Optimisation of use of probiotics for control of *Salmonella* in hens

Final Project Report | **MAY 2020**

A report for Australian Eggs Limited

by Samiullah Khan and Kapil Chousalkar

Australian Eggs Limited Publication 1FS802UA

ISBN 978-1-920835-57-6

Project Title: Optimisation of use of probiotics for control of *Salmonella* in hens

Australian Eggs Limited Project Number 1FS802UA

The views expressed and the conclusions reached in this publication are those of the author and not necessarily those of persons consulted. Australian Eggs Limited shall not be responsible in any way whatsoever to any person who relies in whole or in part on the contents of this report.

Researcher/Author Contact Details
Name: Kapil K. Chousalkar
Address: School of Animal and Veterinary Sciences
The University of Adelaide
Mudla Wirra Road
Roseworthy SA 5371
Phone: 08 8313 1502
Fax: 08 8313 7956
Email: Kapil.chousalkar@adelaide.edu.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: 66 102 859 585
Suite 6.02, Level 6, 132 Arthur St
North Sydney NSW 2060

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

Published in [October 2021]

Foreword

This project was conducted to understand the role of commercial probiotics and synbiotics in the control of *Salmonella* and their interactions with gut microbiota in layer production systems. Both pen and field trials were conducted to understand the effects of short-term and strategic feeding of probiotics and synbiotics on *Salmonella* shedding. The structure of gut microbiota was studied in the presence and absence of *Salmonella* Typhimurium infection.

The short-term trials in chicks showed that short-term feeding of both the probiotics and synbiotics were effective in improving the gut microbial balance displaced by the *Salmonella* Typhimurium challenge, but the products were not effective in significantly reducing *Salmonella* shedding level or invasion into internal organs. The caecal transcriptomic data revealed that Toll-like receptors and cytokines were the main players during *Salmonella* Typhimurium infection. The exposure of chicks to *Salmonella* Mbandaka and *Salmonella* Agona prior to *Salmonella* Typhimurium infection resulted in significant reduction of *Salmonella* Typhimurium. However, this study needs to be repeated at a large scale by understanding the role of non-pathogenic *Salmonella* on gut microbiota. The long-term pen trial with the *Bacillus* based probiotic revealed that continuous feeding of the probiotic was effective in reducing the faecal and organ load of *Salmonella* Typhimurium and balancing the microbial communities displaced by the challenge. The long-term field trial in free range production system showed that the *Bacillus* based probiotic was effective in positively influencing the egg internal quality. However, this trial was continued only up to 36 weeks of flock age, and suggestions are made to perform such trials until the end of the production cycle.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

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.

Most of our publications are available for viewing or downloading through our website:

www.australianeggs.org.au

Printed copies of this report are available for a nominal postage and handling fee and can be requested by phoning (02) 9409 6999 or emailing research@australianeggs.org.au.

Acknowledgments

The authors thank the members of the project's Steering Committee (Professor Bob Swick, University of New England; Dr Santiago Ramierez, DSM International; and Dr Raymond Chia, Australian Eggs Limited) for their valuable insights during the course of the project.

The authors acknowledge the support of an egg producer in South Australia, who generously agreed to allow the execution of the free range *Bacillus* based probiotic trial. The authors would also like to acknowledge the staff at feed mill. The authors acknowledge members of Dr Chousalkar's laboratory – Dr Andrea McWhorter, Ms Sonali Deshmukh, Mr Nitish Joat and Mr Jiawei Zhang, in sampling and DNA extraction during the course of this project.

Australian Eggs Limited provided the funds, which supported this project.

About the Authors

Kapil K. Chousalkar is an Associate Professor at the School of Animal and Veterinary Sciences, the University of Adelaide. Kapil is leading a food safety research group at the University of Adelaide. His research has been funded by AgriFutures, the Poultry CRC, Australian Egg Corporation Limited (AECL), the NSW Food Authority, SA Health, Department of Agriculture, Fisheries and Forestry (DAFF) and ARC. Kapil teaches poultry health, food safety and poultry production to veterinary and animal science students. Kapil also provides veterinary consultancy services to local poultry producers through the production animal clinic at the University of Adelaide.

Samiullah Khan is a postdoctoral fellow in Dr Chousalkar's laboratory and has completed a PhD degree from the University of New England, Australia. As an earlier career researcher, he has successfully completed a project for AECL and is currently working on projects funded by AgriFutures, Australian Eggs and ARC.

Table of Contents

List of Tables

List of Figures

Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
AhR	aryl hydrocarbon receptor
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
AVD	Avidin precursor
BPW	Buffered peptone water
BSA	Brilliance *Salmonella* agar
cDNA	Complementary DNA
CFU	Colony forming unit
CPM	Counts per million
CRC	Cooperative Research Centre
DAFF	Department of Agriculture, Fisheries and Forestry
DEGs	Differentially expressed genes
EDTA	Ethylenediamine tetraacetic acid
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
g	Force
GLM	Generalised linear model
GPR41	G protein-coupled receptor 41
IgA	Immunoglobulin A
ILs	Interleukins
KEGG	Kyoto encyclopedia of genes and genomes
LB	Luria Bertani
MPN	Most probable number
mL	Millilitre
µL	Microlitre
MRS	De Man, Rogosa and Sharpe
OD	Optical density
OTU	Operational taxonomy unit
PBS	Phosphate buffered saline
p.i.	Post-infection
PLSD	Protected least significant difference
qPCR	Quantitative polymerase chain reaction
RDA+	Redundancy analysis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RVS	Rappaport Vassiliadis soya peptone
SAL1	Salmonellosis *resistance* locus
SCFAs	Short-chain fatty acids
*S*T	*Salmonella* Typhimurium
T3SS	Type III secretory system
T4SS	Type IV secretory system
TBP	TATA-box binding protein
TMM	Trimmed mean of M values
TLRs	Toll-like receptors
USDA	U.S. Department of Agriculture
XLD	*Xylose lysine deoxycholate*
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Executive Summary

This project involved a series of pen trials and a field experiment to understand and optimise the role of commercially available probiotics and synbiotics on the Salmonella shedding level in faeces and its invasion into internal organs. Probiotics, prebiotics, or their combinations (synbiotics) are used in poultry production for enhancing overall bird performance.

Chapter 1 investigated the effects of the use of short-term two probiotics and two synbiotics on the composition of gut microbiota, reduction in *Salmonella* Typhimurium shedding, and invasion into internal organs in layer chicks. The data suggested that although the early colonisation of the gut with probiotics was effective in positively modulating the gut microbiota, none of the probiotics and synbiotics were sufficient to significantly reduce a load of *Salmonella* in organs.

Chapter 2 attempted to understand the effect of *Salmonella* Typhimurium on the regulation of the mucosal immune system in chicks in a temporal manner. The transcriptomic data revealed that 103 CFU of *Salmonella* Typhimurium was sufficient to modulate the caecal immune system by mainly up-regulating Toll-like receptors and cytokine pathways. Nested network analysis showed that Interleukin 6 (*IL6*) had a broader role in the immune system regulation, where it was involved in multiple immune system pathways. This experiment also suggested that matrix metalloproteinase 7 (*MMP7*) and matrix metalloproteinase 9 (*MMP9*) might play a defensive role in *Salmonella* Typhimurium infection in chickens.

Chapter 3 investigated the role of *Salmonella* Mbandaka and *Salmonella* Agona in the competitive exclusion of *Salmonella* Typhimurium. The data showed that exposure to both the serovars before *Salmonella* Typhimurium infection resulted in a significant reduction of *Salmonella* Typhimurium load and invasion into internal organs; however, both serovars did not completely inhibit the colonisation caused by *Salmonella* Typhimurium.

Chapter 4 determined the effects of strategic feeding of a *Bacillus* based probiotic on the shedding profile of *Salmonella* Typhimurium, and gut microbiota in layers at point of lay. The data showed that the continuous supplementation of the probiotic was effective in lowering the overall load of *Salmonella* in faeces and organs. The probiotic was effective in restoring various microbial genera displaced by the *Salmonella* challenge. The data also showed that certain microbial genera, particularly *Faecalibacterium*, might confer resistance to *Salmonella* colonisation in the gut.

Chapter 5 determined the effects of *Bacillus* based probiotic on gut health and egg quality in a flock followed from day 1 to 36 weeks of age in the free range production system. Due to the low prevalence of *Salmonella* in the selected experimental flock, the effect of probiotic on the reduction of *Salmonella* could not be determined in the field conditions.

Overall Conclusions

Improving gut health through the strategic feeding of probiotics and synbiotics may be one aspect of enhancing food safety. Although *Salmonella* Typhimurium does not cause clinical disease in layers, it colonises the gut and invades into internal organs for continuous shedding, but the shedding level can be reduced by the supplementation of probiotics.

- Short-term feeding of probiotics did not reduce the *Salmonella* shedding although early feeding of probiotics can modulate the development of gut microbiota.
- Exposure of chicks to Non Typhoidal *Salmonella* serovars such as *Salmonella* Mbandaka and *Salmonella* Agona prior to *Salmonella* Typhimurium infection resulted in significant reduction in *Salmonella* Typhimurium.
- Continuous feeding of a *Bacillus* based probiotic supplement resulted in reduction of *Salmonella* Typhimurium shedding and also reduced bacterial load in vital organs.
- Periodic feeding (four weeks on and four weeks off) did not result in reduction of *Salmonella* Typhimurium shedding, which suggests that continuous feeding is beneficial.
- Partly, probiotics and synbiotics supplementation can be an effective strategy for improving gut microbiota that in turn enhances food safety. Further studies are essential to understand the effects of probiotics on the gut microbiota of hens raised in different housing conditions.
- The continuous supplementation of a *Bacillus* based probiotic improved egg internal quality during early lay. Further studies are essential to investigate the effects of continuous feeding of probiotics on the egg quality of flocks in mid to late lay.

1 Short-term feeding of probiotics and synbiotics modulates caecal microbiota during *Salmonella* Typhimurium infection but does not reduce shedding and invasion in chickens

Samiullah Khan and Kapil K. Chousalkar

Published in *Applied Microbiology and Biotechnology* 104, pp. 319–334; 2020.

1.1 Introduction

Caecal microbiota in chickens is linked with host health and productive traits that reflect its importance in colonisation resistance to zoonotic pathogens (Shini et al. 2013). Positively influencing the host gut microbiota helps in digestion and metabolism (Stanley et al. 2012), regulation of intestinal angiogenesis (Stappenbeck et al. 2002), development and regulation of host immune system (Hooper et al. 2001), and even in brain function (Benakis et al. 2016). Microbial communities are influenced by tissue type, flock age, disease and rearing conditions (Cui et al. 2017; Luoma et al. 2017, Ngunjiri et al. 2019). Studies suggested that the host genotype can exert a strong influence on gut microbiota composition (Goodrich et al. 2016) and, therefore, the microbiome of egg-type birds is not the same as that of broilers (Ocejo et al. 2019). In layer chicks, the lowest complexity of caecal microbiota is around day 1 of life, where it usually consists of five different species (Crhanova et al. 2011). The microbial diversity slowly increases with bird age to 14 species on day 3, and approximately 42 species around day 19 of life (Crhanova et al. 2011). After two weeks post-hatch, the *Ruminococcus* and *Firmicutes* increase to a greater extent than the *Enterobacteriaceae* (Ballou et al. 2016). Newly hatched chicks are prone to colonisation by pathogenic microorganisms present in the rearing environment. Higher microbial diversity is commonly associated with healthy host conditions, while reduced microbial diversity affects the intestinal health negatively (Sommer et al. 2017).

To modulate the gut microbiota composition in chickens, diets are often supplemented with pre- and pro- biotics. Prebiotics are non-living fibrous feed additives (non-digestible oligosaccharides) that promote the growth and multiplication of the indigenous gut microbiota (Macfarlane et al. 2008). Therefore, a prebiotic serves as feed for beneficial indigenous gut bacteria. The proposed mechanisms of action of prebiotics include the production of antimicrobial substances (Chen et al. 2007), modulation of the host immune system (Babu et al. 2012) and improving gut morphology (Pourabedin et al. 2014). In contrast to prebiotics, probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (Fuller 1989; Martin & Langella 2019). The protective effects of probiotics in the gut could be due to the production of organic acids (lactic acid) and adhesion inhibitors, and the secretion of antimicrobial substances such as hydrogen peroxide and bacteriocins (Spinler et al. 2008). Probiotics can also secrete enzymes that hydrolyse bacterial toxins and modify toxin receptors (Buts et al. 1994).

In laying hens, diets supplemented with different strains of probiotics have significantly improved gut microbial balance, blood and yolk cholesterol levels, egg production and overall egg quality. For example, *Lactobacillus* improved the equilibrium of gut microbiota by increasing the population of *Bifidobacteria* and decreased potentially harmful bacteria (Forte et al. 2016). *Lactobacillus* and *Bacillus* improved overall bird performance, caecal microbiota and gut morphology (Forte et al. 2016). *Lactobacillus salivarius* and *Bacillus subtilis* improved egg production, serum low-density cholesterol

and serum antibody level against avian influenza virus (Zhang et al. 2012). *Bacillus licheniformis* and *B. subtilis* improved egg production, damaged egg ratio, egg yolk cholesterol and serum cholesterol (Kurtoglu et al. 2004). *Pediococcus acidilactici* positively influenced overall egg quality, feed efficiency, and yolk fatty acid composition and yolk content (Mikulski et al. 2012). *Enterococcus faecium* improved overall egg quality, gut microbiota, serum cholesterol level, nutrient digestibility and excreta ammonia emission (Park et al. 2016; Zhang and Kim 2013). Apart from competitive exclusion theory, probiotics may offer protection against pathogens by modulating the host immune response. For example, chickens infected with *Salmonella* Typhimurium had reduced production of IFN-γ when fed with *Lactobacillus*, *Bifidobacterium* and *Streptococcus* based probiotic (Haghighi et al. 2008). Similarly, pre-treatment of human intestinal epithelial cells with *Bifidobacterium* or *Lactobacillus* resulted in the inhibition of *Salmonella* induced *IL-8* expression (O'Hara et al. 2006). In laying hens, prebiotics supplementation has been shown to influence gut health and bird performance positively. For example, xylo-oligosaccharides improved intestinal epithelial morphology, caecal Bifidobacterial population, caecal butyrate level, plasma immunoglobulins concentrations and plasma vitamin D3 level (Ding et al. 2017). Isomalto-oligosaccharide improved overall egg quality, egg production, feed intake and serum cholesterol level (Tang et al. 2017). Mannan-oligosaccharides positively influenced egg production, egg weight, liver antioxidant status and feed conversion ratio (Bozkurt et al. 2016). Fructo-oligosaccharides reduced *Salmonella* Enteritidis colonisation in the liver and ovary (Donalson et al. 2008). Inulin reduced yolk cholesterol concentration, caecal pH and coliform bacteria count (Shang et al. 2010).

In healthy adult chickens, *Salmonella* infection generally does not lead to the development of clinical signs (Barrow & Lovell 1991), while in young chicks it can cause morbidity and mortality (Williams & Tucker 1980). However, *Salmonella* infection potentially changes gut microbial communities dominated by *Enterobacteriaceae* (Liu et al. 2018), with more visible effects in younger chicks (Juricova et al. 2013). In one-week old layer chicks, *Salmonella* Enteritidis challenge altered the caecal microbial communities (Mon et al. 2015). The effects of *Salmonella* Enteritidis on changes in gut microbiota was greater in day old chicks compared with 4 and 16-day old Isa-Brown chicks (Juricova et al. 2013). Pre- and pro- biotics are effective in clearing *Salmonella* from the chicken gut through modification of the gut microbiome (Azcarate-Peril et al. 2018; Bratburd et al. 2018) and host immune system modulation (Chang et al. 2019; Haghighi et al. 2008). *Lactobacillus* has been shown to reduce *Salmonella* Enteritidis load in chicken caeca significantly (Penha Filho et al. 2015). In a mouse colitis model, probiotic *Escherichia coli* Nissle 1917 was effective in reducing *Salmonella* Typhimurium colonisation (Deriu et al. 2013). Multiple strains based probiotic was effective in reducing the shedding level of *Salmonella* Typhimurium in pigs (Casey et al. 2007). A significant interaction of pre- and pro- biotics on host immune response against *Salmonella* Typhimurium has been observed in pigs (Naqid et al. 2015). In humans, gastroenteritis caused by *Salmonella* Typhimurium is often traced back to contaminated poultry produce (Fearnley et al. 2011). Hence the poultry industry is under constant pressure to contain this pathogen at farm level. Based on the intended use of probiotics and synbiotics for controlling *Salmonella* in chickens, we hypothesised that, if used in the first week of the chick's hatch, commercial probiotics and synbiotics can provide colonisation resistance through competitive exclusion against *Salmonella* Typhimurium in the caeca at an early age. To test this hypothesis, we used next-generation sequencing targeting hypervariable regions within microbial 16S rRNA genes to compare the caecal luminal microbiota of layer chicks exposed to short-term probiotic and synbiotic supplementation and subsequently challenged with *Salmonella* Typhimurium or left as a control. To understand the effects of different commercial probiotics and synbiotics on *Salmonella* Typhimurium colonisation and invasion into internal organs, culture methods were used for organ load determination. The outcome of the study has broadened our understanding of the interaction of *Salmonella* Typhimurium with gut microbiota in the presence or absence of probiotic and synbiotic supplements.

1.2 Materials and methods

1.2.1 Ethics statement and rearing of birds

The Animal Ethics Committee at the University of Adelaide approved the work (approval number S-2017-080) in accordance with the guidelines specified in the Australian code for the care and use of animals for scientific purposes, 8th edition (2013). Standard Operating Procedures were followed for caring and processing of the experimental chicks.

Fertile eggs from an Isa-Brown parent flock were obtained from a local breeder farm. Following fumigation (by formaldehyde and potassium permanganate (3:1)), the eggs were incubated in clean conditions for hatching in the School of Animal and Veterinary Sciences. From the incubator, the hatching tray papers with chicks' meconium samples were processed by culture methods for *Salmonella* isolation. Before the chicks' placement, the entire experimental facility was cleaned and then tested for *Salmonella*. The hatched chicks were reared in a house with strict biosecurity protocols as per the protocol of the ISA General Management Guide 2009-10. Next, the chicks (n = 90) were equally divided into ten treatment groups (Table 1-1) and reared in pens (different treatment groups in separate rooms), with water and feed provided *ad libitum*. The feed was fumigated as described previously and the drinking water was autoclaved. The fumigated feed was routinely tested by culture enrichment for the presence of *Salmonella* spp. The commercially available probiotics and synbiotics used in this study were selected based on their claimed efficacy for control of *Salmonella* through gut microbiota modulation in poultry. These products are usually used in layer industry for improving birds' performance.

Four commercial probiotic and synbiotic products were purchased and used in this study. 100 mg of each of the probiotics and synbiotics was cultured in De Man, Rogosa and Sharpe (MRS) media and characteristic colonies were gram stained. For approximate CFU count in 1 g of the probiotics and synbiotics, 100 mg of individual products was suspended into PBS, serially diluted and plated (100 µL) on MRS media. For all the probiotic and synbiotic treatment groups, 1 g of each of the product was mixed in either 1 kg of fumigated feed or 1 L of autoclaved water. Every day, a freshly prepared batch of the products was offered to the treatment groups from the day of hatch to day 7 of the chicks' age. At day 8 of the chicks' age, birds from the probiotics and synbiotics supplemented and *Salmonella* Typhimurium treatment and positive control groups (Table 1-1) were challenged via the oral route with *Salmonella* Typhimurium phage type 9. The probiotics, synbiotics and negative control groups received phosphate buffered saline (PBS) only.

Table 1-1 Treatment group distribution and probiotics/synbiotics details used in the study

Code used for supplement	Commercial product composition	Aerobically grown log_{10} CFU	Anaerobically grown log_{10} CFU	Mode of administration	Treatment group[a]
Probiotic A	*Lactobacillus acidophilus*, *L. delbrueckii* subspecies *bulgaricus*, *L. plantarum, L. rhamnosus, Bifidobacterium bifidum, Enterococcus faecium* and *Streptococcus salivarius* subspecies *thermophilus*	11.008	8.411	Water	Probiotic A control
					Probiotic A and *ST*[b] challenge
Probiotic B	*Bacillus subtilis*, *Bacillus subtilis* and *Bacillus amyloliquefaciens*	8.204	4.040	Feed	Probiotic B control
					Probiotic B and *ST* challenge
Synbiotic A	*Enterococcus* sp., *Pediococcus* sp., *Bifidobacterium* sp., *Lactobacillus* sp. and fructooligosaccharides	4.944	2.954	Water	Synbiotic A control
					Synbiotic A and *ST* challenge
Synbiotic B	*Lactobacillus acidophilus, L. casei, L. salivarius, L. plantarum, L. rhamnosus, L. brevis, Bifidobacterium bifidum, B. lactis, S. thermophiles*, prebiotic inulin (chicory root extract), protease, amylase, cellulase, hemicellulase, lipase, papain and bromelain	8.602	8.049	Water	Synbiotic B control
					Synbiotic B and *ST* challenge
Controls in the study					Positive control (normal feed and *ST* challenge)
					Negative control (normal feed, no *ST* challenge)

[a] In each treatment group, there was a total of 9 chicks. At each sampling time-point (days 3, 5 and 7 post-infection), 3 chicks from each treatment group were euthanised for sample collection.

[b] *ST* is *Salmonella* Typhimurium. Each product was aerobically and an-aerobically grown on MRS media to understand the approximate log_{10} CFU per gram of the product.

1.2.2 Inoculum preparation and birds challenge

Salmonella Typhimurium previously isolated from a layer farm (Gole et al. 2014) was used in this study. *Salmonella* Typhimurium inoculum was prepared by following the method described previously (McWhorter & Chousalkar 2018). In the challenge groups, each bird received an oral dose of 10^3 colony forming unit (CFU; 0.1 mL) of *Salmonella* Typhimurium. The inoculation dose was kept low to understand its effects on gut microbiota modulation and colonisation of internal organs. Studies suggest that approximately 10^3 CFU of *Salmonella* per chicken is enough to activate the host immune

system (Chart et al. 1992; Marcq et al. 2011). 100 µL of the original inoculum with serial dilutions was plated onto Xylose Lysine Deoxycholate (XLD; Thermo Fisher Scientific, Victoria, Australia) media to confirm the CFU received by each bird. From each treatment group at each time-point (days 3, 5 and 7 post-infection), three birds were euthanised by cervical dislocation for the collection of caecal contents and tissues. Previous study showed a minor variation in gut microbiota between individual birds (Videnska et al. 2014). A number of other studies also used three birds at each time-point for gut microbiota analysis (Juricova et al. 2013; Kubasova et al. 2019).

1.2.3 *Salmonella* Typhimurium enumeration in tissue

Pieces of liver, spleen and caecal tissues were aseptically collected and weighed into 1.5 mL Eppendorf tubes containing stainless steel beads 0.5–2.0 mm and PBS. The samples were maintained on ice until further use. Tissues were homogenised using a Bullet Blender Storm homogeniser (Next Advance, NY, USA) on full speed for 5–10 min. Serial dilutions were prepared from the original tissue homogenates, plated onto XLD agar and incubated overnight at 37°C. The *Salmonella* Typhimurium colonies were counted to determine the bacterial load (log_{10} CFU) in 1 g of tissue. A 100 µL sample from the original homogenates was also enriched in 900 µL buffered peptone water (BPW; Thermo Fisher, Victoria, Australia) and incubated overnight at 37°C. A 100 µL sample of the incubated BPW samples was added to 10 mL of Rappaport Vassiliadis soya peptone (RVS) broth (Thermo Fisher Scientific, Victoria, Australia) and incubated overnight at 42°C for selective growth of *Salmonella*. The RVS samples were streaked on XLD and incubated overnight at 37°C. Suspected *Salmonella* cultures from XLD agar were sub-cultured on Brilliance *Salmonella* agar (BSA; Thermo Fisher Scientific, Victoria, Australia) plates for confirmation.

1.2.4 Caecal contents DNA extraction

To obtain quality DNA from caecal contents, the manufacturer's protocol of the commercial kit used (QIAamp DNA Stool Mini Kit; Qiagen, Victoria Australia) was modified slightly. Briefly, approximately 180 mg of caecal contents was weighed into a 1.5 mL tube (Eppendorf Safe-Lock). Glass beads (acid-washed ≤106 µm and 425–600 µm; Sigma Aldrich) were added to the samples and maintained on ice. Next, the samples were processed for DNA extraction as per the kit protocol except for the inclusion of the step of homogenisation in a bullet blender. DNA was eluted in 100 µL of buffer ATE (10 mM Tris-Cl pH 8.3, 0.1 mM EDTA and 0.04% NaN_3) as per protocol of QIAamp DNA Stool Mini Kit. The purity (260/280 ratio 1.70–2.05; 230/260 ratio 1.80–2.30) and concentrations (20~300 ng/µL) were measured in a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Victoria, Australia). The DNA samples were stored at -80°C until used for downstream applications. Three samples at each sampling time-point per treatment group were submitted to the Australian Genome Research Facility (Melbourne, Australia) for diversity profiling analysis (16S: 341F - 806R (V3 - V4)) using the forward (CCTAYGGGRBGCASCAG) and reverse (GGACTACNNGGGTATCTAAT) primer pair.

1.2.5 16S rRNA analysis

1.2.5.1 PCR amplification and MiSeq sequencing

PCR amplicons were generated using the primers (forward, CCTAYGGGRBGCASCAG and reverse, GGACTACNNGGGTATCTAAT) from V3-V4 region amplification of bacterial DNA using AmpliTaq Gold 360 master mix (Thermo Fisher Scientific, Victoria, Australia) for the primary PCR. The PCR conditions were: initial heating at 95°C for 7 min and 29 cycles of dissociation at 94°C for 30 sec, annealing at 50°C for 60 sec and extension at 72°C for 60 sec with a final finish of 72°C for 7 min. A secondary PCR to index the amplicons was performed

with TaKaRa Taq DNA Polymerase (Takara Bio, CA, USA). The resulting amplicons were measured by Qubit 4 fluorometer (Thermo Fisher Scientific, Victoria, Australia) and normalised. The equimolar pool was then measured by qPCR (KAPA) followed by sequencing on the Illumina MiSeq Platform (San Diego, CA, USA) with 2 × 300 base pairs paired-end chemistry.

Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al. 2013). From the sequences, primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (qiime 1.8) (Caporaso et al. 2010) USEARCH (version 7.1.1090) (Edgar 2010; Edgar et al. 2011) and UPARSE (Edgar 2013) software. Using USEARCH, sequences were quality filtered, and full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using "rdp_gold" database as a reference. To obtain the number of reads in each operational taxonomic unit (OTU), sequences were mapped back to OTUs with a minimum identity of 97%. In qiime, taxonomy was assigned using GreenGenes database (version 13_8, Aug 2013) (DeSantis et al. 2006). The OTU file was uploaded into Calypso software (version 8.72) (Zakrzewski et al. 2016) and the data were further analysed for group comparisons at false discovery rate (FDR) < 0.05. During data analysis in Calypso, the OTU table was filtered to exclude taxa with low abundance (< 0.01%) and was total sum normalised (TSS) square root transformed. The processed data in Calypso were subsequently used for univariate (one- and two-way ANOVA), multivariate and diversity analyses. The Shannon index was used to calculate the microbial alpha diversity affected by probiotics treatment and sampling time-point (or birds' age) at OTU, genus and family levels. In Calypso, the Shannon index at OTU level measures how the microbes are balanced and how species (evenness) are at a similar or dominant level to each other.

1.3 Statistical Analysis

The *Salmonella* Typhimurium load (log_{10} CFU) per gram of tissue was analysed in Statview v.5.0.1.0 by taking sampling time-point and treatment group as the main effects. Repeated measure analysis was used to investigate the effects of time-point or treatment on *Salmonella* load in the organs. Level of significance was determined by PLSD at $P < 0.05$.

1.4 Results

The double enrichment method used in the current study can detect one viable cell of *Salmonella*. Before the placement of chicks, the experimental facility tested was negative for *Salmonella* spp. Similarly, the fumigated feed tested was also negative for the presence of *Salmonella*. No *Salmonella* was recovered from the chicks' meconium or from chicks sampled at regular intervals until the selected groups were challenged with *Salmonella* Typhimurium (*S*T). The faecal samples collected from the challenged groups on days 3, 5 and 7 post-infection (p.i.) were positive for *Salmonella* by culture method. All the control groups were negative for *Salmonella* throughout the experiment. No mortality or clinical signs of salmonellosis were observed after the chicks were challenged with *S*T. However, during sample collection, some of the challenged birds showed partially emptied caeca with mucous plugs. On day 3 p.i., one bird from the positive control group showed necrotic foci on the liver, while one bird from the probiotic A supplemented and *S*T challenge group showed haemorrhage in the spleen. The characteristic clinical signs observed in the caeca of the *S*T challenged groups have been summarised in Table 1-2.

Table 1-2 Gross lesions observed in the caeca of different treatment groups

Treatment group[a]	Day post-infection	Lesion observed	% of birds affected
Positive control	3	Partially filled caeca and mucous plug	33
Positive control	5	Partially filled caeca	33
Positive control	7	Partially filled caeca	66
Probiotic A and *ST*[b] challenge	7	Mucous plug	33
Probiotic B and *ST* challenge	7	Partially filled caeca	33
Synbiotic A and *ST* challenge	5	Partially filled caeca	33

[a] Details of the treatment groups have been provided in Table 1-1.

[b] *ST* is *Salmonella* Typhimurium.

1.4.1 Microbiome sequencing and quality of generated data

A total of 6.45 Gb sequences data for the ten different treatment groups were generated using Illumina sequencing. The average reads numbers per treatment group and quality have been tabulated in Table 1-3. To further check the depth of microbial communities' coverage, a rarefaction analysis curve was calculated for all the treatment groups. In rarefaction analysis, the number of observed species was counted and plotted as a function of the number of sampled sequences. The slope of the curve indicates how well the sequenced data represent the underlying microbial community. The rarefaction analysis curve showed that the underlying microbial communities in all the treatment groups were well covered by the sequenced data (Appendix Figure 1-1).

Table 1-3 Reads quality generated in the study

Treatment group	Raw average reads	After QC (average)	Mapped at 97%
Negative control	118632.00	93701.22	90890.19
Positive control	143071.40	111496.10	108151.20
Probiotic A control	144692.70	112840.90	109455.70
Probiotic A and *ST*[a] challenge	126533.30	93272.33	90474.16
Probiotic B control	102005.40	80460.56	78046.74
Probiotic B and *ST* challenge	105908.60	81927.33	79469.51
Synbiotic A control	75867.67	59892.33	58095.56
Synbiotic A and *ST* challenge	122043.40	95423.89	92561.17
Synbiotic B control	125146.40	95996.67	93116.77
Synbiotic B and *ST* challenge	126789.00	96383.67	93492.16

[a] *ST* is *Salmonella* Typhimurium.

Details of individual probiotics and synbiotics have been mentioned in Table 1-1.

1.4.2 Caecal luminal microbial communities

A total of 22 known genera was identified with some sequences reads mapped to unclassified bacterial families (Figure 1-1). At family level, the communities of caecal bacteria mainly comprised of *Bacillaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Paenibacillaceae*, *Peptostreptococcaceae*, *Pseudomonadaceae*, *Ruminococcaceae*, *Streptococcaceae* and *Veillonellaceae* in addition to unclassified bacteria. The three most abundant phyla were *Actinobacteria*, *Firmicutes*, and *Proteobacteria*.

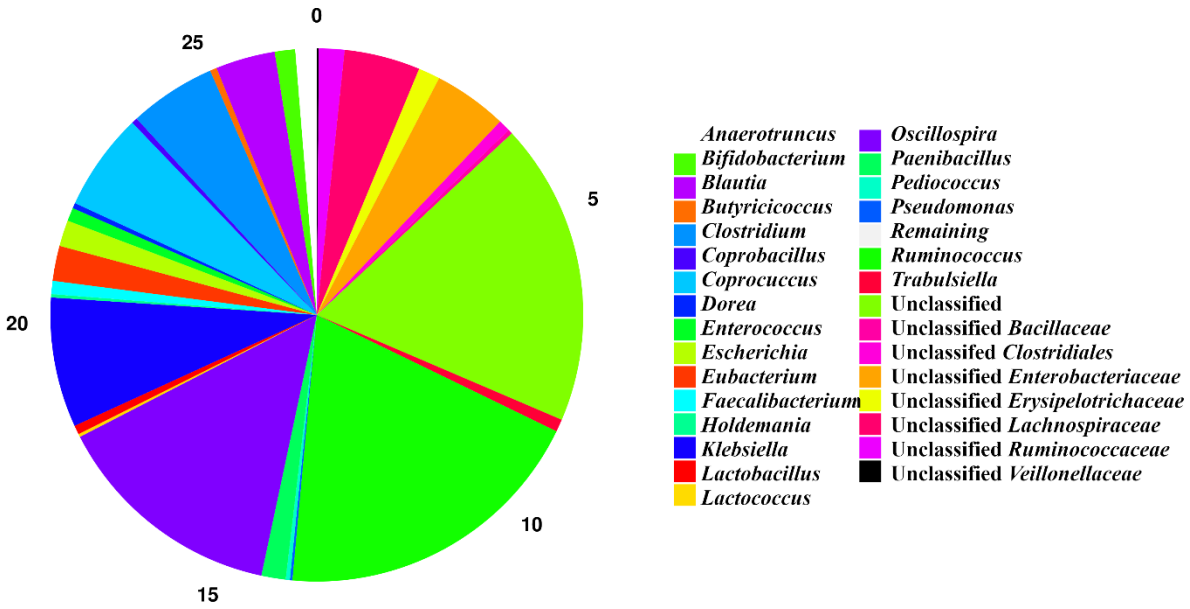


Figure 1-1 Overall genera observed in the caecal lumen of layer chicks

For genera taxa visualisation, OTU data were clustered in all the treatment groups combined in Calypso software.

1.4.3 Caecal luminal bacterial abundance and diversity are affected by probiotic and synbiotic supplementation and *Salmonella* Typhimurium challenge

Overall, the probiotic and synbiotic supplementation and *S*T challenge significantly (FDR < 0.05) affected microbial abundance in the caecal lumen of layer chicks. Within each treatment group, sampling time-point (or flock age) significantly affected (P < 0.05) the microbial diversity. To gain insight into microbial abundance and diversity, individual probiotic or synbiotic supplemented group samples (with or without *S*T challenge) were analysed against the positive control and negative control groups. Data from the different probiotic or synbiotic treatment groups were not compared with one another because the contents of each product were different. Only the genera and families significantly affected (FDR < 0.05) by probiotic, synbiotic treatments or *S*T challenge have been presented here.

1.4.3.1 Effect of probiotic A supplementation

At the genus level, in the probiotic A control group, the abundance of *Trabulsiella* and *Oscillospira* was higher compared to the probiotic A supplemented and *S*T challenged, *S*T negative control and *S*T positive control groups (Figure 1-2a). Unclassified *Ruminococcaceae*, *Klebsiella* and *Anaerotruncus* abundance was higher in the *S*T positive control compared with the other treatment groups. *S*T challenge decreased the abundance of *Paenibacillus* and increased *Anaerotruncus* in the *S*T positive control and probiotic A supplemented and *S*T challenged groups compared with the probiotic A control and *S*T negative control groups. Compared with the *S*T positive control group, in the presence of probiotic A supplementation, *S*T challenge affected the abundance of *Coprococcus*, *Butyricicoccus*, *Eubacterium* and *Blautia* differently as seen in the probiotic A supplemented and *S*T challenged group. A complete list of the significant genera affected by the probiotic A supplementation and/or *S*T challenge is depicted in Figure 1-2a. The core caecal microbiome was affected by the probiotic A supplementation and *S*T challenge (Appendix Figure 1-2). At OTU level, there were 32 common taxa in the probiotic A supplemented group.

In the probiotic A supplemented group, at OTU level, the Shannon index showed that the bacterial communities per group were at the same level (*P = 0.6600*) across all the treatment groups (Appendix Figure 1-3). Within each treatment group, bird age (or sampling time-points) increased (*P = 0.0230*) the diversity of microbial communities in the negative control (*P = 0.0091*) and the probiotic A control (*P = 0.0046*) groups, while *S*T challenge with (*P = 0.2723*) or without (*P = 0.3128*) probiotic A supplementation had no significant effect on the microbial diversity (Figure 1-2b). The Shannon index at the genus and family level produced very similar results to OTU level diversity measurements. The redundancy analysis (RDA+) is a multivariate method that is used to explore complex associations between community composition and multiple explanatory variables. The RDA+ showed that overall the treatment groups clustered separately, showing the significant effects (*P = 0.001*) of probiotic A supplementation and *S*T challenge on the composition of bacterial communities (Figure 1-2c).

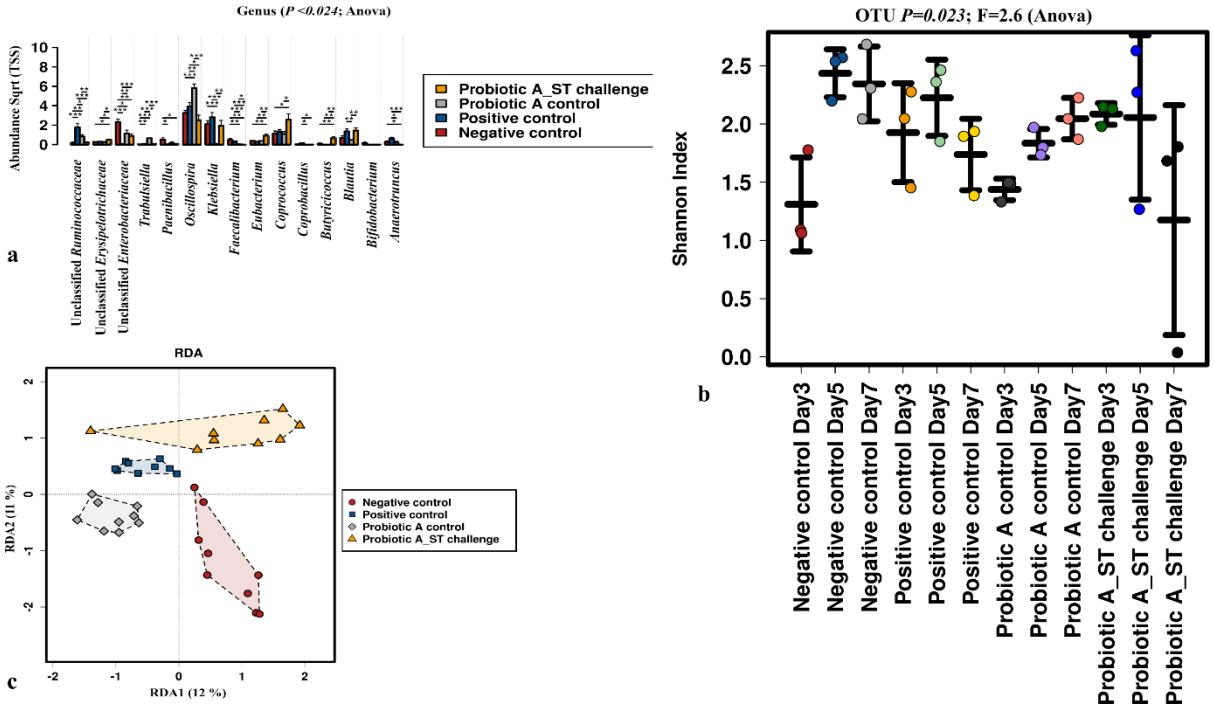


Figure 1-2 Caecal luminal microbial abundance and diversity affected by probiotic A supplementation and *Salmonella* Typhimurium (*ST*) challenge

(a) Microbial abundance at genera level.
(b) Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point).
(c) RDA+ of each of the treatment group.
P < 0.024 was equivalent of FDR < 0.05 in Calypso software.
Probiotic A (protexin) was comprised of *Lactobacillus acidophilus*, *L. delbrueckii* subspecies *bulgaricus*, *L. plantarum, L. rhamnosus, Bifidobacterium bifidum, Enterococcus faecium* and *Streptococcus salivarius* subspecies *thermophilus.*

1.4.3.2 Effect of probiotic B supplementation

Probiotic B supplementation and *S*T challenge had a significant effect (FDR < 0.05) on caecal luminal microbial abundance at the genus and family levels. At the genus level, *S*T challenge increased the abundance of unclassified bacteria and decreased *Ruminococcaceae* both in the probiotic B supplemented and *S*T challenged and *S*T positive control groups (Figure 1-3a). The abundance of *Ruminococcus* and *Bifidobacterium* was higher in the probiotic B supplemented control group compared with the probiotic B supplemented and *S*T challenged, *S*T positive control and negative control groups. *Paenibacillus*, *Lactococcus*, *Eubacterium*, *Enterococcus* and *Coprobacillus* were abundant in the probiotic B supplemented and *S*T challenged group compared with the probiotic B control, *S*T positive control and negative control groups. The core caecal microbiome was affected by

the probiotic B supplementation and *S*T challenge (Appendix Figure 1-4). At OTU level, there were 30 common taxa in the probiotic B supplemented group.

Probiotic B supplementation and *S*T challenge did not significantly affect (*P = 0.0860*) microbial diversity among the treatment groups (Appendix Figure 1-5). Alpha diversity measured by the Shannon index based on sampling time-point (or birds' age) showed a significant ($P = 6e^{-05}$) variation in microbial diversity (Figure 1-3b). Probiotic B supplementation reduced (P = 0.0032) the microbial diversity on day 5 and day 7 compared with day 3 in the probiotic B control group, although it was not altered (*P = 0.1252*) in the probiotic B supplemented and *S*T challenged group. The RDA+ showed that probiotic B supplementation and *S*T challenge changed (*P = 0.001*) the composition of bacterial communities (Figure 1-3c).

