temperature of 30 °C was utilized to capture mesophiles which are likely the dominant group of organisms as described by their temperature growth range. Some psychrotrophs and thermophiles, if present, would also be capable of growth at 30 °C. Inhibition was scored on a +, ++ or +++ scale and described as weak, strong and very strong inhibition. Weak inhibitors required high colony numbers to completely inhibit the *S*. Typhimurium ATCC 14028 strain used for screening.

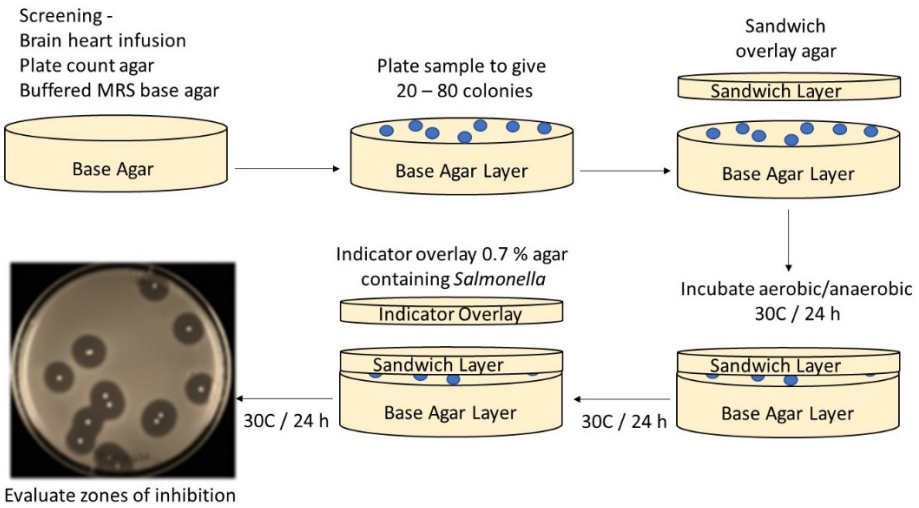

Figure 1: Isolation method adapted from Henning et al., (2015). Initial screening conducted with the three agars stated.

Aerococcus were analysed using pulsed field gel electrophoresis (PFGE) to determine if they were potentially clonal as many were isolated from the third farm visit. PFGE was conducted using the method of Stebbing et al. (2012). Genomic DNA (in plugs) was digested with *Sma*1 for 6 h at 25 °C. Digested plugs were electrophoresed in a 1.3 % agarose gel with run conditions of 6.0 V/cm with switch times of 1 to 30 s at 14 °C for 21 h. The gel was post stained in ethidium bromide before capturing an image using a Gel Doc 2000 gel documentation system.

Candidate isolates were then subjected to 16S rRNA gene sequencing to identify their genus and where possible to species. Genomic DNA was extracted from isolates using the DNeasy Blood and Tissue kit (Qiagen, Australia) according to the manufacturer's instructions. DNA quantity was measured using the Qubit dsDNA HS assay kit (Thermo Fisher, Australia) and an A260/A280 of 1.8 to 2.2 was confirmed using a NanoDrop spectrophotometer (Thermo Fisher, Australia).

A preliminary examination of two strains for inhibitory activity against other *Salmonella* serovars was conducted. An *Aerococcus* isolate from previous CSIRO projects (not isolated from the egg or poultry production chain) and the most inhibitory *Aerococcus* isolate were tested against *S*. Singapore and *S*. Infantis to determine if there was potential for inhibition against other *Salmonella* serovars as demonstrated in section 3.

2.2 Results and Discussion

The number of organisms potentially found on steel surfaces on egg farms is likely lower than that found in feacal and other samples tested. It was thought that by sampling from sources that potentially had more exposure to *Salmonella* that there was an increased chance of isolating an anti-*Salmonella* organism. Investigation of biofilms on steel surfaces within the egg farms may also be a potential source of biocontrol isolates. In this project every sample from the first two farm visits yielded at least one isolate showing some inhibition against *Salmonella*, under at least one of the media and atmosphere combinations tested. More than 200 isolates across all samples were individually assessed against *S*. Typhimurium ATCC 14028 using the agar overlay method. A single isolate from the shed floor collected in the caged egg farm showed strong inhibition against *S.* Typhimurium ATCC 14028; all other inhibitory isolates showed weaker inhibition and required high colony numbers to prevent detection of this *Salmonella* strain. Inhibitory isolates from the third visit were found in only 13 of the 30 samples tested. A total of 74 isolates showing inhibition against *S.* Typhimurium were speciated by 16S rRNA sequencing using the Sanger method. Details of each isolate, from each farm visit, which showed inhibitory activity against *Salmonella* is shown in (Table 2, Table 3, Table 4). The most common genus identified was *Enterococcus (*n=47), followed by *Escherichia* (n=15) and *Aerococcus* (n=9). All but the *Aerococcus* and *Staphylococcus* isolates were isolated from agar plates grown under anaerobic conditions, however all species are either aerotolerant or capable of growth under aerobic conditions.

A single *Aerococcus* isolate demonstrated the greatest inhibition against *S*. Typhimurium ATCC 14028 compared to all other potential biocontrol isolates except the CSIRO *Aerococcus* strain. For the weakly inhibitory isolates, it was noted that zone clearance was small for single colonies, whereas when cell densities were higher no detection of *S.* Typhimurium was noted. MRS plates were buffered with phosphate buffer to ensure this was not due to low pH. In a biocontrol strain cocktail, bacteria would be applied at high concentrations. Based on this, the results suggest these bacteria will thus be capable of inhibiting *Salmonella* since they will be in high numbers. There is scope to use both bacteria themselves as well as any bacteriocins they may be producing that have anti-*Salmonella* properties. It may also be possible to enhance production of these anti-*Salmonella* compounds, as has been previously achieved by optimising the growth matrix used to cultivate bacteriocin-producing isolates to increase yield 20-fold (Suganthi and Mohanasrinivasan, 2015).

Two isolates were further screened for their inhibitory activity against *Salmonella* serovars Singapore and Infantis. This included the *Aerococcus* isolate from this study, as well as an *Aerococcus* isolate in the CSIRO collection shown to inhibit *S.* Typhimurium. As can be seen from Figure 2, both isolates showed strong inhibition of all three serovars screened against. These isolates are both capable of growth under aerobic conditions, suggesting they are suitable candidates for use in the egg farm environment.

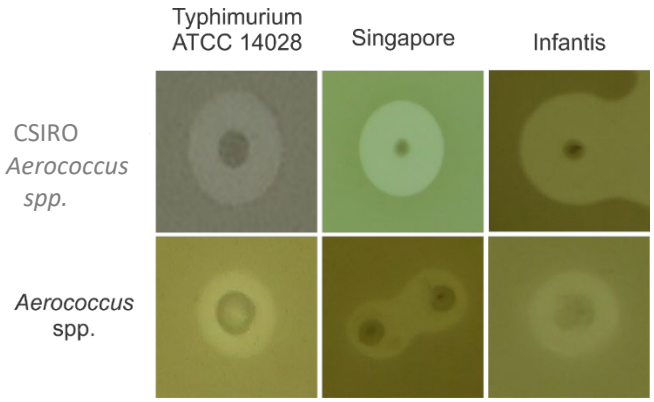

Figure 2: Inhibition of *Salmonella* serovars Typhimurium, Singapore and Infantis by the CSIRO *Aerococcus* isolate, as well as the highest inhibitory *Aerococcus* species isolate from the caged egg farm environment.