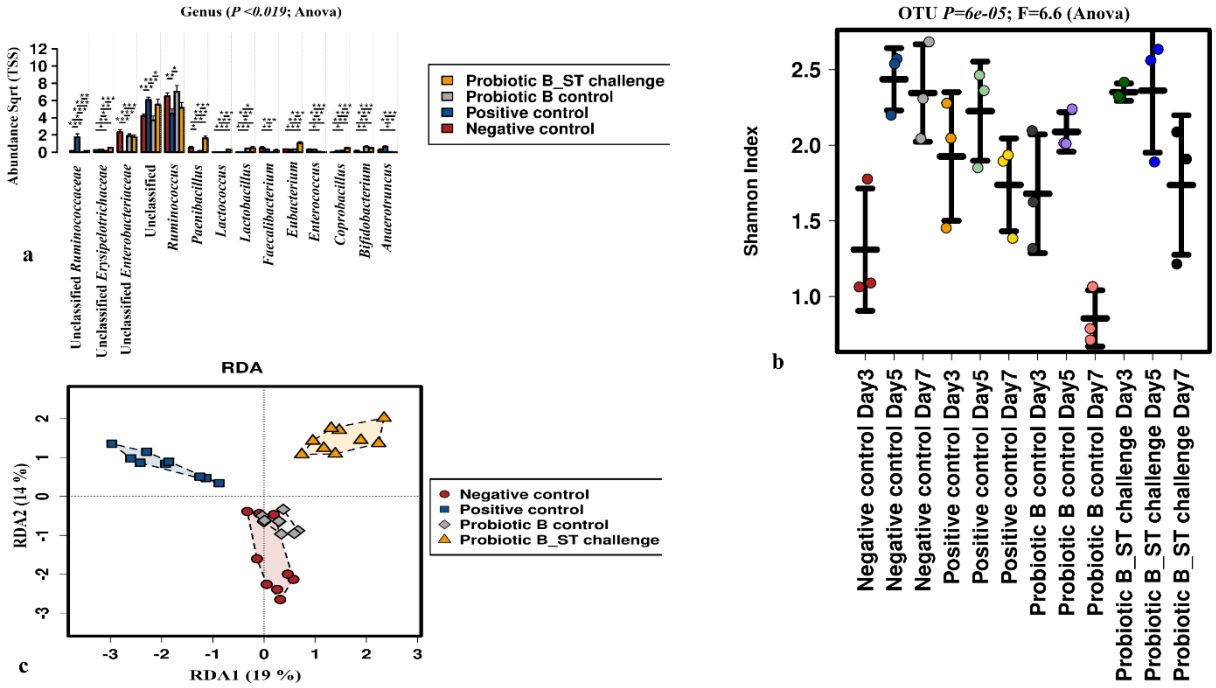


Figure 1-3 Caecal luminal microbial abundance and diversity affected by probiotic B supplementation and *Salmonella* Typhimurium (*S*T) challenge

(a) Microbial abundance at genera level.
(b) Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point).
(c) RDA+ of each of the treatment group.
P < 0.019 was equivalent of FDR < 0.05 in Calypso software.
Probiotic B contained *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* in its composition.

1.4.3.3 Effect of synbiotic A supplementation

At the genus level, in the synbiotic A supplemented and *S*T challenge group, the abundance of *Anaerotruncus*, *Lactococcus*, *Lactobacillus*, *Eubacterium* and *Bifidobacterium* was significantly (FDR < 0.05) higher compared with the synbiotic A control, *S*T positive control and negative control groups (Figure 1-4a). Synbiotic A supplementation without *S*T challenge significantly increased the abundance of *Trabulsiella* and *Ruminococcus* as seen in the synbiotic A control group. A list of all of the significant genera has been provided in Figure 1-4a. The core caecal microbiome was mainly affected by the synbiotic A supplementation interaction with *S*T challenge (Appendix Figure 1-6). At OTU level, there were 28 common taxa in the synbiotic A supplemented group. There were no specific taxa attributed to the synbiotic A control group.

A significant effect (*P = 0.0072*) of synbiotic A supplementation was observed on microbial diversity in different treatment groups (Appendix Figure 1-7). The microbial diversity was significantly lower in the synbiotic A control group compared with the synbiotic A supplemented and *S*T challenged, *S*T positive control and negative control groups. Within each treatment group (*P = 0.0012*), microbial diversity significantly decreased (*P = 0.0191*) with bird age (or sampling time-point) in the synbiotic A control and negative control groups, while in the synbiotic A supplemented and *S*T challenged (*P = 0.4262*) and *S*T positive control (*P = 0.3128*) groups, there was no difference in the microbial diversity (Figure 1-4b). The RDA+ showed that synbiotic A supplementation and *S*T challenge changed (*P = 0.001*) the composition of bacterial communities, although some overlap between the synbiotic A control and negative control groups was observed (Figure 1-4c).

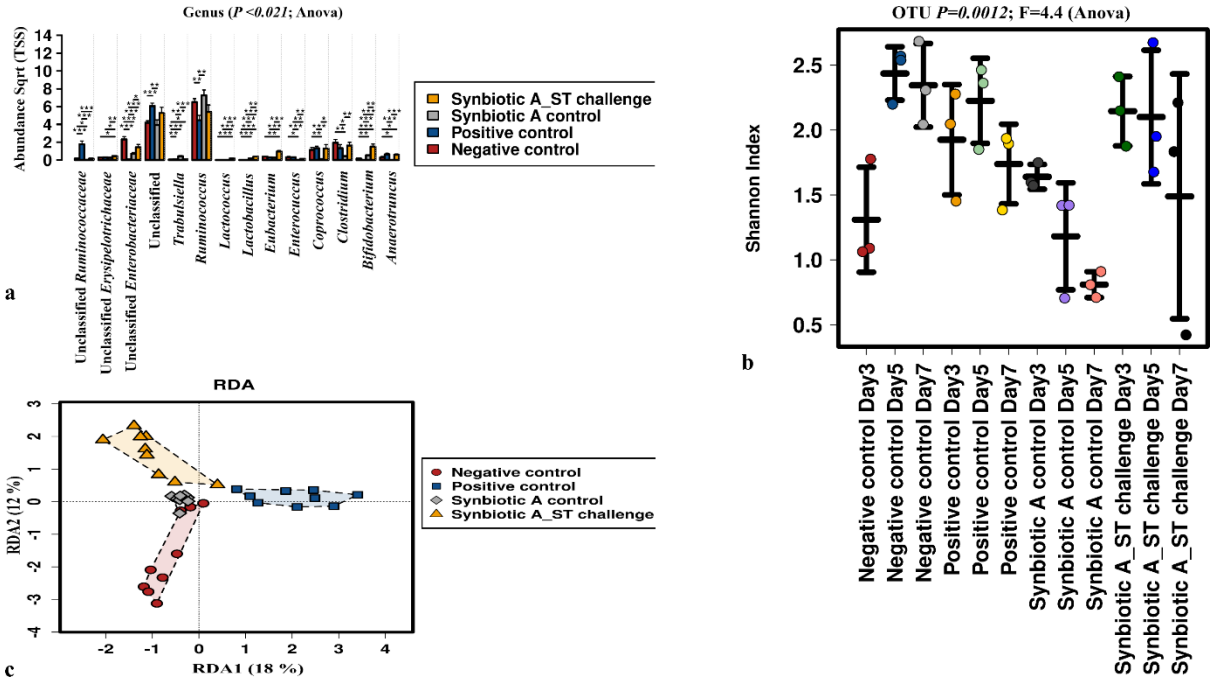


Figure 1-4 Caecal luminal microbial abundance and diversity affected by the synbiotic A supplementation and *Salmonella* Typhimurium (*S*T) challenge

(a) Microbial abundance at genera level.

(b) Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point).

(c) RDA+ of each of the treatment group.

P < 0.021 was equivalent of FDR < 0.05 in Calypso software.

Synbiotic A was composed of *Enterococcus* sp., *Pediococcus* sp., *Bifidobacterium* sp., *Lactobacillus* sp. and fructooligosaccharides.

1.4.3.4 Effect of synbiotic B supplementation

At the genus level, in the synbiotic B control group, the abundance of *Trabulsiella*, *Oscillospira* and *Holdemania* significantly increased compared with the synbiotic B supplemented and *S*T challenged, *S*T positive control and negative control groups (Figure 1-5a). *Escherichia* and *Coprococcus* genera were higher in abundance in the synbiotic B supplemented and *S*T challenged group compared with the other three treatment groups. Overall, the *S*T challenge increased the abundance of unclassified bacteria in the *S*T positive control group. The overall genera significantly affected by the synbiotic B supplementation and *S*T challenge have been depicted in Figure 1-5a. The core caecal microbiome was affected by the synbiotic B supplementation and *S*T challenge (Appendix Figure 1-8). At OTU level, there were 36 common taxa in the synbiotic B supplemented group.

In the synbiotic B supplemented group, at the OTU level, the diversity of the microbial communities was not affected (*P = 0.4200*) by the synbiotic supplementation or *S*T challenge (Appendix Figure 1-9). Overall, sampling time-point (or birds' age) had a significant effect (*P = 0.004*) on microbial diversity (Figure 1-5b) only in the negative control group (*P = 0.0091*). The RDA+ showed that synbiotic B supplementation and *S*T challenge changed (*P = 0.001*) the composition of bacterial communities (Figure 1-5c).

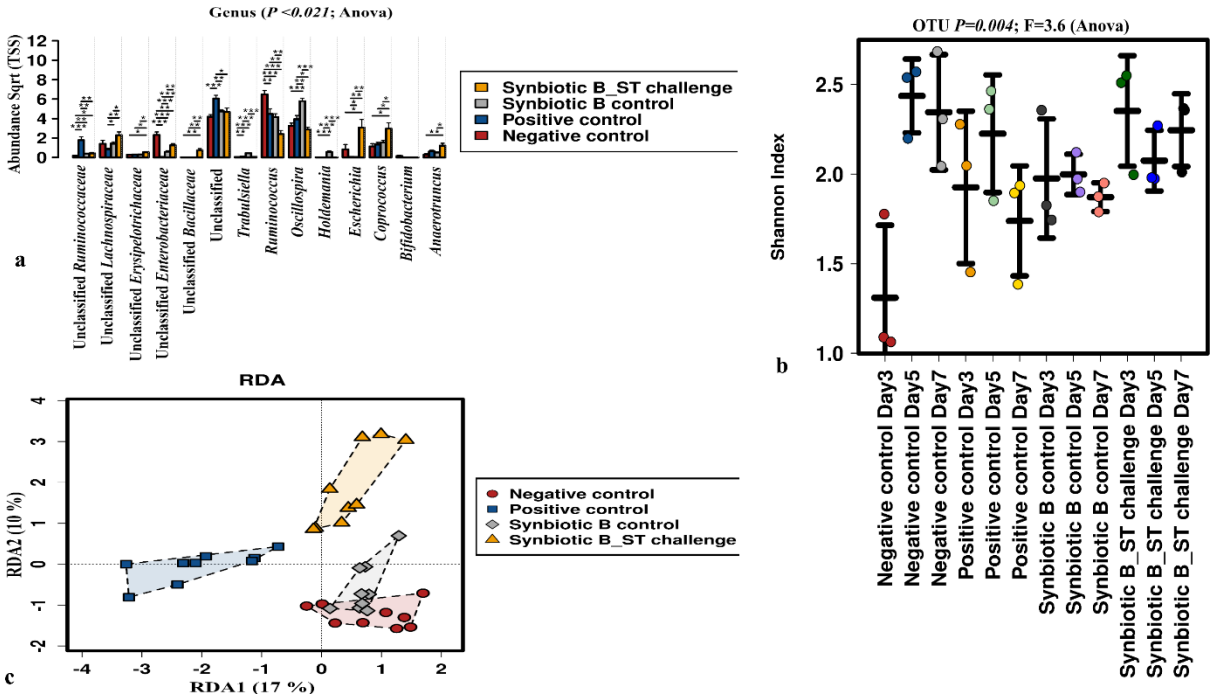


Figure 1-5 Caecal luminal microbial abundance and diversity affected by synbiotic B supplementation and *Salmonella* Typhimurium (*S*T) challenge

(a) Microbial abundance at genera level.

(b) Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point).

(c) RDA+ of each of the treatment group.

P < 0.019 was equivalent of FDR < 0.05 in Calypso software.

Synbiotic B contained *Lactobacillus acidophilus, L. casei, L. salivarius, L. plantarum, L. rhamnosus, L. brevis, Bifidobacterium bifidum, B. lactis, S. thermophiles*, prebiotic inulin (chicory root extract), protease, amylase, cellulase, hemicellulase, lipase, papain and bromelain in its composition.

1.4.4 *Salmonella* Typhimurium load in different organs

Salmonella spp. were not recovered from either the negative control group or the probiotics and synbiotics control groups. Probiotics A and B supplementation in young layer chicks for a week did not significantly reduce (*P > 0.05*) the *S*T load (log_{10} CFU/g of tissue) in the caeca, liver and spleen (Figure 1-6a–f). In the probiotics A and B supplemented and challenged with *S*T groups, the bacterial load in all three organs increased with day p.i. except for the caeca and liver of the probiotic A challenged group (Figure 1-6a–f).

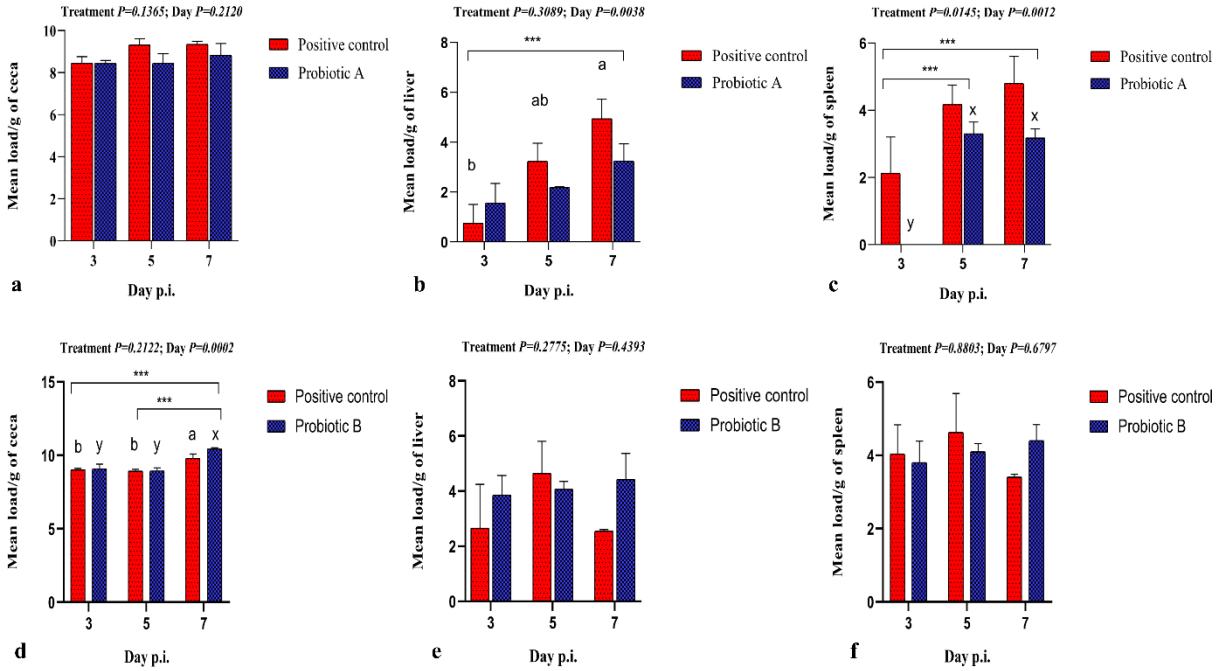


Figure 1-6 Effect of probiotics A and B on mean load (log10 CFU/g of tissue) of *Salmonella* Typhimurium (*S*T) in caeca, liver and spleen collected on days 3, 5 and 7 p.i.

(a) *S*T load in caecal tissue of probiotic A supplemented chicks.
(b) *S*T load in liver tissue of probiotic A supplemented chicks.
(c) *S*T load in spleen tissue of probiotic A supplemented chicks.
(d) *S*T load in caecal tissue of probiotic B supplemented chicks.
(e) *S*T load in liver tissue of probiotic B supplemented chicks.
(f) *S*T load in spleen tissue of probiotic B supplemented chicks.
Superscripts (^{a,b}) represent significant difference affected by days p.i. in positive control groups.
Superscripts (^{x,y}) represent significant difference affected by days p.i. in probiotic supplemented groups.
In each graph, the line across the bars represents significant differences (*P < 0.05; **P < 0.005; ***P < 0.0005) between the respective groups.
Details of probiotics A and B are in Table 1-1.

Feeding synbiotics A and B to layer chicks for a week did not significantly reduce (*P > 0.05*) *S*T load in the caeca, liver and spleen. The *S*T load significantly increased with day p.i. only in the caeca (Figure 1-7a–f). There was a general trend of lower *S*T load in the liver and spleen of the synbiotics A and B supplemented groups; however, these differences were not statistically significant.

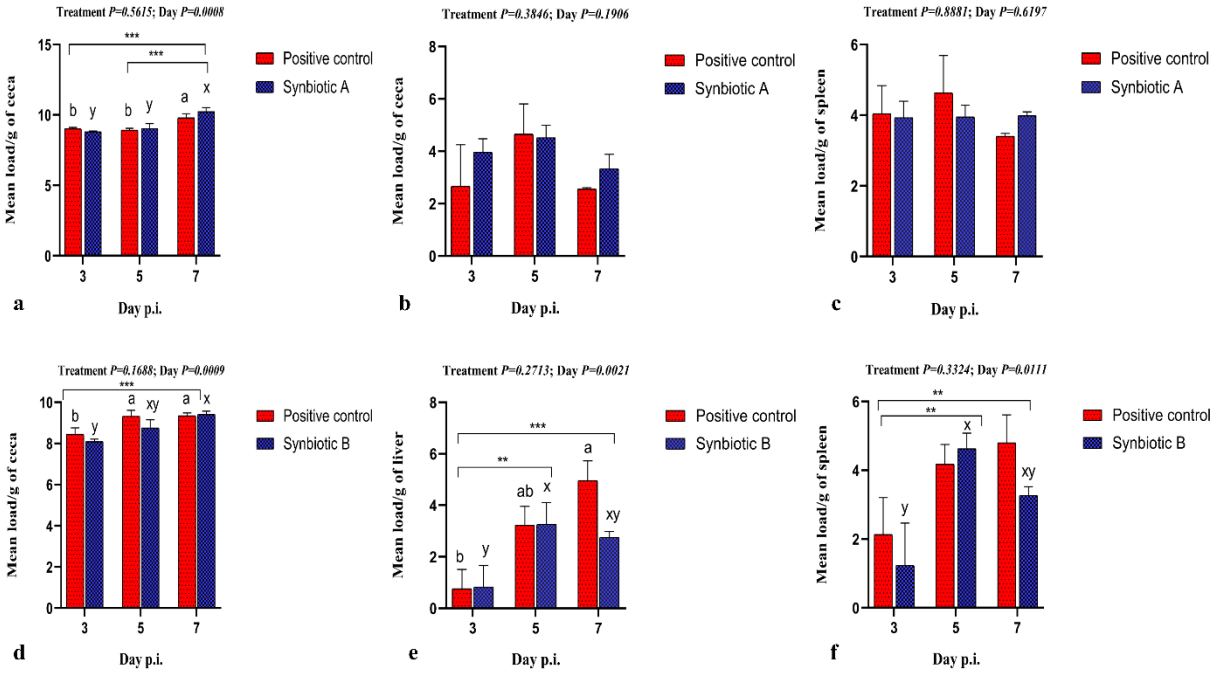


Figure 1-7 Effect of synbiotics A and B on mean load (log10 CFU/g of tissue) of *Salmonella* Typhimurium (*ST*) in caeca, liver and spleen tissues collected on days 3, 5 and 7 p.i.

(a) *ST* load in caecal tissue of synbiotic A supplemented chicks.
(b) *ST* load in liver tissue of synbiotic A supplemented chicks.
(c) *ST* load in spleen tissue of synbiotic A supplemented chicks.
(d) *ST* load in caecal tissue of synbiotic B supplemented chicks.
(e) *ST* load in liver tissue of synbiotic B supplemented chicks.
(f) *ST* load in spleen tissue of synbiotic B supplemented chicks.
Superscripts (^{a,b}) represent significant difference affected by days p.i. in positive control groups.
Superscripts (^{x,y}) represent significant difference affected by days p.i. in probiotic supplemented groups.
In each graph, the line across the bars represents significant differences (***P < 0.0005) between the respective groups.
Details of synbiotics A and B are in Table 1-1.

1.5 Discussion

This study aimed to understand the effects of short-term feeding of four different commercial probiotic and synbiotic supplements on gut health in the presence or absence of *Salmonella* Typhimurium challenge in Isa-Brown layer chicks. During the last century, the incorporation of growth-promoting antibiotics into the feed of production animals has resulted in improvements to health conditions and productivity. Globally there are concerns about the use of in-feed antibiotics for growth promotion due to the development of antimicrobial resistance and the spread of resistance genes (Marshall & Levy 2011). Moreover, the use of antimicrobials can negatively alter gut microbiota (Becattini et al. 2016). Probiotics and synbiotics are favoured in recent days due to their ability to induce a structural change in gut microbiota (Ziemer & Gibson 1998).

The rationale behind discontinuation of the probiotics and synbiotics before *Salmonella* Typhimurium challenge was to understand the effects of the developed gut microbiota on *Salmonella* load and invasion into internal organs. In this study, irrespective of the probiotic and synbiotic supplementation and *Salmonella* Typhimurium challenge, a total of 22 distinct genera were identified in layer chicks; however, we have only discussed the genera significantly affected by the probiotics, synbiotics or *Salmonella* Typhimurium challenge relative to positive and negative control groups.

In the current study, most of the genera that colonised the chicks' caeca are involved in diverse physiological functions. For example, *Bifidobacterium* (Milani et al. 2015), *Clostridium* (Bayer et al. 2008), *Enterococcus* (Robert & Bernalier-Donadille 2003), *Eubacterium* (Montgomery 1988) and *Ruminococcus* (Moon et al. 2011) in the gut are involved in fibre digestion. *Eubacterium* is one of the dominant genera of the caecum in layers (Callaway et al. 2009). Some species of *Eubacterium* (e.g. *Eubacterium hallii*) in the gut are involved in metabolising glycerol to 3-hydroxypropionaldehyde that exists in a multi-compound system called reuterin (Fekry et al. 2016). Reuterin possesses antimicrobial activity against a range of pathogens (Vollenweider et al. 2010). *Eubacterium hallii* in the human gut has been linked with the formation of propionate (Engels et al. 2016). Therefore, the most abundant *Eubacterium* in chicks' caeca could be linked with numerous functions ranging from short chain fatty acids (SCFAs) production to metabolic balance. In the current study, a reduction of *Eubacterium* abundance in the *Salmonella* Typhimurium positive control group compared with the probiotics (A and B) and synbiotic A supplemented and *Salmonella* Typhimurium challenged groups showed that these products were effective in maintaining the *Eubacterium* population in the gut even in the presence of *Salmonella* Typhimurium. This study also demonstrated some interaction between *Eubacterium* and *Salmonella* Typhimurium because, in the absence of challenge, the abundance of *Eubacterium* was not significantly different among the treatment groups.

Oscillospira is another genus of gut microbiota abundantly present in layer chickens' caeca (Volf et al. 2016). *Oscillospira* species help in starch digestion in many different hosts (Mackie et al. 2003) and therefore are butyrate producers (Gophna et al. 2017). Butyrate is one of the three main types of SCFAs and is involved in ATP provision to enterocytes (Treem et al. 1994) and possesses anti-inflammatory (Vinolo et al. 2011) and anti-microbial properties (Cox et al. 1994). In this study, an increased abundance of *Oscillospira* in the probiotic A and synbiotic B supplemented groups without *Salmonella* Typhimurium challenge indicates that these products favoured starch digesting and SCFAs producing bacteria were favoured. It was also observed that, in the presence of the probiotic A and synbiotic B supplementation, *Salmonella* Typhimurium reduced the *Oscillospira* abundance compared with the *Salmonella* Typhimurium positive control group. It is possible that there is an interaction between *Oscillospira* and *Salmonella*, where *Salmonella* Typhimurium depletes its population. *Ruminococcus* is among other genera involved in SCFAs production in chickens (Huang et al. 2018). In the current study, different probiotics and synbiotics affected *Ruminococcus* abundance differently. For example, synbiotic B supplementation reduced *Ruminococcus* abundance in the synbiotic supplemented groups with or without *Salmonella* Typhimurium challenge. Probiotic B and synbiotic A increased the *Ruminococcus* abundance both in the probiotic and synbiotic supplemented and *Salmonella* Typhimurium challenged groups. This shows that one particular probiotic or synbiotic may not favour the abundance of all beneficial bacterial genera in the gut. In the current study, the predominant bacterial genera of *Eubacterium*, *Oscillospira* and *Ruminococcus* positively modulated by the probiotics and synbiotics indicate their role in the overall gut health in young chicks. No significant effect of *Salmonella* Typhimurium and *Salmonella* Enteritidis was observed on the composition of gut microbial communities of laying chickens (Azcarate-Peril et al. 2018; Nordentoft et al. 2011).

Probiotic A and synbiotics A and B supplementation increased the abundance of *Trabulsiella*, while *Salmonella* Typhimurium challenge decreased it. *Trabulsiella* is a member of the *Enterobacteriaceae* with no apparent role in chicken gut microbiota. *Bifidobacterium* is one of the dominant members of gut microbiota that plays a role in complex starch digestion (Milani et al. 2015), preventing the production of pro-inflammatory cytokines (Fanning et al. 2012) and stress reduction (Savignac et al. 2014). In this study, its abundance was affected mainly by the probiotic B and synbiotic A with or without *Salmonella* Typhimurium challenge. These probiotic and synbiotic were effective in maintaining *Bifidobacterium* abundance even in the presence of *Salmonella* Typhimurium. This shows that *Bifidobacterium* is one of the gut bacteria with a possible protective role against *Salmonella* Typhimurium infection. However, at least in the conditions applied in the current study, the protective

environment produced by the *Bifidobacterium* was not sufficient to reduce the load of *Salmonella* Typhimurium significantly in caeca. We suggest further investigation to understand the interaction of *Bifidobacterium* with *Salmonella* Typhimurium as a probiotic candidate for the chicken gut. A reduced abundance of *Klebsiella* and *Escherichia* in the probiotic A and synbiotic B supplemented and no *Salmonella* Typhimurium challenge groups, respectively, showed the positive modulation of gut microbiota by these products. Important diseases of poultry attributed to *E. coli* include cellulitis, septicaemia, colibacillosis, omphalitis and respiratory tract infection (Morley & Thomson 1984; Stromberg et al. 2017).

Different probiotics and synbiotics supplementation in the presence of *Salmonella* Typhimurium challenge showed positive effects on the abundance of certain genera. For example, probiotic A supplementation increased the abundance of *Butyricicoccus*, *Eubacterium*, *Coprococcus* and *Blautia* in the *Salmonella* Typhimurium challenged compared to the positive control or probiotic control groups. Similarly, synbiotic B affected the abundance of *Escherichia*, *Coprococcus* and *Anaerotruncus*, while synbiotic A affected *Lactococcus*, *Lactobacillus*, *Eubacterium*, *Coprococcus*, *Bifidobacterium* and *Anaerotruncus*. Probiotic B mainly influenced *Paenibacillus*, *Eubacterium* and *Coprobacillus*. This further strengthens the notion that, in the presence of *Salmonella* Typhimurium, short-term feeding of probiotics or synbiotics has the potential to influence resident gut bacterial genera positively. However, this short-term treatment may not be sufficient to inhibit *Salmonella* Typhimurium from colonising caeca or invading internal organs. Overall, compared to the negative control group, the microbial diversity (measured at OTU level) was decreased with bird age (or sampling time-point) by synbiotic A supplementation. This shows that the synbiotic A reduced the genera of certain bacteria in the gut of layer chicks. The Shannon index of redundancy analysis (RDA+) showed that probiotic and synbiotic supplementation and *Salmonella* Typhimurium challenge shifted the gut microbiota diversity and therefore it clustered separately among different treatment groups, showing the importance of probiotic or synbiotic supplementation in *Salmonella* Typhimurium infected birds. These results were further supported by the presence of various biomarkers in different treatment groups. Probiotic and synbiotic supplementation increased the abundance of many genera of the gut microbiota in the presence or absence of *Salmonella* Typhimurium; however, these effects varied depending on the products.

The bacteriology results (counted as CFU/g of tissue) showed that feeding the probiotics and synbiotics for a week was not effective in significantly reducing *S*T load in the liver, spleen and caecal tissues of layer chicks. It seems that the early colonisation of caeca with probiotic bacterial strains may not competitively exclude *Salmonella* Typhimurium. Limited studies performed on probiotic or prebiotic supplementation and *Salmonella* Typhimurium challenge in layer chickens presented different results reflecting the importance of the nature and duration of the probiotic being supplemented, genetic strain, age of the bird and the bird rearing environment. For example, the inclusion of fructooligosaccharides (FOS) in an alfalfa moulting diet significantly decreased caecal *Salmonella* Enteritidis counts in laying hens (Donalson et al. 2008). In White Leghorn Hy-Line cockerels challenged with *Salmonella* Enteritidis, FOS supplementation (alone or in combination with probiotic) significantly decreased *Salmonella* load in caeca at days 1, 7 and 14 p.i. (Fukata et al. 1999). In 46-week old White Leghorn laying hens challenged with *Salmonella* Enteritidis, supplementation of *Lactobacillus plantarum* for seven days post-infection did not significantly reduce *Salmonella* load in the caeca (Adhikari et al. 2018). Interestingly, synbiotic supplemented laying hens challenged with *Salmonella* Enteritidis showed no *Salmonella* in the caecal contents on day 10 p.i. (Luoma et al. 2017).

Lactobacillus johnsonii strain R-17504 and *Lactobacillus reuteri* strain R-17485 supplementation into day-old layer chicks significantly reduced *Salmonella* Enteritidis load in caeca on day 6 p.i. (Van Coillie et al. 2007). In this study, no significant difference between the treatment groups for *Salmonella* Typhimurium load in caecal tissue indicate that the probiotics and synbiotics were not effective in

reducing *Salmonella* count at days 3, 5 and 7 p.i. in Isa-Brown layer chicks. Further studies are required to investigate the long-term feeding of probiotic supplements on *Salmonella* Typhimurium colonisation, shedding and/or invasion into the vital organs of hens. Overall, a week long probiotic and synbiotic supplementation to layer chicks was effective in modulating positively the abundance of certain resident gut microbiota. *Salmonella* Typhimurium challenge decreased the abundance of many useful bacterial genera, while the probiotics and synbiotics supplementation increased it. The abundant genera play a pivotal role in maintaining overall gut health. Nevertheless, probiotic and synbiotic supplementation to chicks for one week did not competitively exclude *Salmonella* Typhimurium from caeca or prevent internal organ invasion. Further studies are required to understand the long-term feeding of probiotics on laying chicken gut microbiota and its effects on *Salmonella* Typhimurium shedding.

1.6 References

ADHIKARI, P., LEE, C. H., COSBY, D. E., COX, N. A. & KIM, W. K. 2018. Effect of probiotics on fecal excretion, colonization in internal organs and immune gene expression in the ileum of laying hens challenged with *Salmonella* Enteritidis. *Poultry Science,* 98**,** 1235-1242.

AZCARATE-PERIL, M. A., BUTZ, N., CADENAS, M. B., KOCI, M., BALLOU, A., MENDOZA, M., ALI, R. & HASSAN, H. 2018. An attenuated *Salmonella enterica* serovar Typhimurium strain and galacto-oligosaccharides accelerate clearance of *Salmonella* infections in poultry through modifications to the gut microbiome. *Applied and Environmental Microbiology,* 84**,** e02526-17.

BABU, U. S., SOMMERS, K., HARRISON, L. M. & BALAN, K. V. 2012. Effects of fructooligosaccharide-inulin on *Salmonella*-killing and inflammatory gene expression in chicken macrophages. *Veterinary Immunology and Immunopathology,* 149**,** 92-96.

BALLOU, A. L., ALI, R. A., MENDOZA, M. A., ELLIS, J. C., HASSAN, H. M., CROOM, W. J. & KOCI, M. D. 2016. Development of the chick microbiome: How early exposure influences future microbial diversity. *Frontiers in Veterinary Science,* 3.

BARROW, P. A. & LOVELL, M. A. 1991. Experimental infection of egg-laying hens with *Salmonella* Enteritidis phage type 4. *Avian Pathology,* 20**,** 335-348.

BAYER, E. A., LAMED, R., WHITE, B. A. & FLINT, H. J. 2008. From cellulosomes to cellulosomics. *The Chemical Record,* 8**,** 364-377.

BECATTINI, S., TAUR, Y., & PAMER, E. G. 2016. Antibiotic-induced changes in the intestinal microbiota and disease. *Trends in Molecular Medicine*, *22*, 458-478.

BENAKIS, C., BREA, D., CABALLERO, S., FARACO, G., MOORE, J., MURPHY, M., SITA, G., RACCHUMI, G., LING, L. & PAMER, E. G. 2016. Commensal microbiota affects ischemic stroke outcome by regulating intestinal [gamma][delta] T cells. *Nature Medicine,* 22**,** 516-523.

BOZKURT, M., BINTAŞ, E., KıRKAN, Ş., AKŞIT, H., KÜÇÜKYıLMAZ, K., ERBAŞ, G., CABUK, M., AKŞIT, D., PARıN, U. & EGE, G. 2016. Comparative evaluation of dietary supplementation with mannan oligosaccharide and oregano essential oil in forced molted and fully fed laying hens between 82 and 106 weeks of age. *Poultry Science,* 95**,** 2576-2591.

BRATBURD, J. R., KELLER, C., VIVAS, E., GEMPERLINE, E., LI, L., REY, F. E. & CURRIE, C. R. 2018. Gut microbial and metabolic responses to *Salmonella enterica* serovar Typhimurium and *Candida albicans*. *mBio,* 9**,** e02032-18.

BUTS, J.-P., DE KEYSER, N. & DE RAEDEMAEKER, L. 1994. *Saccharomyces boulardii* enhances rat intestinal enzyme expression by endoluminal release of polyamines. *Pediatric Research,* 36**,** 522-527.

CALLAWAY, T. R., DOWD, S. E., WOLCOTT, R. D., SUN, Y., MCREYNOLDS, J. L., EDRINGTON, T. S., BYRD, J. A., ANDERSON, R. C., KRUEGER, N. & NISBET, D. J. 2009. Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing. *Poultry Science,* 88**,** 298-302.

CAPORASO, J. G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F. D., COSTELLO, E. K., FIERER, N., PENA, A. G., GOODRICH, J. K. & GORDON, J. I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods,* 7**,** 335.

CASEY, P. G., GARDINER, G. E., CASEY, G., BRADSHAW, B., LAWLOR, P. G., LYNCH, P. B., LEONARD, F. C., STANTON, C., ROSS, R. P., FITZGERALD, G. F. & HILL, C. 2007. A five-strain probiotic combination reduces pathogen shedding and alleviates disease signs in pigs challenged with *Salmonella enterica* Serovar Typhimurium. *Applied and Environmental Microbiology,* 73**,** 1858-1863.

CHANG, C. H., TENG, P. Y., LEE, T. T. & YU, B. 2019. The effects of the supplementation of multi-strain probiotics on intestinal microbiota, metabolites and inflammation of young SPF chickens challenged with *Salmonella enterica* subsp. *enterica*. *Animal Science Journal,* 90, 737-746.

CHART, H., BASKERVILLE, A., HUMPHREY, T. & ROWE, B. 1992. Serological responses of chickens experimentally infected with *Salmonella* Enteritidis PT4 by different routes. *Epidemiology & Infection,* 109**,** 297-302.

CHEN, Y.-S., SRIONNUAL, S., ONDA, T. & YANAGIDA, F. 2007. Effects of prebiotic oligosaccharides and trehalose on growth and production of bacteriocins by lactic acid bacteria. *Letters in Applied Microbiology,* 45**,** 190-193.

COX, N. A., MCHAN, F., BAILEY, J. S. & SHOTTS, E. B. 1994. Effect of butyric or lactic acid on the in vivo colonization of *Salmonella* Typhimurium. *The Journal of Applied Poultry Research,* 3**,** 315-318.

CRHANOVA, M., HRADECKA, H., FALDYNOVA, M., MATULOVA, M., HAVLICKOVA, H., SISAK, F. & RYCHLIK, I. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica* serovar Enteritidis infection. *Infection and Immunity,* 79**,** 2755-2763.

CUI, Y., WANG, Q., LIU, S., SUN, R., ZHOU, Y. & LI, Y. 2017. Age-related variations in intestinal microflora of free-range and caged Hens. *Frontiers in Microbiology,* 8**,** 1310-1310.

DERIU, E., LIU, J. Z., PEZESHKI, M., EDWARDS, R. A., OCHOA, R. J., CONTRERAS, H., LIBBY, S. J., FANG, F. C. & RAFFATELLU, M. 2013. Probiotic bacteria reduce *Salmonella* Typhimurium intestinal colonization by competing for iron. *Cell Host & Microbe,* 14**,** 26-37.

DESANTIS, T. Z., HUGENHOLTZ, P., LARSEN, N., ROJAS, M., BRODIE, E. L., KELLER, K., HUBER, T., DALEVI, D., HU, P. & ANDERSEN, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology,* 72**,** 5069-5072.

DING, X. M., LI, D. D., BAI, S. P., WANG, J. P., ZENG, Q. F., SU, Z. W., XUAN, Y. & ZHANG, K. Y. 2017. Effect of dietary xylooligosaccharides on intestinal characteristics, gut microbiota, cecal short-chain fatty acids, and plasma immune parameters of laying hens. *Poultry Science,* 97**,** 874-881.

DONALSON, L. M., MCREYNOLDS, J. L., KIM, W. K., CHALOVA, V. I., WOODWARD, C. L., KUBENA, L. F., NISBET, D. J. & RICKE, S. C. 2008. The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation, *Salmonella* Enteritidis infection, and intestinal shedding in laying hens. *Poultry Science,* 87**,** 1253-1262.

EDGAR, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26**,** 2460-2461.

EDGAR, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods,* 10**,** 996.

EDGAR, R. C., HAAS, B. J., CLEMENTE, J. C., QUINCE, C. & KNIGHT, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics,* 27**,** 2194-2200.

ENGELS, C., RUSCHEWEYH, H.-J., BEERENWINKEL, N., LACROIX, C. & SCHWAB, C. 2016. The common gut microbe *Eubacterium hallii* also contributes to intestinal propionate formation. *Frontiers in Microbiology,* 7**,** 713.

FANNING, S., HALL, L. J. & VAN SINDEREN, D. 2012. Bifidobacterium breve UCC2003 surface exopolysaccharide production is a beneficial trait mediating commensal-host interaction through immune modulation and pathogen protection. *Gut Microbes,* 3**,** 420-425.

FEARNLEY, E., RAUPACH, J., LAGALA, F. & CAMERON, S. 2011. *Salmonella* in chicken meat, eggs and humans; Adelaide, South Australia, 2008. *International Journal of Food Microbiology,* 146**,** 219-227.

FEKRY, M. I., ENGELS, C., ZHANG, J., SCHWAB, C., LACROIX, C., STURLA, S. J. & CHASSARD, C. 2016. The strict anaerobic gut microbe *Eubacterium hallii* transforms the carcinogenic dietary heterocyclic amine 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP). *Environmental Microbiology Reports,* 8**,** 201-209.

FORTE, C., ACUTI, G., MANUALI, E., CASAGRANDE PROIETTI, P., PAVONE, S., TRABALZA-MARINUCCI, M., MOSCATI, L., ONOFRI, A., LORENZETTI, C. & FRANCIOSINI, M. 2016. Effects of two different probiotics on microflora, morphology, and morphometry of gut in organic laying hens. *Poultry Science,* 95**,** 2528-2535.

FUKATA, T., SASAI, K., MIYAMOTO, T. & BABA, E. 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on *Salmonella* colonization of chicks. *Journal of Food Protection,* 62**,** 229-233.

FULLER, R. 1989. Probiotics in man and animals. *Journal of Applied Bacteriology,* 66**,** 365-378.

GOLE, V. C., TOROK, V., SEXTON, M., CARAGUEL, C. G. B. & CHOUSALKAR, K. K. 2014. Association between indoor environmental contamination by *Salmonella enterica* and contamination of eggs on layer farms. *Journal of Clinical Microbiology,* 52**,** 3250-3258.

GOODRICH, J. K., DAVENPORT, E. R., WATERS, J. L., CLARK, A. G. & LEY, R. E. 2016. Cross-species comparisons of host genetic associations with the microbiome. *Science,* 352**,** 532-535.

GOPHNA, U., KONIKOFF, T. & NIELSEN, H. B. 2017. *Oscillospira* and related bacteria–From metagenomic species to metabolic features. *Environmental Microbiology,* 19**,** 835-841.

HAGHIGHI, H. R., ABDUL-CAREEM, M. F., DARA, R. A., CHAMBERS, J. R. & SHARIF, S. 2008. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and *Salmonella* infection. *Veterinary Microbiology,* 126**,** 225-233.

HOOPER, L. V., WONG, M. H., THELIN, A., HANSSON, L., FALK, P. G. & GORDON, J. I. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science,* 291**,** 881-884.

HUANG, P., ZHANG, Y., XIAO, K., JIANG, F., WANG, H., TANG, D., LIU, D., LIU, B., LIU, Y. & HE, X. 2018. The chicken gut metagenome and the modulatory effects of plant-derived benzylisoquinoline alkaloids. *Microbiome,* 6**,** 211.

JURICOVA, H., VIDENSKA, P., LUKAC, M., FALDYNOVA, M., BABAK, V., HAVLICKOVA, H., SISAK, F. & RYCHLIK, I. 2013. Influence of *Salmonella enterica* serovar Enteritidis infection on the development of the cecum microbiota in newly hatched chicks. *Applied and Environmental Microbiology,* 79**,** 745-747.

KUBASOVA, T., KOLLARCIKOVA, M., CRHANOVA, M., KARASOVA, D., CEJKOVA, D., SEBKOVA, A., MATIASOVICOVA, J., FALDYNOVA, M., POKORNA, A. & CIZEK, A. 2019. Contact with adult hen affects development of caecal microbiota in newly hatched chicks. *PloS One,* 14**,** e0212446.

KURTOGLU, V., KURTOGLU, F., SEKER, E., COSKUN, B., BALEVI, T. & POLAT, E. 2004. Effect of probiotic supplementation on laying hen diets on yield performance and serum and egg yolk cholesterol. *Food Additives and Contaminants,* 21**,** 817-823.

LIU, L., LIN, L., ZHENG, L., TANG, H., FAN, X., XUE, N., LI, M., LIU, M. & LI, X. 2018. Cecal microbiome profile altered by *Salmonella enterica*, serovar Enteritidis inoculation in chicken. *Gut Pathogens,* 10**,** 34.

LUOMA, A., MARKAZI, A., SHANMUGASUNDARAM, R., MURUGESAN, G. R., MOHNL, M. & SELVARAJ, R. 2017. Effect of synbiotic supplementation on layer production and cecal *Salmonella* load during a *Salmonella* challenge. *Poultry Science,* 96**,** 4208-4216.

MACFARLANE, G.T., STEED, H. AND MACFARLANE, S. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology*, *104*, 305-344.

MACKIE, R. I., AMINOV, R. I., HU, W., KLIEVE, A. V., OUWERKERK, D., SUNDSET, M. A. & KAMAGATA, Y. 2003. Ecology of uncultivated *Oscillospira* species in the rumen of cattle, sheep, and reindeer as assessed by microscopy and molecular approaches. *Applied and Environmental Microbiology,* 69**,** 6808-6815.

MARCQ, C., COX, E., SZALO, I. M., THEWIS, A. & BECKERS, Y. 2011. *Salmonella* Typhimurium oral challenge model in mature broilers: Bacteriological, immunological, and growth performance aspects. *Poultry Science,* 90**,** 59-67.

MARSHALL, B. M., & LEVY, S. B. 2011. Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews*, *24*, 718-733.

MARTÍN, R. AND LANGELLA, P. 2019. Emerging health concepts in the probiotics field: streamlining the definitions. *Frontiers in Microbiology*, *10*, 1047.

MCWHORTER, A. R. & CHOUSALKAR, K. K. 2018. A Long-Term Efficacy Trial of a Live, Attenuated *Salmonella* Typhimurium Vaccine in Layer Hens. *Frontiers in Microbiology,* 9**,** 1380-1380.

MIKULSKI, D., JANKOWSKI, J., NACZMANSKI, J., MIKULSKA, M. & DEMEY, V. 2012. Effects of dietary probiotic (*Pediococcus acidilactici*) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poultry Science,* 91**,** 2691-2700.

MILANI, C., LUGLI, G. A., DURANTI, S., TURRONI, F., MANCABELLI, L., FERRARIO, C., MANGIFESTA, M., HEVIA, A., VIAPPIANI, A. & SCHOLZ, M. 2015. Bifidobacteria exhibit social behavior through carbohydrate resource sharing in the gut. *Scientific Reports,* 5**,** 15782.

MON, K. K. Z., SAELAO, P., HALSTEAD, M. M., CHANTHAVIXAY, G., CHANG, H.-C., GARAS, L., MAGA, E. A. & ZHOU, H. 2015. *Salmonella enterica* serovars Enteritidis infection alters the indigenous microbiota diversity in young layer chicks. *Frontiers in Veterinary Science,* 2**,** 61.

MONTGOMERY, L. 1988. Isolation of human colonic fibrolytic bacteria. *Letters in Applied Microbiology,* 6**,** 55-57.

MOON, Y. H., IAKIVIAK, M., BAUER, S., MACKIE, R. I. & CANN, I. K. O. 2011. Biochemical analyses of multiple endoxylanases from the rumen bacterium Ruminococcus albus 8 and their synergistic activities with accessory hemicellulose-degrading enzymes. *Applied and Environmental Microbiology,* 77**,** 5157-5169.

MORLEY, A.J. & THOMSON, D.K., 1984. Swollen-head syndrome in broiler chickens. *Avian Diseases*, pp.238-243.

NAQID, I. A., OWEN, J. P., MADDISON, B. C., GARDNER, D. S., FOSTER, N., TCHÓRZEWSKA, M. A., LA RAGIONE, R. M. & GOUGH, K. C. 2015. Prebiotic and probiotic agents enhance antibody-based immune responses to *Salmonella* Typhimurium infection in pigs. *Animal Feed Science and Technology,* 201**,** 57-65.

NGUNJIRI, J. M., TAYLOR, K. J. M., ABUNDO, M. C., JANG, H., ELAISH, M., KC, M., GHORBANI, A., WIJERATNE, S., WEBER, B. P., JOHNSON, T. J. & LEE, C.-W. 2019. Farm stage, bird age and body site dominantly affect the quantity, taxonomic composition, and dynamics of respiratory and gut microbiota of commercial layer chickens. *Applied and Environmental Microbiology***,** AEM.03137-18.

NORDENTOFT, S., MØLBAK, L., BJERRUM, L., DE VYLDER, J., VAN IMMERSEEL, F. & PEDERSEN, K. 2011. The influence of the cage system and colonisation of *Salmonella* Enteritidis on the microbial gut flora of laying hens studied by T-RFLP and 454 pyrosequencing. *BMC Microbiology,* 11**,** 187.

O'HARA, A. M., O'REGAN, P., FANNING, Á., O'MAHONY, C., MACSHARRY, J., LYONS, A., BIENENSTOCK, J., O'MAHONY, L. & SHANAHAN, F. 2006. Functional modulation of human intestinal epithelial cell responses by *Bifidobacterium infantis* and *Lactobacillus salivarius*. *Immunology,* 118**,** 202-215.

OCEJO, M., OPORTO, B. & HURTADO, A. 2019. 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. *Scientific Reports,* 9**,** 2506.

PARK, J. W., JEONG, J. S., LEE, S. I. & KIM, I. H. 2016. Effect of dietary supplementation with a probiotic (*Enterococcus faecium*) on production performance, excreta microflora, ammonia emission, and nutrient utilization in ISA brown laying hens. *Poultry Science,* 95**,** 2829-2835.

PENHA FILHO, R. A. C., DÍAZ, S. J. A., FERNANDO, F. S., CHANG, Y.-F., ANDREATTI FILHO, R. L. & JUNIOR, A. B. 2015. Immunomodulatory activity and control of *Salmonella* Enteritidis colonization in the intestinal tract of chickens by *Lactobacillus* based probiotic. *Veterinary Immunology and Immunopathology,* 167**,** 64-69.

POURABEDIN, M., XU, Z., BAURHOO, B., CHEVAUX, E. & ZHAO, X. 2014. Effects of mannan oligosaccharide and virginiamycin on the cecal microbial community and intestinal morphology of chickens raised under suboptimal conditions. *Canadian Journal of Microbiology,* 60**,** 255-266.

ROBERT, C. & BERNALIER-DONADILLE, A. 2003. The cellulolytic microflora of the human colon: evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. *FEMS Microbiology Ecology,* 46**,** 81-89.

SAVIGNAC, H. M., KIELY, B., DINAN, T. G. & CRYAN, J. F. 2014. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterology & Motility,* 26**,** 1615-1627.

SHANG, H. M., HU, T. M., LU, Y. J. & WU, H. X. 2010. Effects of inulin on performance, egg quality, gut microflora and serum and yolk cholesterol in laying hens. *British Poultry Science,* 51**,** 791-796.

SHINI, S., SHINI, A. & BLACKALL, P. J. 2013. The potential for probiotics to prevent reproductive tract lesions in free-range laying hens. *Animal Production Science,* 53**,** 1298-1308.

SOMMER, F., ANDERSON, J. M., BHARTI, R., RAES, J. & ROSENSTIEL, P. 2017. The resilience of the intestinal microbiota influences health and disease. *Nature Reviews. Microbiology,* 15**,** 630.

SPINLER, J. K., TAWEECHOTIPATR, M., ROGNERUD, C. L., OU, C. N., TUMWASORN, S. & VERSALOVIC, J. 2008. Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. *Anaerobe,* 14**,** 166-171.

STANLEY, D., DENMAN, S. E., HUGHES, R. J., GEIER, M. S., CROWLEY, T. M., CHEN, H., HARING, V. R. & MOORE, R. J. 2012. Intestinal microbiota associated with differential feed conversion efficiency in chickens. *Applied Microbiology and Biotechnology,* 96**,** 1361-1369.

STAPPENBECK, T. S., HOOPER, L. V. & GORDON, J. I. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 15451-15455.

STROMBERG, Z.R., JOHNSON, J.R., FAIRBROTHER, J.M., KILBOURNE, J., VAN GOOR, A., CURTISS 3RD, R. AND MELLATA, M., 2017. Evaluation of Escherichia coli isolates from healthy chickens to determine their potential risk to poultry and human health. *PloS One*, *12*, p.e0180599.

TANG, S. G. H., SIEO, C. C., RAMASAMY, K., SAAD, W. Z., WONG, H. K. & HO, Y. W. 2017. Performance, biochemical and haematological responses, and relative organ weights of laying hens fed diets supplemented with prebiotic, probiotic and synbiotic. *BMC Veterinary Research,* 13**,** 248.

TREEM, W. R., AHSAN, N., SHOUP, M. & HYAMS, J. S. 1994. Fecal short-chain fatty acids in children with inflammatory bowel disease. *Journal of Pediatric Gastroenterology and Nutrition,* 18**,** 159-164.

VAN COILLIE, E., GORIS, J., CLEENWERCK, I., GRIJSPEERDT, K., BOTTELDOORN, N., VAN IMMERSEEL, F., DE BUCK, J., VANCANNEYT, M., SWINGS, J., HERMAN, L. & HEYNDRICKX, M. 2007. Identification of lactobacilli isolated from the cloaca and vagina of laying hens and characterization for potential use as probiotics to control *Salmonella* Enteritidis. *Journal of Applied Microbiology,* 102**,** 1095-1106.

VIDENSKA, P., SEDLAR, K., LUKAC, M., FALDYNOVA, M., GERZOVA, L., CEJKOVA, D., SISAK, F. & RYCHLIK, I. 2014. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. *PLoS One,* 9**,** e115142.

VINOLO, M. A., RODRIGUES, H. G., HATANAKA, E., SATO, F. T., SAMPAIO, S. C. & CURI, R. 2011. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *Journal of Nutritional Biochemistry,* 22**,** 849-855.

VOLF, J., POLANSKY, O., VARMUZOVA, K., GERZOVA, L., SEKELOVA, Z., FALDYNOVA, M., BABAK, V., MEDVECKY, M., SMITH, A. L. & KASPERS, B. 2016. Transient and prolonged response of chicken cecum mucosa to colonization with different gut microbiota. *PloS One,* 11**,** e0163932.

VOLLENWEIDER, S., EVERS, S., ZURBRIGGEN, K. & LACROIX, C. 2010. Unraveling the hydroxypropionaldehyde (HPA) system: an active antimicrobial agent against human pathogens. *Journal of Agricultural and Food Chemistry,* 58**,** 10315-10322.

WILLIAMS, S. H. & TUCKER, J. F. 1980. The virulence of *Salmonella* strains for chickens: their excretion by infected chickens. *Journal of Hygiene,* 84**,** 479-488.

ZAKRZEWSKI, M., PROIETTI, C., ELLIS, J. J., HASAN, S., BRION, M.-J., BERGER, B. & KRAUSE, L. 2016. Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. *Bioinformatics,* 33**,** 782-783.

ZHANG, J., KOBERT, K., FLOURI, T. & STAMATAKIS, A. 2013. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics,* 30**,** 614-620.

ZHANG, J. L., XIE, Q. M., JI, J., YANG, W. H., WU, Y. B., LI, C., MA, J. Y. & BI, Y. Z. 2012. Different combinations of probiotics improve the production performance, egg quality, and immune response of layer hens. *Poultry Science,* 91**,** 2755-2760.

ZHANG, Z. F. & KIM, I. H. 2013. Effects of probiotic supplementation in different energy and nutrient density diets on performance, egg quality, excreta microflora, excreta noxious gas emission, and serum cholesterol concentrations in laying hens. *Journal of Animal Science,* 91**,** 4781-4787.

ZIEMER, C.J. & GIBSON, G.R. 1998. An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. *International Dairy Journal*, *8*, 473-479.

2 Transcriptome profiling analysis of caeca in chicks challenged with *Salmonella* Typhimurium reveals differential expression of genes involved in host mucosal immune response

Samiullah Khan and Kapil K. Chousalkar

This chapter has been accepted for publication in *Applied Microbiology and Biotechnology*.

2.1 Introduction

In Australia, eggs or egg products related to foodborne outbreaks in humans are often associated with *Salmonella* Typhimurium (Ford et al. 2018). *Salmonella* Typhimurium infected chicks do not always exhibit clinical signs of salmonellosis; however, blood-tinged faeces were recorded in a previous study (Howard et al. 2018). The pattern of pathogenesis in the chicken gut varies with different *Salmonella* serovars (Henderson et al. 1999). For example, infection with *Salmonella* Pullorum causes limited inflammation, while *Salmonella* Typhimurium results in an influx of heterophils in gut epithelial tissue (Henderson et al. 1999). Two days post-challenge, *Salmonella* Typhimurium elicits heterophilic infiltration, individual cell necrosis and formation of crypt abscesses in the lamina propria of the caecal epithelium (Henderson et al. 1999). After colonisation, *Salmonella* Typhimurium resides in vacuoles inside intestinal epithelial and mononuclear cells in the lamina propria of caecal epithelium (Henderson et al. 1999). As a general concept, during *Salmonella* infection the pathogen and host interact at several points with varying results that are determined by the strength of the host immune response.

Salmonella encounters phagocytes either within the host gut (Rescigno et al. 2001) or during the invasion of the mucosal epithelium (Vazquez-Torres et al. 1999). Uptake of *Salmonella* by antigen-presenting cells (APCs) results in either the death of the survival of the APCs. The death of the APCs results in the release of pro-inflammatory cytokines (e.g. *IL1β*, *IL18*, *IFNγ*) and bacterial antigen uptake by other APCs for presentation to macrophages. Upon survival, APCs present the antigens to the major histocompatibility complex (MHC), but *Salmonella* has the potential to divert this process through *Salmonella* pathogenicity island 2 (SPI2) dependent pathways. Protected from host antibody detection, intracellular *Salmonella* utilises APCs for invasion and replication in internal organs (Salcedo et al. 2001). After activation by the antigen, helper T cells help in isotype class switching, cytokine, and humoral immune response, while cytokine production supports Th1 dependent T cell differentiation (Harris et al. 2000). Therefore, the outcome of *Salmonella* infection is the cumulative result of complex interactions between host and pathogen (Fields et al. 1986).

As a part of host immune system response, specialised epithelial cells produce antimicrobial peptides and stimulate the release of pro-inflammatory cytokines that attract immune cells such as macrophages, granulocytes and dendritic cells (Wick 2004). Chickens respond to *Salmonella enterica* infections by regulating their immune system in the gut (Berndt et al. 2007; Withanage et al. 2005). After *Salmonella* Typhimurium infection in chickens, the expressions of pro-inflammatory cytokines, such as *IL1β*, *TGFβ*, and *IFNγ* are activated in the caeca (Beal et al. 2004). The infection elicits a significant increase in granulocytes, $TCR1^+$ (γδ), $CD4^+$ and $CD8\alpha^+$ cell proliferation or infiltration into

caecal tissue from day 1 to day 9 post-infection (Berndt et al. 2007). Other molecules that are regulated in the caeca of chickens during *Salmonella* Typhimurium infection include *iNOS*, *IL12*, *IL18*, *LITAF* and *MIP1β* (Berndt et al. 2007). Overall, it takes approximately three weeks for chickens to clear *Salmonella* Typhimurium from the gut (Beal et al. 2006). However, the persistence of *Salmonella* Typhimurium in the gut for longer periods has been shown (McWhorter & Chousalkar 2018; Pande et al. 2016). The clearance of *Salmonella* Typhimurium from infected chickens is dependent on the age of the birds at infection (Barrow et al. 1987), whereas Th1 plays a crucial role in pathogen clearance from the gut (Withanage et al. 2005). The host immune system regulation in *Salmonella* infections in non-chicken models has been widely studied; however, it is not completely understood how the gut regulates its immune functions against *Salmonella*, as laying chickens act as asymptomatic carriers. Here, we performed RNA sequencing on caecal tissue to understand the chick caecal immune response to *Salmonella* Typhimurium challenge in a temporal manner. The primary objective of this study was to understand the basics of the mucosal immune system of the *Salmonella* Typhimurium challenged chicks that can be used as a platform for devising measures for *Salmonella* control in poultry.

2.2 Materials and methods

2.2.1 Animal ethics and rearing of *Salmonella* free layer chicks

The experimental work was approved by the Animal Ethics Committee (approval number S-2017-080) at the University of Adelaide. The experimental procedures complied with the guidelines specified in the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th edition (2013). Isa-Brown laying chicks (n = 18) were hatched and the meconium on the day of hatch and faeces from day-old until day 7 were tested fortnightly for *Salmonella* using a culture enrichment method. Briefly, 1 g of faeces was suspended in 9 mL of buffered peptone water (BPW; Thermo Fisher Scientific, Australia) and incubated overnight at 37°C. 100 µL of the incubated samples was enriched in 10 mL of Rappaport Vassiliadis soya peptone (RVS) broth (Thermo Fisher Scientific, Australia), incubated overnight at 42°C, and streaked on xylose lysine deoxycholate (XLD; Thermo Fisher Scientific, Australia) and Brilliance *Salmonella* agar (BSA; Thermo Fisher Scientific, Australia) media plates. The plates were incubated overnight at 37°C and were then read as positive or negative for *Salmonella* on the basis of characteristics colony shape and colour. Chicks were equally divided into *Salmonella* challenged and negative control groups. All the chicks were reared in pens as per the ISA General Management Guide 2009-10. Before placing the chicks, environmental swabs (Whirl-Pak Speci-Sponge Environmental Surface Sampling Bag) from rearing pens and rooms were collected for *Salmonella* testing using the enrichment method described earlier. Water and feed were regularly tested for the presence of *Salmonella* using this same enrichment method.

2.2.2 *Salmonella* Typhimurium inoculum preparation and chicks challenge

Salmonella Typhimurium phage type 9 previously isolated from a layer flock was streaked on XLD agar plates and a single colony was enriched in Luria-Bertani (LB; Thermo Fisher Scientific, Australia) broth by incubating the sample at 37°C in a shaking incubator for 12 hours. The *Salmonella* culture broth was centrifuged at 4200 ×*g* for 10 min, the bacterial pellet was resuspended in phosphate buffered saline (PBS) and the inoculum dose was prepared from its optical density (OD) reading at 600 nm in a spectrophotometer (Eppendorf BioPhotometer Plus). A week after the hatch, individual chicks in the infected group orally received 10^3 colony forming units (CFUs) of *Salmonella* Typhimurium, while chicks in the control group received PBS. The low inoculum dose was used to understand the host caecal immune system modulation by *Salmonella*. The faeces on days 3, 5 and 7 post-infection (p.i.) were processed for the isolation of *Salmonella* by the culture enrichment method as previously described.

2.2.3 Caecal tissue and contents collection

From each of the control and infected groups, three chicks on days 3, 5 and 7 p.i. were euthanised by cervical dislocation for the collection of caeca. The caecum was selected for studying intestinal immune response, as it plays a more important role in host defence compared with other segments of the gut. The caecal tissue was collected in RNALater and stored at -80°C until used for total ribonucleic acid (RNA) extraction. Previous studies show that for RNA sequencing analysis, a minimum of 3 biological replicates per treatment group are sufficient to obtain robust data (Li et al. 2018; Li et al. 2017; Sah et al. 2018). Caecal contents were collected for the quantification of *Salmonella* Typhimurium load through quantitative polymerase chain reaction (qPCR).

2.2.4 Caecal tissue RNA extraction and purification

TRIzol Reagent (Invitrogen, Australia) was used for total RNA extraction from the stored caecal tissue following a previously described method (Khan et al. 2019). The RNA clean-up was performed using an RNeasy Mini Kit (Qiagen, Australia) and the procedure included an RNase-Free DNase (Qiagen, Australia) digestion step. The total RNA was tested for integrity and purity in an Agilent 2200 TapeStation (Integrated Sciences, Australia) as per the manufacturer's protocol for an RNA Screen Tape. The RNA samples (n = 18) were processed by the Australian Genome Research Facility for RNA sequencing and basic bioinformatics analyses. For optimisation of the RNA sequencing data through qPCR, approximately 1 µg RNA from individual samples was reversely transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Australia) as per the manufacturer's protocol.

2.2.5 RNA sequencing of caecal tissue

2.2.5.1 cDNA synthesis

Illumina's TruSeq Stranded mRNA Library Prep (Cat. No. 20020594) kit was used for the processing of total RNA samples. The process included mRNA purification via poly-T oligo attached magnetic beads, fragmentation of mRNA into small pieces using divalent cations under appropriate temperature, copying of cleaved mRNA into first strand cDNA using random primers and reverse transcriptase and second strand cDNA synthesis using RNase H and DNA Polymerase I. During the second strand complementary DNA (cDNA) synthesis, strand specificity was achieved by replacing deoxythymidine triphosphate (dTTP) with deoxyuridine triphosphate (dUTP) in the Second Strand Marking Mix (SMM) as per protocol of the kit.

2.2.5.2 cDNA library preparation

cDNA libraries were prepared by DNA fragment end repair (blunt ending of DNA fragments), 3' adenylation of DNA fragments, sequence adaptor ligation (utilising T-A pairing of adapter and DNA fragments) and amplification of library via PCR. One library for each sample was constructed for sequencing – three samples per sampling for a total of three sampling time-points and two treatment groups. Sequencing of libraries using 100 bp single read was performed on an Illumina NovaSeq 6000 sequencing system. The primary sequence data were generated using the Illumina bcl2fastq 2.20.0.422 pipeline. The sequence files were generated in a standard FASTQ format. Next, the data were processed through RNA sequencing expression analysis workflow, which included alignment, transcript assembly, quantification and normalisation. Differential gene expression analysis was performed at the end and false discovery rate (FDR) was used for determining the level of significance between the treatment groups.

2.2.5.3 Bioinformatics analysis

2.2.5.3.1 Quality control and sequence data evaluation

Image analysis was performed in real time by the NovaSeq Control Software v1.6.0 and Real Time Analysis v3.4.4. Initial quality control of the RNA sequences was evaluated by the FastQC v0.11.5 (Andrews 2010). The per base sequence quality of all the samples was >96% bases above Q30. The raw reads were screened for the presence of any cross-species contamination, Illumina adaptor or overrepresented sequences, empty reads and low-quality sequences. Trim_galore v0.4.4 was used for removing Illumina adaptors and contaminated sequences (Martin 2011).

2.2.5.3.2 Reads mapping

The cleaned sequence reads were aligned against the reference genome of *Gallus gallus* v6 (Anonymous 2018). The Spliced Transcripts Alignment to a Reference (STAR) aligner v2.5.3a was used to map reads to the genomic sequences. The features summary table was created that provided an overall mapping rate with the genome and genome feature (feature=exons).

2.2.5.3.3 Transcriptome assembly

2.2.5.3.3.1 Raw gene counts and reference guided transcript assembly

The counts of reads mapping to each known gene were summarised at gene level using featureCounts v1.5.3 utility of the subread package (Liao et al. 2019). The raw gene counts or the DGE (digital gene expression) values were used in edgeR (v3.26.5) for computing differential gene expression in counts per million (CPM). The transcripts were assembled with the StringTie tool v1.3.3 (Pertea et al. 2015) utilising the reads alignment and reference annotation based assembly option (RABT). This option generated assembly for known and potentially novel transcripts.

2.2.5.3.3.2 Differential gene expression analysis

To identify differentially expressed genes (DEGs) between the *Salmonella* infected and control groups on days 3, 5 and 7 p.i., CPM values were used in edgeR (v3.26.5) of R packages v3.6.0. The edgeR is a package used to detect and quantify differential expression of digital gene expression data, that is, counts of reads mapped for each gene of a given organism. During the analysis, the default trimmed mean of M values (TMM) normalisation method of edgeR was used to normalise the counts between the treatment groups. A Generalised Linear Model (GLM) was used to quantify the DEGs between the *Salmonella* challenged and control groups on days 3, 5 and 7 p.i. For assessing the regulation of genes affected by *Salmonella* Typhimurium, the control group was used a reference control. Within the infected and control groups, data were also compared between days 3, 5 and 7 p.i. to understand the effects of the age of the chicks on gene regulation in the caecal tissue.

2.2.6 Functional annotation of significantly regulated DEGs

The DEGs (log_2 fold change > 1 or < -1; False Discovery Rate (FDR) < 0.05) from days 3, 5 and 7 p.i. were subjected to functional analysis using ClueGO v2.5.6 (Bindea et al. 2009) and CluePedia (Bindea et al. 2013) plugins in Cytoscape v3.7.2. Previously, ClueGO and CluePedia have been used for the functional analysis of RNA sequencing data (Khan et al. 2019). The DEGs were enriched for terms specific for *Gallus gallus* Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved in diseases. In the

ClueGO, the right-sided hypergeometric test with Benjamini-Hochberg was used for significant ($P < 0.05$) enrichment of pathway analysis. The association strength between the pathway terms was calculated using a corrected kappa score of 0.4. The created network showed the pathway terms as nodes and size of the nodes reflected the enrichment significance. A nested network of the significantly regulated genes for individual functional group terms was created in CluePedia. Efforts were also made to study the transcriptome regulation of *Salmonella* Typhimurium in the caeca of laying chicks temporally; however, the sequences generated were only 0.2% of the total sequences. Only 10% of the generated sequences matched to the reference genome of *Salmonella* Typhimurium, and this was considered insufficient for DEGs data analysis.

2.2.7 RNA sequencing results validation by qPCR

2.2.7.1 Primer design and validation

Using the option for exon-exon spanning in the *National Center for Biotechnology Information* (NCBI) software, primers for the candidate target and reference genes were designed (Table 2-1). The primer sequences were further confirmed for containing minimum secondary structure characteristics in Beacon Designer and NetPrimer software (Premier Biosoft). For candidate target genes, at least two genes that were significantly up- or down-regulated on days 3, 5 and 7 p.i. were selected. *TBP* and *YWHAZ* were used as reference genes due to their stable expression in different treatment conditions in chickens (Khan et al. 2017; Hassanpour et al. 2018). Using qPCR and gel electrophoresis with 2% agarose gel, primers were optimised for the amplification efficiency and target specificity as previously described (Khan et al. 2017).

Table 2-1 Primer sequence used for RNA sequencing data validation

Gene Symbol	Primer sequence (5'-3')	Exon junction (bp)	Fragment size (bp)	Annealing (°C)	NCBI Accession
IL8L2	F-CCTAACCATGAACGGCAAGC R-CGTCCTACCTTGCGACAGAG	138/139 (reverse primer)	76	60	NM_205498.1
MMP7	F- AGCCCTGATGTACCCTCTCT R-CATCACGTACTTGCGGATGC	822/823 (reverse primer)	107	60	NM_001006278.1
CALB1	F: CTTCGAGATCTGGCACCACT R: TGTTAAGTCCAAGCCTGCCT	261/262 (reverse primer)	118	60	NM_205513.1
FABP2	F- AAGCAATGGGCGTGAATGTG R- GTCGATGGTACGGAAGTTGC	67/68 (forward primer)	122	60	NM_001007923.1
ACOD1	F- ATGTGGGCAAAGACAATTACAGG R- TACAGAGGAGGCAGTCGGAA	68/69 (forward primer)	239	60	NM_001030821.1
EXFABP	F- GACTCTACAGCAGAAGCCGT R- TGTAGTTCCGCTCCCTAGCA	427/428 (forward primer)	78	60	NM_205422.1
IL4I1	F- CCGGCCATAAGGTCACGAT R- TGTCATCTGTCTGGCGGAAC	228/229 (forward primer)	183	60	NM_001099351.3
MYL1	F- GCCCGTCCGCTCTACTTTT R- AGGCCTCCTTGAAGTCATTGAT	80/81 (reverse primer)	75	60	NM_001044632.1
CYP1A1	F- GAGCTGGATCAGACCATCGG R- GCTTGAAGGAAGGAGGGTCC	1146/1147 (forward primer)	250	60	NM_205147.1

IL6	F- GCAGGACGAGATGTGCAAGA R- ATTTCTCCTCGTCGAAGCCG	295/296 (forward primer); 409/410 (reverse primer)	131	60	NM_204628.1
CCLL4	F- TCCTCAACTCCACTTGCTGTC R- TGTGTGCTTCTCCACGTTCA	73/74 (forward primer)	213	60	NM_001045831.1
NOS2	F- CCTGTACTGAAGGTGGCTATT R- AGGCCTGTGAGAGTGTGCAA	2649/2650 (forward primer)	66	60	NM_204961.1
ALB	F- GCAGCCATGAAGTGGGTAAC R- ACTCTTGTGCTCTGCATCACG	116/117 (reverse primer)	102	60	NM_205261.2
*TBP**	F- CCACGGTGAATCTTGGTTGC R- GCAGCAAAACGCTTGGGATT	534/535 (reverse primer)	88	60	NM_205103.1
*YWHAZ**	F- ACTTGACATTGTGGACATCGGA R- GAGGCAGACGGAAGTTGGAA	761/762 (forward primer)	94	60	NM_001031343.1

* Used as reference genes for relative expression data analysis.

Exon junctions represent the spanning of exons on respective genes sequence, necessary for primers to specifically amplify cDNA and not genomic DNA.

2.2.7.2 Quantitative PCR

Quantitative PCR was performed on all caecal tissue cDNA samples (n = 18) using the QuantiTect SYBR Green PCR Kit (Qiagen, Australia). PCR master mix (in a 20 µL reaction volume) was prepared as per the manufacturer's protocol, and 2 µL of the cDNA was added into each reaction well (in duplicate) using a robot (Corbett Research, Sydney, Australia). Thermocycling conditions in Rotor-Gene Q were: polymerase activation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30s and extension at 72°C for 30s. A melting step from a ramp of 50 to 99°C was included to assess the specificity of amplification. For relative expression data analysis, the expression levels of the candidate target genes were normalised against the reference genes *TBP* and *YWHAZ*.

2.2.8 *Salmonella* Typhimurium quantification from caecal contents

Total DNA was extracted from approximately 180 mg of caecal contents using the QIAamp DNA Stool Mini Kit (Qiagen, Australia). To get quality DNA, a bead bashing step was added to the extraction method (Khan & Chousalkar 2020). The quality and purity of DNA were assessed in a Nanodrop-1000 (Thermo Fisher, Australia). For DNA copy number calculation, a standard curve was constructed (R^2 = 0.99234; amplification efficiency = 1.01) from different dilutions (10^{-2} to 10^{-9}) of a purified qPCR product (using QIAquick PCR Purification Kit) of *Salmonella* Typhimurium DNA fragment. A primer sequence (F: 5'-TTTACCTCAATGGCGGAACC-'3; R: 5'-CCCAAAAGCTGGGTTAGCA-'3; 303 bp; Accession Number: AAL23311.1) was optimised for target specificity and efficiency for the amplification of *Salmonella* Typhimurium LT2 putative cytoplasmic protein gene (STM4493) fragment. qPCR was performed as per the protocol of QuantiTect SYBR Green PCR Kit (Qiagen, Australia) in a Rotor-Gene Q thermal cycler. DNA copy number in the standard curve dilutions was calculated based on the number of different nucleotides in the DNA fragment sequence, nucleotide molecular weight and the purified qPCR product DNA concentration in ng/µL. To quantify *Salmonella*, DNA from both the *Salmonella* challenged and negative control groups was amplified by qPCR along with standard curve dilutions. The known DNA copy number of the standard curve dilutions was used to calculate the

genomic DNA copy number of *Salmonella* in the samples. The $\log_{10}$ DNA copy number per gram of caecal contents was expressed as *Salmonella* Typhimurium mean load/g of caecal contents. All of the qPCR-amplified samples were run on 2% agarose gel for amplicon size confirmation.

2.3 Statistical analysis

For the validation of RNA sequencing data, the relative expression of candidate target genes was calculated by $2^{\wedge-\Delta\Delta Ct}$ method. The Ct values of the target genes were normalised against the reference genes *YWHAZ* and *TBP* (Khan et al. 2017). A regression analysis was performed in StatView (v 5.0.1.0) to calculate the correlation between the RNA sequencing and qPCR data. The qPCR *Salmonella* Typhimurium genomic DNA copy number data were expressed as $\log_{10}$ DNA copy number/g of caecal contents, and analysed in IBM SPSS and Amos 26.0 by one-way analysis of variance (ANOVA). Level of significance was determined by Tukey's test at $P < 0.05$.

2.4 Results

2.4.1 Gross pathological lesions in caeca and *Salmonella* Typhimurium load in caecal contents

In this study, clinical signs were not observed in the *Salmonella* Typhimurium infected chicks. All birds from the negative control group were *Salmonella* negative throughout the experiment. From the infected group, the faecal samples collected on days 3, 5 and 7 p.i. were positive for *Salmonella* Typhimurium (Appendix Table 2-1). The post mortem findings revealed partially filled caeca (in 33.33% of chicks on each of the day 3 and 5 p.i.) and the presence of a mucous plug (in 33.33% of chicks on day 3 p.i.). On day 7 p.i, 66.66% of the chicks had partially filled caeca.

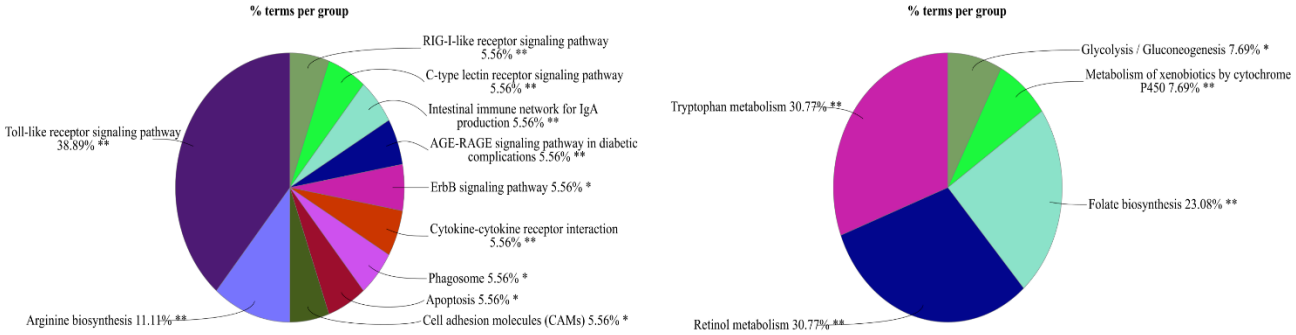
Using qPCR assay, all the caecal contents samples from the *Salmonella* negative control group tested negative for *Salmonella* Typhimurium. The mean load (expressed as $\log_{10}$ DNA copy number/g of caecal contents) of *Salmonella* Typhimurium on days 3, 5 and 7 p.i. was 9.253, 9.586 and 9.553, respectively (Appendix Figure 2-1). The *Salmonella* Typhimurium load was not significantly ($P = 0.7320$) different between days 3, 5 and 7 p.i. (Appendix Figure 2-1). All of the qPCR amplified products from the *Salmonella* challenged groups showed the expected band size (303 bp) on 2% agarose gel electrophoresis (Appendix Figure 2-2).

2.4.2 Transcriptomic analysis of caecal tissue

All the RNA samples showed distinct 18S and 28S bands with an average RNA integrity number (RIN) of > 8.9 (Appendix Figure 2-3). The transcriptome data feature summary showed that the percentage of the clean reads mapped to *the Gallus gallus* genome was ≥ 95% (Appendix Table 2-2). Normalised reads in a multi-dimensional scaling plot indicated that there was a significant effect of *Salmonella* infection on the expressed genes in the caecal tissue, at different time-points. A total of 5584 gene transcripts were assessed for differential gene expression after filtering. There were no significantly (FDR > 0.05; $\log_2$ fold change > 1 or < -1) regulated differently expressed genes (DEGs) in the control group between days 3, 5 and 7 p.i. In the *Salmonella* Typhimurium challenged group, 23 genes were significantly regulated between days 3 and 5 p.i., 184 genes between days 3 and 7 p.i., and 89 genes between days 5 and 7 p.i. Most of the significantly regulated genes were from pathways involved in immune system regulation.

2.4.2.1 Genes differentially expressed following infection with *Salmonella* Typhimurium and their functional annotation

Compared to the control group, on day 3 p.i, 321 genes were up-regulated (FDR < 0.05; log_2 fold change > 1) and 112 genes were down-regulated (FDR < 0.05; log_2 fold change < -1) in the *Salmonella* Typhimurium infected group. Most of the up-regulated genes were from a broad list of genes that play direct or indirect roles in immune system regulation. Most of the down-regulated genes observed in this experiment are involved in basic cell functional activities and metabolism. Functional pathway analysis of the up-regulated DEGs was grouped into 11 Kyoto Encyclopedia of Genes and Genomes (KEGG) disease-associated pathway terms (Figure 2-1A). Amongst these, the six major pathway terms were Toll-like receptor signalling pathway, arginine biosynthesis, cell adhesion molecules, apoptosis, phagosome and cytokine-cytokine receptor interaction (Figure 2-1A). The down-regulated genes on day 3 p.i. mapped into pathway terms tryptophan metabolism, retinol metabolism, folate biosynthesis, metabolism of xenobiotics by cytochrome P450 and glycolysis/gluconeogenesis (Figure 2-1B).



A. Up-regulated at day 3 p.i. **B.** Down-regulated at day 3 p.i.

Figure 2-1 KEGG pathway functional group terms associated with significantly (FDR < 0.05; log2 fold change > 1 or < -1) up- or down- regulated DEGs in caeca of *Salmonella* Typhimurium challenged chicks

A Terms associated with the up-regulated genes on day 3 p.i.
B Terms associated with the down-regulated DEGs on day 3 p.i.
The DEGs list was obtained by comparing the *Salmonella* Typhimurium challenged samples with the control group.

The annotated genes to the individual pathway terms and their associations that up-regulated on day 3 p.i. are presented in Figure 2-2. The annotated genes to the individual pathway terms and their associations that down-regulated on day 3 p.i. are shown in Appendix Figure 2-4.

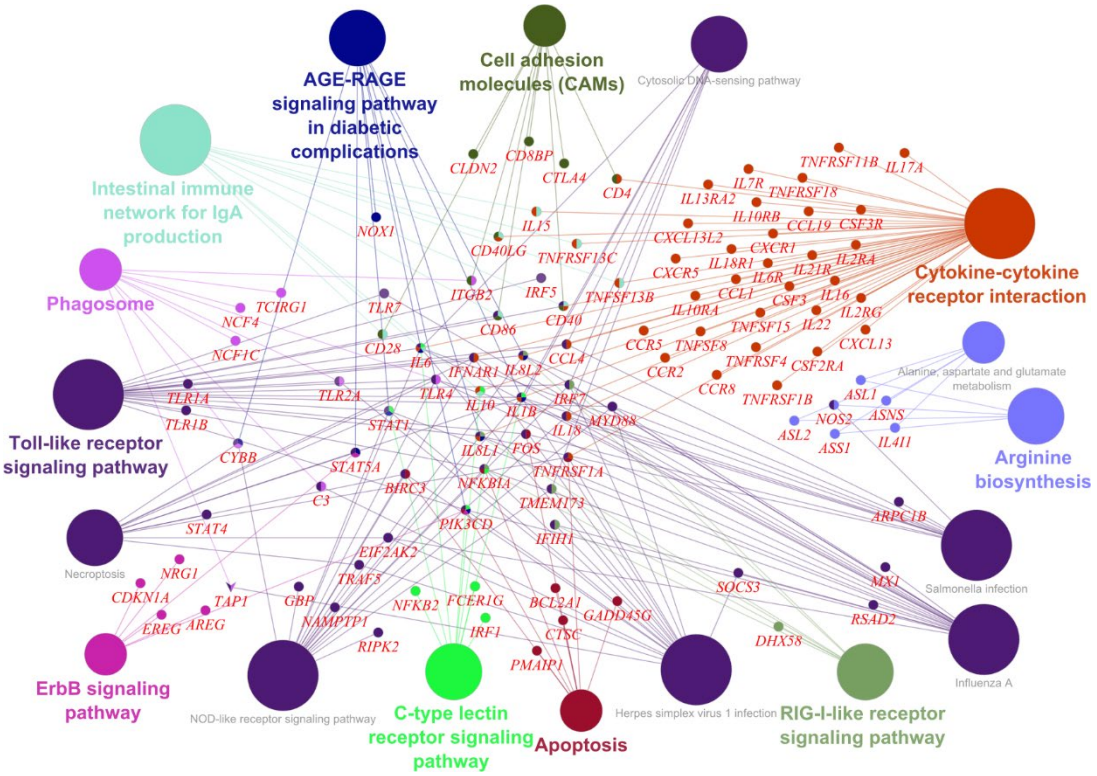
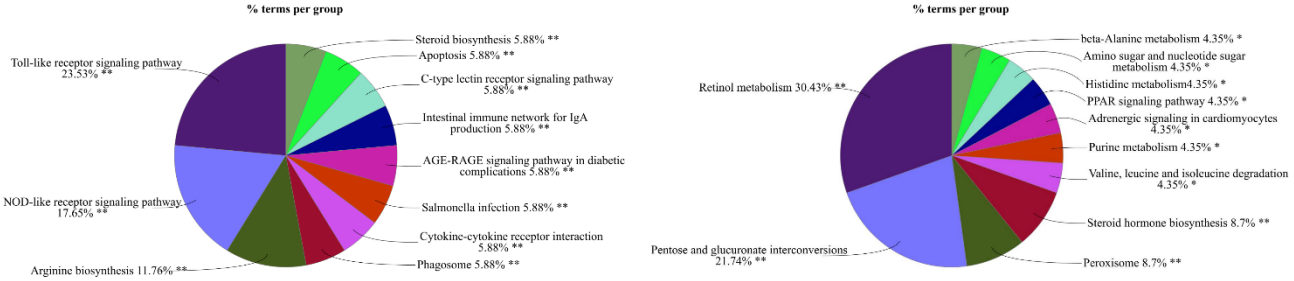


Figure 2-2 Enriched KEGG pathway terms and their associated genes obtained from the mapping of significantly up-regulated genes on day 3 post-infection

In ClueGO, pathways only significantly (p-value < 0.05) enriched were visualised.
Size of the nodes indicates the number of associated genes affected by *Salmonella* Typhimurium challenge.