Table 2: Isolation information for inhibitory isolates (n=21) from samples collected during the first farm visit. Inhibition was scored on a +, ++ or +++ scale and described as weak, strong and very strong inhibition.

Farm	Genus Species	Original Sample	Media	Atmosphere	Inhibition Score[c]
1	*Aerococcus species*	Shed floor	BHIA	O_2[a]	++
1	*Enterococcus faecalis*	Faecal material, 22 week hens	PCA	AnO_2[b]	+
1	*Enterococcus faecalis*	Faecal material, 70 week hens	PCA	AnO_2	+
1	*Enterococcus faecalis*	Faecal material, 70 week hens	PCA	AnO_2	+
1	*Enterococcus faecalis*	Shed floor	MRSA	AnO_2	+
1	*Enterococcus faecalis*	Faecal material, 70 week hens	MRSA	AnO_2	+
1	*Enterococcus faecalis*	Faecal material, 70 week hens	MRSA	AnO_2	+
1	*Enterococcus faecium*	Faecal conveyor belt under cages	MRSA	AnO_2	+
1	*Enterococcus faecium*	Faecal conveyor belt under cages	MRSA	AnO_2	+
1	*Enterococcus faecium*	Faecal conveyor belt under cages	MRSA	AnO_2	+
1	*Enterococcus faecium*	Faecal material, 70 week hens	PCA	AnO_2	+
1	*Enterococcus faecium*	Faecal material, 57 week hens	MRSA	AnO_2	+
1	*Enterococcus faecium*	Faecal material, 70 week hens	MRSA	AnO_2	+
1	*Enterococcus faecium*	Faecal material, 70 week hens	MRSA	AnO_2	+
1	*Escherichia coli*	Faecal conveyor belt under cages	MRSA	AnO_2	+
1	*Escherichia coli*	Faecal conveyor belt under cages	BHIA	AnO_2	+
1	*Escherichia coli*	Faecal material, 43 week hens	BHIA	AnO_2	+
1	*Escherichia coli*	Faecal material, 43 week hens	BHIA	AnO_2	+
1	*Escherichia coli*	Faecal material, 43 week hens	BHIA	AnO_2	+
1	*Escherichia coli*	Faecal material, 57 week hens	BHIA	AnO_2	+
1	*Escherichia coli*	Faecal material, 70 week hens	BHIA	AnO_2	+

[a] O_2 standard atmosphere; [b]AnO_2 anaerobic atmosphere; [c] use of a 3+ scale

Table 3: Isolation information for inhibitory isolates (n=36) from samples collected during the second farm visit. Inhibition was scored on a +, ++ or +++ scale and described as weak, strong and very strong inhibition.

Farm	Genus species	Original Sample	Media	Atmosphere	Inhibition Score[b]
2	*Enterococcus faecium*	Litter	PCA	AnO_2[a]	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter	PCA	AnO_2	+
2	*Enterococcus faecium*	Litter and feathers	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Enterococcus faecium*	Litter	MRSA	AnO_2	+
2	*Escherichia coli*	Litter	PCA	AnO_2	+
2	*Escherichia coli*	Litter and feathers	BHIA	AnO_2	+
2	*Escherichia coli*	Litter	BHIA	AnO_2	+
2	*Escherichia coli*	Litter	BHIA	AnO_2	+
2	*Escherichia coli*	Litter	BHIA	AnO_2	+
2	*Escherichia coli*	Litter	BHIA	AnO_2	+
2	*Escherichia coli*	Litter	BHIA	AnO_2	+
2	*Escherichia coli*	Litter	MRSA	AnO_2	+
2	*Lactobacillus plantarum*	Litter	MRSA	AnO_2	+

[a]AnO_2 anaerobic atmosphere; [b] use of a 3+ scale

Table 4: Isolation information for inhibitory isolates (n=17) from samples (n=13) collected during the third farm visit (Farm 1, visit 2). Inhibition was scored on a +, ++ or +++ scale and described as weak, strong and very strong inhibition.

Farm	Genus Species	Original Sample	Media	Atmosphere	Inhibition Score[c]
1	*Lactococcus lactis*	Shed floor	PCA	AnO_2[b]	+
1	*Aerococcus*	Shed floor	bMRSA	O_2[a]	+
1	*Enterococcus faecium*	Shed floor	bMRSA	AnO_2	+
1	*Staphylococcus* *Enterococcus faecium*	Shed floor	bMRSA	O_2 AnO_2	+ +
1	*Enterococcus faecium*	Faecal conveyor belt	bMRSA	AnO_2	+
1	*Aerococcus*	Faecal conveyor belt	bMRSA	O_2	+
1	*Staphylococcus*	Faecal conveyor belt	bMRSA	O_2	+

	Enterococcus			AnO_2	+
1	*Staphylococcus* *Enterococcus*	Faecal conveyor belt	bMRSA	O_2 AnO_2	+ +
1	*Staphylococcus gallinarum*	Faecal material	PCA	O_2	+
1	*Aerococcus species*	Faecal material	bMRSA	O_2	+
1	*Enterococcus species*	Faecal material	PCA	AnO_2	+
1	*Aerococcus* *Aerococcus (different colony)*	Faecal material	bMRSA bMRSA	O_2 O_2	++ +
1	*Enterococcus faecium*	Faecal material	bMRSA	AnO_2	+

[a] O_2 standard atmosphere; [b]AnO_2 anaerobic atmosphere; [c] use of a 3+ scale

As there were a number of *Aerococcus* isolated from the third farm visit these were first analysed using pulsed field gel electrophoresis (PFGE) to investigate if they were potentially clonal. Previous *Aerococcus* isolates were also included in the analysis. The finding that six of the seven *Aerococcus* examined using PFGE did not have the same banding pattern (Figure 3) ensured that these isolates could be considered as individual isolates for the purposes of further testing.

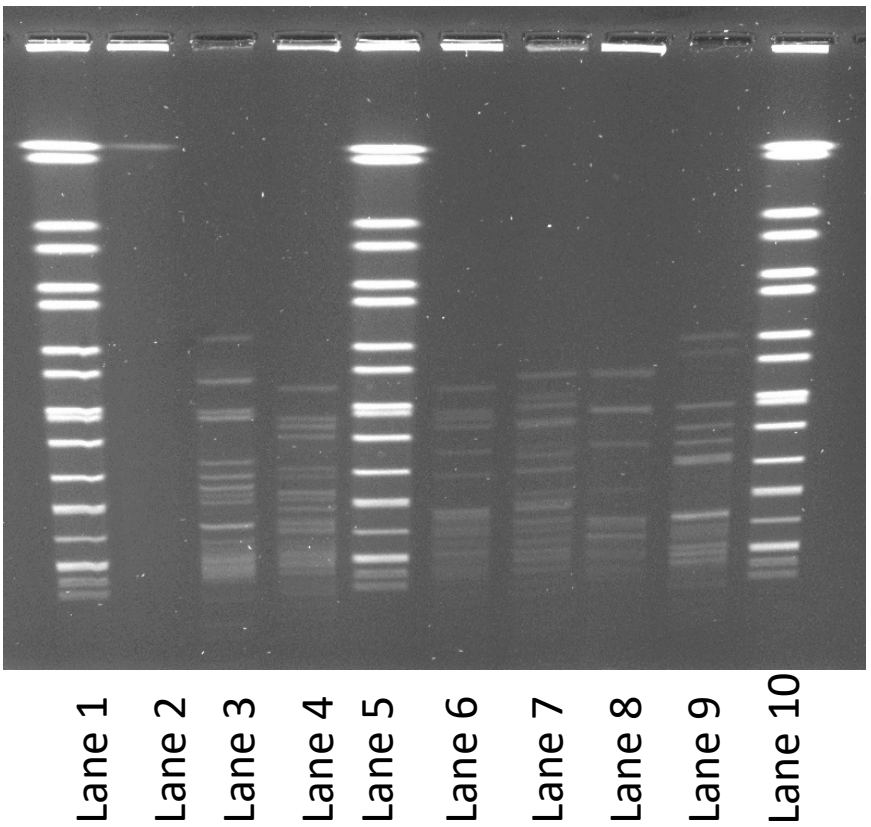

Figure 3: Pulsed Field Gel Electrophoresis of seven isolates, all initially tested as *Aerococcus*. Lane 1 control H9812, Lane 2 isolate 46, lane 3 isolate 183, lane 4 isolate 184, lane 5 control H9812, lane 6 isolate 186, lane 7 isolate 190, lane 8 isolate 197, lane 9 isolate 200 and lane 10 control H9812. The control isolate H9812 is *S*. Branderup.

3 Screening of potential biocontrol isolates against five *Salmonella* strains belonging to four serovars

There are over 2500 *Salmonella enterica* serovars although a limited number of these are responsible for the majority of human disease. Of these, the highest concern for the Australian egg industry is *S*. Typhimurium. Of the 166 *Salmonella* outbreaks linked to eggs from 2001 to 2011 in Australia, 90 % were caused by *S*. Typhimurium (Moffatt et al., 2016). *S*. Typhimurium was also found to make up the highest number of food related poultry/poultry+eggs *Salmonella* isolates other than the non-virulent S. Sofia from New South Wales (Simpson et al., 2018). There may be differing responses within the Typhimurium serovar as well between serovars, and these should be assessed in a much broader study.

3.1 Screening Method

From all of the inhibitory isolates collected a sub-group were chosen to further investigate their capacity to inhibit different serovars of *Salmonella*. This included representatives from the genera *Aerococcus, Enterococcus, Staphylococcus and Escherichia*. The sub-group comprised 20 isolates to enable both screening against further *Salmonella* isolates and competitive exclusion on stainless steel studies, to be completed in triplicate. An *Aerococcus* isolate from a CSIRO collection was also included. Using a modified overlay method utilised in the isolation procedure this sub-group of isolates was tested against the following five *Salmonella enterica* subsp. *enterica* isolates; *S*. Typhimurium (ATCC 14028), *S*. Kiambu (1368, Broiler), *S*. Agona (2351, spent layer), *S*. Montevideo (2177, Broiler), *S*. Typhimurium PT 135 a (2327, spent layer). Each isolate was spread plated onto Tryptone Soya Agar (TSA; Thermo Fisher) at two dilutions to ensure a plate of between 100 and 1000 colonies and immediately overlayed with TSA. After 24 h incubation at 37 °C the plates were overlayed with two dilutions of *Salmonella* within 5 mL of TSA to ensure approximately 10^7 *Salmonella* were added. Plates were then further incubated for another 24 h at 37 °C. Plates were examined for any zones of inhibition. Inhibition was graded from + to +++ and is listed in Table 2.

3.2 Results and Discussion

The zones of inhibition for all isolates against most *Salmonella* strains was small (Table 5). A single *Aerococcus* had a zone of inhibition rated ++ against *S*. Typhimurium ATCC 14028. The method utilised for the screening of multiple serovars incorporated TSA instead of BHI. While these agar media are not greatly different it may be sufficient to alter the inhibitory affects observed in the initial isolation phase. When the inhibition assay was repeated, two *Aerococcus* isolates did not produce any zones of inhibition (Table 5).

The indicator overlay method has been previously utilised to isolate a number of bacteriocin producing organisms from a variety of foods and faecal material (Henning et al., 2015). The isolation of organisms with inhibitory action to *Salmonella* was successful with *Serratia ficaria* CEL-1 isolated from Chinese celery found to have activity against *S*. Typhimurium H3380 (Henning et al., 2015). *Enterococcus* sp. represented the largest group of organisms in both this project and in the Henning et al., study (2015). A limited number of isolates in the original study were also found to be inhibitory to other pathogens such as *Staphylococcus aureus* ATCC 12600 and to a limited extent *Enterococcus faecalis* ATCC 19433 when these were used as secondary indicators. Assessing the effectiveness of the layer farm isolates against indicators other than *Salmonella* was not within the scope of this

project, however it would be useful to assess this in any further work. Further work would also examine the chemical nature of the inhibitors with the highest potential.

Table 5: Inhibition of multiple *Salmonella* serovars by the 20 selected isolates using the initial screening method.

ID # (Farm/visit)	ID	Initial screen 14028 *S.* Typhimurium (BHI agar)	14028 *S.* Typhimurium (TSA)	1368 *S.* Kiambu	2351 *S.* Agona	2177 *S.* Montevideo	2327 *S.* Typhimurium (PT135a)
4 (-)	*Aerococcus*	++	+	+	+	+	-
43 (1/1)	*Enterococcus faecium*	+	+	-	-	-	-
46 (1/1)	*Aerococcus* spp.	+	+	+	+	-	+
49 (1/1)	*E. coli*	+	+	+	+	+	+
55 (1/1)	*Enterococcus faecium*	+	-	-	-	-	-
59 (1/1)	*E. coli*	+	+	+	+	+	+
76 (2/1)	*Enterococcus faecium*	+	+	+	+	+	+
87 (2/1)	*Enterococcus* spp.	+	+	+	+	+	+
91 (2/1)	*Enterococcus*	+	+	+	+	+	+
115 (2/1)	*E. coli*	+	+	+	+	+	+
134 (2/1)	*E. coli*	+	+	-	-	-	-
145 (1/2)	*Enterococcus*	+	+	+	+	-	-
183 (1/2)	*Staphylococcus* spp.	+	+	+	+	+	+
186 (1/2)	*Staphylococcus* spp.	+	+	+/-[a]	-	-	-
190 (1/2)	*Aerococcus* spp.	+	-	+	+	+	+
197 (1/2)	*Staphylococcus* spp.	+	+	+/-	+/-	+/-	+/-
202 (1/2)	*Enterococcus*	+	+	+	+	+	+
206 (1/2)	*E. coli*	+	+	+	+	+	+
222 (1/2)	*Enterococcus faecium*	+	+	-	-	-	-
224 (1/2)	*Enterococcus* spp.	+	+	+	+	+	+

[a]Repeat assays were variable against these *Salmonella* strains

4 Competitive exclusion on stainless steel of five *Salmonella* strains by selected isolates

Administration of micoorganisms that represent adult-type flora from healthy poultry have been shown to prevent the colonisation of pathogenic *Salmonella* by means of competitive exclusion (De Cort et al., 2017; Wigley and Barrow, 2017). While not precluding the use of the isolates found in this project as a form of probiotic, one of the aims of the project was to undertake preliminary experiments to determine if the isolates could be used in the form of an environmental application. The idea of competitive exclusion is not new and formed the basis of the initial application for this project. Antagonistic bacteria or their metabolites have been shown to be inhibitory to various foodborne pathogens on abiotic surfaces in food production and food processing environments. While other foodborne pathogens have been challenged using this approach there has been little published on the competitive exclusion of *Salmonella* in relation to attachment to stainless steel.