Compared to the control group, on day 5 p.i., 371 genes were significantly up-regulated and 249 genes were down-regulated in the infected group. Functional pathway analysis of the up-regulated genes was grouped into 11 pathway terms (Figure 2.3A). The six major pathway terms were Toll-like receptor signalling pathway, NOD-like receptor signalling pathway, arginine biosynthesis, phagosome, cytokine-cytokine receptor interaction and *Salmonella* infection (Figure 2-3A). The down-regulated genes on day 5 p.i. mapped into 11 pathway terms that included retinol metabolism, pentose and glucuronate interconversions, peroxisome, steroid hormone biosynthesis, and valine, leucine and isoleucine degradation (Figure 2-3B).



A. Up-regulated at day 5 p.i. **B.** Down-regulated at day 5 p.i.

Figure 2-3 KEGG pathway functional group terms associated with significantly (FDR < 0.05; log2 fold change > 1 or < -1) up- or down- regulated DEGs in caeca of *Salmonella* Typhimurium challenged chicks

A Terms associated with the up-regulated genes on day 5 p.i.
B Terms associated with the down-regulated DEGs on day 5 p.i.
The DEGs list was obtained by comparing the *Salmonella* Typhimurium challenged samples with the control group.

The annotated genes to the individual pathway terms and their associations that up-regulated on day 5 p.i. are shown in Figure 2-4. The annotated genes to the individual functional group terms and their associations that down-regulated on day 5 p.i. are shown in Appendix Figure 2-5.

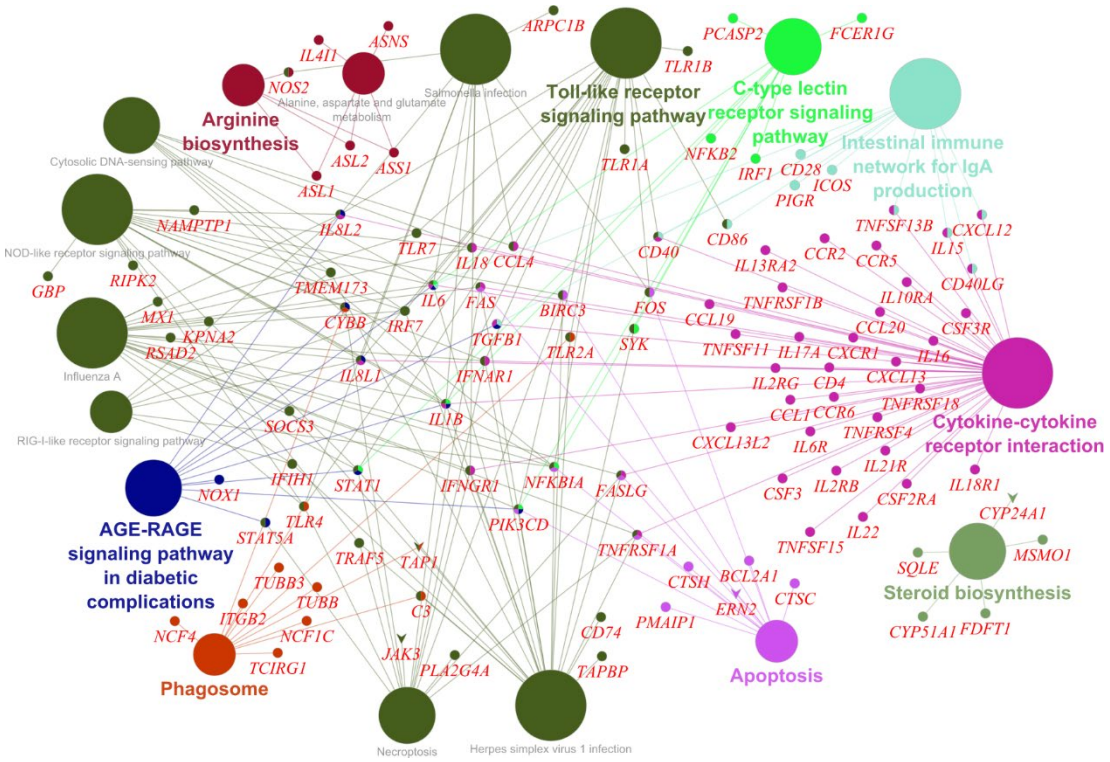
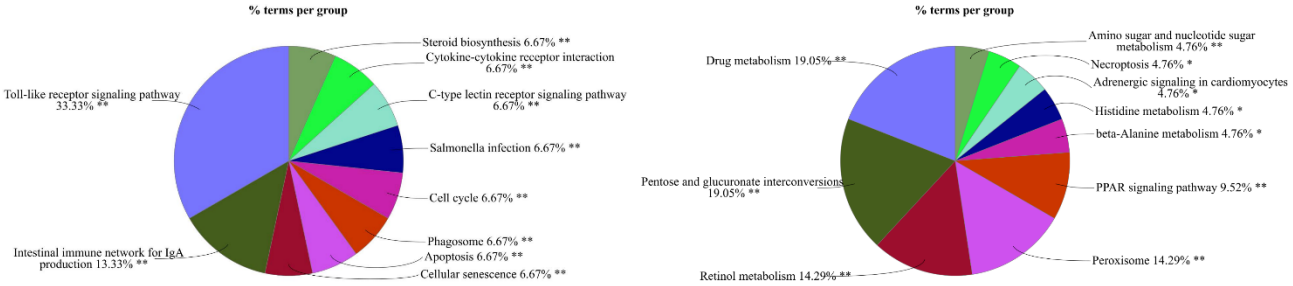


Figure 2-4 Enriched KEGG pathway terms and their associated genes obtained from the mapping of significantly up-regulated genes on day 5 post-infection

In ClueGO, pathways only significantly (p-value < 0.05) enriched were visualised.
Size of the nodes indicates the number of associated genes affected by *Salmonella* Typhimurium challenge.

Compared with the control group, on day 7 p.i., 632 genes were significantly up-regulated and 325 genes were down-regulated in the *Salmonella* Typhimurium infected group. Functional pathway analysis of the up-regulated genes was grouped into 10 pathway terms that included the Toll-like receptor signalling pathway, the intestinal immune network for IgA production, apoptosis, phagosome, cell cycle and *Salmonella* infection (Figure 2-5A). The down-regulated genes on day 7 p.i. mapped into 10 pathway terms that included drug metabolism, pentose and glucuronate interconversions, retinol metabolism, peroxisome and PPAR signalling pathway (Figure 2-5B).



A. Up-regulated at day 7 p.i. **B.** Down-regulated at day 3 p.i.

Figure 2-5 KEGG pathway functional group terms associated with significantly (FDR < 0.05; log2 fold change > 1 or < -1) up- or down- regulated DEGs in caeca of *Salmonella* Typhimurium challenged chicks

A Terms associated with the up-regulated genes on day 7 p.i.

B Terms associated with the down-regulated DEGs on day 7 p.i.

The DEGs list was obtained by comparing the *Salmonella* Typhimurium challenged samples with the control group.

The up-regulated genes on day 7 p.i. annotated to individual pathway terms and/or associated with other pathway terms showed that the immune system was modulated by the interactions of multiple genes (Figure 2-6).

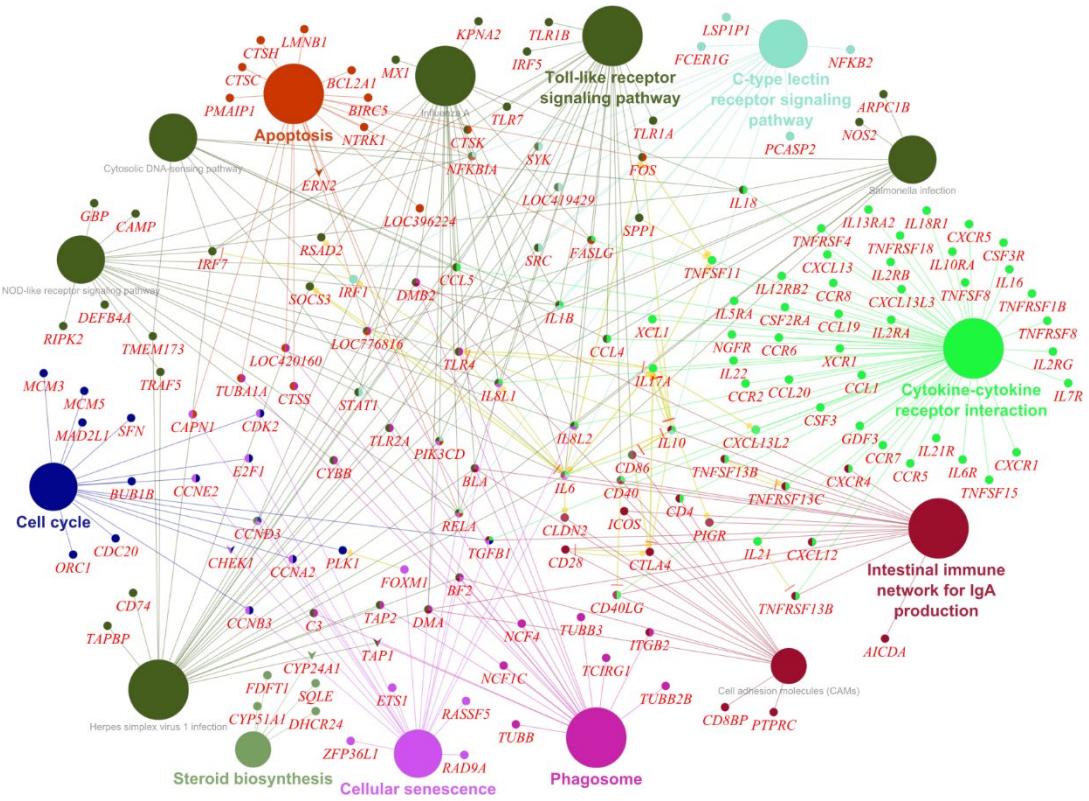


Figure 2-6 Enriched KEGG pathway terms and their associated genes obtained from the mapping of significantly up-regulated genes on day 7 post-infection

In ClueGO, pathways only significantly (p-value < 0.05) enriched were visualised.

Size of the nodes indicates the number of associated genes affected by *Salmonella* Typhimurium challenge.

Yellow line with circular end between genes shows association, while red bar at the end of yellow line indicates the inhibition of one gene by another.

Nested network analysis of the pathway terms cytokine-cytokine receptor interaction (Figure 2-7A), Toll-like receptor signalling pathway (Figure 2-7B), the intestinal immune network for IgA production (Figure 2-7C), *Salmonella* infection (Figure 2-7D), cellular senescence (Figure 2-7E) and C-type lectin receptor signalling pathway showed a strong association of *IL6* with other genes. The annotated genes

to the individual pathway terms and their associations that down-regulated on day 7 p.i. are shown in Appendix Figure 2-6.

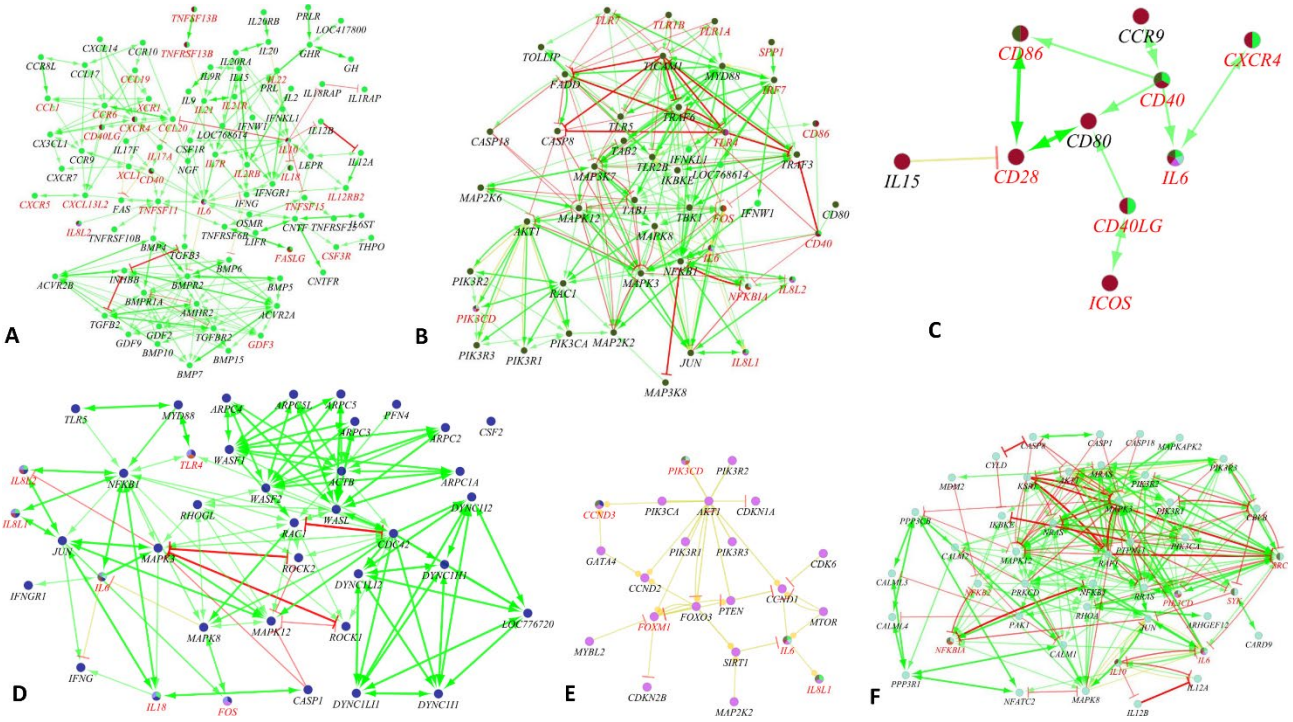


Figure 2-7 Nested network of individual pathway term based on the significantly up-regulated genes (red colour font) on day 7 p.i. and all associated genes (black colour font) with the term

A Cytokine-cytokine receptor interactions.
B Toll-like receptor signalling pathway.
C Intestinal immune network for IgA production.
D *Salmonella* infection.
E Apoptosis.
F C-type receptor signalling pathway.
The nested network was created in CluePedia for the association of activation (green line with arrow at the end), expression (yellow line with circle at the end) and inhibition (red line with bar at the end) of the significantly up-regulated genes with all the associated genes in individual functional group term.
Within each pathway term, colour fragments of the node represent the percentage of the individual genes involved in more than one pathway as shown in Figure 2-6.
Not all of the significantly up-regulated term associated with the individual function group term were mapped in the nested network visualisation in CluePedia software.

2.4.2.2 Consistently regulated genes across days 3, 5 and 7 post-infection

A total of 214 genes were consistently up-regulated (FDR < 0.05; $\log_2$ fold change > 1) on days 3, 5 and 7 p.i. Thirty seven genes were up-regulated both on day 3 and day 7 but did not up-regulate on day 5 p.i. Out of the 37 genes, *IRF5*, *TNFRSF13C*, *TNFSF8*, *CXCR5*, *IL6*, *LRP8*, *IRF4*, *CD79B*, *STAT4*, *CCR8*, *CHIR-B4*, *IFITM5*, *IL10*, *IL2RA*, *IL7R* and *IRF5* are known to be involved in immune system function. A total of 94 genes were up-regulated both on days 5 and 7 p.i. but did not up-regulate on day 3 p.i. The genes that were up-regulated on day 5 and 7 p.i. and involved in immune system regulation were *CCL20*, *CCR6*, *CD14*, *CD5*, *CD7*, *CD74*, *CXCL12*, *FASLG*, *ICOS*, *GNLY*, *IL2RB*, *JAK3*, *MST1R*, and *TNFSF11*. A total of 287 genes were up-regulated on day 7 p.i. but these genes were not up-regulated at either day 3 or day 5 p.i. Some of the genes that were up-regulated only on day 7 p.i. and are known to be involved in immune system regulation included *AICDA*, *AvBD1*, *AvBD2*, *AvBD6*, *AvBD7*, *BF2*, *BLA*, *BLEC1*, *BLEC2*, *CCL5*, *CCR7*, *CD3D*, *CISH*, *CXCR4*, *DMB1*, *IGLL1*, *IL12RB2*, *IL21*, *IL5RA*, *ISLR2*, *LEF1*, *LSP1*, *LYZ*, *SIVA1*, *TNFRSF13B*, *TNFRSF8*, *TP63*, *XLC1*, *XCR1* and *CXCL13L3*.

A total of 75 genes were consistently down-regulated (FDR < 0.05; log_2 fold change < -1) on days 3, 5 and 7 p.i. Eleven genes were down-regulated on days 3 and 7 p.i., while 91 genes were down-regulated on days 5 and 7 p.i. The down-regulated genes list both on days 5 and 7 p.i. contained *TLR3*, *ICOSLG*, and *MAPKAPK5* that are involved in immune system regulation. A total of 148 genes were down-regulated on day 7 p.i. but did not change in regulation on days 5 and 3 p.i. On day 7 p.i., the down-regulated genes involved in immune system regulation were *CD200R1*, *CD19*, *CXCL14*, *LY86*, *TLR5* and *TPD52L1*. Overall, the down-regulated genes were involved in activities relevant to cell nutrient synthesis and metabolism functions.

2.4.2.3 Genes associated with pathway term Salmonella infection

Functional analysis of the significantly up-regulated genes in KEGG resulted in the association of genes with the pathway term *Salmonella* infection. Most of the associated genes were up-regulated on days 3, 5 or 7 p.i. in the *Salmonella* Typhimurium challenged group (Table 2-2). *MYD88* was up-regulated only on day 3, while *RELA* and *ACTC2L* were up-regulated on day 7 p.i.

Table 2-2 Significantly up-regulated genes annotated to KEGG pathway term *Salmonella* infection

Gene symbol	Day post-infection						Location on chromosome (position in bp)
	3		5		7		
	Fold change	FDR	Fold change	FDR	Fold change	FDR	
ARPC1B	2.324	0.0363	3.087	0.0192	3.666	0.0021	14: 4,766,374-4,772,041 forward strand
CCL4	6.350	0.0008	6.966	0.0017	5.384	0.0039	19: 539,445-570,383 reverse strand
FOS	1.639	0.0291	1.948	0.0214	1.742	0.0105	5: 38,430,793-38,433,702 forward strand
IFNGR1	-	-	1.059	0.0085	-	-	3: 54,895,190-54,914,161 forward strand
IL18	4.255	0.0077	3.983	0.0198	3.535	0.0082	24: 6,169,486-6,173,722 forward strand
IL1B	5.095	0.0151	4.626	0.0361	4.619	0.0108	22: 5,171,645-5,173,295 reverse strand
IL6	10.636	0.0427	9.163	0.0100	7.931	0.0045	2: 30,863,310-30,866,004 forward strand
IL8L1	4.415	0.0002	3.547	0.0016	3.288	0.0005	4: 50,925,077-50,928,369 forward strand
IL8L2	5.394	0.0000	5.452	0.0001	5.282	0.0000	4: 50,937,659-50,940,883 forward strand
MYD88	1.285	0.0058	-	-	-	-	2: 4,892,458-4,905,115 forward strand
NOS2	5.650	0.0008	6.837	0.0014	5.731	0.0005	19: 9,342,031-9,361,245 reverse strand
TLR4	2.192	0.0114	2.370	0.0135	1.865	0.0108	17: 3,938,812-3,944,462 forward strand
RELA	-	-	-	-	2.020	0.0059	33: 6,490,052-6,498,311 reverse strand
ACTC2L	-	-	-	-	1.074	0.0289	18: 9,187,889-9,190,934 forward strand

The RNA sequencing data are expressed in log_2 fold change.

For fold change calculation, the *Salmonella* Typhimurium challenged groups sampled on days 3, 5 and 7 post-infection were compared with their respective negative control groups.

Empty cells indicate that the respective genes were not significantly up-regulated.

2.4.3 Quantitative PCR validation of RNA sequencing data

The primers used for the amplification of the selected genes fragments that were either up- or down-regulated at different time-points were highly specific (Appendix Figure 2-7). The gene amplification efficiency (%) ranged from 0.89 to 1.20. Regression analysis of the qPCR versus the RNA sequencing data showed a positive correlation ($P < 0.0001$; $R^2 = 0.720$) (Table 2-3).

Table 2-3 Comparative gene expression data of RNA sequencing and qPCR

Gene	Mean log_2 fold change post *Salmonella* Typhimurium challenge						Linear regression
	RNA sequencing			qPCR			
	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7	
IL8L2	5.394	3.548	5.283	5.341	5.143	5.667	
MMP7	11.155	14.143	11.503	3.049	2.721	2.943	
ACOD1	6.978	8.224	7.346	0.089	0.728	1.257	
EXFABP	8.256	8.051	7.242	6.908	7.438	6.210	
IL4I1	8.431	8.011	7.487	7.401	5.893	6.385	
IL6	10.637	9.163	7.931	4.786	7.786	2.277	$R^2 = 0.720$;
CCLL4	10.561	6.966	5.385	3.618	3.989	1.645	$P < 0.0001$
NOS2	5.651	6.838	5.731	3.163	3.434	3.453	
ALB	-4.349	-0.795	-0.102	-3.923	-5.151	-7.733	
CALB1	-2.397	-3.971	-5.193	-9.418	-8.018	-8.161	
FABP2	-2.248	-4.526	-5.959	-4.003	-5.229	-6.372	
MYL1	-5.073	-10.069	-11.524	-4.058	-6.524	-8.797	

For linear regression analysis between the RNA sequencing and qPCR data, the log_2 fold change values were analysed in StatView software.

The log_2 fold change was calculated by comparing the *Salmonella* Typhimurium infected samples with the control group.

qPCR was performed on caecal tissue cDNA samples, the mean relative expression was calculated by $2^{-\Delta\Delta Ct}$ method and the values were converted into log_2 fold change.

For qPCR data analysis, *YWHAZ* and *TBP* were used as reference genes.

Plus (+) and minus (-) signs show log_2 fold change values for the up- or down- regulated genes, respectively.

2.5 Discussion

The primary objective of this study was to understand the regulation of caecal mucosal immune response during *Salmonella* Typhimurium infection in chicks. Validation results of the qPCR assay indicated that the data obtained through RNA sequencing were robust for scientific interpretations. The *Salmonella* Typhimurium load quantified by qPCR was comparable to the load of *Salmonella* obtained through culture method from caecal tissue containing caecal contents (Khan & Chousalkar 2020). Almost all of the terms in the KEGG pathway analysis of the up-regulated genes were highly relevant to the terms associated with the immune system, indicating the role of *Salmonella* Typhimurium in activating the host immune system response in the caeca. These findings were also supported by the pathological lesions found in the caeca of infected chicks.

The down-regulated genes mapped to pathways involved in cellular functions and nutrient metabolism indicated that the *Salmonella* infection disrupted normal cell functions. The non-significant difference in the regulation of genes in the control group between days 3, 5 and 7 p.i. showed that the regulation of genes in the infected group was mainly due to the *Salmonella* colonisation/invasion in the caecal tissue. It is not clear why chickens do not exhibit clinical signs similar to human or murine models during *Salmonella* Typhimurium infection. However, *Salmonella* resistance associated quantitative trait loci (QTLs) on chromosomes 2, 3, 5, 12 and 25 were linked to the severity of infection in disease resistant and susceptible chickens (Fife et al. 2009). In chickens,

SAL1 loci on chromosome 5 contain 14 genes that confer resistance to *Salmonella* infection. In the current study, genes, such as *SLC11A1* (*NRAMP1*), *SIVA1* and *TNC* that have been implicated in *Salmonella* resistance (Hu et al. 1997*),* were not significantly up-regulated except for *SIVA1* on day 7 p.i. This finding suggests that other genes may be involved in the gut mucosal immune response to *Salmonella* infection in chickens. This hypothesis was also supported by the 208 genes consistently up-regulated on days 3, 5 and 7 p.i. Therefore, the immune response developed against *Salmonella* Typhimurium is a cumulative process involving multiple immune system pathways.

While the up-regulated genes on days 3, 5 and 7 p.i. annotated to KEGG pathway terms remained consistent, the number of genes associated with individual pathway terms varied considerably. This shows that some of the genes either down-regulated or up-regulated clustered to specific pathway terms on days 3, 5 or 7 p.i. The major pathway term Toll-like receptor signalling pathway on days 3, 5 and 7 p.i. shows the role of TLRs in the caecal mucosal immune system activation against *Salmonella* Typhimurium in young chicks. Amongst TLRs, *TLR4* recognises the presence of *Salmonella* lipopolysaccharide leading to the activation of macrophages, expression of various cytokines, chemokine and antibacterial peptides genes (Kogut et al. 2005). In the current study, the consistent up-regulation of *TLR4* implicated *Salmonella* activated immune response. The significant up-regulation of *CCL4* on days 3, 5 and 7 p.i. suggests that it could be a potential marker for quantifying caecal immune response to *Salmonella* Typhimurium. *CCL4* acts as an effector molecule in the regulation of immune response against *Salmonella* infection (Kogut et al. 2012). Based on the nested network analysis of individual pathway terms, it can be concluded that the immune response was an accumulative process of the involvement of multiple genes. The role of *IL6* was important, as it showed a wider association with other genes in the respective pathway terms.

The non-consistent up-regulation of *CCL5*, *CTSK*, *MYD88* and *SPP1* on days 3, 5 and 7 p.i. indicated their specific role in the regulation of immune response during *Salmonella* Typhimurium infection. Secreted phosphoprotein-1 (*SPP1*) also known as osteopontin (*OPN*) or early T-lymphocyte activation 1 (*ETA-1*) is a cytokine and macrophage chemoattractant that was found to be upregulated in the gut of *Salmonella* Enteritidis infected chickens (Luan et al. 2012). In the current study, the up-regulation of *ETA-1* on day 7 showed its role in the activation of the immune response at later stages of infection. *TLR15* was not annotated to any KEGG pathway term; however, its consistent up-regulation on days 3, 5 and 7 p.i. indicates its role in immunity against *Salmonella*. *TLR15* was associated with the caecal immune response against *Salmonella* Typhimurium (Higgs et al. 2006).

Several genes were annotated to the pathway term intestinal immune network for IgA production. The up-regulation of *AICDA* (*AID*) only on day 7 p.i. shows its role in the immunity in later stages of *Salmonella* Typhimurium infection. The expression of *AICDA* is induced by activated B-cell *CD40* signalling, and is further enhanced by IL4 secreted from CD4+ T cells or TLR activating bacterial molecules (Xu et al. 2007). In the current study, *CD40* and various TLRs were up-regulated. These TLRs may play a role in the regulation of *AICDA* for IgA production in the chicken caecal tissue. The role of *AICDA* in the regulation of IgA can be further supported by the up-regulation of *BLA* on day 7 p.i., *PIGR* on days 5 and 7 p.i. and *MHCDMA* on days 5 and 7 p.i.

Most of the genes annotated to the KEGG pathway term phagosome on days 3 and 5 p.i. were *C3*, *CYBB*, *ITGB2*, *NCF1*, *NCF4*, *TAP1*, *TCIRG1*, *TLR2A* and *TLR4* in addition to *TUBB* and *TUBB*3 up-regulated on day 5 p.i. only. On day 7 p.i., in addition to these genes, genes such as *ACTC2L*, *BF2*, *BLA*, *CTSS*, *DMB2*, *LOC420160*, *MHCDMA*, *TAP2*, *TUBA1A* and *TUBB2B* were also annotated to phagosome. This shows that the function of phagocytic cells increased with number of days post-infection. *MHCDMA* and *DMB2* are shown to be part of MHC class II system (Parker & Kaufman 2017). *CD28* is expressed on NK and T cells and provides costimulatory signals for T cells (Mittrücker et al. 1999). In this study,

the up-regulation of *CD28* could be attributed to the stimulation of T cells in the caeca of *Salmonella* Typhimurium challenged chicks.

Overall, 59 genes annotated to the KEGG pathway term cytokine-cytokine receptor interaction. This pathway involve the regulation of genes from CC subfamily, CX subfamily, C subfamily, the class I and II helicase cytokines, interferon family, TNF family and TGF-β family. Amongst them, *CCL1* is one of the several chemokine genes involved in immunoregulatory and inflammatory processes. *CCL20* interacts with *CCR6* while *CCL19* interacts with *CCR7* and, therefore, *CCL20* plays a vital role in the regulation of mucosal immune response through the recruitment of lymphocytes and dendritic cells to the sites of inflammation (Schutyser et al. 2003). In the current study, the up-regulations of *CCL1, CCL19*, *CCL20* and *CCR7* demonstrate their role in cytokine-cytokine receptor interaction pathway activated by *Salmonella* Typhimurium challenge. *CCR6* is a chemokine receptor that mediates the homing of dendritic and CD4+ T cells to the gut mucosal lymphoid tissue as shown in human and mouse studies (Ito et al. 2011). Flagellin of *Salmonella* induces the secretion of the inflammatory chemokine *CCL20*, a vital ligand for *CCR6* (Sierro et al. 2001). Caspase genes involved in apoptosis produced mixed results as only *PCASP2* was significantly up-regulated on days 3, 5 and 7 p.i. *CASP2* and *CASP18* up-regulated only on day 7 p.i. indicating their role in apoptosis at the later stage of *Salmonella* Typhimurium infection.

Genes that were significantly and consistently up-regulated on days 3, 5 and 7 p.i., but did not annotate to any known KEGG term during pathway analysis, included *MMP7*, *MMP9*, *EXFABP*, *AVD*, *LYG2*, *ACOD1*, *IFNγ*, *OASL*, *CD72AG*, *TLR15*, *CD3E*, *EOMES*, *GNLY* (*TLA519*), *FUT4, IRF4, SASH3* and *RSFR*. These genes may be implicated in the regulation of the caecal immune system triggered by *Salmonella* Typhimurium infection. Both the *MMP7* and *MMP9* are expressed by different cells including macrophages and are involved in immune system regulation (Parks et al. 2004). We suggest future investigation into the mechanistic role of *MMP7, MMP9* and *TLR15* in chicken resistance to *Salmonella* infection. The up-regulation of *MMP7* in chicken caeca has been associated with *Salmonella* Enteritidis infection (Rychlik et al. 2014). *EXFABP, LYG2* and *AVD* are expressed in the caeca of chickens in response to *Salmonella* Enteritidis infection (Matulova et al. 2012). In the current study, the up-regulation of *EXFABP, LYG2* and *AVD* on days 3, 5 and 7 p.i. confirmed their important roles in the modulation of the caecal immune system in *Salmonella* Typhimurium infection. *CD3E* is involved in early T lymphocytes and NK cells development (Wang et al. 1994). In the current study, the significant up-regulation of *CD3E* highlights its role in caecal immune system modulated by *Salmonella* Typhimurium challenge. The up-regulation of *EOMES* shows its role in the differentiation of effector CD8+ T cells, which are involved in defence against pathogens. RSFR protein possesses antibacterial activity and the gene *RSFR* has been implicated in chicken caecal immune response triggered by *Salmonella* Enteritidis (Ma et al. 2014). The role of *GNLY* and *FUT4* in the regulation of the chicken immune system is not clear and further studies are essential to investigate their specific roles during *Salmonella* Typhimurium infection.

Intestinal epithelial cells are held together through the regulation of junction proteins that include junctional adherent molecules, occludins, claudins and cadherins. Amongst the tight junction proteins, mucins cover epithelial surfaces of the gut and form a protective layer. The protective function is achieved by both the secreted and membrane-bound mucins. Secreted mucins are controlled by *MUC2*, *MUC5ac*, *MUC5b* and *MUC6*. In the current study, the significant up-regulation of *MUC2* on days 5 and 7 p.i. shows its role in maintaining the function of tight junctions during *Salmonella* Typhimurium infection. Amongst the claudins, the up-regulation of *CLDN2* on days 3 and 7 p.i. is important in *Salmonella* infection, as the other claudins were not significantly regulated. The down-regulation of *GJB6* on day 7 p.i. indicates that *Salmonella* was still affecting the function of GJB6 protein involved in the regulation of gap junction for nutrient transport. The function of *CAMP* ranges from antimicrobial activities to inflammatory response regulation and cell chemotaxis, and its up-

regulation only on day 7 p.i. confirms its role in multiple pathways. The down-regulation of *CALB1* on day 7 p.i. shows that calcium absorption from the intestinal epithelium was disrupted by the *Salmonella* Typhimurium infection.

In the current study, Toll-like receptor signalling pathway seemed to be the major immune system regulator. Specific TLRs dependent vaccine adjuvants against *Salmonella* may improve the efficacy of *Salmonella* vaccine, as TLR adjuvants can enhance host immune response (Steinhagen et al. 2011). There were more genes regulated by *Salmonella* Typhimurium on day 7 p.i. compared to days 3 and 5 p.i. The current study confirmed that 10^3 CFU of *Salmonella* Typhimurium was sufficient to cause gross pathological lesions in caeca and modulate the caecal immune response in a week old chicks. The load of *Salmonella* Typhimurium on rearing farms depends on individual farm management. Hence, further studies are required to determine a dose dependent relationship with the severity of clinical disease and immune response in laying chickens. In conclusion, the caecal mucosal immune response of chicks to *Salmonella* Typhimurium infection was modulated by multiple pathways that regulated the genes involved in the Toll-like signalling pathway, cytokine, chemokine production and IgA production. Future research should focus on adding TLR adjuvants to improve the efficacy of *Salmonella* vaccine for a robust host immune response.

2.6 References

ANDREWS, S. 2010. *FastQC: a quality control tool for high throughput sequence data.* http://www.bioinformatics.babraham.ac.uk/projects/fastqc [Online]. [Accessed].

ANONYMOUS, 2018. Genome Reference Consortium Chicken Build 6a. Accessed: 27/03/2018.

https://www.ncbi.nlm.nih.gov/assembly/GCF_000002315.6

BARROW, P. A., HUGGINS, M. B., LOVELL, M. A. & SIMPSON, J. M. 1987. Observations on the pathogenesis of experimental *Salmonella* typhimurium infection in chickens. *Research in Veterinary Science,* 42**,** 194-199.

BEAL, R. K., POWERS, C., DAVISON, T. F., BARROW, P. A. & SMITH, A. L. 2006. Clearance of enteric *Salmonella enterica* serovar Typhimurium in chickens is independent of B-cell function. *Infection and Immunity,* 74**,** 1442-1444.

BEAL, R. K., POWERS, C., WIGLEY, P., BARROW, P. A. & SMITH, A. L. 2004. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with *Salmonella enterica* serovar Typhimurium. *Avian Pathology,* 33**,** 25-33.

BERNDT, A., WILHELM, A., JUGERT, C., PIEPER, J., SACHSE, K. & METHNER, U. 2007. Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. *Infection and Immunity,* 75**,** 5993-6007.

BINDEA, G., GALON, J. & MLECNIK, B. 2013. CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data. *Bioinformatics,* 29**,** 661-663.

BINDEA, G., MLECNIK, B., HACKL, H., CHAROENTONG, P., TOSOLINI, M., KIRILOVSKY, A., FRIDMAN, W.-H., PAGÈS, F., TRAJANOSKI, Z. & GALON, J. 2009. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics,* 25**,** 1091-1093.

FIELDS, P. I., SWANSON, R. V., HAIDARIS, C. G. & HEFFRON, F. 1986. Mutants of *Salmonella* Typhimurium that cannot survive within the macrophage are avirulent. *Proceedings of the National Academy of Sciences of the United States of America,* 83**,** 5189-5193.

FIFE, M., SALMON, N., HOCKING, P. & KAISER, P. 2009. Fine mapping of the chicken salmonellosis resistance locus (SAL1). *Animal Genetics,* 40**,** 871-877.

FORD, L., MOFFATT, C. R. M., FEARNLEY, E., MILLER, M., GREGORY, J., SLOAN-GARDNER, T. S., POLKINGHORNE, B. G., BELL, R., FRANKLIN, N. & WILLIAMSON, D. A. 2018. The epidemiology of *Salmonella enterica* outbreaks in Australia, 2001–2016. *Frontiers in Sustainable Food Systems,* 2**,** 86.

HARRIS, D. P., HAYNES, L., SAYLES, P. C., DUSO, D. K., EATON, S. M., LEPAK, N. M., JOHNSON, L. L., SWAIN, S. L. & LUND, F. E. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nature Immunology,* 1**,** 475.

HASSANPOUR, H., BAHADORAN, S., FARHADFAR, F., CHAMALI, Z. F., NAZARI, H. & KAEWDUANGTA, W. 2018. Identification of reliable reference genes for quantitative real-time PCR in lung and heart of pulmonary hypertensive chickens. *Poultry Science,* 97**,** 4048-4056.

HENDERSON, S. C., BOUNOUS, D. I. & LEE, M. D. 1999. Early events in the pathogenesis of avian salmonellosis. *Infection and Immunity,* 67**,** 3580-3586.

HIGGS, R., CORMICAN, P., CAHALANE, S., ALLAN, B., LLOYD, A. T., MEADE, K., JAMES, T., LYNN, D. J., BABIUK, L. A. & O'FARRELLY, C. 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. *Infection and Immunity,* 74**,** 1692-1698.

HOWARD, A. J., CHOUSALKAR, K. K. & MCWHORTER, A. R. 2018. *In vitro* and *in vivo* efficacy of a live attenuated *Salmonella* Typhimurium vaccine at preventing intestinal colonization in chicks. *Zoonoses and Public Health,* 65**,** 736-741.

HU, J., BUMSTEAD, N., BARROW, P., SEBASTIANI, G., OLIEN, L., MORGAN, K. & MALO, D. 1997. Resistance to salmonellosis in the chicken is linked to NRAMP1 and TNC. *Genome Research,* 7**,** 693-704.

ITO, T., CARSON IV, W. F., CAVASSANI, K. A., CONNETT, J. M. & KUNKEL, S. L. 2011. CCR6 as a mediator of immunity in the lung and gut. *Experimental Cell Research,* 317**,** 613-619.

KHAN, S. & CHOUSALKAR, K. K. 2020. Short-term feeding of probiotics and synbiotics modulates caecal microbiota during *Salmonella* Typhimurium infection but does not reduce shedding and invasion in chickens. *Applied Microbiology and Biotechnology,* 104**,** 319-334.

KHAN, S., ROBERTS, J. & WU, S.-B. 2017. Reference gene selection for gene expression study in shell gland and spleen of laying hens challenged with infectious bronchitis virus. *Scientific Reports,* 7**,** 14271.

KHAN, S., WU, S.-B. & ROBERTS, J. 2019. RNA-sequencing analysis of shell gland shows differences in gene expression profile at two time-points of eggshell formation in laying chickens. *BMC Genomics,* 20**,** 89.

KOGUT, M., CHIANG, H.-I., SWAGGERTY, C. & ZHOU, H. 2012. Gene expression analysis of Toll-like receptor pathways in heterophils from genetic chicken lines that differ in their susceptibility to *Salmonella* enteritidis. *Frontiers in Genetics,* 3**,** 121.

KOGUT, M., HE, H. & KAISER, P. 2005. Lipopolysaccharide binding protein/CD14/TLR4-dependent recognition of *Salmonella* LPS induces the functional activation of chicken heterophils and up-regulation of pro-inflammatory cytokine and chemokine gene expression in these cells. *Animal Biotechnology*, *16*, 165-181.

LI, P., FAN, W., EVERAERT, N., LIU, R., LI, Q., ZHENG, M., CUI, H., ZHAO, G. & WEN, J. 2018. Messenger RNA sequencing and pathway analysis provide novel insights into the susceptibility to *Salmonella* Enteritidis infection in chickens. *Frontiers in Genetics,* 9**,** 256.

LI, P., FAN, W., LI, Q., WANG, J., LIU, R., EVERAERT, N., LIU, J., ZHANG, Y., ZHENG, M. & CUI, H. 2017. Splenic microRNA expression profiles and integration analyses involved in host responses to *Salmonella* Enteritidis infection in chickens. *Frontiers in Cellular and Infection Microbiology,* 7**,** 377.

LIAO, Y., SMYTH, G. K. & SHI, W. 2019. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Research,* 47**,** e47-e47.

LUAN, D., CHANG, G., SHENG, Z., ZHANG, Y., ZHOU, W., LI, Z., LIU, Y. & CHEN, G. 2012. Analysis of gene expression responses to a *Salmonella* infection in Rugao chicken intestine using genechips. *Asian-Australasian Journal of Animal Sciences,* 25**,** 278.

MA, T., CHANG, G., CHEN, R., SHENG, Z., DAI, A., ZHAI, F., LI, J., XIA, M., HUA, D. & XU, L. 2014. Identification of key genes in the response to *Salmonella enterica* Enteritidis, *Salmonella enterica* Pullorum, and poly (I: C) in chicken spleen and caecum. *BioMed Research International,* 2014, 154946.

MARTIN, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal,* 17**,** 3.

MATULOVA, M., RAJOVA, J., VLASATIKOVA, L., VOLF, J., STEPANOVA, H., HAVLICKOVA, H., SISAK, F. & RYCHLIK, I. 2012. Characterization of chicken spleen transcriptome after infection with *Salmonella enterica* serovar Enteritidis. *PLoS One,* 7, e48101.

MCWHORTER, A. R. & CHOUSALKAR, K. K. 2018. A long-term efficacy trial of a live, attenuated *Salmonella* Typhimurium vaccine in layer hens. *Frontiers in Microbiology,* 9**,** 1380-1380.

MITTRÜCKER, H.-W., KÖHLER, A., MAK, T. W. & KAUFMANN, S. H. 1999. Critical role of CD28 in protective immunity against *Salmonella* Typhimurium. *The Journal of Immunology,* 163**,** 6769-6776.

PANDE, V. V., DEVON, R. L., SHARMA, P., MCWHORTER, A. R. & CHOUSALKAR, K. K. 2016. Study of *Salmonella* Typhimurium infection in laying hens. *Frontiers in Microbiology,* 7**,** 203.

PARKER, A. & KAUFMAN, J. 2017. What chickens might tell us about the MHC class II system. *Current Opinion in Immunology,* 46**,** 23-29.

PARKS, W. C., WILSON, C. L. & LÓPEZ-BOADO, Y. S. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature Reviews Immunology,* 4**,** 617-629.

PERTEA, M., PERTEA, G. M., ANTONESCU, C. M., CHANG, T.-C., MENDELL, J. T. & SALZBERG, S. L. 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature Biotechnology,* 33**,** 290.

RESCIGNO, M., URBANO, M., VALZASINA, B., FRANCOLINI, M., ROTTA, G., BONASIO, R., GRANUCCI, F., KRAEHENBUHL, J.-P. & RICCIARDI-CASTAGNOLI, P. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology,* 2**,** 361.

RYCHLIK, I., ELSHEIMER-MATULOVA, M. & KYROVA, K. 2014. Gene expression in the chicken caecum in response to infections with non-typhoid *Salmonella*. *Veterinary Research,* 45**,** 119.

SAH, N., KUEHU, D. L., KHADKA, V. S., DENG, Y., PEPLOWSKA, K., JHA, R. & MISHRA, B. 2018. RNA sequencing-based analysis of the laying hen uterus revealed the novel genes and biological pathways involved in the eggshell biomineralization. *Scientific Reports,* 8**,** 16853.

SALCEDO, S. P., NOURSADEGHI, M., COHEN, J. & HOLDEN, D. W. 2001. Intracellular replication of *Salmonella* Typhimurium strains in specific subsets of splenic macrophages *in vivo*. *Cellular Microbiology,* 3**,** 587-597.

SCHUTYSER, E., STRUYF, S. & VAN DAMME, J. 2003. The CC chemokine CCL20 and its receptor CCR6. *Cytokine & Growth Factor Reviews,* 14**,** 409-426.

SIERRO, F., DUBOIS, B., COSTE, A., KAISERLIAN, D., KRAEHENBUHL, J.-P. & SIRARD, J.-C. 2001. Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proceedings of the National Academy of Sciences,* 98**,** 13722-13727.

STEINHAGEN, F., KINJO, T., BODE, C. & KLINMAN, D. M. 2011. TLR-based immune adjuvants. *Vaccine,* 29**,** 3341-3355.

VAZQUEZ-TORRES, A., JONES-CARSON, J., BÄUMLER, A. J., FALKOW, S., VALDIVIA, R., BROWN, W., LE, M., BERGGREN, R., PARKS, W. T. & FANG, F. C. 1999. Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature,* 401**,** 804-808.

WANG, B., BIRON, C., SHE, J., HIGGINS, K., SUNSHINE, M.-J., LACY, E., LONBERG, N. & TERHORST, C. 1994. A block in both early T lymphocyte and natural killer cell development in transgenic mice with high-copy numbers of the human CD3E gene. *Proceedings of the National Academy of Sciences,* 91**,** 9402-9406.

WICK, M. J. 2004. Living in the danger zone: innate immunity to *Salmonella*. *Current Opinion in Microbiology,* 7**,** 51-57.

WITHANAGE, G. S. K., WIGLEY, P., KAISER, P., MASTROENI, P., BROOKS, H., POWERS, C., BEAL, R., BARROW, P., MASKELL, D. & MCCONNELL, I. 2005. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. *Infection and Immunity,* 73**,** 5173-5182.

XU, Z., PONE, E. J., AL-QAHTANI, A., PARK, S.-R., ZAN, H. & CASALI, P. 2007. Regulation of aicda expression and AID activity: relevance to somatic hypermutation and class switch DNA recombination. *Critical Reviews™ in Immunology,* 27.

3 Effects of non-pathogenic *Salmonella* serovars on the shedding and invasion profile of *Salmonella* Typhimurium in laying chicks

3.1 Introduction

The leading causes of foodborne human gastroenteritis in Australia and other countries are *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Salmonella* associated gastroenteritis in humans is often traced back to the consumption of contaminated poultry products. In Australia, *Salmonella* Typhimurium is frequently associated with poultry production (Ford et al. 2018; Gole et al. 2017), while *Salmonella* Enteritidis has been recently isolated from layer farms in different States of the country. Once colonised in chickens, *Salmonella* Typhimurium has the potential to invade internal organs resulting in prolonged faecal shedding (Pande et al. 2016; McWhorter & Chousalkar 2018). However, the shedding profile of *Salmonella* in chickens is affected by factors that include gut microbiota composition (Khan & Chousalkar, 2020) and environmental conditions (Gole et al. 2017).

In layers, coinfection with *Salmonella* Typhimurium and *Salmonella* Mbandaka indicates that *Salmonella* Mbandaka could provide a competitive and immunoprotective mechanism against *Salmonella* Typhimurium infection (Pande et al. 2016). Leghorn chicks either challenged with Typhimurium or Enteritidis followed by the reciprocal serovar challenge showed that the initially inoculated serovars predominantly colonised the gut (Yang et al. 2018). Similarly, in mice with different gut microbiota composition, the endogenous Enterobacteriaceae population resulted in the reduced colonisation of gut by *Salmonella* Typhimurium (Velazquez et al. 2019). In day-old Light Sussex chicks, oral administration of live strains of virulent *Salmonella* produced inhibition to the subsequent caecal colonisation by *Salmonella* Typhimurium administered one day later (Barrow et al. 1987). However, a recent study confirmed that co-infection of layer chickens with *Salmonella* Typhimurium and *Salmonella* Mbandaka results in a minor variation in the genome of *Salmonella* Typhimurium (McWhorter et al. 2019). It is important to note that prior infection of layer chickens with a other serotypes of *Salmonella* can reduce *Salmonella* Enteritidis, but does not eliminate it completely from the gut (Holt & Gast 2004). Therefore, it seems that there is a competition among *Salmonella* serovars for gut colonisation. If this competition exists, some strains (particularly non-pathogenic strains) can be used as a tool for competitive exclusion of pathogenic *Salmonella* from poultry production for improving food safety.

An important source of variation in animal experiments is the microbiome, but little is known about specific changes in the microbiota composition that cause phenotypic differences. Day-old hatched chicks in commercial hatcheries lack natural gut microbiota as they are hatched in a clean environment and are therefore highly susceptible to pathogens colonisation (Crhanova et al. 2011). The maturation of gut microbiota enhances the immune system and ultimately increases resistance to infection by pathogens; however, it does not completely inhibit the colonisation of pathogens such as *Salmonella* Enteritidis and *Salmonella* Typhimurium. How the composition of gut microbiota in the presence of non-pathogenic *Salmonella* varies, needs to be investigated. Based on the potential capabilities of non-pathogenic *Salmonella* in providing gut colonisation resistance, we hypothesised that *Salmonella* Mbandaka and *Salmonella* Agona might act as competitive inhibitors in *Salmonella* Typhimurium challenge model. Therefore, the main objective of this study was to investigate the effectiveness of *Salmonella* Mbandaka and *Salmonella* Agona on the colonisation and invasion profile of *Salmonella* Typhimurium in laying chicks.

3.2 Materials and methods

3.2.1 Animal ethics and rearing of *Salmonella* free chicks

All experimental work was approved by the University of Adelaide, Animal Ethics Committee (approval number S-2017-080) in accordance with the guidelines specified in the Australian code for the care and use of animals for scientific purposes, 8th edition (2013). Standard Operating Procedures were followed for caring and processing of the experimental chicks. Fertile eggs from an Isa-Brown laying flock were obtained from a local farm with no obvious history of *Salmonella*. After fumigating (formaldehyde and $KMnO_4$), the eggs were incubated in clean conditions for hatching in the School of Animal and Veterinary Sciences. The hatched chicks were reared as per the protocol of the ISA General Management Guide 2009-10. From the incubator, the hatching trays paper with the chicks' meconium samples were processed through an enrichment method for *Salmonella* detection (if any). Prior to the chicks' placement, the rearing house was sampled for *Salmonella* presence through the enrichment method. The chicks were equally divided into 6 treatment groups and reared in pens for 15 days with water and feed provided *ad libitum*. For the *Salmonella* Mbandaka and *Salmonella* Agona treatment groups, bacterial pellets were suspended in PBS and added into 500 mL of autoclaved water with a final 10^3 CFU/mL. At day 1 of hatch, the chicks were allowed to drink the *Salmonella* containing water for 20–24 hrs. The *Salmonella* shedding was monitored on days 3, 5 and 7 p.i. in faeces through culture method.

3.2.2 *Salmonella* Typhimurium inoculum preparation and chicks challenge

Pure isolate of *Salmonella* Typhimurium was cultured on nutrient agar (Thermo Fisher Oxoid, CM3, Hampshire, UK) and then individual colony was grown to a log phase in *Luria-Bertani* (*LB*) *broth*. The culture was centrifuged and the pellet was resuspended in PBS. The CFU of *Salmonella* in per mL of PBS was calculated from the optical density (OD) of the *Salmonella* suspension. In the challenge groups, an individual bird received an oral dose of 10^3 CFU (0.1 mL) of *Salmonella* Typhimurium, while birds in the control groups received PBS only. A 100 µL of the original inoculum and its serial dilutions were plated onto xylose lysine deoxycholate (XLD; Thermo Fisher Scientific, Australia) media to confirm the CFU received by an individual bird. From each treatment group at each time-point (days 3, 5 and 7 p.i.), 3 birds were processed by cervical dislocation for the collection of ceca, liver and spleen.

3.2.3 *Salmonella* Typhimurium enumeration in tissue

Small pieces of liver, spleen and caeca aseptically collected into 1.5 mL Eppendorf tubes containing stainless steel beads 0.5–2.0 mm and PBS were maintained on ice. After weighing, tissues were homogenised using a bullet blender (Next Advance, United States) on full speed for 5–10 min. From the original tissue homogenates or serially diluted samples (caeca), 100 µL was plated onto XLD and incubated overnight at 37°C. The *Salmonella* colonies (black with sheen metal appearance) were counted for enumeration of bacterial load (log_{10}) in 1 g of tissue. *Salmonella* Typhimurium colonies were distinguished from Mbandaka and Agona based on the size and morphology of the colonies as previously established (Howard et al. 2018). A 100 µL from the original homogenates was also enriched into 900 µL buffered peptone water (BPW; Oxoid, Australia) and incubated overnight at 37°C. 100 µL of the incubated BPW samples were added into 10 mL of Rappaport Vassiliadis soya peptone (RVS) broth (Oxoid, Australia) and incubated overnight at 42°C for selective growth of *Salmonella*. *Salmonella* appearance colonies on XLD were sub-cultured on Brilliance *Salmonella* agar (BSA; Thermo Fisher Scientific, Australia) plates for confirmation. Incubated plates were read as positive or negative for *Salmonella* based on the characteristics colour development on the XLD (black with mucoid surrounding) and Brilliance *Salmonella* Agar (BSA; deep pink) media.

3.3 Statistical analysis

The log_{10} CFU/g of tissue data were analysed in StatView software by taking treatment group and day effect as independent variables. Level of significance was determined by PLSD at $P < 0.05$. Graphs were prepared in GraphPad Prism version 8.0.0.

3.4 Results and discussion

Faeces collected from the chicks that received *Salmonella* Agona or *Salmonella* Mbandaka were *Salmonella* positive at days 3, 5 and 7 post-infection. This shows that both the *Salmonella* Agona and *Salmonella* Mbandaka had the potential to effectively colonise the gut and shed in the faeces. The *Salmonella* Typhimurium load in the caeca of chicks that received *Salmonella* Agona, and in the spleen of chicks that received either *Salmonella* Mbandaka or *Salmonella* Agona was significantly lower compared with the positive control group (Figure 3-1a, c). Interestingly, there was a significant effect of treatment and day post-infection on the overall load of *Salmonella* Typhimurium in the liver, but the mean values were not significantly different at different days post-infection. A significantly lower *Salmonella* Typhimurium load in the caeca of the *Salmonella* Agona and *Salmonella* Typhimurium challenged birds showed that the serovar Agona competitively reduced the colonisation of *Salmonella* Typhimurium but did not completely inhibit it from caecal colonisation. However, both the *Salmonella* Mbandaka and *Salmonella* Agona were equally effective in conferring resistance to the invasion potential of *Salmonella* Typhimurium in the spleen. In layers, co-infection with *Salmonella* Mbandaka and *Salmonella* Typhimurium resulted in colonisation resistance of Mbandaka to Typhimurium (Pande et al. 2016). However, a more recent study indicated that this co-infection model results in a minor variation in the genome of *Salmonella* Typhimurium that increases its invasion capacity (McWhorter et al. 2019).

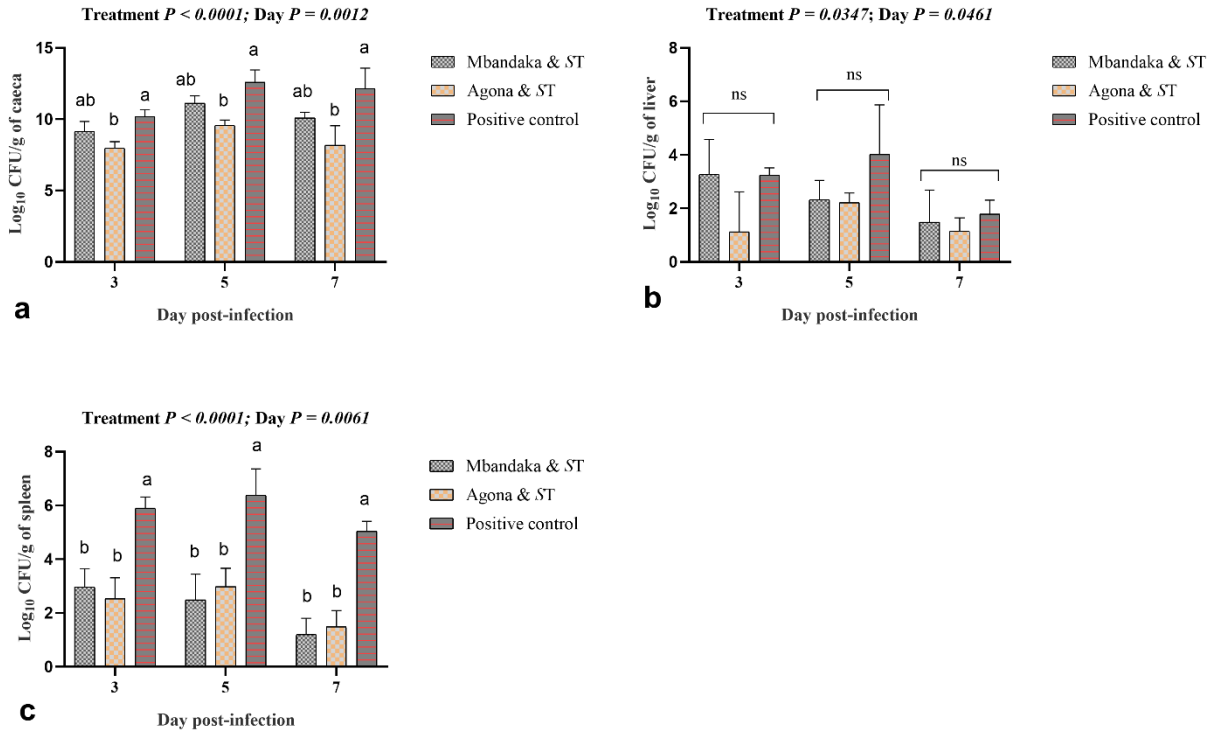


Figure 3-1 *Salmonella* Typhimurium load in organs of chicks

The chicks received *Salmonella* Mbandaka or *Salmonella* Agona at day 1 of hatch and were challenged with *Salmonella* Typhimurium at day 8 of hatch.

Salmonella Typhimurium load was determined in a) caeca, b) liver and c) spleen at days 3, 5 and 7 post *Salmonella* Typhimurium challenge.

Values are mean ± SD. Different superscripts across the bar show significant difference.

The load of *Salmonella* Mbandaka and *Salmonella* Agona was also quantified from the *Salmonella* Typhimurium challenged chicks (Figure 3-2). The data showed that both the *Salmonella* Mbandaka and *Salmonella* Agona had a similar pattern of potential to colonise the caecal tissue (Figure 3-2a) and invade liver (Figure 3-2b) and spleen (Figure 3-2c). This shows that both the *Salmonella* Mbandaka and *Salmonella* Agona regulate their *Salmonella* pathogenicity islands (SPIs) machinery for invasion into internal organs. *Salmonella* Mbandaka is frequently present in layer production (Gole et al. 2014); however, its role in the competitive exclusion against *Salmonella* Typhimurium has not been determined. Based on the preliminary findings of the current study, it seems that *Salmonella* Agona performs better in competitively excluding *Salmonella* Typhimurium in the gut and invasion into internal organs. *Salmonella* Agona and *Salmonella* Typhimurium possess a common O-antigen (Varmuzova et al. 2016) that might enable them to affect the colonisation of each other in chickens' gut. Studies confirm that both the *Salmonella* Typhimurium and *Salmonella* Agona possess a similar profile of multi-drug resistance (Cloeckaert et al. 2000; Boyd et al. 2001). Therefore, future studies should focus on characterising the interaction between *Salmonella* Agona and *Salmonella* Typhimurium with the aim of using both the serovars in developing vaccine for poultry.

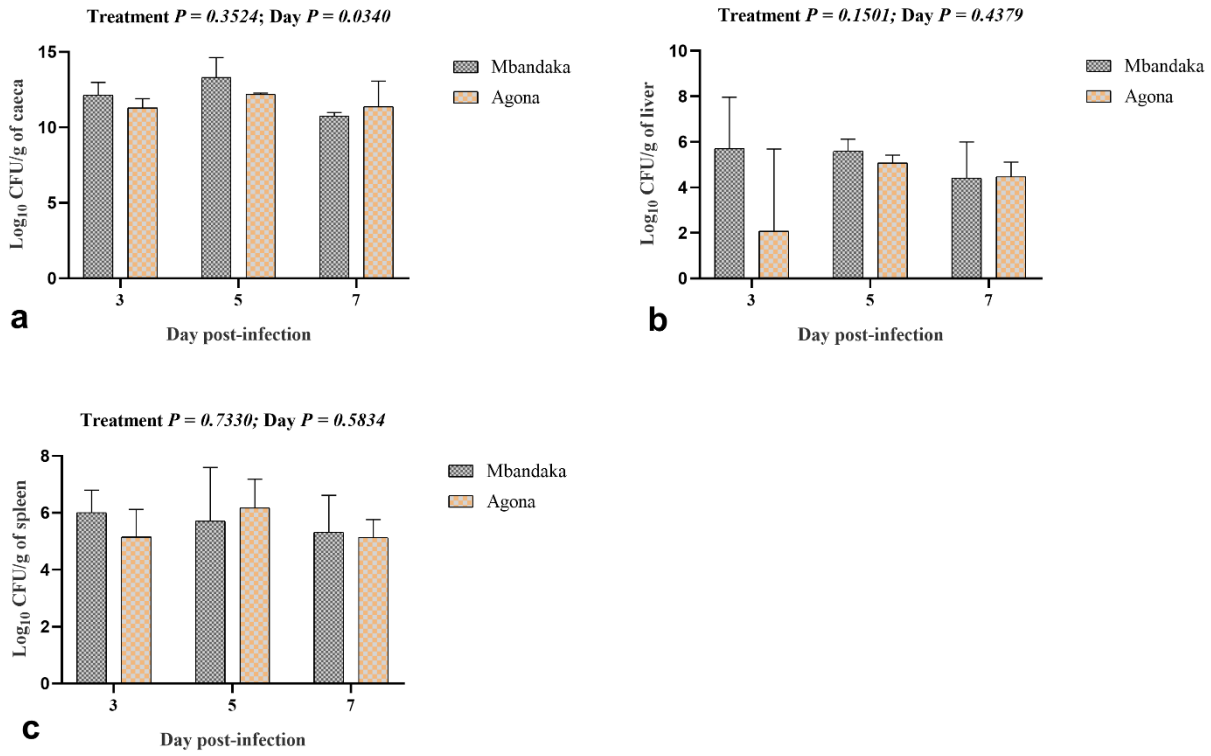


Figure 3-2 *Salmonella* Mbandaka and *Salmonella* Agona load in chicks challenged with *Salmonella* Typhimurium at day 8 of birds age

The chicks received *Salmonella* Mbandaka or *Salmonella* Agona at day 1 of hatch and were challenged with *Salmonella* Typhimurium at day 8 of hatch.

Salmonella Mbandaka and *Salmonella* Agona load was determined in a) caeca, b) liver and c) spleen at days 3, 5 and 7 post *Salmonella* Typhimurium challenge.

Values are mean ± SD.

3.5 References

BARROW, P., TUCKER, J. & SIMPSON, J. 1987. Inhibition of colonization of the chicken alimentary tract with *Salmonella* Typhimurium gram-negative facultatively anaerobic bacteria. *Epidemiology & Infection,* 98**,** 311-322.

BOYD, D., PETERS, G. A., CLOECKAERT, A., BOUMEDINE, K. S., CHASLUS-DANCLA, E., IMBERECHTS, H. & MULVEY, M. R. 2001. Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *Journal of Bacteriology,* 183**,** 5725-5732.

CLOECKAERT, A., BOUMEDINE, K. S., FLAUJAC, G., IMBERECHTS, H., D'HOOGHE, I. & CHASLUS-DANCLA, E. 2000. Occurrence of a *Salmonella enterica* Serovar Typhimurium DT104-Like Antibiotic Resistance Gene Cluster Including thefloR Gene in S. *enterica* Serovar Agona. *Antimicrobial Agents and Chemotherapy,* 44**,** 1359-1361.

CRHANOVA, M., HRADECKA, H., FALDYNOVA, M., MATULOVA, M., HAVLICKOVA, H., SISAK, F. & RYCHLIK, I. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica* serovar Enteritidis infection. *Infection and Immunity,* 79**,** 2755-2763.

FORD, L., MOFFATT, C. R., FEARNLEY, E., MILLER, M., GREGORY, J., SLOAN-GARDNER, T. S., POLKINGHORNE, B. G., BELL, R., FRANKLIN, N. & WILLIAMSON, D. A. 2018. The epidemiology of *Salmonella enterica* outbreaks in Australia, 2001–2016. *Frontiers in Sustainable Food Systems,* 2**,** 86.

GOLE, V. C., CARAGUEL, C. G., SEXTON, M., FOWLER, C. & CHOUSALKAR, K. K. 2014. Shedding of *Salmonella* in single age caged commercial layer flock at an early stage of lay. *International Journal of Food Microbiology,* 189**,** 61-66.

GOLE, V. C., WOODHOUSE, R., CARAGUEL, C., MOYLE, T., RAULT, J.-L., SEXTON, M. & CHOUSALKAR, K. 2017. Dynamics of *Salmonella* shedding and welfare of hens in free-range egg production systems. *Applied and Environtal Microbiology,* 83**,** e03313-16.

HOLT, P. S. & GAST, R. K. 2004. Effects of prior coinfection with different *Salmonella* serovars on the progression of a *Salmonella enterica* serovar enteritidis infection in hens undergoing induced molt. *Avian Diseases,* 48**,** 160-166.

HOWARD, A. J., CHOUSALKAR, K. K. & MCWHORTER, A. R. 2018. *In vitro* and *in vivo* efficacy of a live attenuated *Salmonella* Typhimurium vaccine at preventing intestinal colonization in chicks. *Zoonoses and Public Health,* 65**,** 736-741.

KHAN, S. & CHOUSALKAR, K. K. 2020. *Salmonella* Typhimurium infection disrupts but continuous feeding of *Bacillus* based probiotic restores gut microbiota in infected hens. *Journal of Animal Science and Biotechnology,* 11**,** 1-16.

MCWHORTER, A. R. & CHOUSALKAR, K. K. 2018. A long-term efficacy trial of a live, attenuated *Salmonella* Typhimurium vaccine in layer hens. *Frontiers in Microbiology,* 9**,** 1380.

MCWHORTER, A. R., TEARLE, R., MOYLE, T. S. & CHOUSALKAR, K. K. 2019. In vivo passage of *Salmonella* Typhimurium results in minor mutations in the bacterial genome and increases in vitro invasiveness. *Veterinary Research,* 50**,** 71.

PANDE, V. V., DEVON, R. L., SHARMA, P., MCWHORTER, A. R. & CHOUSALKAR, K. K. 2016. Study of *Salmonella* Typhimurium infection in laying hens. *Frontiers in Microbiology,* 7**,** 203.

VARMUZOVA, K., FALDYNOVA, M., ELSHEIMER-MATULOVA, M., SEBKOVA, A., POLANSKY, O., HAVLICKOVA, H., SISAK, F. & RYCHLIK, I. 2016. Immune protection of chickens conferred by a vaccine consisting of attenuated strains of *Salmonella* Enteritidis, Typhimurium and Infantis. *Veterinary Research,* 47**,** 94.

VELAZQUEZ, E. M., NGUYEN, H., HEASLEY, K. T., SAECHAO, C. H., GIL, L. M., ROGERS, A. W., MILLER, B. M., ROLSTON, M. R., LOPEZ, C. A. & LITVAK, Y. 2019. Endogenous Enterobacteriaceae underlie variation in susceptibility to *Salmonella* infection. *Nature Microbiology,* 4**,** 1057-1064.

YANG, Y., TELLEZ, G., LATORRE, J. D., RAY, P. M., HERNANDEZ, X., HARGIS, B. M., RICKE, S. C. & KWON, Y. M. 2018. *Salmonella* excludes *Salmonella* in poultry: confirming an old paradigm using conventional and barcode-tagging approaches. *Frontiers in Veterinary Science,* 5**,** 101.

4 *Salmonella* Typhimurium infection disrupts but continuous feeding of *Bacillus* based probiotic restores gut microbiota in laying chickens

Samiullah Khan and Kapil K. Chousalkar

Published in the *Journal of Animal Science and Biotechnology* 11:29 (2020).

4.1 Introduction

The chicken gut microbiome is composed of multiple microorganisms and their genetic materials. These microorganisms (microbiota) are involved in functions that are critical to bird health and performance. The gut microbiota helps in digestion and metabolism (Stanley et al. 2012), regulation of enterocytes (Stappenbeck et al. 2002), vitamin synthesis and development and regulation of the host immune system (Talham et al. 1999). The chicken gut microbiota is mainly composed of the phyla Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Oakley et al. 2014). The host gut microbiota is affected by multiple factors such as disease, diet, husbandry conditions and age (Cui et al. 2017). *Salmonella* Typhimurium causes clinical disease in many animals and humans; however, chickens are often asymptomatic carriers. *Salmonella* present in laying production systems often result in gastroenteritis in humans after the consumption of contaminated food (Ford et al. 2018). In the chicken gut, *Salmonella* elicits inflammation through the activation of *Salmonella* pathogenicity island 1 (SPI1) for encoding the type III secretion system (Dieye et al. 2009). In the inflamed gut, motility allows *Salmonella* Typhimurium to utilise available nutrients for its enhanced growth (Stecher et al. 2008). To escape nutrient limitation caused by the intestinal microbiota, *Salmonella* uses specific metabolic traits for the utilisation of compounds that are not metabolised by gut microbiota (Staib & Fuchs 2015). Gram-negative bacteria dominate the gut at an early age, while gram positive *Firmicutes*, particularly *Clostridia* taxa, become more prominent at later ages (Ballou et al. 2016).

A previous study demonstrated a negative correlation between *Enterobacteriaceae* and *Lachnospiraceae*, *Ruminococcaceae, Erysipelotrichaceae* and *Peptostreptococcaceae* in *Salmonella* Enteritidis challenged chicks (Mon et al. 2015), thus causing gut dysbiosis. Gut dysbiosis results from microbial imbalance due to impaired microbiota (Myers 2004). The mechanism by which the gut microbiome affects pathogen colonisation is partly mediated by the production of short-chain fatty acids (SCFAs) that are the metabolites of bacterial fermentation of undigested dietary fibre (Morrison & Preston 2016). SCFAs activate G protein-coupled receptors (GPCRs) including free fatty-acid receptors 2 and 3 (FFAR2 and FFAR3) (Sun et al. 2017), inhibit histone deacetylases (Fellows et al. 2018) and provide energy to enterocytes (Scheppach 1994). Although the roles of GPCRs (e.g. FFAR3/GPR41 and FFAR2/GPR43) are not well established, they have been implicated in regulation of leukocytes (Senga et al. 2003) and leptin production (Lu et al. 2016) in murine models. GPR41 and GPR43 play a role in lowering body weight through the down-regulation of leptin mRNA (Lu et al. 2016). Previous research in broiler chickens showed that the activation of GPR41 and GPR43 by gut microbiota derived SCFAs resulted in the production of Glucagon-like peptide-1 (GLP-1), which suppressed lipid accumulation in the liver (Zhang et al. 2019). Therefore, the host microbiome constitutes an attractive target for manipulation, as it can be modified for pathogen colonisation resistance to reduce disease risk.

To strengthen and improve the gut microbiota composition in chickens, pre- and pro- biotics are often supplemented as a part of the feeding regimen. Prebiotics are host non-digestible complex carbohydrates that help to increase the resident gut microbiota through fermentation (Macfarlane et al. 2008). Examples of prebiotics are pectin, xylooligosaccharides, galactooligosaccharides, fructooligosaccharides and inulin. Probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (Fuller 1989; Martin & Langella 2019). The representative bacterial genera in probiotics include *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus* and *Bacillus*. Apart from gram-positive bacteria, some probiotics are also composed of yeast and moulds. Some of the proposed functions of probiotics include competitive exclusion (Lloyd et al. 1977), antagonism (Laport et al. 2018), bacterial interference (Reid et al. 2001), barrier effect (Honda & Littman 2012), modulation of host immune system (Hooper et al. 2001) and colonisation resistance (Buffie & Pamer 2013). These actions are achieved mainly through bacteria-bacteria and host-bacteria interactions. The bacteria-bacteria interactions result in the production of SCFAs (Rechkemmer & Rönnau 1988), modification of redox potential (Million et al. 2016), production of antimicrobial compounds, competition for epithelial receptors, quorum sensing (Ismail et al. 2016) and production of an ecosystem harmful for pathogenic organisms. The reduced luminal pH due to organic acids restricts the growth of many pathogens. Probiotic bacteria secrete enzymes that hydrolyse bacterial toxins and modify toxin receptors (Wilson & Perini 1988). Attachment of probiotic bacteria to cell surface receptors of enterocytes initiates signalling events that result in the synthesis of cytokines (Tejada-Simon & Pestka 1999) and stimulation of Toll-like receptors (Brisbin et al. 2010).

In laying hens, probiotics are generally used as feed supplements for improving flock performance and egg quality (Mikulski et al. 2012; Panda et al. 2008). From the food safety perspective, *Salmonella* is an important foodborne pathogen that is often present in the gut of chickens. *Salmonella* reduction in layers, for the production of safer egg and egg products, has always been a priority for the egg industry. In Australia, *Salmonella* Typhimurium has been responsible for the majority of the egg related foodborne outbreaks (Ford et al. 2018). The supplemental use of probiotics lowers the incidence of *Salmonella* in poultry production (Van Coillie et al. 2007). Given the longer commercial life span of egg laying hens, in order to achieve the cost-effective reduction in *Salmonella* shedding, it is critical to optimise the use of probiotics and to understand the dynamics of gut microbiota during probiotic treatment. Previous studies of probiotics use for *Salmonella* control in laying chickens have mainly focused either on young chicks, using different serovars of *Salmonella* or have used a short duration trial where the effect of *Salmonella* was not tested on gut microbiota dysbiosis at different time-points while chickens were laying (Luoma et al. 2017; Wang et al. 2018; Pascual et al. 1999). In this study, we raised *Salmonella* free birds to understand the role of *Salmonella* Typhimurium in gut microbiota dysbiosis and its subsequent restoration through the use of a *Bacillus* based probiotic in laying chickens from point of laying until 30 weeks of age. Based on the role of microbiota in the clearance of gut pathogens, we hypothesised that, if used strategically, a *Bacillus* based probiotic could be effective in positively modulating the microbiota for gut health during *Salmonella* Typhimurium infection.

4.2 Methods

4.2.1 Ethics approval and rearing of laying chickens

All experimental work was approved by the Animal Ethics Committee at the University of Adelaide under approval number S-2017-080 in accordance with the guidelines specified in the Australian code for the care and use of animals for scientific purposes, 8th edition (2013). Eggs from an Isa-Brown parent breeder flock were obtained from a hatchery, fumigated and hatched at the School of Animal and Veterinary Sciences. Meconium samples were tested through standard culture methods for the presence of *Salmonella* spp. (if any). Before placement of day-old laying chicks, the rearing facility was

tested for the presence of *Salmonella* spp. The day-old female chicks were divided into six treatment groups (7 chickens in each treatment group), reared in pens until week 14 and then transferred into individual cages. The treatment groups were: negative control (NC), *Salmonella* challenge (SX), continuous probiotic supplemented and *Salmonella* challenge (CPX), continuous probiotic supplemented control (CPC), intermittent probiotic supplemented and *Salmonella* challenge (IPX) and intermittent probiotic control (IPC). The feeding regime was as per the protocol of the ISA General Management Guide. Before adding the probiotic, the feed was fumigated and regularly tested for the presence of *Salmonella*. For the probiotic-supplemented groups, 1 g of *Bacillus* based probiotic (*Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens*) was mixed with 1 kg of fumigated feed. The intermittent probiotic supplemented groups were on the probiotic supplement for alternate 4 weeks (4 weeks ON/OFF strategy). Faeces from all the treatment groups were tested fortnightly for *Salmonella* isolation until the specific group chickens were challenged with *Salmonella* Typhimurium. At 18 weeks of age, pullets from the selected groups were orally inoculated with 10^6 colony forming units (CFUs) per mL of *Salmonella* Typhimurium phage type 9, while the control groups received phosphate buffered saline (PBS). For the preparation of bacterial inoculum, *Salmonella* Typhimurium was grown on xylose lysine deoxycholate (XLD; Thermo Fisher Scientific, Australia) agar and a single colony was cultured in Luria-Bertani (LB) broth. The inoculum was prepared by re-suspending the washed bacterial pellet in PBS. Ten-fold serial dilutions of the original inoculum were plated onto XLD to confirm the CFU received by the individual chickens.

4.2.2 Faecal shedding profile of *Salmonella* Typhimurium challenged chickens

Individual chickens were monitored for the faecal shedding profile of *Salmonella* Typhimurium by sampling the faeces on days 3, 5 and 7 and then weeks 2, 4, 6, 8, 10 and 12 post-challenge. Fresh faecal samples were collected in sterile zip lock bags from individual chickens including the control groups. Faecal samples were also collected in 1.5 mL and 5 mL tubes and stored at -80°C until used for microbial DNA extraction and quantification of SCFAs, respectively. The SCFA analysis was performed on samples collected at weeks 1, 4, 8 and 12 post-challenge. A miniaturised most probable number (mMPN) method was used for the enumeration of *Salmonella* Typhimurium in individual positive faecal samples. The mMPN method was originally developed by the U.S. Department of Agriculture (USDA) and U.S. Food and Drug Administration (FDA), validated on chicken faecal samples for *Salmonella* enumeration (Pavic et al. 2010) and has been used frequently in similar studies (Gole et al. 2017; Pande et al. 2016). The bacterial culture and mMPN procedures were performed following the methods previously described (Gole et al. 2017).

4.2.3 Processing of eggs for *Salmonella* enumeration

Once the chickens were in lay, eggs from all the treatment groups were aseptically collected every fortnight in Whirl-Pack plastic bags and processed for the enumeration of *Salmonella* Typhimurium on the eggshell surface and in egg internal contents following the methods previously described (Pande et al. 2016; Gole et al. 2013). An mMPN was performed on the samples positive for *Salmonella* Typhimurium.

4.2.4 Short chain fatty acids quantification in faeces

The faecal samples stored at -80°C (≤ 3 month-old samples) were processed for SCFA (acetate, propionate and butyrate) quantification using gas chromatography (Hewlett-Packard6890; Palo Alto, CA, USA) equipped with a BP21 capillary column 10 mm, I.D. 0.32 mm, film thickness 0.25 mm (SGE Pty Ltd., Australia) and a flame ionisation detector (FID). Briefly, 0.1 g of individual faecal samples were weighed into 1.5 mL centrifuge tubes into which 1 mL of water containing 2 % orthophosphoric acid was dispensed. 20 µL of internal standard (1 mmol/L of 4-methyl valerate) was added to each sample,

which was then briefly vortexed and incubated for 30 min at room temperature. The samples were centrifuged at 12,000 rpm for 10 min. The supernatants were transferred with a disposable glass Pasteur pipette to their corresponding 6 mL scintillation vials and 2 mL of diethyl ether was added into each sample, which was then briefly vortexed. The upper layer of diethyl ether was transferred into corresponding gas chromatography vials and run for SCFA analysis. A programmed temperature ramp (50–220°C) was used. Helium gas was utilised as a carrier at a flow rate of 3 mL/min in the column and the inlet split ratio was set at 20:1. The identification and quantitation of SCFAs were achieved by comparing the retention times and a peak area of unknown samples to that of commercial lipid standard (4-methylvaleric acid) as an internal control.

4.2.5 *Salmonella* Typhimurium enumeration in organs

At week 30 of flock age, the laying chickens were humanely euthanised by cervical dislocation and tissue pieces of various organs (spleen, liver, ovary, infundibulum/magnum, shell gland, jejunum and caecum) were aseptically collected into 1.5 mL Safe-Lock Eppendorf tubes containing stainless steel beads 0.5–2.0 mm and 500 µL PBS. After weighing, tissues were homogenised using a bullet blender (Next Advance, USA) on full speed for 5–10 min. From the original tissue homogenates or serially diluted samples (caecum), 100 µL was plated onto XLD agar and incubated overnight at 37°C. *Salmonella* load was expressed as $\log_{10}$ CFU/g of tissue. A 100 µL from the original homogenates was also enriched into 900 µL buffered peptone water (BPW) and processed for *Salmonella* isolation through the enrichment method (Gole et al. 2017). Putative *Salmonella* colonies on XLD were streaked on Brilliance *Salmonella* agar (BSA; Oxoid, Australia) plates and incubated overnight at 37°C for confirmation. Incubated plates were read as positive (scored as 1) or negative (scored as 0) for *Salmonella* based on the colony characteristics.

4.2.6 Faecal DNA extraction and 16S rRNA sequencing

Faecal DNA was extracted following the protocol of QIAamp FAST DNA Mini Kit with the inclusion of homogenisation step with glass beads (acid-washed ≤ 106 µm and 425–600 µm; Sigma Aldrich, Australia). The DNA quality was tested using a Nanodrop-1000 and the samples (n = 378) were submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for 16S rRNA sequencing and generation of operational taxonomic units (OTUs) table. For generating 2 × 300 bp paired - end reads in Illumina, V3-V4 region specific primer pair (341F: 5'-CCTACGGGNGGCWGCAG-3'; 805R: 5'-GACTACHVGGGTATCTAATCC-3') was used.

4.2.6.1 16S rRNA library preparation and Illumina sequencing

The library was prepared using barcoding PCR in a 25 µL reaction volume that contained 12.5 µL KAPA HiFi HotStart Readymix (Kapa Biosystems), 1 µL of each the primers, 1 µL DNA template and 10.5 µL PCR grade water. The thermal cycling conditions in SimpliAmp Thermal Cycler (Applied Biosystems) were: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, ending with a final elongation at 72°C for 5 min. The PCR products were normalised and pooled using SequalPrep™ Normalization Plate Kit (Thermo Fisher Scientific, Australia) according to the manufacturer's instructions. The library was purified using Axygen AxyPrep Mag PCR Clean-Up Kit (Fisher Biotec, Australia) as per the manufacturer's instructions. Concentration and quality of the pooled library were checked with Qubit and the library size on an Agilent 2200 TapeStation instrument. The Agencourt AMPure XP Bead Clean-up kit was used on the pool to reduce/remove the presence of primer dimers. The library pool was sequenced on Illumina MiSeq using a MiSeq Reagent Kit v3 with a 2 × 300 bp run format, using default run parameters including adaptor trimming. For these runs, custom primers were added to the reagent cartridge for Read1, Index and Read2.