Stainless steel is commonly used in many food production environments including layer farms, egg wash facilities, grading floors and continuing into facilities such as restaurants, and commercial or domestic kitchens. This material was therefore chosen to determine if attachment of the potential biocontrol isolates to stainless steel could competitively exclude introduced poultry related *Salmonella*.

4.1 Competitive Exclusion biofilm assay

The competitive exclusion (CE) assay is described fully in Table 6. Stainless Steel (SS) coupons of grade 304, mill finish (5 mm diameter by 0.9 mm thick; surface area 0.53 cm^2) were cleaned in a solution of 3% sodium hydroxide for 20 minutes, then 0.1% peracetic acid for two minutes. Coupons were rinsed with sterile water three times between washes and then sterilised in the autoclave. Briefly each assay was conducted in microtiter plates, which contained wells for *Salmonella* only, BC isolates only, media blank only and CE wells. Briefly biocontrol (BC) isolates were grown to stationary growth phase by incubating without shaking for 24 h in Tryptone Soya Broth (TSB; Thermo Fisher) at 37 °C before addition to the CE assay by dilution to 10^5 CFU/mL in 1/10 TSB. Initial counts were conducted to ensure the dilution was made to 10^5 CFU/mL thereby also ensuring that stationary phase had been achieved. Media blank controls contained only stainless steel coupons (SSC) and TSB. *Salmonella* controls contained SSC and 1/10 TSB for the first 24 h followed by removal of liquid and addition of approximately 10^3 CFU/mL *Salmonella*. BC isolate controls contained SSC and 10^5 CFU/mL BC for 48 h. CE assays contained SSC and 10^5 CFU/mL BC for 24 h, followed by removal of liquid and addition of 10^5 CFU/mL *Salmonella*. All incubations of the CE assay were conducted at 20 °C as this more closely represents the temperature found within egg farms dependent on season.

After the second 24 h incubation the media was removed from all wells on the microtiter plate and the SSC transferred to a new microtiter plate. The SSC were washed three times in phosphate buffered saline (PBS) to remove any loosely adhered cells. A 270 µL portion of PBS was added to each well containing a coupon. The microtiter plate was sealed with parafilm before sonicating for 5 min at 80 % power (Soniclean, Thermo Fisher). Sonication has previously been found to be more successful in removing bacteria attached to steel surfaces (Bjerkan et al., 2009). Serial dilutions were prepared from each well (-1 to -4) and 100 µL of each dilution, in addition to the undiluted well sample were then individually plated on TSA and Xylose Lysine Deoxycholate agar (XLD; Thermo Fisher). Plates were incubated at 37 °C for 48 h to allow the slower growing *Aerococcus* and *Enterococcus* isolates to more fully develop. This temperature is optimum (35 °C *Enterococcus*) for recovery of all isolates except Aerococcus which has an optimum of 30 °C. Counts of each colony type were recorded and where necessary, a representative (n=4) number of colonies were confirmed as *Salmonella* by use of a *Salmonella* latex agglutination kit (Thermo Fisher). All counts were Log_{10} transformed before

calculation of the number of *Salmonella* competitively excluded from pre-formed biocontrol isolate biofilms calculated as follows:

> (Number of *Salmonella* in biofilm on sterile SSC after 24 h) minus (number of *Salmonella* on SS coupons with preformed biocontrol strain biofilms after 24 h) = Number of *Salmonella* competitively excluded by biocontrol isolate (Results are shown in Table 7).

To further investigate the efficacy of mixed biocontrol isolates cocktails were made of four isolates. Each BC isolate was ranked according to the reduction in *Salmonella* attachment achieved (Table 8 and Table 9). The isolates were grown at 37 °C for 24 h before dilution to 10^5 CFU/mL. A 1 mL portion of each of the four isolates was combined before using the mix to inoculate the CE biofilm assay as described. BC isolates included in the cocktails were selected to ensure the cocktail had high potential activity against all of the five tested *Salmonella* strains and are listed in Table 9. Statistical differences in the number of *Salmonella* competitively excluded by individual isolates were determined by one-way analysis of variance (Tukey's method) using Minitab software (Minitab 17, Minitab Inc., Minneapolis, USA).

Table 6: Explanation of competitive exclusion assay method conducted in microtiter plates at 20 °C.

	Day 0	Day 1 (20°C)	Day 2	Day 3	Day 4	Day 5
CE wells	Incubate biocontrol isolates at 37 °C for 24 h	Add 150 µL of 10^5 BC to well with SSC Incubate microtiter plate at 20 °C	Remove liquid from CE wells (containing BC) and from *Salmonella* +ve control wells Add 150 µL of ~10^3 *Salmonella*	Remove liquid from all wells Wash all SSC three times in PBS Sonicate for 3 min Plate on TSA and XLD (37 °C/48 h)	Continue incubation at 37 °C to ensure smaller colonies grow	Count, confirm *Salmonella*, record
Salmonella +ve control wells		Add 150 µL of 1/10 TSB to *Salmonella* +ve control well Incubate *Salmonella* at 37 °C for 24 h	Add 150 µL of ~10^3 *Salmonella*	As for CE wells		
BC +ve control wells	As for CE wells	Add 150 µL of 10^5 BC to well with SSC	Continue incubation at 20 °C	As for CE wells		
-ve control wells		Add 150 µL of 1/10 TSB to negative control well		As for CE wells		

BC – biocontrol isolate
CE – competitive exclusion

SSC – stainless steel coupons
PBS – phosphate buffered saline
TSB - Tryptone Soya Broth
XLD - Xylose Lysine Deoxycholate agar

4.2 Results

The recorded change in the count of *Salmonella* attached to the SSC after pre-exposure to the potential biocontrol isolates compared to the count of *Salmonella* attached to non-exposed steel coupons is listed in Table 7. Isolates 49 (*E. coli*) and 46 (*Aerococcus*) were consistently the best performing isolates with the highest reduction in *Salmonella* attachment numbers for all *Salmonella* strains. Significant ($P<0.01$) differences in the number of *Salmonella* attached were noted across some isolates for each *Salmonella* strain. These differences between isolates varied depending on the combination of BC isolate and *Salmonella* strain.

While all tested isolates were capable of growing to stationary phase after 24 h at 20 °C, some isolates attached to the SSC in very low numbers. The low attaching isolates were either *Aerococcus* or *Enterococcus* species (43, 55, 76, 183, 190, 197, 202, 222, 224). None of these isolates produced high levels of exclusion of *Salmonella* attachment.

Table 7: Change in the Log_{10} CFU/mL of attached *Salmonella* when exposed to potential biocontrol isolates.

Salmonella	Potential Biocontrol Isolate Number															
	4	**4**	**4**	**43**	**43**	**43**	**43**	**43**	**43**	**46**	**46**	**46**	**49**	**49**	**49**
14028	-3.27	-3.18	-3.88	-0.16	-0.27	-0.26	-0.71	-0.26	0.04	NR	NR	NR	NR	NR	NR
1368	-1.62	-2.35	1.01	-0.10	0.00	0.47	-0.60	-0.14	-0.62	NR	NR	NR	NR	NR	NR
2351	0.56	0.54	1.70	-0.46	-0.76	-1.29	0.10	0.28	0.30	-3.32	NR	NR	NR	NR	NR
2177	-3.32	-3.15	-2.92	1.55	0.52	0.64	0.34	0.08	-0.01	-2.45	NR	-4.15	NR	NR	NR
2327	NT	NT	NT	0.30	-0.23	0.18	0.51	0.50	0.70	NT	NT	NT	NR	NR	NR
	55	**55**	**55**	**76**	**76**	**76**	**87**	**87**	**87**	**91**	**91**	**91**	**115**	**115**	**115**
14028	-0.84	-0.14	0.05	0.32	-0.17	0.15	-3.33	-3.11	-3.73	0.42	0.19	0.17	-6.41	-3.60	-2.41
1368	-0.29	-0.16	-0.28	0.58	0.58	0.25	-2.1	-2.00	-1.95	-0.38	0.29	0.82	NR	NR	NR
2351	-0.81	-1.07	-1.42	-0.90	-1.03	-0.72	-2.05	-1.61	-1.54	0.62	-1.68	0.92	-3.9	-3.42	-3.00
2177	0.63	0.25	0.88	0.72	0.07	-0.09	-1.20	-0.84	-2.38	0.03	-0.20	-0.10	-4.47	-3.19	-3.90
2327	-0.23	-0.48	-0.78	-0.93	-0.9	-0.25	-3.63	-3.63	-2.63	NT	NT	NT	-2.34	-2.02	NR
	134	**134**	**134**	**145**	**145**	**145**	**183**	**183**	**183**	**183**	**183**	**183**	**186**	**186**	**186**
14028	-1.94	-0.16	-1.05	-3.01	-2.89	-2.29	-0.28	-0.6	-0.34	0.44	0.42	0.06	0.59	0.49	0.61
1368	-0.32	0.12	-4.78	-3.26	-3.26	-2.23	-0.37	0.20	0.11	-0.01	-0.69	-0.88	-0.61	-1.95	-0.51
2351	-1.90	0.66	0.57	-3.67	-1.15	-4.30	0.00	-0.94	-0.39	0.28	0.37	0.38	0.08	0.23	0.00
2177	-2.51	-2.42	-1.6	-0.91	-2.01	-0.85	0.06	-0.06	0.07	-0.35	0.10	-0.01	-0.18	-0.08	0.15
2327	-5.76	-2.97	-2.8	-0.75	-0.8	0.06	0.45	0.26	0.11	0.44	0.44	0.11	0.36	0.45	0.47
	186	**186**	**186**	**190**	**190**	**190**	**190**	**190**	**190**	**197**	**197**	**197**	**197**	**197**	**197**
14028	-3.08	-3.1	-2.53	-0.89	-0.20	-0.17	-0.62	1.04	0.16	0.51	0.45	0.40	NR	NR	NR
1368	NR	NR	NR	-0.43	-0.24	-5.85	-0.91	-1.16	-0.69	-0.82	-0.57	-0.70	NR	NR	NR
2351	0.56	0.54	1.70	-0.24	-0.75	-1.67	-0.02	-0.43	-0.11	-0.24	0.22	0.09	-0.69	-0.32	-5.90
2177	NR	NR	NR	0.37	-0.03	0.20	0.09	-0.34	0.11	0.16	-0.08	0.34	-6.94	-0.69	-3.86
2327	NT	NT	NT	-0.36	0.06	-0.33	0.11	0.48	0.41	0.39	-0.15	0.48	-4.76	-1.61	-0.25
	202	**202**	**202**	**206**	**206**	**206**	**222**	**222**	**222**	**224**	**224**	**224**			
14028	0.17	0.19	0.2	-1.57	-3.74	-0.88	-0.19	0.12	-0.34	0.09	0.61	0.47			
1368	-1.16	-0.06	0.06	0.12	-0.19	0.20	0.00	0.61	0.57	-0.76	-0.99	-0.54			
2351	-2.48	-2.23	-1.62	-2.45	0.15	-0.32	-1.02	-1.00	-1.23	-0.26	0.21	0.00			
2177	-0.03	0.04	-0.22	-1.30	-0.60	-0.21	1.19	0.99	1.30	-0.06	0.07	-0.20			
2327	NT	NT	NT	-0.42	-0.59	-0.77	-0.97	-0.98	-0.42	0.46	0.36	0.19			

Combinations of isolates and *Salmonella* strains are marked as high potential in red, medium in blue and low in green (Replicates all > 2.0 Log_{10} CFU reduction in red, all replicates > 0.5 Log_{10} CFU/mL reduction in blue and at least two replicates with some reduction in green). Where *Salmonella* was not recovered at the end of the competitive exclusion assay, suggesting complete exclusion, the result is stated as NR (not recovered). NT represents Not Tested. Each isolate was analysed between three and six times.

Table 8: Change in the Log_{10} CFU/mL of attached *Salmonella* when exposed to isolates potential biocontrol isolates.

Salmonella	**Cocktail 1 (46, 4, 202, 49)**			**Cocktail 2 (134, 145, 186, 222)**			**Cocktail 3 (43, 87, 115, 197)**		
14028	-0.03	-0.45	0.15	-0.54	-0.89	-1.24	0.13	-0.15	0.26
1368	-0.85	-2.85	-1.85	-3.31	NR	NR	-2.22	-3.15	-3.35
2351	-2.67	NR	-2.85	-4.03	-2.70	-3.87	-2.30	-2.73	-2.97
2177	NR	-1.95	NR	-1.38	-2.37	-3.57	-2.19	-2.80	-1.57
2327	-2.40	-3.40	NR	-3.44	-3.22	-3.25	-2.92	-1.80	-2.06

Combinations of isolates and *Salmonella* strains are marked as high potential in red, medium in blue and low in green (Replicates all > 2.0 Log_{10} CFU reduction in red, all replicates > 0.5 Log_{10} CFU/mL reduction in blue and at least two replicates with some reduction in green). Where *Salmonella* was not recovered at the end of the competitive exclusion assay, suggesting complete exclusion, the result is stated as NR (not recovered). Each combination was analysed three times.

Table 9: Overall rating of isolates (with identification from the whole genome sequencing completed in section 5) that demonstrated some exclusion activity against *Salmonella* and the cocktail mix the isolates were assigned to. Ratings are summaries from the reduction values listed in Table 7; +++ red, ++ blue, + green, - black.