4.2.6.2 Microbial community data analysis for generation of OTU table

Reads were processed with mothur (v1.39.5) (Schloss et al. 2009) according to the MiSeq protocol. Briefly, the reads were quality filtered and assigned to their respective samples. Samples were trimmed and only those with a length between 405 and 495 bp were retained. Samples with homopolymers longer than 8 bp were removed. Chimeric sequences were removed using the chimera.vsearch script in mothur (Quast et al. 2012). The sequences were aligned and classified against the SILVA reference alignment (v132) (Quast et al. 2012) and lineages not targeted by the primer pair (i.e. archaea, chloroplast, eukaryote, mitochondria and unknown) were removed. Sequences were grouped into OTUs based on 97% similarity using the OptiClust algorithm (Westcott & Schloss 2017) and subsampled based on the sample with the lowest number of sequences, i.e. 25556 sequences. Sequencing error was assessed using the ZymoBIOMICS Microbial Community Standard as control in each sequencing run. Interactive OTU plots were created with Krona (Ondov et al. 2011) from the subsampled data. OTU richness plot was generated with the mothur_krona_XML.py script. Diversity plots were generated by using the OTUsamples2krona.sh script by providing a reformatted mothur biom file.

4.3 Statistical analysis

The *Salmonella* Typhimurium load data in faeces (log_{10} mMPN) and in organs (mean percent value) were analysed in Statview software (Version 5.0.1.0) by taking treatment and sampling time-point as main effects. Level of significance was determined by Fisher's protected least significant difference (PLSD) at $P < 0.05$. For microbial community profiling (abundance and diversity), the OTU table was analysed in Calypso software (Zakrzewski et al. 2016) using one- and two-way ANOVA, redundancy analysis (RDA+), regression and diversity analyses. To remove the non-independence of relative microbial abundance, the data were transformed using the total sum normalisation (TSS) method (Badri et al. 2018; Zakrzewski et al. 2016). TSS normalises count data by dividing feature read counts by the total number of reads in each sample for obtaining relative abundance (Zakrzewski et al. 2016). RDA is used to calculate complex association between microbial community composition and explanatory variables. In Calypso, a false discovery rate (FDR) of < 0.05 was used for level of significance between the treatment groups.

4.4 Results

4.4.1 16S rRNA data and its quality

The sequenced reads quality was as per Q30 standard and the average reads generated per sample were enough for genome alignment and the generation of the OTU table for downstream analysis. The rarefaction analysis showed that the sequenced data covered well the diversity of the studied microbiota (Appendix Figure 4-1). Overall, at phylum level, the microbial communities were clustered into Actinobacteria, Bacteroidetes, Cyanobacteria, Deferribacteres, Firmicutes, Proteobacteria, Synergistetes, Tenericutes and Verrucomicrobia (Appendix Figure 4-2).

4.4.2 Gut microbiota abundance and diversity are affected by *Salmonella* Typhimurium challenge

To understand the effects of *Salmonella* Typhimurium on gut microbiota diversity and the abundance levels of different genera, the faecal microbiota data of the challenged laying chickens were analysed against the negative control group. Compared with the negative control, *Salmonella* challenge

significantly (FDR < 0.05) reduced the abundance of various bacterial genera that included *Subdoligranulum*, *Shuttleworthia*, *Sellimonas*, Ruminiclostridium_9, *Intestinimonas*, Gastranaerophilales_ge, *Faecalibacterium*, *Enorma* and *Blautia* (Figure 4-1).

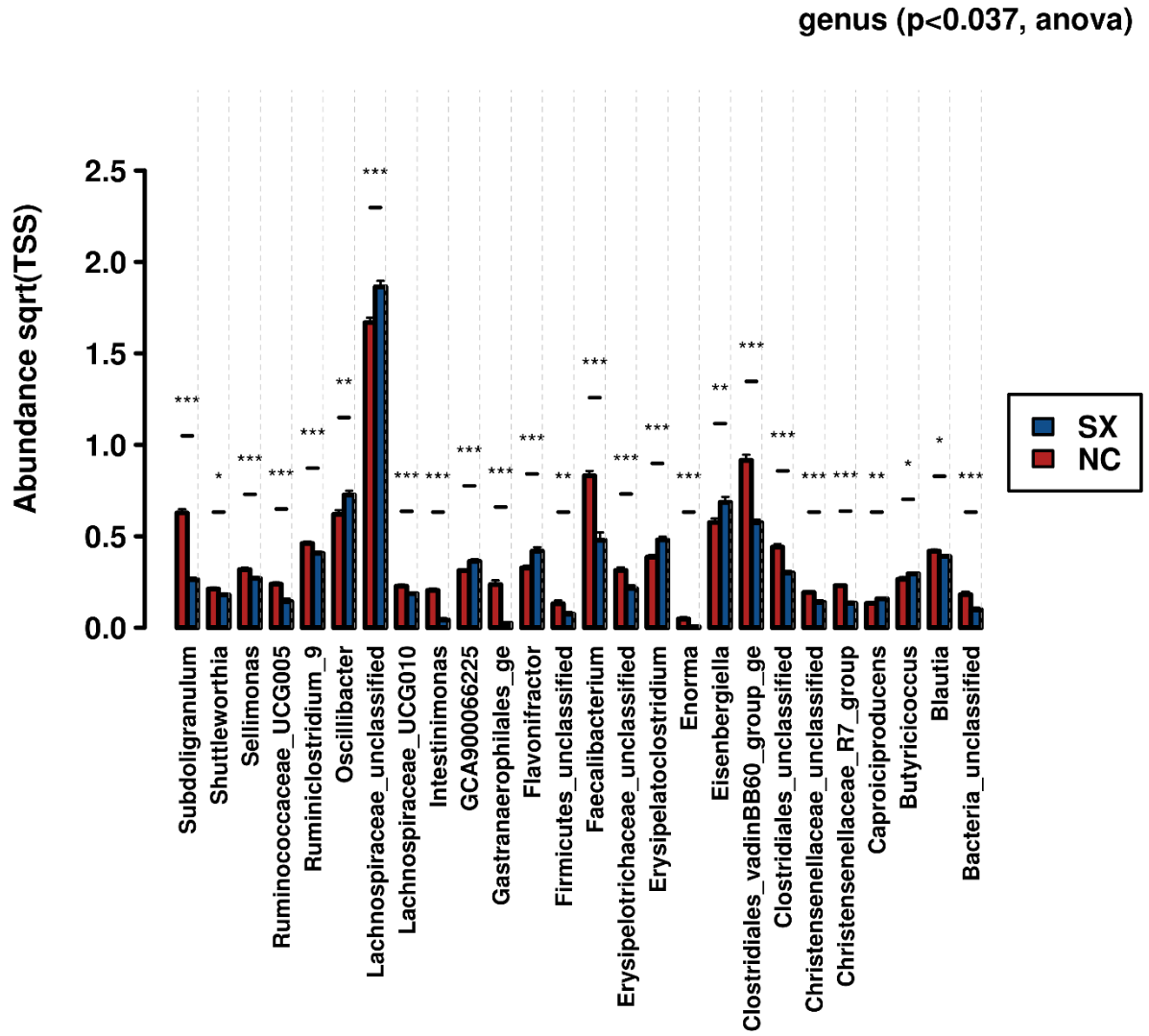


Figure 4-1 Effect of *Salmonella* Typhimurium challenge on the abundance of gut microbial communities

Compared with the negative control (NC), the abundance levels of different genera were significantly different in the *Salmonella* Typhimurium challenged (SX) group.
Only significant genera (* indicates the level of significance) between the two treatment groups have been depicted here.
In the Calypso software, P < 0.037 was equivalent to FDR < 0.05.

The abundance levels of *Oscillibacter*, GCA900066225, *Flavonifractor*, *Erysipelatoclostridium*, *Eisenbergiella*, *Caproiciproducens* and *Butyricicoccus* were significantly increased in the *Salmonella* Typhimurium challenged group compared with the negative control group. The abundance of these genera was also visualised in individual samples of the same chickens obtained at different sampling time-points (Appendix Figure 4-3). The abundance of *Bacteroides* increased after week 8 post-challenge both in the negative control and *Salmonella* challenged groups.

A significant (FDR < 0.05) effect of sampling time-point was observed on the abundance of multiple genera between the negative control and *Salmonella* Typhimurium challenged groups (Figure 4-2; Appendix Figure 4-4).

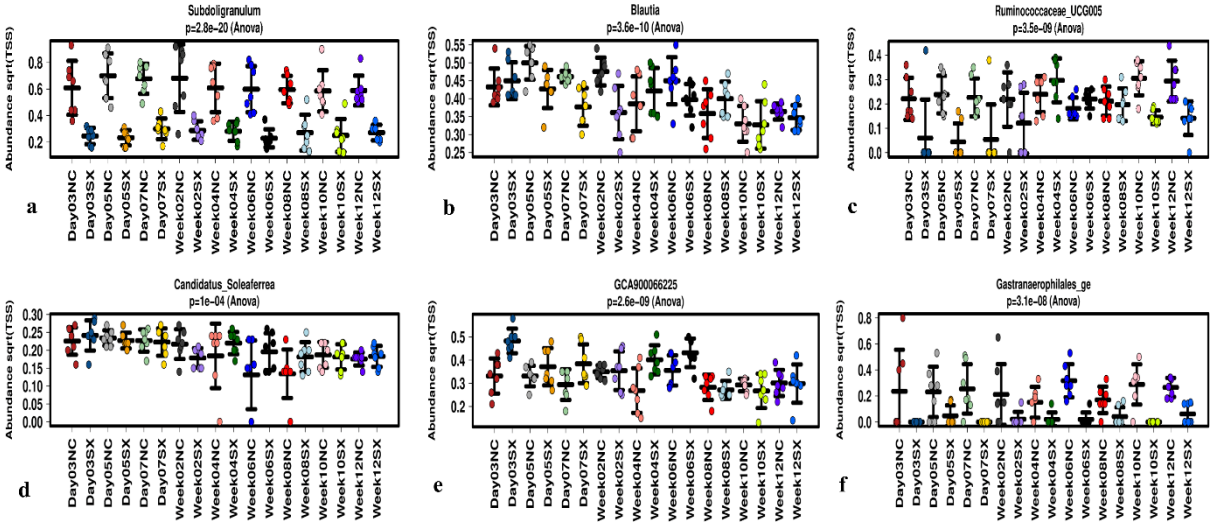


Figure 4-2 The microbial abundance of individual genera affected by *Salmonella* Typhimurium challenge

The microbial abundance at the genera level of the negative control (NC) group was compared with the *Salmonella* Typhimurium challenged (SX) group.
The data from the samples collected at nine different sampling time-points (days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12) were visualised between the NC and SX groups.

The abundance levels of different genera varied differently with sampling time-points. Genera such as *Subdoligranulum*, Gastranaerophilales_ge, *Intestinimonas*, Ruminococcaceae_UCG005 and *Sellimonas* were consistently lower in abundance in the *Salmonella* Typhimurium challenged group at all sampling time-points. A correlation heatmap was used to understand the effects of the sampling time-points and *Salmonella* Typhimurium challenge on the abundance of individual genera of gut microbial communities. A clear pattern of representation of individual microbial communities at different time-points both in the negative control and *Salmonella* Typhimurium challenged groups shows that *Salmonella* challenge affected the abundance of multiple microbial genera (Figure 4-3).

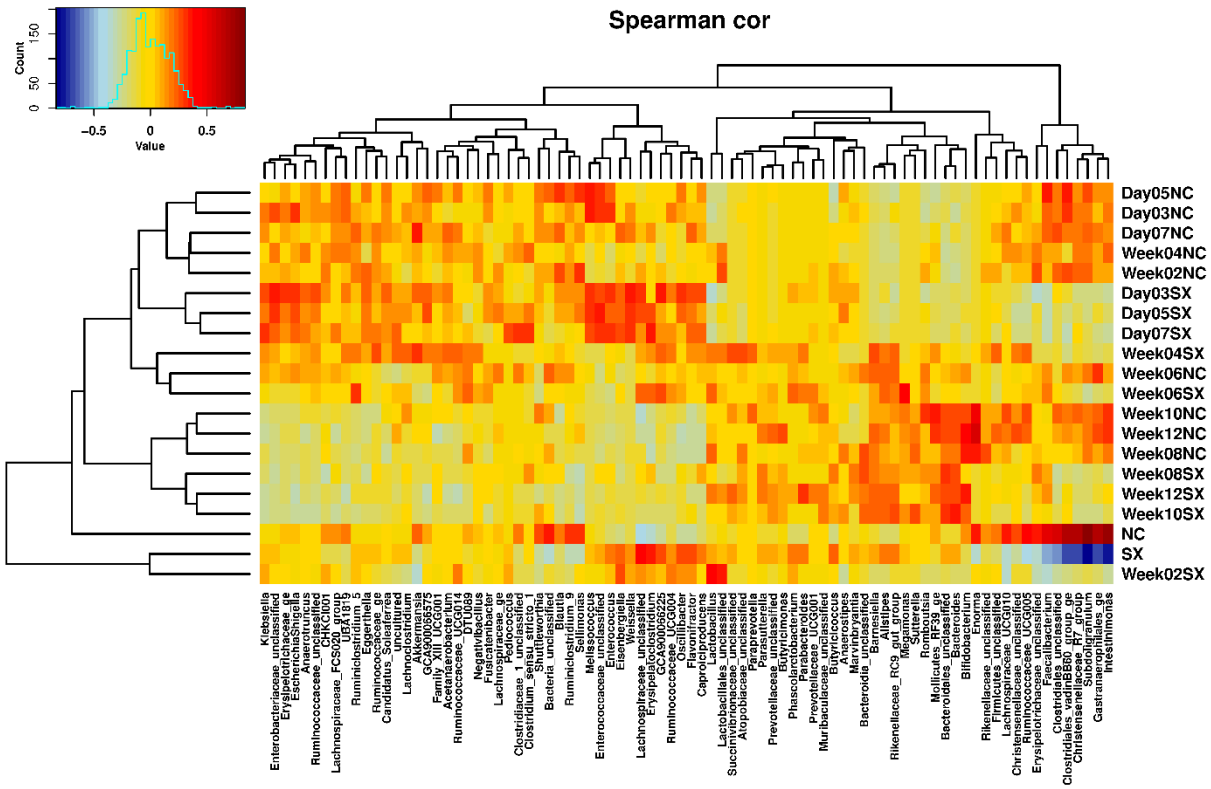


Figure 4-3 Heatmap showing the abundance of individual microbial communities affected by *Salmonella* Typhimurium challenge and sampling time-points

The abundance levels of different microbial genera of the negative control group (NC) were clearly separated from the *Salmonella* challenged (SX) group.

Data obtained from the faecal samples collected on days 3, 5 and 7, and weeks 2, 4, 6, 8, 10, and 12 post-challenge were visualised.

Measured by redundancy analysis (RDA+), there was a significant ($P < 0.05$) effect of *Salmonella* challenge on the microbial community composition (Figure 4-4a). The microbial alpha diversity (measured as Shannon index at genera level) was significantly different between the negative control (NC) and the *Salmonella* Typhimurium challenged (SX) group (Figure 4-4b).

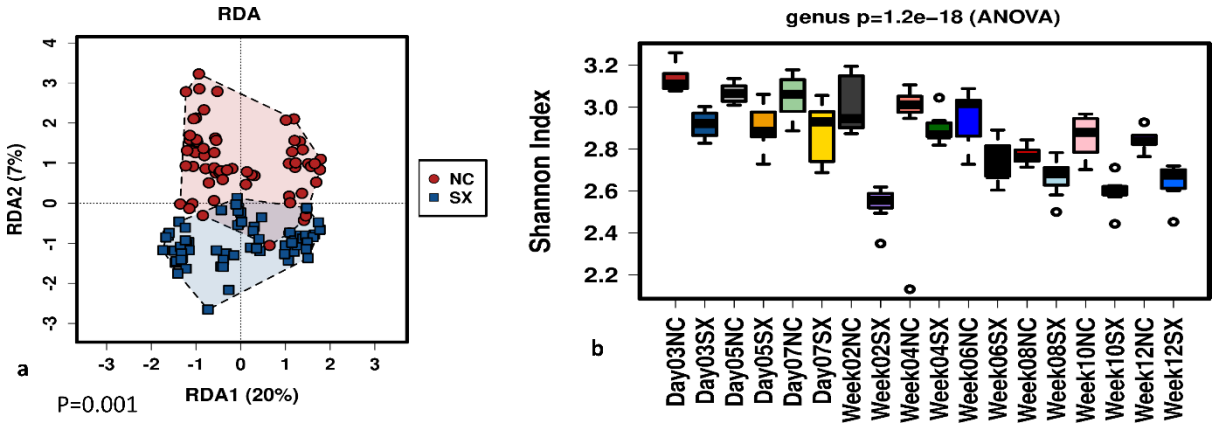


Figure 4-4 Microbial community composition and diversity affected by *Salmonella* Typhimurium challenge

(a) Microbial community composition between the negative control (NC) and *Salmonella* Typhimurium challenged (SX) groups.

(b) Microbial diversity between the NC and SX at different time-points (days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12) post-challenge.

The microbial diversity was significantly lower in the SX group across all the sampling time-points. Around week 4 post-challenge, two out of seven chickens were consistently negative for *Salmonella* Typhimurium. The gut microbiota analysis of the two *Salmonella* negative chickens showed a significantly higher abundance of *Faecalibacterium*, Erysipelotrichaceae_unclassified, Rikenellaceae_RC9_gut_group and *Intestinimonas* (Appendix Figure 4-5).

To understand the effects of the probiotic on gut microbiota in the presence of *Salmonella* Typhimurium, the abundance of microbial genera was compared between the CPC and CPX and between the IPC and IPX groups. Compared with the CPC, *Salmonella* Typhimurium challenge significantly decreased the abundance levels of *Acetanaerobacterium*, *Akkermansia*, *Anaerostipes*, *Bacteroides*, *Blautia*, *Eggerthella*, *Eisenbergiella*, *Enterococcus*, *EscherichiaShigella*, *Faecalibacterium*, *Lactobacillus*, *Melissococcus*, *Oscillibacter*, *Pediococcus*, Ruminiclostridium_9, Ruminococcaceae_UCG014, *Sellimonas*, *Subdoligranulum* and *Weissella*, while increasing the abundance levels of *Alistipes*, *Barnesiella*, *Bifidobacterium*, *Butyricimonas*, *Enorma*, *Intestinimonas*, *Megamonas*, *Parabacteroides*, *Paraprevotella*, *Parasutterella*, *Phascolarctobacterium* and *Sutterella* (Appendix Figure 4-6).

The microbial alpha diversity of the CPC was significantly separated from the CPX group (Appendix Figure 4-7a). The microbial diversity was significantly lower in the CPC compared with the CPX across all the sampling time-points (Appendix Figure 4-7b). The abundance levels of microbial genera in the IPC and IPX treatment groups were comparable to the CPC and CPX treatment groups, but there were fewer genera significantly affected between the two treatment groups of IPC and IPX (Appendix Figure 4-8). The microbial community composition of the IPX group was clearly separated from the IPC group and diversity of the IPX treatment group was significantly lower than the IPC across all the sampling time-points (Appendix Figure 4-9a, b).

To determine the effects of probiotic supplementation on microbial abundance and diversity, data were analysed and compared between the negative control and the probiotic supplemented control groups (excluding *Salmonella* Typhimurium challenge). Compared to the negative control, the continuous supplementation of the probiotic decreased the diversity of microbiota (Appendix Figure 4-10a) and the abundance of *Eisenbergiella*, *EscherichiaShigella*, *Blautia*, *Flavonifractor* and *Subdoligranulum* (Appendix Figure 4-10b). Compared with the negative control, the intermittent supplementation of probiotic decreased the diversity of microbiota (Appendix Figure 4-11a) and the abundance of microbial genera, such as *Faecalibacterium*, *EscherichiaShigella*, *Blautia*, *Sellimonas* and *Subdoligranulum* (Appendix Figure 4-11b).

4.4.3 Gut microbiota displaced by *Salmonella* Typhimurium was restored by *Bacillus* based probiotic supplementation

To understand the effects of the *Bacillus* based probiotic in restoring the gut microbial community abundance, we analysed the data obtained from the chickens continuously or intermittently fed with probiotic supplement and challenged with *Salmonella* Typhimurium or left as probiotic controls. The data were analysed against each of the respective treatment groups. The abundance levels of microbial genera that were significantly decreased or increased by the *Salmonella* Typhimurium challenge (SX) compared with the negative control (NC) group, were assessed for the effects of the probiotic. Probiotic supplementation restored (FDR > 0.05) the abundance levels of microbial genera, such as Bacteria_unclassified, Christensenellaceae_R7_group, Christensenellaceae_unclassifed, Lachnospiraceae_UCG010, Ruminiclostridium_9, Erysipelotrichaceae_unclassified, Firmicutes_unclassified, Ruminococcaceae_UCG005, Clostridiales_unclassified and Gastranaerophilales_ge (Figure 4-5a–j). Compared with the negative control, *Salmonella* challenge significantly increased the abundance of *Eisenbergiella*, *Erysipelatoclostridium*, *Flavonifractor*,

GCA900066225 and *Oscillibacter* (Figure 4-5k–o). When the effects of the continuously and intermittently supplemented *Bacillus* based probiotic on the restoration of the abundance of these microbial communities were assessed, the data showed that the continuously and intermittently supplemented probiotic restored microbiota with clearer effects observed for the continuously supplemented probiotic (Figure 4-5a–o).

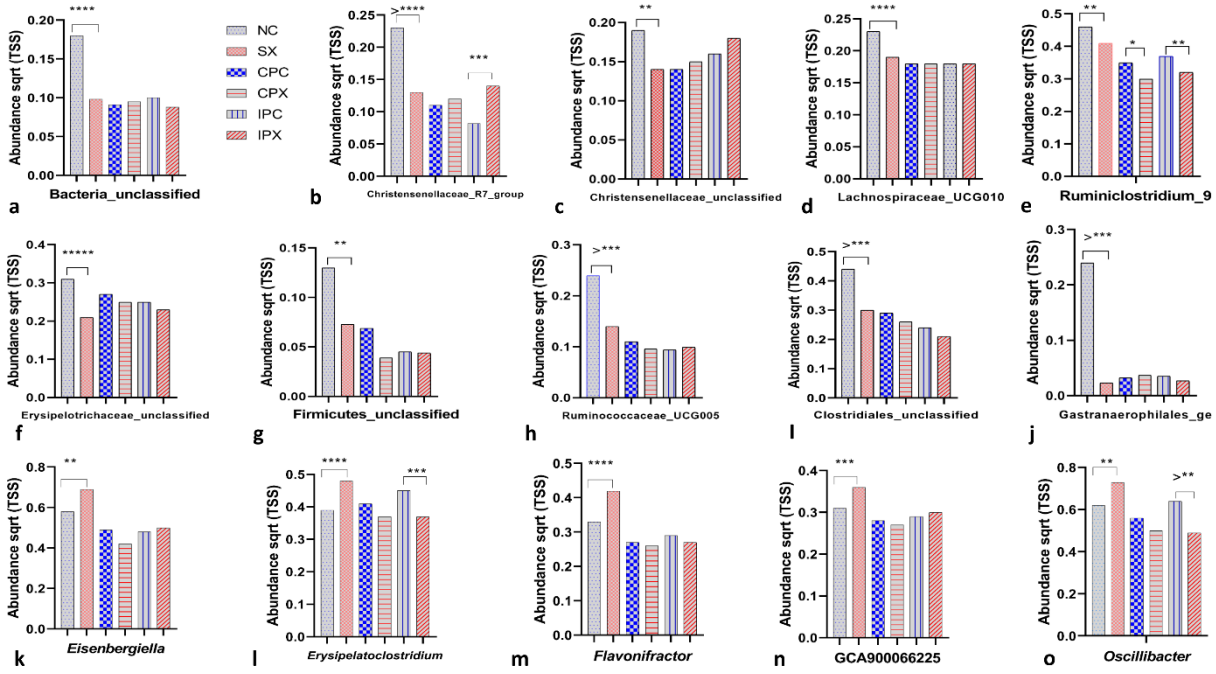


Figure 4-5 Bacillus based probiotic can restore the abundance of microbial communities (genera level analysis) that are displaced by *Salmonella* Typhimurium challenge

For direct group comparisons, the level of significance (if any) has shown between: the negative control (NC) and *Salmonella* challenged (SX); the continuously supplemented probiotic control (CPC) and continuous supplemented probiotic and *Salmonella* challenged (CPX); and the intermittent supplemented probiotic control (IPC) and intermittent supplemented probiotic and *Salmonella* challenged (IPX) groups.

The number of asterisks shows the level of significance (Tukey's *P* value).

The graphs show the abundance levels of (a) Bacteria_unclassified; (b) Christensenellaceae_R7_group; (c) Christensenellaceae_unclassified; (d) Lachnospiraceae_UCG010; (e) Ruminiclostridium_9; (f) Erysipelotrichaceae_unclassified; (g) Firmicutes_unclassified; (h) Ruminococcaceae_UCG005; (i) Clostridiales_unclassified; (j) Gastranaerophilales_ge; (k) *Eisenbergiella*; (l) *Erysipelatoclostridium*; (m) *Flavonifractor*; (n) GCA900066225 and (o) *Oscillibacter* between the treatment groups NC and SX, CPC and CPX, and IPC and IPX.

4.4.4 Microbial abundance affected by *Salmonella* Typhimurium was different in the presence of probiotic

The effect of the *Bacillus* based probiotic on the abundance of microbial communities at genera level in the presence and absence of *Salmonella* Typhimurium challenge was also assessed. Compared with the probiotic supplemented control groups, *Salmonella* challenge significantly reduced the abundance of *Acetanaerobacterium*, *Pediococcus*, *Anaerostipes*, *Eggerthella*, *Bacteroides* and *Lactobacillus* in the probiotic supplemented and *Salmonella* Typhimurium challenged groups (Figure 4-6a–f). This effect was highly significant for the continuously supplemented probiotic compared with the intermittently supplemented probiotic group (Figure 4-6a–f). Interestingly, the abundance of *Butyricimonas*, *Anaerotruncus*, *Barnesiella*, *Megamonas*, *Parabacteroides*, *Paraprevotella*, *Parasutterella*, *Alistipes*, *Phascolarctobacterium* and *Sutterella* was significantly higher in the probiotic supplemented and *Salmonella* challenged groups compared with the probiotic supplemented control groups (Figure 4-6g–p). The abundance of these microbial communities was not significantly different between the negative control and *Salmonella* challenged groups (Figure 4-6a–p).

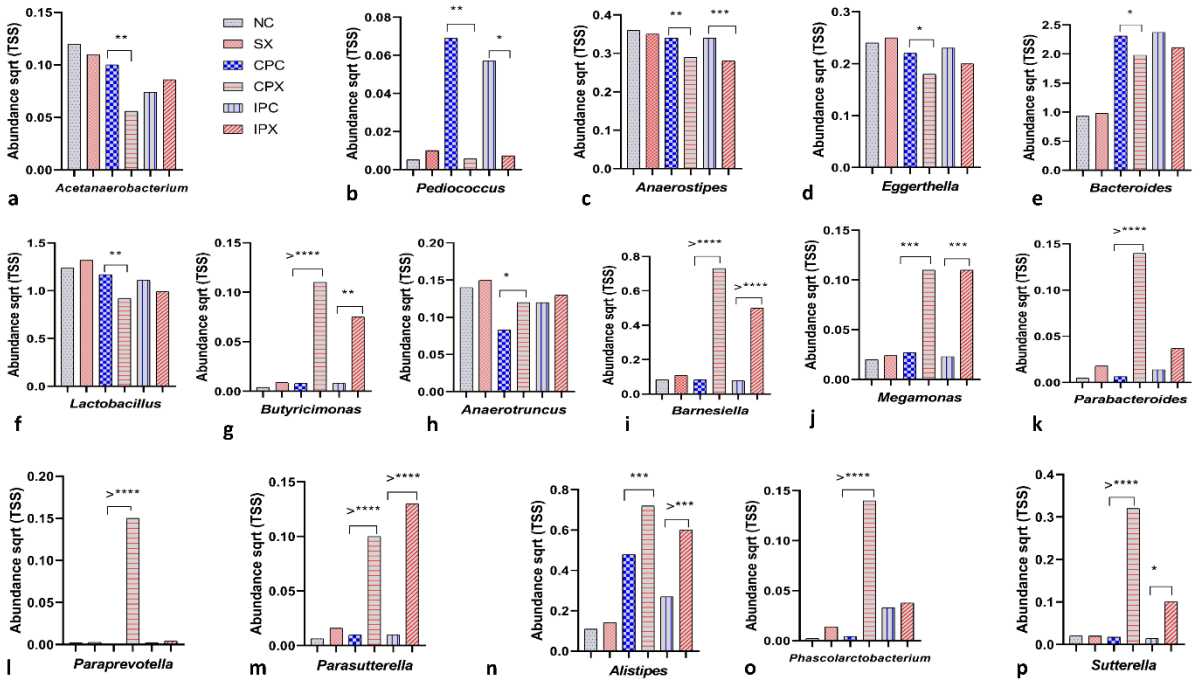


Figure 4-6 The abundance of microbial genera affected by *Salmonella* Typhimurium in the presence of *Bacillus* based probiotic

For direct group comparisons, the level of significance (if any) has shown between: the negative control (NC) and *Salmonella* challenged (SX); the continuous supplemented probiotic control (CPC) and continuously supplemented probiotic and *Salmonella* challenged (CPX); and the intermittent supplemented probiotic control (IPC) and intermittent supplemented probiotic and *Salmonella* challenged (IPX) groups.

The number of asterisks shows the level of significance (Tukey's *P* value).

The graphs show the abundance levels of (a) *Acetanaerobacterium*; (b) *Pediococcus*; (c) *Anaerostipes*; (d) *Eggerthella*; (e) *Bacteroides*; (f) *Lactobacillus*; (g) *Butyricimonas*; (h) *Anaerotruncus*; (i) *Barnesiella*; (j) *Megamonas*; (k) *Parabacteroides*; (l) *Paraprevotella*; (m) *Parasutterella*; (n) *Alistipes*; (o) *Phascolarctobacterium* and (p) *Sutterella* between the treatment groups NC and SX, CPC and CPX, and IPC and IPX.

4.4.5 Gut microbiota drives *Salmonella* Typhimurium load

To understand the interaction of *Salmonella* Typhimurium load with the gut microbiota at individual genera level, a regression analysis was performed on the $\log_{10}$ mMPN values of individual birds against each of the genera in the *Salmonella* challenged (SX) group. The load of *Salmonella* Typhimurium in the gut significantly ($P < 0.05$) affected the abundance of different microbial genera. The abundance of 30 microbial genera showed a significant weak negative correlation with the *Salmonella* Typhimurium load in the gut (Table 4-1). These genera included important gut resident microbiota members such as *Lactobacillus*, *Megamonas*, *Enorma*, *Barnesiella*, *Butyricimonas*, *Faecalibacterium*, *Intestinimonas* and *Parabacteroides*. The abundance of 24 microbial genera showed a significant weak positive correlation with the *Salmonella* Typhimurium load in the gut (Table 4-1). These microbial communities included genera such as *Acetanaerobacterium*, *Akkermansia*, *Anaerostipes*, *Blautia*, *Eggerthella*, *Pediococcus* and *EscherichiaShigella*.

Table 4-1 Correlation of *Salmonella* Typhimurium load with abundance of microbial genera in faeces

Microbe	R value	P value	Microbe	R value	P value
Alistipes	-0.341	1.60E-06	Prevotellaceae_UCG001	-0.323	5.70E-06
Atopobiaceae_unclassified	-0.442	1.80E-10	Prevotellaceae_unclassified	-0.297	3.30E-05
Bacteroidales_unclassified	-0.279	1.00E-04	Rikenellaceae_RC9-gut-group	-0.258	3.30E-04
Bifidobacterium	-0.531	3.70E-10	*Acetanaerobacterium*	0.181	0.013
Barnesiella	-0.433	5.00E-10	*Akkermansia*	0.212	0.0034
Butyricimonas	-0.233	1.30E-03	*Anaerostipes*	0.153	0.036
Christensenellaceae_R7_group	-0.146	4.40E-02	*Anaerotruncus*	0.179	0.014
Clostridiales_vadinB860_group_ge	-0.346	1.10E-06	*Blautia*	0.258	0.00033
Enorma	-0.403	9.10E-09	*Caproiciproducens*	0.192	0.0082
Faecalibacterium	-0.213	3.30E-03	Clostridiaceae_1_unclassified	0.299	2.90E-05
Family_XIII_UCG001	-0.318	8.10E-06	Clostridium_sensu_stricto_1	0.25	0.00051
Intestinimonas	-0.301	2.50E-05	*Eggerthella*	0.39	2.90E-08
Lactobacillus	-0.384	4.80E-08	*Eisenbergiella*	0.416	2.70E-09
Megamonas	-0.309	2.60E-05	Enterococcaceae_unclassified	0.397	1.60E-08
Negativibacillus	-0.168	2.10E-02	*Erysipelatoclostridium*	0.391	2.70E-08
Parabacteroides	-0.301	2.60E-05	Erysipelotrichaceae_ge	0.29	5.00E-05
Paraprevotella	-0.242	7.90E-04	*EscherichiaShigella*	0.542	8.90E-16
Parasutterella	-0.304	2.10E-05	*Flavonifractor*	0.268	1.90E-04
Phascolarctobacterium	-0.194	7.50E-03	*Fusicatenibacter*	0.21	3.70E-03
Romboutsia	-0.304	2.10E-05	GCA900066575	0.422	1.50E-09
Sutterella	-0.377	9.20E-08	Lachnospiraceae_unclassified	0.267	0.00021
Succinivibrionaceae_unclassified	-0.26	3.00E-04	*Melissococcus*	0.252	4.60E-04
Ruminococcaceae_UCG005	-0.355	5.50E-07	*Pediococcus*	0.22	2.40E-03
Rikenellaceae_unclassified	-0.318	8.40E-06	*Ruminiclostridium_5*	0.279	1.00E-04
Bacteroidia_unclassified	-0.186	1.00E-02	Ruminococcaceae_unclassified	0.146	4.60E-02
Mollicutes_RF39_ge	-0.281	8.90E-05	*Sellimonas*	0.333	2.90E-06
Muribaculaceae_unclassified	-0.309	1.80E-05	*Weissella*	0.352	6.70E-07

The faecal load of *Salmonella* Typhimurium (in $\log_{10}$ mMPN) was regressed against the abundance of individual genera of gut microbiota.
Minus (-) sign shows negative correlation.

4.4.6 Short chain fatty acids quantification from faeces

The levels of acetate, butyrate and propionate were significantly ($P < 0.05$) affected over time following *Salmonella* Typhimurium infection (Figure 4-7). Among the treatment groups, the levels of acetate and butyrate were significantly higher in the continuously supplemented probiotic control (CPC) compared with the continuously supplemented probiotic and *Salmonella* Typhimurium challenged (CPX) group. However, within each treatment group, at each sampling time-point, there was no significant ($P > 0.05$) difference in the acetate content of the faeces (Figure 4-7a). Within each treatment group, the level of butyrate in the faeces was significantly higher in the CPC and intermittent

supplemented probiotic control (IPC) groups compared with the CPX and the intermittent supplemented probiotic and *Salmonella* Typhimurium challenged (IPX) groups. Within each treatment group, the level of butyrate in the faeces at weeks 1, 4 and 8 post-challenge was significantly higher in the CPC compared with the IPX group (Figure 4-7b). The propionate level was significantly affected by the sampling time-point post *Salmonella* Typhimurium challenge but was not consistent with the levels of acetate and butyrate (Figure 4-7c). Within each treatment group, the level of propionate in the faeces was significantly lower in the CPC and IPC compared with the CPX and IPX groups.

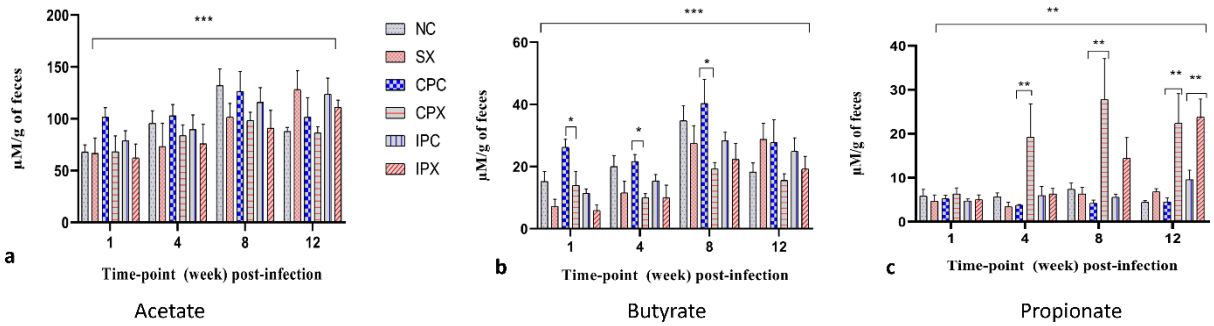


Figure 4-7 Short-chain fatty acids levels in the faeces of chickens fed with probiotic and challenged with *Salmonella* Typhimurium

The respective treatment groups were compared with each other at each sampling time-point of *Salmonella* Typhimurium post-challenge.
(a) acetate, (b) butyrate and (c) propionate levels in faeces at different sampling time-points (weeks 1, 4, 8 and 12 post-challenge).
Bar (with asterisks) across the individual graph shows a significant effect of treatment group on short-chain fatty acids production.
NC is negative control; SX is *Salmonella* challenge; CPX is a continuous probiotic supplemented and *Salmonella* challenge; CPC is a continuous probiotic supplemented control; IPX is intermittent probiotic supplemented and *Salmonella* challenge; IPC is intermittent probiotic control.

4.4.7 Effects of probiotic supplementation on *Salmonella* Typhimurium load in faeces and organs

To understand the effects of gut microbiota modulation through the probiotic on *Salmonella* Typhimurium shedding levels in faeces, an mMPN method (log_{10}) was performed. Faeces from the negative and probiotic control groups were negative for *Salmonella*. Irrespective of the probiotic supplementation, some chickens from the *Salmonella* challenged groups turned negative for *Salmonella* Typhimurium load in faeces around week 4 post-challenge. However, not all of these chickens were consistently negative for faecal load of *Salmonella* at different sampling time-points. A significant effect of time-point and treatment was observed on the shedding level of *Salmonella* Typhimurium in the faeces (Figure 4-8a, b). Within each sampling time-point, the continuously supplemented probiotic and *Salmonella* Typhimurium challenged group (CPX) showed a significantly lower bacterial load compared with the intermittent supplemented probiotic and *Salmonella* Typhimurium challenged group (IPX) at week 8 post-challenge (Figure 4-8a). Overall, the load of *Salmonella* Typhimurium was significantly lower in the CPX compared with the *Salmonella* challenged (SX) and IPX groups (Figure 4-8b).

The load of *Salmonella* Typhimurium in organs was determined at the point of termination of the experiment (week 30 of flock age). *Salmonella* was not recovered from various organs collected from the negative and probiotic control groups. For the *Salmonella* Typhimurium challenged groups, organ homogenates directly plated on XLD and BSA media were negative; however, some samples turned positive when an enrichment method was followed. Therefore, the load of *Salmonella* Typhimurium in organs was expressed as mean percent value per treatment group. The mean percent value of

Salmonella Typhimurium for caecum was significantly lower in the CPX compared with the SX group (Figure 4-8c) The mean percent value of *Salmonella* Typhimurium for shell gland was significantly lower in the CPX and IPX compared with the SX group (Figure 4-8h). *Salmonella* Typhimurium was not recovered from the caecum, jejunum, liver, magnum/infundibulum and shell gland of the CPX group (Figure 4-8c, d, e, g, h). Similarly, *Salmonella* Typhimurium was not recovered from the liver, magnum/infundibulum and shell gland of the IPX group (Figure 4-8e, g, h). No *Salmonella* was isolated from the internal contents of the eggs. Shell wash samples positive for *Salmonella* through the enrichment method showed no measurable load by the mMPN method.

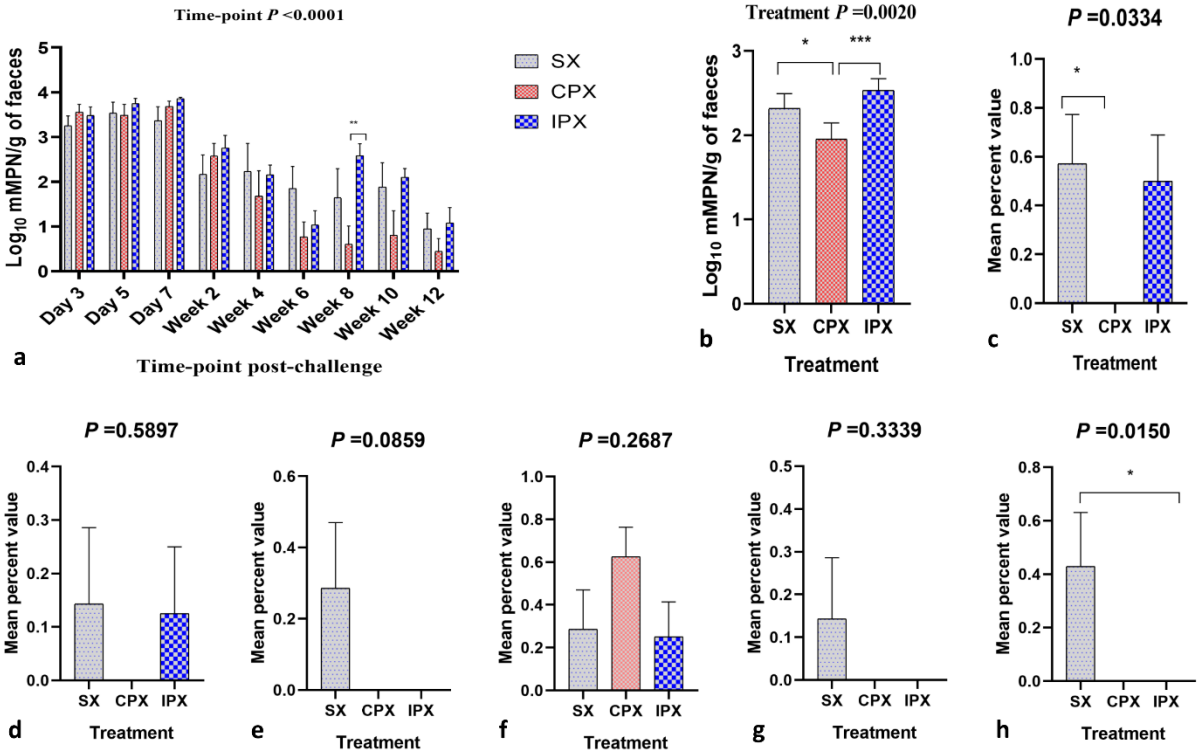


Figure 4-8 Load of *Salmonella* Typhimurium in faeces affected by sampling time-points and mean percent value of *Salmonella* Typhimurium in organs at week 12 post-challenge

(a) *Salmonella* load in $\log_{10}$ mMPN per gram of faeces at different sampling time-points in three treatment groups (SX- *Salmonella* Typhimurium challenged; CPX- *Salmonella* Typhimurium challenged and continuously supplemented feed with probiotic; IPX- *Salmonella* Typhimurium challenged and continuously supplemented feed with probiotic).