ID	Genus species (where known)	14028	1368	2351	2177	2327	Cocktail
4	*Aerococcus*	+++	+	-	+++		1
43	*Enterococcus faecium*	+	+/-	+/-	-	-	3
46	*Aerococcus*	+++	+++	+++	+++	NT	1
49	*E. coli*	+++	+++	+++	+++	+++	1
55	*Enterococcus faecium*	+	+	++	-	+	-
76	*Enterococcus faecium*	-	-	++	-	+	-
87	*Enterococcus sp.*	+++	+++	++	++	+++	3
91	*Enterococcus sp.*	-	-	-	+		-
115	*E. coli*	+++	+++	+++	+++	+++	3
134	*E. coli*	+	+	-	+++	+++	2
145	*Enterococcus sp.*	+++	+++	++	++	++	2
183	*Staphylococcus*	+	+	+	+	-	-
186	*Staphylococcus*	++	++	-	++	-	2
190	*Aerococcus*	+	++	+	-	-/+	-
197	*Staphylococcus*	++	++	+/-	+	+	3
202	*Enterococcus sp.*	-	+	++	+		1
206	*E. coli*	++	-	+	+	+	-
222	*Enterococcus faecium*	+	-	++	-	+	2
224	*Enterococcus sp.*	+	-	+	-		-

4.3 Discussion

Competitive exclusion is where one bacterial species competes with another over resources and/or space in a habitat, successfully reducing the number of cells or excluding that species (Hibbing et al., 2010). Competitive exclusion can take three approaches; competition between planktonic cells of both species co-cultured, exclusion where the potential control isolate is grown to a biofilm before addition of the target organism, and displacement where the organism of concern is grown to a biofilm before addition of the potential control isolates (Gray et al., 2018). As this project is preliminary and potential pathway to market is yet to be defined, only the role of exclusion was examined for each of the isolates individually and in three cocktails of isolates. The ability of the BC isolates to form biofilms was not assessed and this would provide further interpretation to the generated data. The approach used suggests the protective culture application is preventative and has not been investigated as a sanitiser analogue. All potential biocontrol isolates and the cocktails were tested against the five *Salmonella enterica* subsp. *enterica* isolates; *S*. Typhimurium (ATCC 14028), *S*. Kiambu (1368, Broiler), *S*. Agona (2351, spent layer), *S*. Montevideo (2177, Broiler), *S*. Typhimurium PT 135 a (2327, spent layer) retrieved from the CSIRO culture collection.

The reduction in *Salmonella* attachment achieved by the BC isolates varied by isolate and by *Salmonella* serovar. This suggests that there are differences in the ability of the BC isolates to exclude the *Salmonella* from attaching to SSC that have pre-formed biofilms. The inhibitory effects of naturally occurring microorganisms in biofilms on the growth of pathogens has been demonstrated previously. Natural biofilms formed on wooden shelves used for storing cheese during the ripening phase were found to inhibit the growth of *Listeria* (Mariani et al., 2011). No antimicrobials were identified in that study and the inhibitory effects were linked to non-specific competition for nutrients. Lactic acid bacteria that produce bacteriocins and acidic fermentation end-products also have anti-listerial properties in biofilms (Guerrieri et al., 2009). Reductions in pH from acid production as well as competition from nutrients was also suggested (Guerrieri et al., 2009). No assessment of the production of potential antimicrobial substance was undertaken in this study therefore the underlying reason for the competitive exclusion of *Salmonella* by some BC isolates was not reported.

The study of the inactivation of *Salmonella* on abiotic surfaces by inhibitory microorganisms has been limited. Cell-free supernatants of *Hafnia alvei* have been used to inhibit the formation of *Salmonella* Enteritidis biofilm on SSC (Chorianopoulos et al., 2010). This project examined the competitive exclusion of *Salmonella* attachment to SSC after 24 h at 20 °C. The formation of *Salmonella* biofilm can be time dependent (Chorianopoulos et al., 2010). The effective exclusion of pathogenic bacteria has also been shown to be time dependent, varying with the choice of BC isolate. Further work that examines the effectiveness over time would be valuable in assessing the usefulness for industry applications. Switching the role of *Salmonella* and BC isolates with *Salmonella* grown as a biofilm first and the ability of the BC isolates to out compete on the basis of growth and space acquisition would provide information on the possibility of the BC isolates to be utilised as a sanitiser.

The CE assays were completed against all five *Salmonella* strains used in assessment of the zones of inhibition by the overlay method (Section 2). The formation of biofilm on polystyrene surfaces by *Salmonella* has been shown to be influenced by serovar and incubation conditions (Wang et al., 2013). Within a single serovar, such as *S*. Typhimurium, the numbers of attached cells can vary between isolates and between surfaces (Chia et al., 2009). A wide range of serovars can be found in egg layer environments with 17 serovars isolated from egg shell wash and egg farm environment between 2010 and 2013 in Australia (Australian *Salmonella* Reference Centre, 2010, 2011, 2012, 2013). This project examined only five strains representing four serovars isolated from poultry. Further serovars would need to be tested in developing a path to market as a biocontrol surface

application. This work requires further assessment in the areas mentioned to develop a natural system that would be effective in a processing environment application.

The potential BC isolates were sent for whole genome sequencing (WGS) at the beginning of the CE assays. Many of the BC isolates used contain antimicrobial resistance genes and potential virulence genes (Section 5) making them initially unsuitable for use in the egg layer environment. However, the demonstrated basis of this project that some naturally occurring microorganisms from the egg layer environment can reduce the attachment of *Salmonella* to stainless steel surfaces could be utilised to screen further isolates and develop improved conditions for applications. Other surfaces utilised along the egg production chain that may contact the egg surface such as those used in conveyor belts could be investigated in developing application protocols.

5 Whole Genome Sequencing of potential biocontrol isolates

The use of 'good' bacteria as biocontrol agents that may inhibit the attachment of *Salmonella* to surfaces in the egg production chain must be balanced against the possible introduction of bacterial elements that may have potential for negative impact on the chickens or on public safety. Those elements carrying known antimicrobial resistance markers or potential virulence genes are not recommended for use as biocontrol agents.

5.1 Method

Initial isolation of potential BC isolates (Section 2) was conducted at CSIRO Werribee. After the use of 16S rRNA gene sequencing for genus identification the isolates were regrown from the -80 °C freezer and sent to the CSIRO Coopers Plains laboratory for screening against other *Salmonella* serovars and forCE biofilm assays. The isolates were regrown, DNA extracted using DNeasy Blood and Tissue kit following the manufacturer's protocol (Qiagen, Australia) and sent for whole genome sequencing (WGS) by staff at the Ramaciotti Center of Genomics at the University of New South Wales.

Genome data in the form of paired-end Illumina DNA sequencing reads was used to confirm the isolate strain identification as well as for the detection of antibiotic resistance markers and known virulence genes. The KmerFinder application[1] was used to compare blocks of sequence data (K-mers) with known reference genomes. These identifications were confirmed by online Blast search analysis of random segments of genome. Antibiotic resistance markers were detected using the ResFinder application[2] which searches for both chromosomal mutations that lead to antibiotic resistance as well as acquired resistance genes. The presence of virulence factors was determined using the Virulence[3]Finder application. ResFinder and VirulenceFinder applications were run using raw read data to reduce the chance of low quality genome assembly masking the detection of known marker genes in the databases.