(b) *Salmonella* load in $\log_{10}$ mMPN per gram of faeces affected by treatment groups.

Mean percent value of *Salmonella* Typhimurium in (c) caecum; (d) jejunum; (e) liver; (f) spleen; (g) magnum/infundibulum and (h) shell gland.

For *Salmonella* load in the organs, small pieces of the tissues with known weight were homogenised in 500 µL PBS and a 100 µL was plated on XLD media. From the same tissue homogenates, a 100 µL was enriched in BPW followed by RVS and streaked on XLD/BSA media. The XLD/BSA plates were read as positive (1) and negative (0).

The data were analysed in Statview software for getting mean percent values that represent the load of *Salmonella* Typhimurium in organs.

4.5 Discussion

The main objectives of this study were to understand the dynamics of the gut microbiota in *Salmonella* Typhimurium infected laying chickens, and to study the effects of continuous and intermittent feeding of probiotic on *Salmonella* Typhimurium shedding. A balanced gut microbiota can resist pathogen colonisation and subsequent clearance from the gut (Pickard et al. 2017). In this study, we reared *Salmonella* spp. free laying chickens to understand the true effects of this pathogen on gut microbiota displacement as other species of *Salmonella,* if already colonised in gut, can significantly influence the

shedding of *Salmonella* Typhimurium. The results showed that both the *Salmonella* Typhimurium and the *Bacillus* based probiotic significantly affected the composition and diversity of the gut microbial communities. The data also showed that continuous supplementation of the *Bacillus* based probiotic reduced the load of *Salmonella* Typhimurium in the faeces (overall) and in organs tested at the end of the experiment. The decrease in abundance levels of *Eisenbergiella*, *EscherichiaShigella*, *Blautia*, *Flavonifractor* and *Subdoligranulum* by the probiotic supplementation shows that the *Bacillus* based probiotic has the potential to affect gut microbial abundance. Of the reduced microbial genera, *Escherichia* and *Shigella* have the potential to cause infection in certain conditions, while other genera such as *Blautia*, *Flavonifractor* and *Subdoligranulum* are vital for gut health. The probiotic supplementation also increased the abundance levels of good bacteria, such as *Bacteroides* and *Alistipes*. Further investigation is necessary to determine the effects of the decreased abundance of the above mentioned microbial genera on the host gut.

In chickens, the composition of gut microbiota varies considerably with bird age, with more complex microbiota present in older birds (Videnska et al. 2014). *Salmonella* Typhimurium induces inflammation of intestinal epithelia (Wang et al. 2018) and displacement of gut microbiota in laying chicks (Azcarate-Peril et al. 2018). However, the long-term effects of *Salmonella* Typhimurium on the gut microbiota in laying chickens have not been investigated. To understand the role of *Salmonella* Typhimurium colonisation on the abundance and diversity of gut microbiota, the microbial communities of the faeces of individual birds collected at nine different time-points post-challenge from the *Salmonella* negative control and *Salmonella* challenged groups were compared. Overall, the *Salmonella* Typhimurium infection reduced the abundance of many bacterial genera including *Blautia*, Bacteria_unclassified, Christensenellaceae_R7_group, *Enorma*, *Faecalibacterium*, Christensenellaceae_unclassifed, Lachnospiraceae_UCG010, Ruminiclostridium_9, *Subdoligranulum* and Firmicutes_unclassified. This shows that not all members of the gut microbiota have the potential to compete with the *Salmonella* Typhimurium. Most of these bacterial genera play a vital role in maintaining gut health through the production of organic acids and vitamins. For example, *Christensenellaceae* contains bacteria that secrete β-glucosidase, β-galactosidase and α-arabinosidase and therefore help in polysaccharide digestion (Morotomi et al. 2012). As *Salmonella* lacks the enzyme β1-4 linkage, which is required for polysaccharide fermentation, *Christensenellaceae* can ferment these polysaccharides. However, *Salmonella* Typhimurium challenge reduces the abundance of *Christensenellaceae* and *Lachnospiraceae* (Azcarate-Peril et al. 2018). Therefore, *Salmonella* infection could lead to the interruption of the Christensenellaceae based polysaccharide fermentation. Ruminiclostridium_9 and Ruminococcaceae_UCG005 are members of *Ruminococcaceae* that are common gut microbes involved in the breakdown of complex carbohydrates. A decreased abundance of *Erysipelotrichaceae* was observed in Crohn's disease (Dey et al. 2013). Some species in *Clostridiales* degrade a variety of fibre and have been identified as producing propionate, acetate and butyrate. *Gastranaerophilales_ge* obtains its energy by obligate fermentation resulting in the production of organic acids in the gut. A previous study suggested that *Salmonella* Enteritidis reduced the abundance level of *Faecalibacterium* in the chicken gut (Liu et al. 2018). The functions of *Faecalibacterium* in the chicken gut are not well characterised; however, it is one of the most abundant resident gut microbes in a human gut (Qin et al. 2010). In the current study, the lower abundance levels of the above mentioned microbial communities show that *Salmonella* Typhimurium establishes its niche in the gut at the expense of displacing these bacterial communities leading to *Salmonella* driven dysbiosis. In the current study, the increased abundance of *Faecalibacterium* in the gut of hens that turned negative for *Salmonella* suggest its potential role to be characterised as a probiotic candidate for gut health.

Compared with the negative control group, *Salmonella* Typhimurium challenge increased the abundance of *Eisenbergiella, Erysipelatoclostridium, Flavonifractor, GCA900066225 and Oscillibacter. Eisenbergiella is a* rod-shaped, non-proteolytic, non-motile, anaerobic bacteria in the *Lachnospiraceae*

that produces succinate, lactate, butyrate and acetate during fermentation (Amir et al. 2014). *Erysipelatoclostridium* is a part of normal gut microbiota but could become an opportunistic pathogen and has been identified as a gut microbiota biomarker in human patients suffering from Crohn's disease and *Clostridium difficile* infection (Mancabelli et al. 2017). In the current study, the non-significant difference in the abundance levels of *Eisenbergiella, Flavonifractor, GCA900066225, Oscillibacter* and *Erysipelatoclostridium* between the continuously supplemented probiotic control and the continuously supplemented probiotic and Salmonella Typhimurium challenged groups shows the positive effect of the probiotic on gut microbiota. These results are further supported by the positive correlation of the abundance levels of the above-mentioned genera with *Salmonella* load in the gut.

The regression analysis of the *Salmonella* Typhimurium load (measured as $\log_{10}$ mMPN/gram of faeces) against the abundance of gut microbial genera showed that more genera were negatively affected by the *Salmonella* Typhimurium infection. This indicates that, as the *Salmonella* Typhimurium load decreased over time, these microbial genera had the potential to restore normal abundance. The negatively correlated genera, such as *Lactobacillus*, *Megamonas*, *Negativibacillus*, *Parabacteroides*, *Paraprevotella*, *Parasutterella*, *Phascolarctobacterium*, *Romboutsia*, *Bifidobacterium*, *Butyricimonas*, *Barnesiella*, *Faecalibacterium* and *Intestinimonas* perform vital functions ranging from vitamin synthesis to organic acid production. *Megamonas* contains a gene cluster that encodes secreted cellobiose phosphotransferase system, endo-glucanases and 6-phospho-beta-glucocidase that potentially degrade non-starch polysaccharides to cellobiose in the chicken gut (Sergeant et al. 2014). *Negativibacillus* belongs to *Ruminococcaceae* with no known functions. *Parabacteroides* improves host metabolism through the production of succinate and secondary bile acids in the gut as shown in mice (Wang et al. 2019); however, its functions in chickens have not been investigated. For propionate production, *Parabacteroides*, *Alistipes* and *Paraprevotella* express cobalamin-binding methylmalonyl-CoA mutase and/or methylmalonyl-CoA epimerase (Polansky et al. 2016). In Firmicutes, *Phascolarctobacterium, Megamonas and Blautia produce propionate through* epimerase, decarboxylase and methylmalonyl-CoA mutase pathways (Polansky et al. 2016). Faecalibacterium, *Subdoligranulum,* and *Phascolarctobacterium* produce butyrate through acetyl/propionyl-CoA carboxylase pathway. In the current study, the reduced abundance of the useful microbial genera by the *Salmonella* Typhimurium challenge would have affected their normal functions vital for maintaining gut health through fermentation. Moreover, most of these microbial communities were positively influenced when the probiotic was supplemented in the diet. The effects of the probiotic on the abundance at the genera level were clearer in the continuously supplemented, rather than the intermittent supplemented group. For example, the continuously supplemented probiotic restored the abundance of Christensenellaceae_R7_group, *Erysipelatoclostridium* and *Oscillibacter*, while the intermittent supplementation merely improved it compared with the *Salmonella* challenged groups. This shows that the continuous supplementation of the probiotic produced better results.

On the other hand, the increased load of *Salmonella* Typhimurium favoured a large number of microbial communities of the gut microbiota by increasing their abundance. The bacterial genera that were positively correlated with the *Salmonella* Typhimurium load included *Flavonifractor*, *Akkermansia*, *Anaerostipes*, *Blautia*, *Caproiciproducens*, *Eggerthella*, *Eisenbergiella*, *Erysipelatoclostridium*, *Melissococcus*, *Pediococcus*, Ruminiclostridium_5, *Sellimonas*, *Weissella* and some unclassified genera. Although these genera are part of normal gut microbiota, some of them can become opportunistic pathogens causing dysbiosis and subsequent infections. The precise molecular mechanisms underlying how *Salmonella* Typhimurium causes the increased abundance of these genera are not known; however, in this study, we showed that the *Salmonella* driven dysbiosis favours a large number of resident gut microbiota to increase in abundance thereby affecting the abundance of other resident gut microbial community members. *Flavonifractor* is a member of resident gut microbiota but has been shown to cause infection in an immunocompromised patient (Berger et al.

2018). The precise role of *Flavonifractor* in the dysbiosed gut of chickens needs to be investigated.

In the current study, the levels of acetate, butyrate and propionate in faeces were quantified at weeks 1, 4, 8 and 12 post-challenge to understand the effects of the probiotic treatment in *Salmonella* challenged or non-challenged hens. The higher level of butyrate in response to the supplementation of the probiotic shows that the probiotic treatment increased its production, while the Salmonella infection decreased it possibly due to the displaced microbial communities. The microbiota produced gut metabolites such as acetate, butyrate and propionate. These metabolites play an important role in gut health ranging from the provision of energy to host enterocytes and regulation of the immune system (LeBlanc et al. 2017). The propionate level was higher in *Salmonella* challenged and probiotic supplemented groups compared to the probiotic control groups. It seems that certain organic acid producing genera that increased in abundance in response to *Salmonella* infection may have produced propionate. However, this needs further investigation.

The inconsistency in the *Salmonella* positive faecal samples from the infected groups with the *Salmonella* status of the caeca (at point of termination of the experiment) might highlight the importance of *Salmonella* persistence in other parts of the gut, such as the colon, which requires further investigation. Irrespective of the probiotic supplementation status, the faeces of some *Salmonella* challenged chickens turned negative for *Salmonella* around week 4 post-challenge but were inconsistent in shedding profile. However, around week 8 post-challenge, more hens turned negative for *Salmonella* Typhimurium shedding in the faeces in the continuous supplemented probiotic (n = 5) compared with the intermittent supplemented probiotic (n = 2) and *Salmonella* challenged (n = 3) groups. This shows that the *Salmonella* challenged chickens could harbour the bacteria in the gut for intermittent shedding. Probiotic treatment can reduce the level of shedding but continuous or intermittent feeding of probiotics does not eliminate the pathogen.

4.6 Conclusions

Salmonella Typhimurium affects the microbial abundance of certain genera that play a role in maintaining a healthy gut. Microbial genera that are increased in abundance in the *Salmonella* populated gut might play a role either in the *Salmonella* driven dysbiosis or in maintaining a normal gut function. The displaced gut microbiota can be partly restored by supplementing the feed with a *Bacillus* based probiotic, thus lowering the mean load of *Salmonella* in faeces.

4.7 References

MiSeq SOP [Online]. Available: https://www.mothur.org/wiki/MiSeq_SOP [Accessed 10 Aug 2019].

Script to convert mothur taxonomy summary files to Krona XML format [Online]. Available: https://github.com/accaldwell/mothur_krona [Accessed 10 Aug 2019].

Simple script to generate Krona charts for all samples in an OTU table file [Online]. Available: https://github.com/GenomicaMicrob/OTUsamples2krona [Accessed 10 Aug 2019].

AMIR, I., BOUVET, P., LEGEAY, C., GOPHNA, U. & WEINBERGER, A. 2014. Eisenbergiella tayi gen. nov., sp. nov., isolated from human blood. *International Journal of Systematic and Evolutionary Microbiology,* 64**,** 907-914.

AZCARATE-PERIL, M. A., BUTZ, N., CADENAS, M. B., KOCI, M., BALLOU, A., MENDOZA, M., ALI, R. & HASSAN, H. 2018. An attenuated *Salmonella enterica* serovar Typhimurium strain and galacto-oligosaccharides accelerate clearance of *Salmonella* infections in poultry through modifications to the gut microbiome. *Applied and Environmental Microbiology,* 84**,** e02526-17.

BADRI, M., KURTZ, Z., MULLER, C. & BONNEAU, R. 2018. Normalization methods for microbial abundance data strongly affect correlation estimates. *bioRxiv***,** 406264.

BALLOU, A. L., ALI, R. A., MENDOZA, M. A., ELLIS, J. C., HASSAN, H. M., CROOM, W. J. & KOCI, M. D. 2016. Development of the chick microbiome: How early exposure influences future microbial diversity. *Frontiers in Veterinary Science,* 3.

BERGER, F. K., SCHWAB, N., GLANEMANN, M., BOHLE, R. M., GÄRTNER, B. & GROESDONK, H. V. 2018. *Flavonifractor* (*Eubacterium*) *plautii* bloodstream infection following acute cholecystitis. *IDCases,* 14**,** e00461.

BRISBIN, J. T., GONG, J., PARVIZI, P. & SHARIF, S. 2010. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. *Clinical and Vaccine Immunology,* 17**,** 1337-1343.

BUFFIE, C. G. & PAMER, E. G. 2013. Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews Immunology,* 13**,** 790.

CUI, Y., WANG, Q., LIU, S., SUN, R., ZHOU, Y. & LI, Y. 2017. Age-related variations in intestinal microflora of free-range and caged hens. *Frontiers in Microbiology,* 8**,** 1310.

DEY, N., SOERGEL, D. A., REPO, S. & BRENNER, S. E. 2013. Association of gut microbiota with post-operative clinical course in Crohn's disease. *BMC Gastroenterology,* 13**,** 131.

DIEYE, Y., AMEISS, K., MELLATA, M. & CURTISS, R. 2009. The *Salmonella* Pathogenicity Island (SPI) 1 contributes more than SPI2 to the colonization of the chicken by *Salmonella enterica* serovar Typhimurium. *BMC Microbiology,* 9**,** 3.

FELLOWS, R., DENIZOT, J., STELLATO, C., CUOMO, A., JAIN, P., STOYANOVA, E., BALÁZSI, S., HAJNÁDY, Z., LIEBERT, A. & KAZAKEVYCH, J. 2018. Microbiota derived short chain fatty acids promote histone crotonylation in the colon through histone deacetylases. *Nature Communications,* 9**,** 105.

FORD, L., MOFFATT, C., FEARNLEY, E., MILLER, M., GREGORY, J., SLOAN-GARDNER, T., POLKINGHORNE, B. G., BELL, R., FRANKLIN, N. & WILLIAMSON, D. A. 2018. The epidemiology of *Salmonella enterica* outbreaks in Australia, 2001-2016. *Frontiers in Sustainable Food Systems,* 2**,** 86.

FULLER, R. 1989. Probiotics in man and animals. *Journal of Applied Bacteriology,* 66**,** 365-378.

GOLE, V. C., CHOUSALKAR, K. K. & ROBERTS, J. R. 2013. Survey of Enterobacteriaceae contamination of table eggs collected from layer flocks in Australia. *International Journal of Food Microbiology,* 164**,** 161-165.

GOLE, V. C., WOODHOUSE, R., CARAGUEL, C., MOYLE, T., RAULT, J.-L., SEXTON, M. & CHOUSALKAR, K. 2017. Dynamics of *Salmonella* shedding and welfare of hens in free-range egg production systems. *Applied and Environmental Microbiology,* 83**,** e03313-16.

HONDA, K. & LITTMAN, D. R. 2012. The microbiome in infectious disease and inflammation. *Annual Review of Immunology,* 30**,** 759-795.

HOOPER, L. V., WONG, M. H., THELIN, A., HANSSON, L., FALK, P. G. & GORDON, J. I. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science,* 291**,** 881-884.

ISMAIL, A. S., VALASTYAN, J. S. & BASSLER, B. L. 2016. A host-produced autoinducer-2 mimic activates bacterial quorum sensing. *Cell Host & Microbe,* 19**,** 470-480.

LAPORT, M. S., BAUWENS, M., COLLARD, M. & GEORGE, I. 2018. Phylogeny and Antagonistic Activities of Culturable Bacteria Associated with the Gut Microbiota of the Sea Urchin (*Paracentrotus lividus*). *Current Microbiology,* 75**,** 359-367.

LEBLANC, J. G., CHAIN, F., MARTÍN, R., BERMÚDEZ-HUMARÁN, L. G., COURAU, S. & LANGELLA, P. 2017. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial Cell Factories,* 16**,** 79.

LEBLANC, J. G., MILANI, C., DE GIORI, G. S., SESMA, F., VAN SINDEREN, D. & VENTURA, M. 2013. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Current Opinion in Biotechnology,* 24**,** 160-168.

LIU, L., LIN, L., ZHENG, L., TANG, H., FAN, X., XUE, N., LI, M., LIU, M. & LI, X. 2018. Cecal microbiome profile altered by *Salmonella enterica*, serovar Enteritidis inoculation in chicken. *Gut Pathogens,* 10**,** 34.

LLOYD, A. B., CUMMING, R. B. & KENT, R. D. 1977. Prevention of *Salmonella* Typhimurium infection in poultry by pretreatment of chickens and poults with intestinal extracts. *Australian Veterinary Journal,* 53**,** 82-87.

LU, Y., FAN, C., LI, P., LU, Y., CHANG, X. & QI, K. 2016. Short chain fatty acids prevent high-fat-diet-induced obesity in mice by regulating G protein-coupled receptors and gut microbiota. *Scientific Reports,* 6**,** 37589.

LUOMA, A., MARKAZI, A., SHANMUGASUNDARAM, R., MURUGESAN, G., MOHNL, M. & SELVARAJ, R. 2017. Effect of synbiotic supplementation on layer production and cecal *Salmonella* load during a *Salmonella* challenge. *Poultry Science,* 96**,** 4208-4216.

MACFARLANE, G.T., STEED, H. AND MACFARLANE, S. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology*, *104*, 305-344.

MANCABELLI, L., MILANI, C., LUGLI, G. A., TURRONI, F., COCCONI, D., VAN SINDEREN, D. & VENTURA, M. 2017. Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. *FEMS Microbiology Ecology,* 93**,** fix153.

MARTÍN, R. AND LANGELLA, P. 2019. Emerging health concepts in the probiotics field: streamlining the definitions. *Frontiers in Microbiology*, *10*, 1047.

MIKULSKI, D., JANKOWSKI, J., NACZMANSKI, J., MIKULSKA, M. & DEMEY, V. 2012. Effects of dietary probiotic (*Pediococcus acidilactici*) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poultry Science,* 91**,** 2691-2700.

MILLION, M., ALOU, M. T., KHELAIFIA, S., BACHAR, D., LAGIER, J.-C., DIONE, N., BRAH, S., HUGON, P., LOMBARD, V. & ARMOUGOM, F. 2016. Increased gut redox and depletion of anaerobic and methanogenic prokaryotes in severe acute malnutrition. *Scientific Reports,* 6**,** 26051.

MON, K. K. Z., SAELAO, P., HALSTEAD, M. M., CHANTHAVIXAY, G., CHANG, H.-C., GARAS, L., MAGA, E. A. & ZHOU, H. 2015. *Salmonella enterica* serovars Enteritidis infection alters the indigenous microbiota diversity in young layer chicks. *Frontiers in Veterinary Science,* 2**,** 61.

MOROTOMI, M., NAGAI, F. & WATANABE, Y. 2012. Description of Christensenella minuta gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order Clostridiales, and proposal of Christensenellaceae fam. nov. *International Journal of Systematic and Evolutionary Microbiology,* 62**,** 144-149.

MORRISON, D. J. & PRESTON, T. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes,* 7**,** 189-200.

MYERS, S. P. 2004. The causes of intestinal dysbiosis: a review. *Alternative Medicine Review,* 9**,** 180-197.

OAKLEY, B. B., LILLEHOJ, H. S., KOGUT, M. H., KIM, W. K., MAURER, J. J., PEDROSO, A., LEE, M. D., COLLETT, S. R., JOHNSON, T. J. & COX, N. A. 2014. The chicken gastrointestinal microbiome. *FEMS Microbiology Letters,* 360**,** 100-112.

ONDOV, B. D., BERGMAN, N. H. & PHILLIPPY, A. M. 2011. Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics,* 12**,** 385.

PANDA, A. K., RAMA RAO, S. S., RAJU, M. V. L. N. & SHARMA, S. S. 2008. Effect of probiotic (*Lactobacillus sporogenes*) feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. *Journal of the Science of Food and Agriculture,* 88**,** 43-47.

PANDE, V. V., DEVON, R. L., SHARMA, P., MCWHORTER, A. R. & CHOUSALKAR, K. K. 2016. Study of *Salmonella* Typhimurium infection in laying hens. *Frontiers in Microbiology,* 7**,** 203.

PASCUAL, M., HUGAS, M., BADIOLA, J. I., MONFORT, J. M. & GARRIGA, M. 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella* Enteritidis colonization in chickens. *Applied and Environmental Microbiology,* 65**,** 4981-4986.

PAVIC, A., GROVES, P. J., BAILEY, G. & COX, J. M. 2010. A validated miniaturized MPN method, based on ISO 6579: 2002, for the enumeration of *Salmonella* from poultry matrices. *Journal of Applied Microbiology,* 109**,** 25-34.

PICKARD, J. M., ZENG, M. Y., CARUSO, R. & NÚÑEZ, G. 2017. Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunological Reviews,* 279**,** 70-89.

POLANSKY, O., SEKELOVA, Z., FALDYNOVA, M., SEBKOVA, A., SISAK, F. & RYCHLIK, I. 2016. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Applied and Environmental Microbiology,* 82**,** 1569-1576.

QIN, J., LI, R., RAES, J., ARUMUGAM, M., BURGDORF, K. S., MANICHANH, C., NIELSEN, T., PONS, N., LEVENEZ, F. & YAMADA, T. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* 464**,** 59.

QUAST, C., PRUESSE, E., YILMAZ, P., GERKEN, J., SCHWEER, T., YARZA, P., PEPLIES, J. & GLÖCKNER, F. O. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research,* 41**,** D590-D596.

RECHKEMMER, G. & RÖNNAU, K. 1988. Fermentation of polysaccharides and absorption of short chain fatty acids in the mammalian hindgut. *Comparative Biochemistry and Physiology. A, Comparative Physiology,* 90**,** 563-568.

REID, G., HOWARD, J. & GAN, B. S. 2001. Can bacterial interference prevent infection? *Trends in Microbiology,* 9**,** 424-428.

SCHEPPACH, W. 1994. Effects of short chain fatty acids on gut morphology and function. *Gut,* 35**,** S35-S38.

SCHLOSS, P. D., WESTCOTT, S. L., RYABIN, T., HALL, J. R., HARTMANN, M., HOLLISTER, E. B., LESNIEWSKI, R. A., OAKLEY, B. B., PARKS, D. H. & ROBINSON, C. J. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology,* 75**,** 7537-7541.

SENGA, T., IWAMOTO, S., YOSHIDA, T., YOKOTA, T., ADACHI, K., AZUMA, E., HAMAGUCHI, M. & IWAMOTO, T. 2003. LSSIG is a novel murine leukocyte-specific GPCR that is induced by the activation of STAT3. *Blood,* 101**,** 1185-1187.

SERGEANT, M. J., CONSTANTINIDOU, C., COGAN, T. A., BEDFORD, M. R., PENN, C. W. & PALLEN, M. J. 2014. Extensive microbial and functional diversity within the chicken cecal microbiome. *PloS One,* 9**,** e91941.

STAIB, L. & FUCHS, T. M. 2015. Regulation of fucose and 1, 2-propanediol utilization by *Salmonella enterica* serovar Typhimurium. *Frontiers in Microbiology,* 6**,** 1116.

STANLEY, D., DENMAN, S. E., HUGHES, R. J., GEIER, M. S., CROWLEY, T. M., CHEN, H., HARING, V. R. & MOORE, R. J. 2012. Intestinal microbiota associated with differential feed conversion efficiency in chickens. *Applied Microbiology and Biotechnology,* 96**,** 1361-1369.

STAPPENBECK, T. S., HOOPER, L. V. & GORDON, J. I. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Science of the United States of America,* 99**,** 15451-15455.

STECHER, B., BARTHEL, M., SCHLUMBERGER, M. C., HABERLI, L., RABSCH, W., KREMER, M. & HARDT, W. D. 2008. Motility allows S. Typhimurium to benefit from the mucosal defence. *Cellular Microbiology,* 10**,** 1166-1180.

SUN, M., WU, W., LIU, Z. & CONG, Y. 2017. Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. *Journal of Gastroenterology,* 52**,** 1-8.

TALHAM, G. L., JIANG, H.-Q., BOS, N. A. & CEBRA, J. J. 1999. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infection and Immunity,* 67**,** 1992-2000.

TEJADA-SIMON, M. V. & PESTKA, J. J. 1999. Proinflammatory cytokine and nitric oxide induction in murine macrophages by cell wall and cytoplasmic extracts of lactic acid bacteria. *Journal of Food Protection,* 62**,** 1435-1444.

VAN COILLIE, E., GORIS, J., CLEENWERCK, I., GRIJSPEERDT, K., BOTTELDOORN, N., VAN IMMERSEEL, F., DE BUCK, J., VANCANNEYT, M., SWINGS, J. & HERMAN, L. 2007. Identification of lactobacilli isolated from the cloaca and vagina of laying hens and characterization for potential use as probiotics to control *Salmonella* Enteritidis. *Journal of Applied Microbiology,* 102**,** 1095-1106.

VIDENSKA, P., SEDLAR, K., LUKAC, M., FALDYNOVA, M., GERZOVA, L., CEJKOVA, D., SISAK, F. & RYCHLIK, I. 2014. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. *PLoS One,* 9**,** e115142.

WANG, K., LIAO, M., ZHOU, N., BAO, L., MA, K., ZHENG, Z., WANG, Y., LIU, C., WANG, W. & WANG, J. 2019. Parabacteroides distasonis alleviates obesity and metabolic dysfunctions via production of succinate and secondary bile acids. *Cell reports,* 26**,** 222-235. e5.

WANG, L., LI, L., LV, Y., CHEN, Q., FENG, J. & ZHAO, X. 2018. *Lactobacillus plantarum* restores intestinal permeability disrupted by *Salmonella* infection in newly-hatched chicks. *Scientific Reports,* 8**,** 2229.

WESTCOTT, S. L. & SCHLOSS, P. D. 2017. OptiClust, an improved method for assigning amplicon-based sequence data to operational taxonomic units. *mSphere,* 2**,** e00073-17.

WILSON, K. H. & PERINI, F. 1988. Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. *Infection and Immunity,* 56**,** 2610-2614.

ZAKRZEWSKI, M., PROIETTI, C., ELLIS, J. J., HASAN, S., BRION, M.-J., BERGER, B. & KRAUSE, L. 2016. Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. *Bioinformatics,* 33**,** 782-783.

ZHANG, J.-M., SUN, Y.-S., ZHAO, L.-Q., CHEN, T.-T., FAN, M.-N., JIAO, H.-C., ZHAO, J.-P., WANG, X.-J., LI, F.-C., LI, H.-F. & LIN, H. 2019. SCFAs-Induced GLP-1 Secretion Links the Regulation of Gut Microbiome on Hepatic Lipogenesis in Chickens. *Frontiers in Microbiology,* 10.

5 Determining the effects of *Bacillus* based probiotic on *Salmonella* load in the free range production system

5.1 Introduction

Salmonella is a gram negative foodborne pathogen frequently associated with poultry production (Ford et al. 2018). While many food items can become contaminated with *Salmonella*, raw eggs and egg based food products are frequently identified as the bacterial source during trace back epidemiological investigation of human salmonellosis (Ford et al. 2018). In the egg production environment, various serovars of *Salmonella* are present (Moffatt et al. 2017). However, *Salmonella* Enteritidis and *Salmonella* Typhimurium, are of particular concern as they cause gastroenteritis in humans. In Australia, *Salmonella* Enteritidis has recently been implicated in egg associated foodborne salmonellosis in humans, while *Salmonella* Typhimurium is the most frequently implicated in egg associated salmonellosis (Ford et al. 2018; Moffatt et al. 2016). In Australia, due to public demand, free range egg production is increasing (Australian Eggs Report 2019). In free range production systems, other common serovars of *Salmonella* frequently isolated include *Salmonella* Mbandaka and *Salmonella* Agona (Gole et al. 2017). The prevalence of *Salmonella* in the poultry environment is influenced by proper sanitisation, the status of the hatched chicks being placed on a farm, stress and genetics of the bird. However, no positive correlation between faecal corticosterone level and *Salmonella* prevalence exists at least in a free range production system (Gole et al. 2017).

Gut health is maintained partly by the resident gut microbiota that provides first line of resistance to pathogen colonisation (Khan et al. 2020). However, the composition and diversity of gut microbiota vary with genotype (Zhao et al. 2013), rearing conditions, age (Cui et al. 2017; Ballou et al. 2016) and stress factors. For example, in a broiler study, temperature stress negatively affected the composition of ileal microbiota and enhanced the colonisation of *Salmonella* Enteritidis (Burkholder et al. 2008). Therefore, strategies that help in improving gut health will result in lower colonisation of the gut by *Salmonella,* leading to the improvement of product safety. The pen experiments conducted as part of this project (see Chapter 5) suggested that diet supplemented with probiotics can modulate the gut microbiota and reduce the colonisation of *Salmonella* Typhimurium DT9 (Khan & Chousalkar 2020). Prebiotics are host non-digestible polysaccharides fermented by the resident gut microbiota members for increasing their population and as a result organic acids are produced (Macfarlane et al. 2008). Probiotics are viable bacteria that maintain gut health through the production of organic acids, priming the immune system and saturation of enterocytes for pathogen colonisation resistance (Fuller 1989, Martin & Langella 2019). Our pen trials conducted as part of this project suggested that *Bacillus* based probiotic (*Bacillus subtilis* spp. and *Bacillus amyloliquefaciens*) improve the abundance and diversity of many gut microbial genera (Khan & Chousalkar 2020). However, as the rearing conditions of a cage system are different from free range, an optimisation of the most effective probiotic is required. Also, the *Salmonella* load dynamics are different between the cage and free range production systems (McWhorter & Chousalkar 2019; McWhorter & Chousalkar 2020). The main objective of this study was to test the effects of *Bacillus* based probiotic on the *Salmonella* load in faeces and shed environment and egg quality in the field conditions, in a free range production system.

5.2 Materials and methods

5.2.1 Animal ethics and experimental design

The experimental work was approved by the Animal Ethics Committee at the University of Adelaide, (approval number S-2019-109) in accordance with the guidelines specified in the Australian code for the care and use of animals for scientific purposes, 8th edition (2013). Faecal swab collections were performed as per standard operating procedures approved by the Animal Ethics Committee.

Prior to placing the chicks, swabs were collected from an empty shed before and after the clean-up to determine the contamination level of *Salmonella* (if any). The experimental flock was divided into two rearing sheds on a pullet rearing farm from day 1 of chicks placement with approximately 10,000 chicks in each shed. Shed A acted as a control, while birds in Shed B received a premix of *Bacillus* based probiotic at the rate of 1 g/kg of feed from day 1 until the termination of the experiment. Prior to shifting the pullets to the production farm, the sheds at the egg production farm were also swabbed to determine the load of *Salmonella* contamination (if any). The flocks were shifted to free range production sheds (Shed A - control; Shed B - probiotic fed) at 16 weeks of flock age. Faecal swabs (n = 20 from each flock at each time-point) and environmental swabs (n = 10 from each flock at each time-point) were collected at days 1 (meconium samples), 5, and 21, and weeks 6, 12, 16 (Day 1 and 5 after shifting), 18, 24, 30 and 36 of flock age. Once in lay, eggs (n = 30) from each flock were collected at 24, 30 and 36 weeks of flock age, and processed for egg quality measurements. Faecal swab samples were collected in 4 mL of buffered peptone water (BPW, Thermo Fisher Scientific, Australia), while environmental swabs (Whirl–Pak Speci-Sponge, Thermo Fisher Scientific, Australia) were soaked in 20 mL BPW and an individual swab was dragged to cover at least 1 m^2 area in the shed including exhaust fans and the covering board of the nest boxes. Shoe covers from each shed were soaked in 150 mL BPW and processed for *Salmonella* isolation.

5.2.2 Qualitative and quantitative assessment of *Salmonella* in faeces and shed environment

At least 0.5 mL from the individual faecal, shoe cover and environmental swab samples were stored at 5°C for the quantitative assessment of *Salmonella* by using the most probable number (MPN) method. MPN was performed only on *Salmonella* positive samples. For *Salmonella* detection, an enrichment method was followed that included the overnight incubation at 37°C of the swab samples collected in BPW, then 0.1 mL of the incubated BPW enrichment into 10 mL Rappaport Vassiliadis Soya Peptone Broth (RVS; Thermo Fisher Scientific, Australia) and overnight incubation at 42°C for the selective growth of *Salmonella*. The incubated RVS samples were streaked into both the *xylose lysine deoxycholate (*XLD; Thermo Fisher Scientific, Australia) and brilliance *Salmonella* (BS) agar, and incubated overnight at 37°C. The plates were read for the presence of *Salmonella* characteristic colony colour and morphology. A single colony from the *Salmonella* positive plates was sub-cultured in Luria Bertani (LB; Thermo Fisher Scientific, Australia) broth and stored in 50% glycerol at -80°C for serovars determination through traditional PCR.

5.2.2.1 Enumeration of *Salmonella* through MPN

The micro-dilution tube MPN method previously described (Pavic et al. 2010) was used to enumerate *Salmonella* in positive samples. Briefly, 0.1 mL of the BPW samples were serially diluted (10^{-1} to 10^{-8}) in 0.9 mL PBS, and 0.1 mL of each dilution was added (in triplicate) to the micro-dilution tubes containing 0.9 mL semi-solid RVS medium with the MSRV *Salmonella* selective agent (Thermo Fisher Scientific, Australia) and incubated overnight at 42°C. White colour development indicated

presumptive positive *Salmonella* growth, which was confirmed by sub-culturing the samples on Brilliance *Salmonella* agar (BSA) plates. A combination of positive and negative micro-dilution tubes gave the MPN result. MPN/mL was determined using the MPN tables sourced from the FDA Laboratory Methods (Blodgett 2010).

5.2.2.2 PCR characterisation of *Salmonella* serovars

The stored isolates were revived on nutrient agar and a single colony was grown in LB broth at 37°C in a shaking incubator. To obtain the bacterial pellet, samples were centrifuged at 10 000 ×*g* for 5 min, the supernatant discarded and the pellet re-suspended in 0.2 mL of 6 % Chelex (Bio-Rad, Australia) prepared in Tris-EDTA (TE) buffer. The samples were incubated at 56°C for 20 min, vortexed and incubated at 100°C for 8 minutes. The samples were incubated on ice for 5 minutes, centrifuged briefly and the supernatant that contained DNA was stored at -20 °C until used for traditional PCR. A duplex PCR (Akiba et al. 2011; McWhorter & Chousalkar 2019) was performed to identify the serotype of the isolates collected during the sampling. Isolates were confirmed as *Salmonella* through the amplification of an *invA* gene fragment (Forward: 5'-AAACCTAAAACCAGCAAAGG-3'; Reverse: 5'-TGTACCGTGGCATGTCTGAG-3'). To confirm for *Salmonella* Typhimurium serotype, primers designed from the *TSR3* gene (Forward: 5'-TTTACCTCAATGGCGGAACC-3'; Reverse: 5'-CCCAAAAGCTGGGTTAGCAA-3') were used in the same reaction well. PCR reactions were performed in a total volume of 20 µL that contained 4 µL 5× MyRed Taq Buffer (Bioline, Australia), 0.3 µL of MyRed Taq Polymerase, 0.5 µL of each of the forward and reverse primers for *invA* and *TSR3* and 2 µL of DNA template. PCR cycling conditions were: initial denaturation at 94°C for 2 minutes, then 40 cycles of 95°C for 30 s and 60°C for 30 s, followed by an extension of 72°C for 5 min at the end. The PCR products were visualised on 2% agarose gel electrophoresis to confirm the size and specificity of the bands.

5.2.3 Egg quality measurements

The collected eggs were tested for measuring egg weight, shell weight, shell thickness, albumen height, Haugh unit and yolk colour. Technical supplies and services (TSS, UK) QCH albumen height gauge was used for albumen height measurement, while yolk colour was measured by the DSM Yolk Colour fan (scale 1-16). Shell thickness was measured by Mitutoyo Dial Comparator Gauge Model 2109–10 (Kawasaki, Japan). Haugh unit was measured from the egg weight and albumen height by using the following equation (Eisen et al. 1962; Samiullah et al. 2014, 2017):

HU = 100 * log10(HT - 1.7 * EW^0.37 + 7.6)

5.3 Statistical analysis

Where appropriate, data were analysed in StatView v5.0.1.0 with one- or two- way ANOVA.

5.4 Results and discussion

5.4.1 Effect of *Bacillus* based probiotic on *Salmonella* in free range layer production

No *Salmonella* was isolated from the rearing and production sheds prior to placing the day old chicks or point of lay pullets. This demonstrates that the decontamination procedures performed on the rearing and production sheds prior to placing the chickens were appropriate. Effective cleaning and disinfection of poultry sheds reduces the levels of *Salmonella* contamination; however, the recovery of *Salmonella* spp. from surfaces such as dropping boards and floors in cleaned and disinfected sheds

is variable (Carrique-Mas et al. 2009). Throughout the sampling period, *Salmonella* was isolated from one faecal sample at 18 weeks and one environmental sample at 36 weeks of flock age from the control shed. At week 36, a shoe cover sample from the control shed was also positive for *Salmonella* spp. During the sampling period (day 1 to week 36 of flock age), no *Salmonella* was isolated from the probiotic supplemented shed. In a previous study, different *Salmonella* serovars – such as *Salmonella* Mbandaka and *Salmonella* Typhimurium – were isolated from a free range layer production system (Carrique-Mas et al. 2009). In the current study, the lack of *Salmonella* isolation from the probiotic supplemented shed could possibly be attributed to the beneficial effects of probiotics; however, a conclusive statement could not be made as the flock was not followed until the termination of the production cycle. Moreover, the number of *Salmonella* positive samples was too low to confidently determine the effects of probiotics on the flock. In a *Salmonella* Typhimurium challenged pen trial, the B*acillus* based probiotic reduced the overall load of *Salmonella* in faeces but not all of the birds turned negative for *Salmonella* in the 12 week period of sampling (Khan & Chousalkar 2020). A previous study reported < 20% of *Salmonella* positive dust and faecal samples obtained from a free range layer production (McWhorter & Chousalkar 2019).

Measured through the MPN method, the load of *Salmonella* in the faecal swab, environmental swab and shoe cover was 7.3, 11.1 and 15.1 per mL of BPW, respectively.

5.4.2 *Salmonella* serotype confirmation through PCR

The PCR and agarose gel electrophoresis results confirmed that one faecal pure culture isolate collected from the control shed at 18 weeks of flock age was *Salmonella* Typhimurium, while one each of the environmental and shoe cover samples collected at 36 weeks of flock age from the control shed were non Typhimurium *Salmonella* serovars (Figure 5-1). These results showed that *Salmonella* spp. and *Salmonella* Typhimurium could be more frequently isolated in the non-probiotic supplemented shed based on the sampling duration of the study. However, it is worth noting that such a low number of *Salmonella* isolates could be random biological variation.