5.2 Results and Discussion

The final list contained two *Aerococcus* strains closely aligned with *Aerococcus urinaeequi.* These isolates did not have any detectable antibiotic resistance markers nor virulence genes detected. *Aerococcus* such as these are generally considered to be opportunistic pathogens. A third *Aerococcus* strain contained the *inuA* gene which provides for lincosamide resistance. Of the six *E. coli* strains two carry the *tsh* gene which encodes an autoinducer that is often seen in avian pathogenic *E. coli* strains. Two other *E. coli* contain long polar fimbrae genes (*lpfA*) associated with pathogenicity. None of the *E. coli* strains encode toxins or other significant pathogenicity genes but a wide suite of virulence

[1] https://cge.cbs.dtu.dk/services/KmerFinder/
http://www.ncbi.nlm.nih.gov/pubmed/24172157
http://www.ncbi.nlm.nih.gov/pubmed/24574292

[2] https://cge.cbs.dtu.dk/services/ResFinder/
http://www.ncbi.nlm.nih.gov/pubmed/22782487

[3] https://cge.cbs.dtu.dk/services/VirulenceFinder/
http://www.ncbi.nlm.nih.gov/pubmed/24574290

associated genes are present in these strains. All *E. coli* have at least one acquired antibiotic resistance gene present across a range of antibiotic classes. There is a single isolate of *Lactobacillus* which would could be classified as "generally regarded as safe", however this was not included in the original inhibition and CE assays. There are 14 *Enterococcus* isolates either of an undetermined species or *Enterococcus faecium*. All of the *Enterococcus* strains contain at least one acquired antibiotic resistance gene and most also have point mutations that produce ampicillin resistance. Several of the acquired antibiotic resistance genes are for medically important antibiotics such as tetracycline, vancomycin, and erythromycin. A further three isolates were most closely aligned with *Staphylococcus*. These isolates had no detectable virulence genes and a single isolate carried the resistance gene, *blaZ*, for ampicillin resistance. The genomic databases are less reliable for unknown organisms and as such care should be taken in interpreting results for these *Staphylococcus*-like organisms.

Table 10: Genus and species identification, virulence genes and antibiotic resistance genes detected using whole genome sequencing. Samples indicated with a * were not included in the subset utilised in the assays.

Sample number	Kmer_ID	Virulence genes	Virulence of interest	Pathogen status	Antibiotic resistance
46	*Aerococcus sp.*	None		probable opportunistic pathogen	none
101*	*Aerococcus sp.*	None		probable opportunistic pathogen	none
190	*Aerococcus sp.*	None		Possible opportunistic pathogen	lnuA
49	*E. coli*	gad, iroN, iss, lpfA, mchF	lpfA	possible human pathogen - lacks most virulence genes	mdfA, tetB
59*	*E. coli*	capU, cba, cma, gad, iha, iroN, iss, mchB, mchC, mchF, mcmA, tsh	tsh - APEC plasmid	possible avian pathogen	aadA1, mdfA, sul1
115	*E. coli*	iroN, iss, lpfA, mchF	lpfA	possible human pathogen - lacks most virulence genes	mdfA, tetB
134	*E. coli*	capU, cba, cma, gad, iha, iroN, iss, mchB, mchC, mchF, mchF, mcmA, tsh	tsh - APEC plasmid	possible avian pathogen	aadA1, mdfA, sul1
184*	*E. coli*	gad, iss		limited pathogenicity potential	mdfA
206	*E. coli*	gad		limited pathogenicity potential	mdfA, parC_mut
43	*Ent. faecium*	acm		potential avian pathogen	aac, msrC, pdp5_muts
55	*Ent. faecium*	acm, efaAfm		potential avian pathogen	aac, msrC, pdp5_muts
76	*Ent. faecium*	acm, efaAfm	efaAfm	potential avian pathogen	aac, msrC, lnuA, pdp5_muts

Sample number	Kmer_ID	Virulence genes	Virulence of interest	Pathogen status	Antibiotic resistance
85*	*Ent. faecium*	None			aac, ant, msrC, lnuB, lsaE, pdp5_muts
91	*Ent. faecium*	Acm, efaAfm	efaAfm	potential avian pathogen	aac, msrC
117*	*Ent. faecium*	acm		potential avian pathogen	aac, msrC, pdp5_muts
136*	*Ent. faecium*	acm		potential avian pathogen	acc, lnuA, msrC, pdp5_muts
145	*Ent. faecium*	None			acc, lnuA, lsaE, msrC, pdp5_muts
202	*Ent. faecium*	acm		potential avian pathogen	aac, ant, lsaE, lnuB, tetM, tetL, pdp5_muts
218*	*Ent. faecium*	acm		potential avian pathogen	aac, lnuA, tetM, pdp5_muts
220*	*Ent. faecium*	acm		potential avian pathogen	aac, ant, lsaE, lnuB, pdp5_muts
224	*Ent. faecium*	None		potential avian pathogen	aac, tetS
4	*Enterococcus sp.*	None		potential avian pathogen	vanC, tetM
87	*Enterococcus sp.*	None		potential avian pathogen	vanC, tetM
200*	*Lactobacillus*	None		-	None
222	*Salmonella + E. faecium*	acm		*Salmonella* in mixture	aac, ant, ermA,
183	*Staphylococcus sp.*	None		Unlikely pathogen	blaZ
186	*Staphylococcus sp.*	None		Unlikely pathogen	None
197	*Staphylococcus sp.*	None		Unlikely pathogen	None

parC - Nalidixic acid,Ciprofloxacin
pdp5_muts - Point mutations potentially leading to ampicillin
aac, ant - Aminoglycoside resistance
lsaE, lnuB - Lincosamide resistance
ermA – erythromycin
tetS, tetM – tetracyclin
msrC - Macrolide, Lincosamide and Streptogramin B resistance
sul1 - Sulphonamide resistance
aadA1 - aminoglycoside resistance
blaZ – ampicillin resistance

6 Conclusions

Bacteria capable of inhibiting *Salmonella* can be readily isolated from the layer farm environment. Many require high cell numbers to exert strong inhibition of *Salmonella* growth as demonstrated in the agar overlay method. The remaining stored isolates collected from the layer farms could be assessed for inhibition or competitive exclusion, therefore negating the necessity to re-isolate microorganisms from the layer environment.

Total exclusion of at least one strain of *Salmonella* from attachment to stainless steel was achieved by five potential biocontrol isolates with a further four demonstrating high levels of competitive exclusion using pre-formed biofilms on stainless steel.

By using a cocktail of isolates there is good potential to develop an application that can exclude numerous *Salmonella* serovars from attaching to abiotic surfaces that may precede the development of a biofilm.

Aerococcus isolate 46 has the highest potential for use as a biocontrol agent in the layer farm environment. The isolate contains no virulence factors and no antibiotic resistance genes as well as demonstrating a high level of competitive exclusion of the *Salmonella* serovars tested. *Aerococcus* was readily isolated from farm 1 and this offers further opportunity to isolate, or test already stored isolates, of similar microorganisms.

The two isolates of unspeciated *Enterococcus* (4 and 87), while recognised as potential avian pathogens, presented a high level of competitive exclusion for multiple *Salmonella* serovars and were included in the initial cocktail of isolates.

E. coli isolates demonstrating a high level of competitive exclusion carried a number of antimicrobial resistance genes or potential virulence genes. While not explored within the scope of this project it may be possible to cure isolates of the avian pathogenic *E. coli* plasmid and/or knock out the potential virulence genes.

References

Australian *Salmonella* Reference Centre. 2010. Quarterly Report. Australian *Salmonella* Reference Centre, Adelaide, South Australia, Australia.

Australian *Salmonella* Reference Centre. 2011. Quarterly Report. Australian *Salmonella* Reference Centre, Adelaide, South Australia, Australia.

Australian *Salmonella* Reference Centre. 2012. Quarterly Report. Australian *Salmonella* Reference Centre, Adelaide, South Australia, Australia

Australian *Salmonella* Reference Centre. 2013. Quarterly Report. Australian *Salmonella* Reference Centre, Adelaide, South Australia, Australia

Bjerkan, G., Witso, E., Bergh, K. 2009. Sonication is superior to scraping for retrieval of bacteria in biofilm on titanium and steel surfaces in vitro. *Acta Orthop* 80:245-250.

Casey, A., Adams, M. C., La Ragione, R. M., Woodward, M. J. 2017. Colonisation of poultry by *Salmonella* Enteritidis S1400 is reduced by combined adminisration of *Lactobacillus salivarius* 59 and *Enterococcus faecium* PVN-33. *Vet Microbiol* 199:100-107.

Chia, T. W. R., Goulter, R. M., McMeekin, T., Dykes, G. A., Fegan, N. 2009. Attachment of different Salmonella serovars to materials commonly used in a poultry processing plant. *Food Microbiol* 26:853-859.

Chorianopoulos, N. G., Giaouris, E. D., Kourkoutas, Y., Nychas, G. J. E. 2010. Inhibition of the early stage of *Salmonella enterica* serovar Enteritidis biofilm development on stainless steel by cell-free supernatant of a *Hafnia alvei* culture. *Appl Environ Microb* 76:2018-2022.

De Cort, W., Ducatelle, R., Immerseel. 2017. Preharvest measures to improve the safety of eggs. In: Ricke, S.C., Gast, R.K., (Eds.), Producing safe eggs: Microbial ecology of Salmonella. Elsevier Inc., Sydney, Australia. 71-85.

Ford, L., Moffatt, C. R. M., Fearnley, E., Miller, M., Gregory, J., Sloan-Gardner, T. S., Polkinghorne, B. G., Bell, R., Franklin, N., Willianson, D. A., Glass, K., Kirk, M. D. 2018. The epidemiology of Salmonella enterica outbreaks in Australia, 2001-2016. *Frontiers in Sustainable Food Systems*:e00086.

Fox, E. M., Solomon, Moore, S., Wall, J. D., Fanning, S. 2014. Phylogenetic profile of in-house microflora in drains at a food production facility: comparisons of Listeria positive and negative containing bacteial populations, and its implications for biocontrol. *Appl Environ Microb* 80:3369-3374.

FSANZ. 2019. Food recall statistics (Australia only) [Online]. Available at (Accessed: May 2019, 2019).

Glass, K., Fearnley, E., Hocking, H., Raupach, J., Veitch, M., Ford, L., Kirk, M. D. 2016. Bayesian source attribution of salmonellosis in South Australia. *Risk Anal* 36:561-570.

Gray, J. A., Chandry, P. S., Kaur, M., Kocharunchitt, C., Bowman, J. P., Fox, E. M. 2018. Novel biocontrol methods for *Listeria monocytogenes* biofilms in food production facilities 9:e00605.

Guerrieri, E., de Niederhausern, S., Messi, P., Sabia, C., Iseppi, R., Anacarso, I., Bondi, M. 2009. Use of lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes* in a small-scale model. *Food Control* 20:861-865.

Henning, C., Vijayakumar, P., Adhikari, R., Jagannathan, B., Gautam, D., Muriana, P. M. 2015. Isolation and taxonomic identity of bacteriocin producing lactic acid bacteria from retail foods and animal sources. *Microorganisms* 3:80-93.

Hibbing, M. E., Fuqua, C., Parsek, M. R., Peterson, S. B. 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology* 8:15-25.

Mariani, C., Oulahal, N., Chamba, J. F., Dubois-Brissonnet, F., Notz, E., Briandet, R. 2011. Inhibition of Listeria monocytogenes by resident biofilms present on wooden shelves used for cheese ripening. *Food Control* 22:1357-1362.

Moffatt, C. R. M., Musto, J., Pingault, N., Miller, M., Stafford, R., Gregory, J., Polkinghorne, B. G., Kirk, M. D. 2016. *Salmonella* Typhimurium and outbreaks of egg-associated disease in Australia, 2001 to 2011. *Foodborne Pathog Dis* 13:379-385.

Simpson, K. M. J., Hill-Cawthorne, G. A., Ward, M. P., Mor, S. M. 2018. Diversity of *Salmonella* serotypes from humans, food, domestic animals and wildlife in New South Wales, Australia. *Bmc Infect Dis* 18:e623.

Stebbing, P. D., Pond, M. J., Peeler, E., Small, H. J., Greenwood, S. J., Verner-Jeffreys, D. 2012. Limited prevalance of gaffkaemia (*Aerococcus viridans* var. *homari*) isolated from wild-caught European lobsters H*omarus gammarus* in England and Wales. *Dis Aquat Organ* 100:159-167.

Suganthi, V. and Mohanasrinivasan, V. 2015. Optimization studies for enhanced bacteriocin production by Pediococcus pentosaceus KC692718 using response surface methodology. *Journal of Food Science and Technology* 52:3773-3783.

Wigley, P. and Barrow, P. A. 2017. *Salmonella* in preharvest chickens: current understanding and approaches to control. In: Ricke, S.C., Gast, R.K., (Eds.), Producing safe eggs: Microbial ecology of *Salmonella*. Elsevier Inc., Sydney, Australia. 71-85.

Yohanes, K. 2002. A journey in public health. Master of Applied Epidemiology, The Australian National University.

Plain English Summary

Project Title:	**Promotion of competitive exclusion by 'good' bacteria against *Salmonella***
Australian Eggs Limited Project No	1FS801CO
Researchers Involved	L.L. Duffy and E. Fox
Organisations Involved	CSIRO Agriculture and Food 39 Kessels Road, Coopers Plains Brisbane, Queensland, 4108
Phone	07 3214 2055
Fax	
Email	Lesley.Duffy@csiro.au
Objectives	This project aimed to determine if biocontrol of *Salmonella* in egg production through the use of 'good' bacteria could be achieved hence presenting an approach which will be favourably viewed by consumers.
Background	'Good' bacteria capable of reducing specific 'bad' bacteria present a relatively new biocontrol strategy, which has key advantages in that it is seen as a natural process. The use of probiotic bacteria in a food production environment means 'good' bacteria are viewed favourably by consumers and retail, and minimising the need to use chemical sanitisers is seen as a priority for both consumers and retail.
Research	Potential control organisms were first isolated from the layer farm environment by demonstration of inhibition to a single *Salmonella* strain. A subset of these were further characterised for inhibition of growth of *Salmonella* across four other *Salmonella* serovars. This subset was also assessed for the competitive exclusion of *Salmonella* attachment to stainless steel coupons by the use of pre-formed biofilms. A number of potential biocontrol isolates were whole genome sequenced to identify the organisms and assess the carriage of antimicrobial resistance genes and potential virulence genes.
Outcomes	The approach used to isolate potential biocontrol organisms has demonstrated that bacteria capable of inhibiting *Salmonella* can be readily isolated from the layer farm environment. Total exclusion of at least one strain of *Salmonella* from attachment to stainless steel was achieved by five isolates with a further four demonstrating high levels of competitive exclusion using pre-formed biofilms on stainless steel. By using a cocktail of isolates there is good potential to develop an application that can exclude numerous *Salmonella* serovars from attaching to abiotic surfaces that may precede the development of a biofilm.
Implications	There is potential for the biocontrol of *Salmonella* on layer farms. Microorganisms inhibitory to the attachment of *Salmonella* to stainless steel by pre-formed biofilms can be readily isolated from the layer farm environment.
Key Words	*Salmonella*, Biocontrol, Inhibition
Publications	List publications