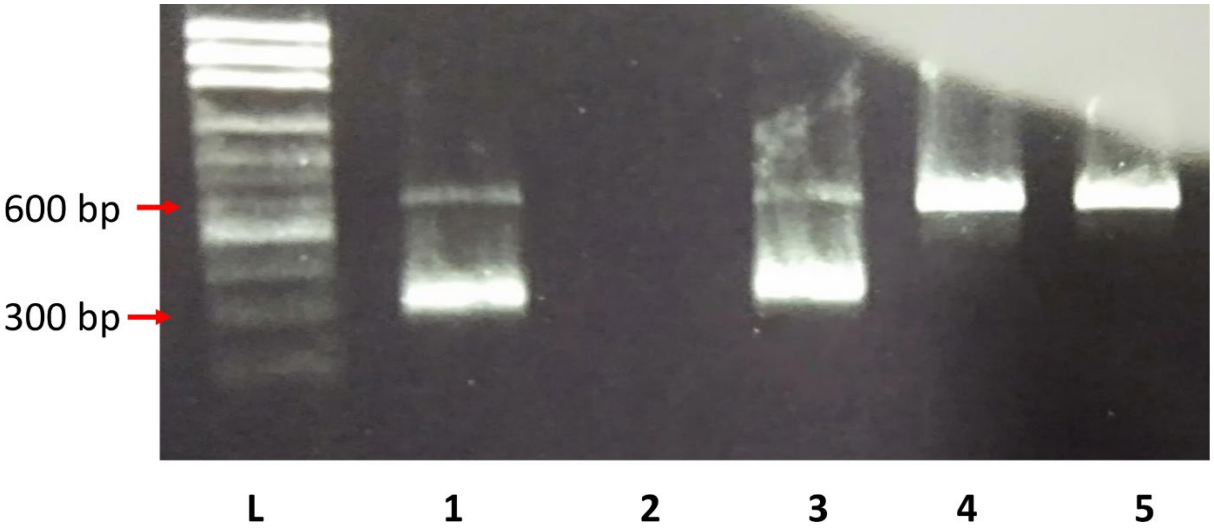


Figure 5-1 PCR products of *Salmonella* positive samples visualised on 2% agarose gel

L Ladder.
1 *Salmonella* Typhimurium as positive control.
2 No template negative control.
3 *Salmonella* Typhimurium isolated from faecal swab at weeks 18 of flock age, from the control shed.
4 *Salmonella* spp. isolated from environmental swab isolated at week 36 of flock age, from the control shed.
5 *Salmonella* spp. isolated from environmental swab isolated at week 36 of flock age, from the control shed.
For *Salmonella* spp. typing primers (605 bp) amplifying the fragment of *invA* gene were used, while for *Salmonella* Typhimurium confirmation, primers (303 bp) amplifying the fragment of *TSR3* gene were used.

5.4.3 Effect of *Bacillus* based probiotic on egg quality

Egg quality was measured on eggs collected at 24, 30 and 36 weeks of flock age. Feeding *Bacillus* based probiotic significantly ($P < 0.05$) affected egg internal quality. The overall quality of albumen height, Haugh unit and yolk colour (Figure 5-2) was improved in the probiotic treated flock. Probiotics are generally used as feed supplements for improvement in flock performance. In laying hens, diet supplemented with different probiotics significantly improved egg production and overall egg quality. For example, the cholesterol level in egg yolk was reduced, and tibial bone mineralisation increased in *Pediococcus acidilactici* fed hens (Mikulski et al. 2012). The feeding of *Lactobacillus sporogenes* to White Leghorn layer breeders from 25–40 weeks flock age lowered both the serum and yolk cholesterol levels without affecting most of the egg quality traits (Panda et al. 2008). Feeding *Enterococcus faecium* and fructooligosaccharides based synbiotic supplemented diet to Hy-Line brown laying hens at 24–36 weeks of flock age significantly reduced serum cholesterol, and positively influenced some egg quality parameters (Abdel-Wareth 2016). Feeding multiple strains or *Enterococcus faecium* based synbiotic to laying chickens for periods of 6 or 12 weeks did not significantly influence the level of blood cholesterol (Mohammadian et al. 2013; Zarei et al. 2011). Feeding protexin probiotic (contains multiple bacterial/fungal strains) to layer chickens for 12 weeks did not significantly influence egg quality, feed conversion and blood immune system parameters (Balevi et al. 2001). Feeding PoultryStar® that contains multiple strains of probiotic and fructooligosaccharide, to laying chickens from day of hatch up to 28 weeks of flock age and challenged with *Salmonella* Enteritidis at 24 weeks of age reduced *Salmonella* shedding from day 5 p.i. onwards (Luoma et al. 2017). Feeding prebiotic (isomaltooligosaccharide), probiotic (PrimaLac) and their combination to 20 to 52 week old laying hens improved performance, serum total cholesterol, and egg production (Tang et al. 2017). Feeding *Bacillus subtilis* and *Enterococcus faecium* to heat stressed laying hens for about 20 days improved gut microbiota and overall egg quality (Zhang et al. 2017). Based on the previous literature focusing on probiotic or prebiotic supplementation in layers and the findings of the current study, it seems that different probiotics affect egg quality parameters differently. The positive effects of the probiotics could be due to improved nutrient absorption in the gut of probiotics supplemented hens (Mikulski et al. 2012; Panda et al. 2008) or through the production of metabolites, enzymes or synthesis of vitamins (Crittenden et al. 2003; Hill 1997). Egg quality deteriorates with flock age and, therefore, it is recommended that future research can focus on the use of probiotics for improving gut health and bird performance from hatch to termination of the production cycle.

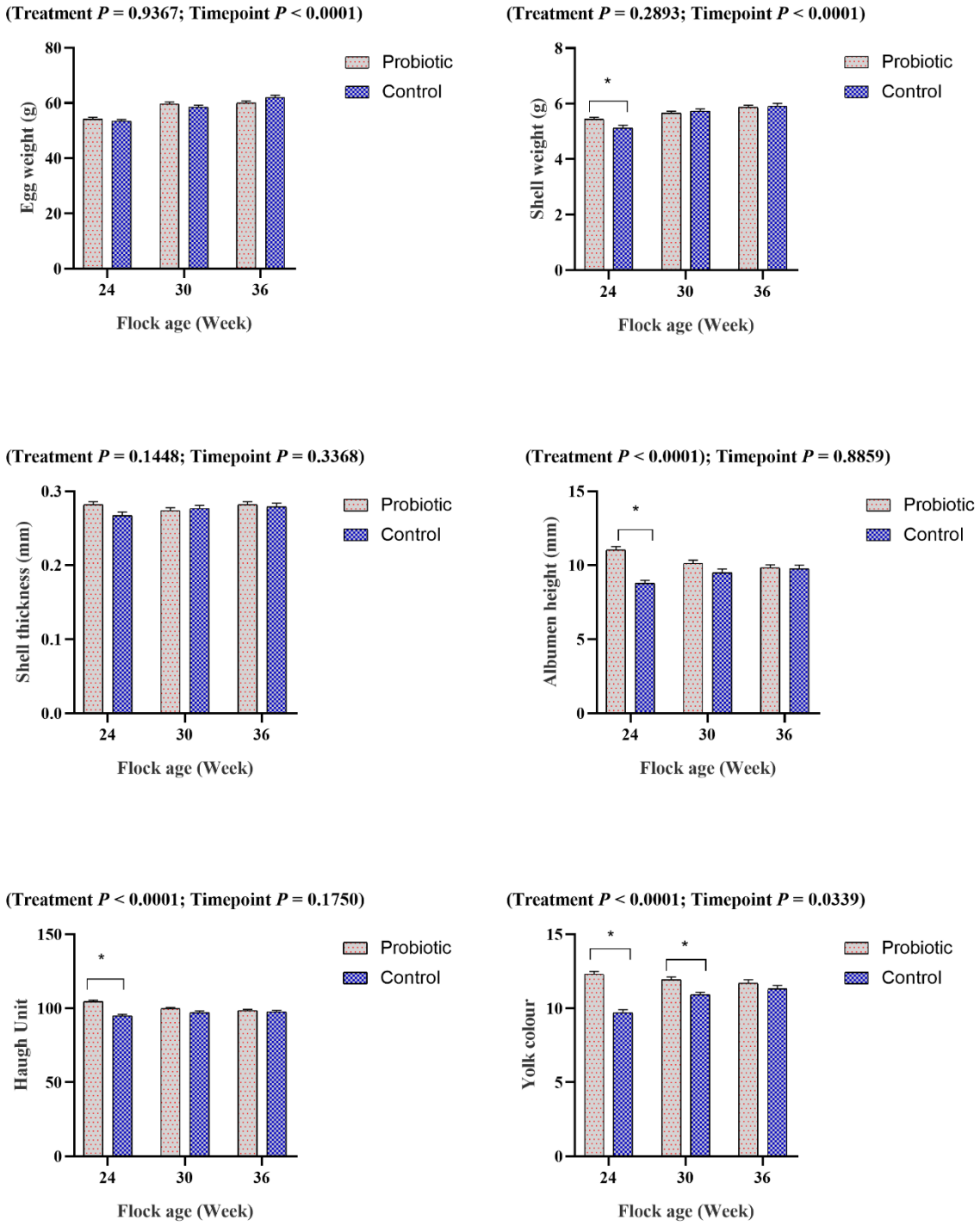


Figure 5-2 Effect of *Bacillus* based probiotic on the overall egg quality measurements

Eggs were collected from both the control and the probiotic supplemented sheds at weeks 24, 30 and 36 week of flock age.

5.5 References

ABDEL-WARETH, A. A. A. 2016. Effect of dietary supplementation of thymol, synbiotic and their combination on performance, egg quality and serum metabolic profile of Hy-Line Brown hens. *British Poultry Science,* 57**,** 114-122.

Akiba, M., Kusumoto, M. and Iwata, T., 2011. Rapid identification of *Salmonella enterica* serovars, typhimurium, choleraesuis, infantis, hadar, enteritidis, dublin and gallinarum, by multiplex PCR. *Journal of Microbiological Methods*, *85*, 9-15.

Australian Eggs Report. 2019. Annual Report 2018/19. Retrieved from: https://www.australianeggs.org.au/who-we-are/annual-reports/#item-1058

BALEVI, T., UÇAN, U. S., COŞUN, B., KURTOĞU, V. & ÇETINGÜL, İ. S. 2001. Effect of dietary probiotic on performance and humoral immune response in layer hens. *British Poultry Science,* 42**,** 456-461.

BALLOU, A. L., ALI, R. A., MENDOZA, M. A., ELLIS, J., HASSAN, H. M., CROOM, W. & KOCI, M. D. 2016. Development of the chick microbiome: how early exposure influences future microbial diversity. *Frontiers in veterinary science,* 3**,** 2.

BLODGETT, R. 2010. BAM Appendix 2: most probable number from serial dilutions, Bacteriological Analytical Manual. *Food and Drug Administration, Silver Spring, MD. https://www. fda. gov/food/foodscienceresearch/laboratorymethods/ucm109656. htm*.

BURKHOLDER, K., THOMPSON, K., EINSTEIN, M., APPLEGATE, T. & PATTERSON, J. 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella* enteritidis colonization in broilers. *Poultry Science,* 87**,** 1734-1741.

CARRIQUE-MAS, J. J., MARIN, C., BRESLIN, M., MCLAREN, I. & DAVIES, R. 2009. A comparison of the efficacy of cleaning and disinfection methods in eliminating *Salmonella* spp. from commercial egg laying houses. *Avian pathology,* 38**,** 419-424.

CRITTENDEN, R. G., MARTINEZ, N. R. & PLAYNE, M. J. 2003. Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology,* 80**,** 217-222.

CUI, Y., WANG, Q., LIU, S., SUN, R., ZHOU, Y. & LI, Y. 2017. Age-related variations in intestinal microflora of free-range and caged hens. *Frontiers in microbiology,* 8**,** 1310.

EISEN, E.J., BOHREN, B.B. AND MCKEAN, H.E. 1962. The Haugh unit as a measure of egg albumen quality. *Poultry Science*, *41*, 1461-1468.

FORD, L., MOFFATT, C. R., FEARNLEY, E., MILLER, M., GREGORY, J., SLOAN-GARDNER, T. S., POLKINGHORNE, B. G., BELL, R., FRANKLIN, N. & WILLIAMSON, D. A. 2018. The Epidemiology of *Salmonella enterica* Outbreaks in Australia, 2001–2016. *Frontiers in Sustainable Food Systems,* 2**,** 86.

FULLER, R. 1989. Probiotics in man and animals. *Journal of Applied Bacteriology,* 66**,** 365-378.

GOLE, V. C., WOODHOUSE, R., CARAGUEL, C., MOYLE, T., RAULT, J.-L., SEXTON, M. & CHOUSALKAR, K. 2017. Dynamics of *Salmonella* shedding and welfare of hens in free-range egg production systems. *Appl. Environ. Microbiol.,* 83**,** e03313-16.

HILL, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. *European Journal of Cancer Prevention,* 6**,** S43-S45.

KHAN, S. & CHOUSALKAR, K. K. 2020. *Salmonella* Typhimurium infection disrupts but continuous feeding of *Bacillus* based probiotic restores gut microbiota in infected hens. *Journal of Animal Science and Biotechnology,* 11**,** 1-16.

KHAN, S., MOORE, R. J., STANLEY, D. & CHOUSALKAR, K. K. 2020. Gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety. *Applied and Environmental Microbiology*, *86*, e00600-20.

LUOMA, A., MARKAZI, A., SHANMUGASUNDARAM, R., MURUGESAN, G., MOHNL, M. & SELVARAJ, R. 2017. Effect of synbiotic supplementation on layer production and cecal *Salmonella* load during a *Salmonella* challenge. *Poultry Science,* 96**,** 4208-4216.

MACFARLANE, G.T., STEED, H. & MACFARLANE, S. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology*, *104*, 305-344.

MARTÍN, R. & LANGELLA, P. 2019. Emerging health concepts in the probiotics field: streamlining the definitions. *Frontiers in Microbiology*, *10*, 1047.

MCWHORTER, A. R. & CHOUSALKAR, K. K. 2019. From hatch to egg grading: monitoring of *Salmonella* shedding in free-range egg production systems. *Veterinary Research,* 50**,** 58.

MCWHORTER, A. R. & CHOUSALKAR, K. K. 2020. *Salmonella* on Australian cage egg farms: Observations from hatching to end of lay. *Food Microbiology,* 87**,** 103384.

MIKULSKI, D., JANKOWSKI, J., NACZMANSKI, J., MIKULSKA, M. & DEMEY, V. 2012. Effects of dietary probiotic (*Pediococcus acidilactici*) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poultry Science,* 91**,** 2691-2700.

MOFFATT, C. R., MUSTO, J., PINGAULT, N., COMBS, B., MILLER, M., STAFFORD, R., GREGORY, J., POLKINGHORNE, B. G. & KIRK, M. D. 2017. Recovery of *Salmonella enterica* from Australian layer and processing environments following outbreaks linked to eggs. *Foodborne Pathogens and Disease,* 14**,** 478-482.

MOFFATT, C. R., 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 Pathogens and Disease,* 13**,** 379-385.

MOHAMMADIAN, A., MEHDIZADEH, S. M., LOTFOLLAHIAN, H., MIRZAEI, F. & NOROOZIAN, H. 2013. Influence of dietary Probiotic (Biomin Imbo) on performance of laying hen. *Agricultural Sciences,* 4**,** 23.

PANDA, A. K., RAMA RAO, S. S., RAJU, M. V. L. N. & SHARMA, S. S. 2008. Effect of probiotic (Lactobacillus sporogenes) feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. *Journal of the Science of Food and Agriculture,* 88**,** 43-47.

PAVIC, A., GROVES, P., BAILEY, G. & COX, J. 2010. A validated miniaturized MPN method, based on ISO 6579: 2002, for the enumeration of *Salmonella* from poultry matrices. *Journal of Applied Microbiology,* 109**,** 25-34.

SAMIULLAH, S. ROBERTS, J.R. & CHOUSALKAR, K.K. 2014. Effect of production system and flock age on egg quality and total bacterial load in commercial laying hens. *Journal of Applied Poultry Research*, *23*, 59-70.

SAMIULLAH, S., OMAR, A.S., ROBERTS, J. & CHOUSALKAR, K. 2017. Effect of production system and flock age on eggshell and egg internal quality measurements. *Poultry Science*, *96*, 246-258.

TANG, S. G. H., SIEO, C. C., RAMASAMY, K., SAAD, W. Z., WONG, H. K. & HO, Y. W. 2017. Performance, biochemical and haematological responses, and relative organ weights of laying hens fed diets supplemented with prebiotic, probiotic and synbiotic. *BMC Veterinary Research,* 13**,** 248.

ZAREI, M., EHSANI, M. & TORKI, M. 2011. Dietary inclusion of probiotics, prebiotics and synbiotic and evaluating performance of laying hens. *American Journal of Agricultural and Biological Science*, *6*, 249-255.

ZHANG, P., YAN, T., WANG, X., KUANG, S., XIAO, Y., LU, W. & BI, D. 2017. Probiotic mixture ameliorates heat stress of laying hens by enhancing intestinal barrier function and improving gut microbiota. *Italian Journal of Animal Science,* 16**,** 292-300.

ZHAO, L., WANG, G., SIEGEL, P., HE, C., WANG, H., ZHAO, W., ZHAI, Z., TIAN, F., ZHAO, J. & ZHANG, H. 2013. Quantitative genetic background of the host influences gut microbiomes in chickens. *Scientific reports,* 3**,** 1163.

6 Plain English Summary

Project Title:	**Optimisation of use of probiotics for control of *Salmonella* in hens**
Australian Eggs Limited Project No	1FS802UA
Researchers Involved	K. Chousalkar and S. Khan
Organisations Involved	School of Animal and Veterinary Sciences The University of Adelaide Mudla Wirra Road Roseworthy South Australia 5371
Phone	08 8313 1502
Fax	08 8313 7956
Email	kapil.chousalkar@adelaide.edu.au
Objectives	The main objectives of this project were: • To understand the role of probiotics and synbiotics in *Salmonella* control in layers. • To determine the effectiveness of probiotics and synbiotics in gut health. • To investigate how effective is the strategic feeding of *Bacillus* based probiotic in reducing the shedding and invasion of *Salmonella* in layers. • To investigate if other serovars of *Salmonella* can be used for inhibiting the colonisation of *Salmonella* Typhimurium in gut that can be used for developing multiserovars vaccine. • To investigate the effectiveness of *Bacillus* based probiotic in *Salmonella* reduction and egg quality in free range production system.
Background	*Salmonella* serovars, such as Typhimurium and Enteritidis are the leading causes of human gastroenteritis. In Australia, most gastroenteritis is associated with the consumption of contaminated eggs or egg based products. In field conditions, *Salmonella* persists and can colonise chickens for continuous or intermittent shedding that can result in the contamination of eggs. The control of *Salmonella* in layer production is multi-faceted and factors such as biosecurity, stress, shed decontamination, the use of feed supplements and vaccination affect its overall load. To date, current vaccines are not effective in lowering the load of *Salmonella* Typhimurium. Feed supplements such as probiotics and prebiotics are mainly used for improving birds' performance and have not been optimised for gut health in layer production. Therefore, a better understanding of the use of prebiotics and probiotics for the control of *Salmonella* and their effects on gut health is needed to improve food safety.
Research	A total of 5 experiments was conducted to understand the role of probiotics and synbiotics in the control of *Salmonella* in layer production.

	The short-term pen trial aimed to understand the role of two probiotics and two synbiotics on the shedding and invasion profile of *Salmonella* Typhimurium in chicks. RNA sequencing was performed to get insights into the regulation of the caecal immune system in a temporal manner of chicks challenged with *Salmonella* Typhimurium. Based on the findings of previous studies, *Salmonella* Mbandaka and *Salmonella* Agona were used as competitive exclusion agents against *Salmonella* Typhimurium. A long-term pen trial was conducted to understand the effects of strategic supplementation of *Bacillus* based probiotic on gut microbiota, SCFAs, *Salmonella* Typhimurium load and invasion in layers at point of lay. Based on the findings that Bacillus based probiotic is effective in reducing the overall load of *Salmonella* and restoring the microbial genera displaced by *Salmonella* Typhimurium, this probiotic was tested for its effectiveness in free range field conditions.
Outcomes	The short-term supplementation of probiotics and synbiotics is effective in improving the gut microbiota balance but does not significantly reduce the colonisation and invasion of *Salmonella* Typhimurium in chicks. The caecal immune system in *Salmonella* Typhimurium is regulated by the involvement of Toll-like receptors and cytokine pathways with a broader role of *IL6*. Both *Salmonella* Mbandaka and *Salmonella* Agona are effective in lowering the load of *Salmonella* Typhimurium, with serovar Agona performing better than Mbandaka. The long-term strategic supplementation of *Bacillus* based probiotic is effective in lowering the overall load of *Salmonella* Typhimurium or *Salmonella* spp. and restores the microbial genera displaced by the *Salmonella* challenge. Data obtained in the pen trial (see Chapter 5) need to be confirmed by long-term probiotic feeding trial as only 3 *Salmonella* isolates were obtained from the control birds, which could be due to biological variation.
Implications	The findings in the current study support the use of the given probiotics and synbiotics for improving gut health in layer production.
Key Words	Laying hen, gut health, *Salmonella*, probiotics, prebiotics, caecal immune system, gut microbiota
Publications	Journal articles: • Samiullah Khan and Kapil Chousalkar (2020). Transcriptome profiling analysis of caeca in chicks challenged with *Salmonella* Typhimurium reveals differential expression of genes involved in host mucosal immune response. **Applied Microbiology and Biotechnology** (Accepted). • Samiullah Khan and Kapil Chousalkar (2020). *Salmonella* Typhimurium infection disrupts but continuous feeding of *Bacillus* based probiotic restores gut microbiota in infected hens. **Journal of Animal Science and Biotechnology**; 11:29. • Samiullah Khan and Kapil Chousalkar (2020). Short-term feeding of probiotics and synbiotics modulates caecal microbiota during *Salmonella* Typhimurium infection but does not reduce shedding and invasion in chickens. **Applied Microbiology and Biotechnology**; 104, 319-334.

Conference paper/abstract:

- Samiullah Khan and Kapil K. Chousalkar (2019). Caecal luminal microbial diversity profiling of laying chicks is manipulated by probiotics supplementation and *Salmonella* Typhimurium challenge. Oral presentation at: Australian Society of Microbiology annual meeting 30th June to 3rd July, 2019.
- Samiullah Khan and Kapil K. Chousalkar (2019). Understanding the caecal immune response of laying chicks to *Salmonella* Typhimurium. Abstract accepted in **World Poultry Congress,** to be held in Paris, France from 16th to 20th August, 2021.

7 Appendix

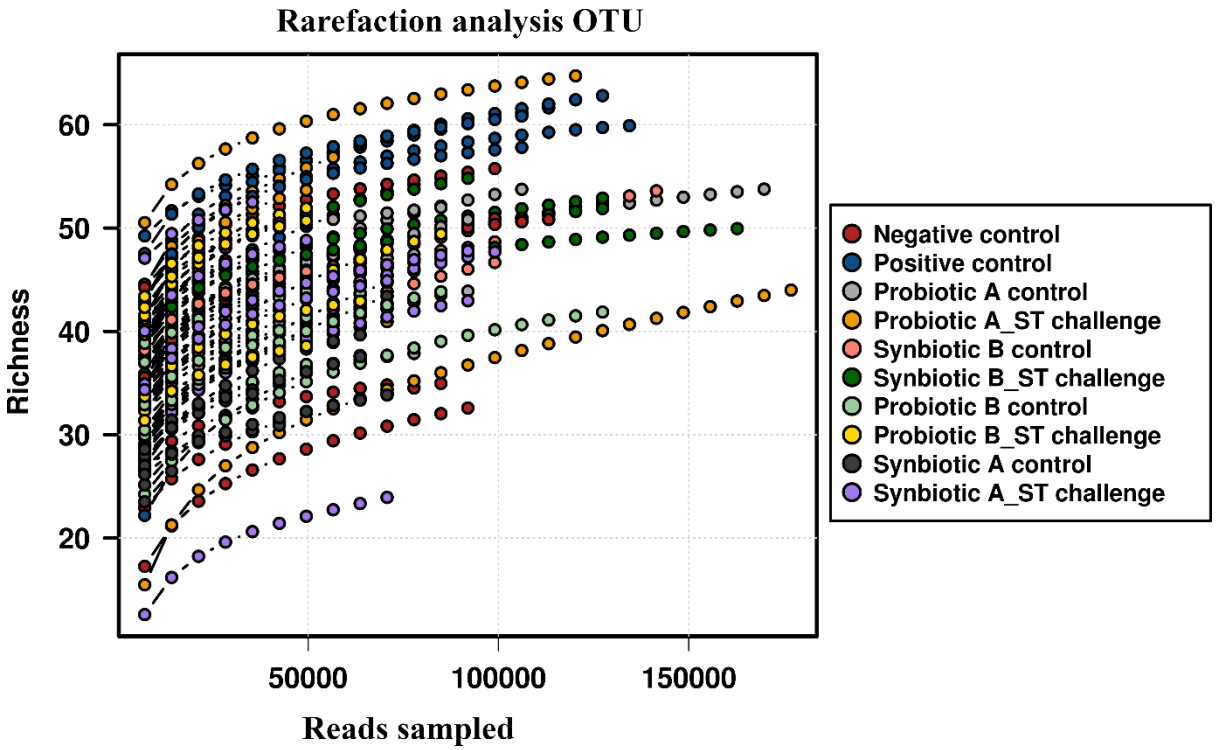


Figure 1-1 Rarefaction curve analysis affected by probiotic supplementation and *Salmonella* Typhimurium challenge shows a good coverage of underlying microbial communities by the sequenced data

Treatment group details have been given in Table 2-1.

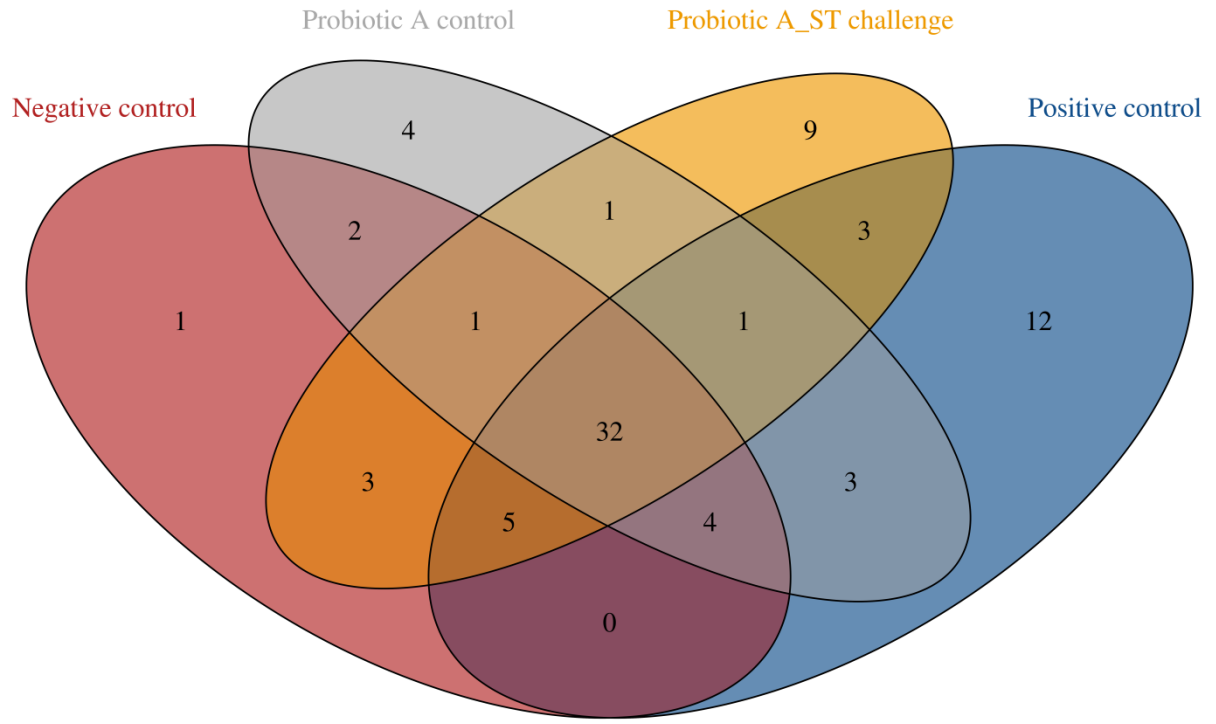


Figure 1-2 Caecal core microbiome (at OTU level) affected by probiotic A (Protexin) supplementation and *Salmonella* Typhimurium challenge

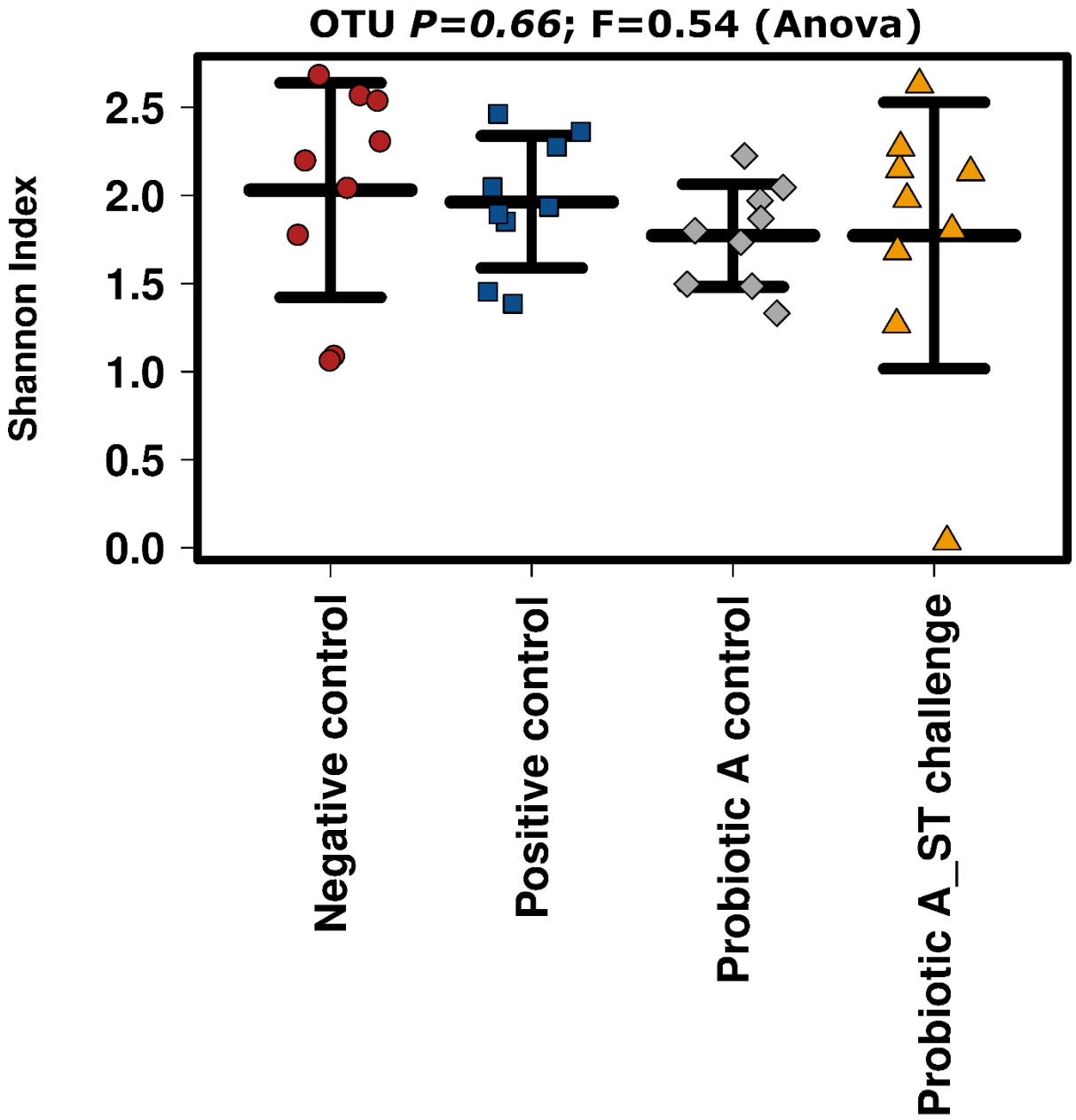


Figure 1-3 Diversity of microbial communities affected by probiotic A supplementation and *Salmonella* Typhimurium challenge

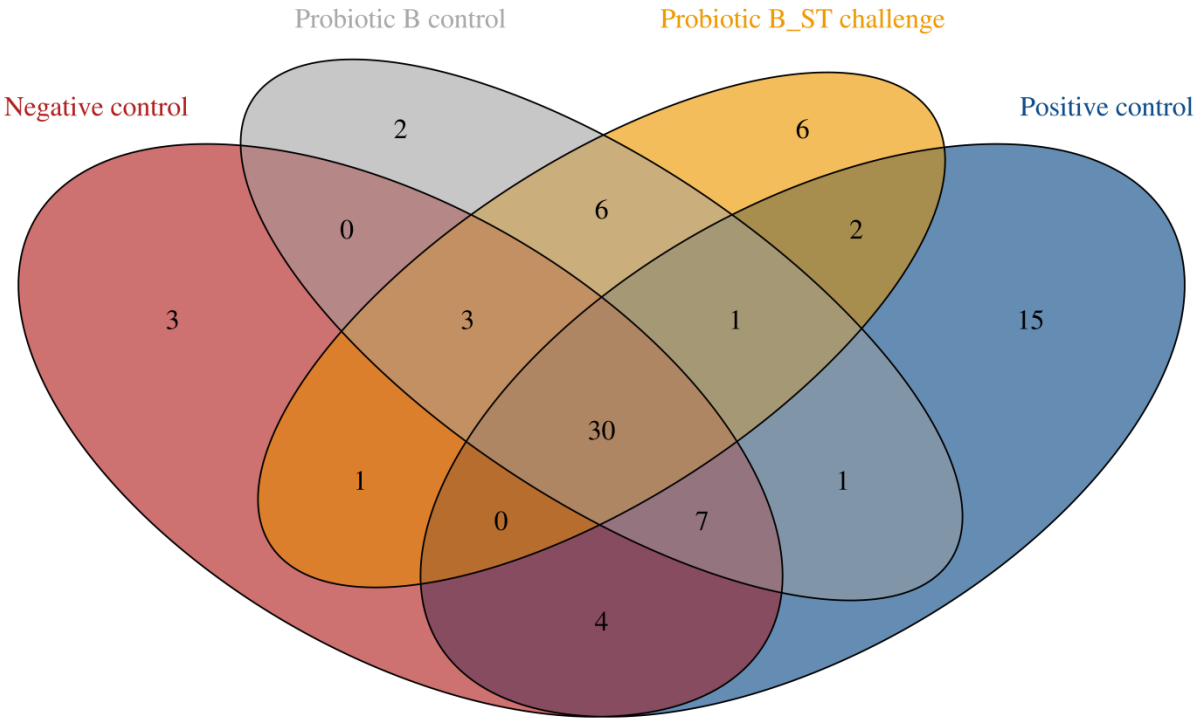


Figure 1-4 Caecal core microbiome (at OTU level) affected by probiotic B supplementation and *Salmonella* Typhimurium challenge

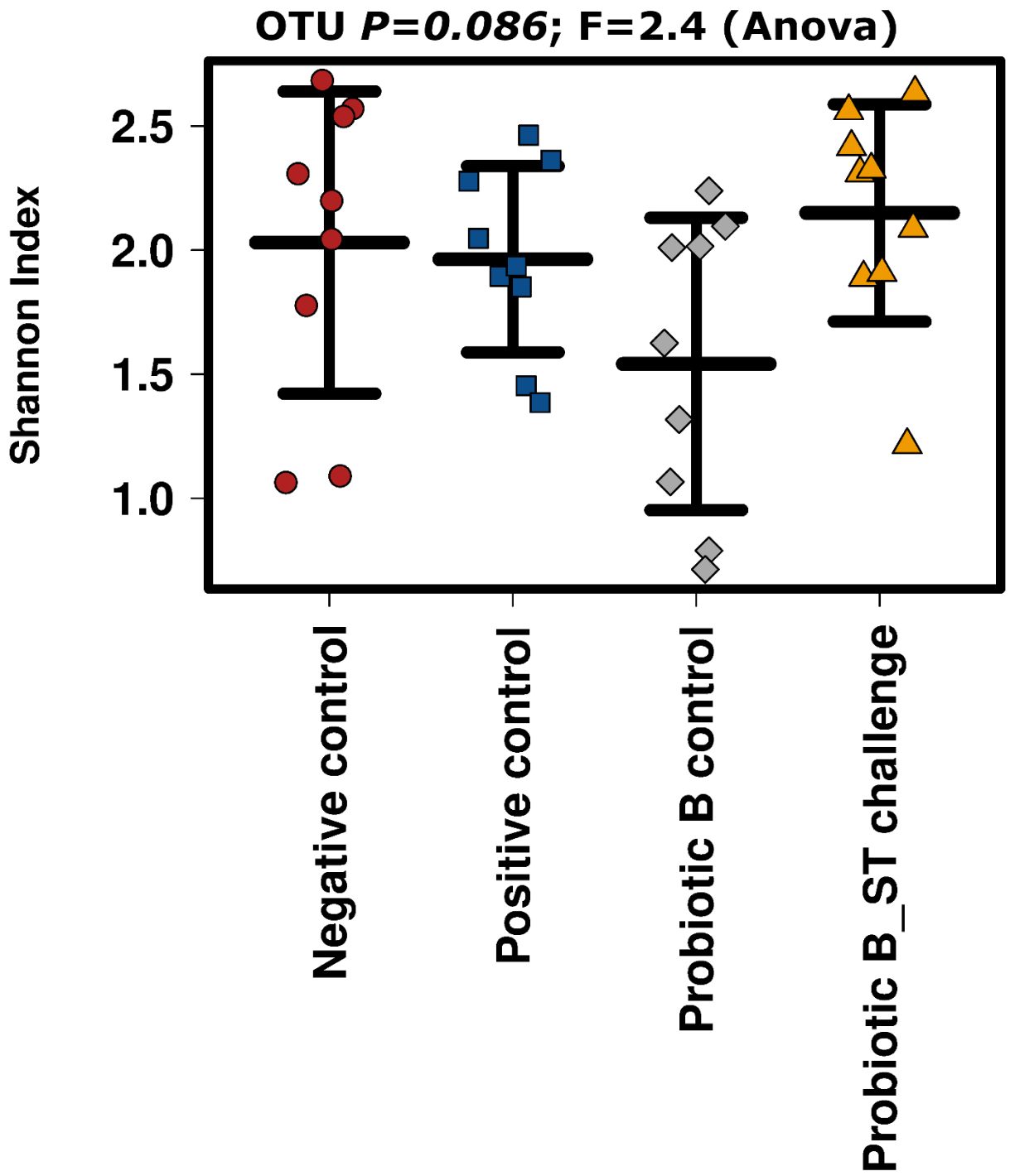


Figure 1-5 Diversity of microbial communities affected by probiotic B (GalliPro Fit) supplementation and *Salmonella* Typhimurium challenge

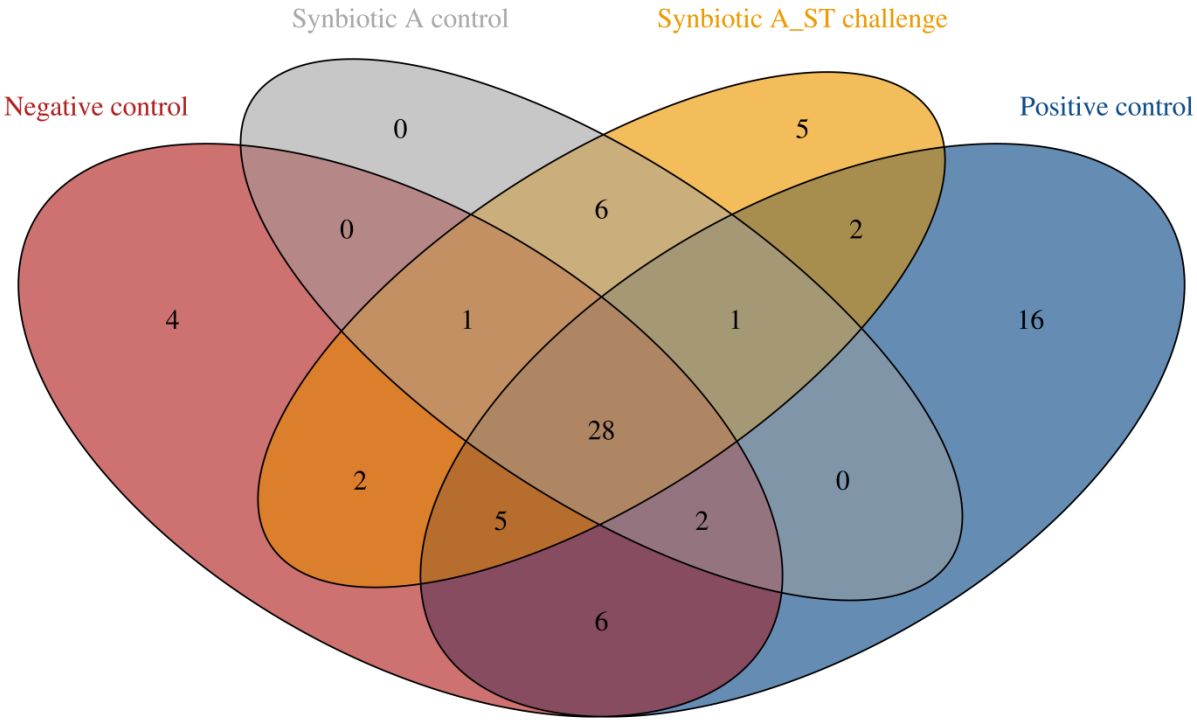


Figure 1-6 Caecal core microbiome (at OTU level) affected by synbiotic A supplementation and *Salmonella* Typhimurium challenge

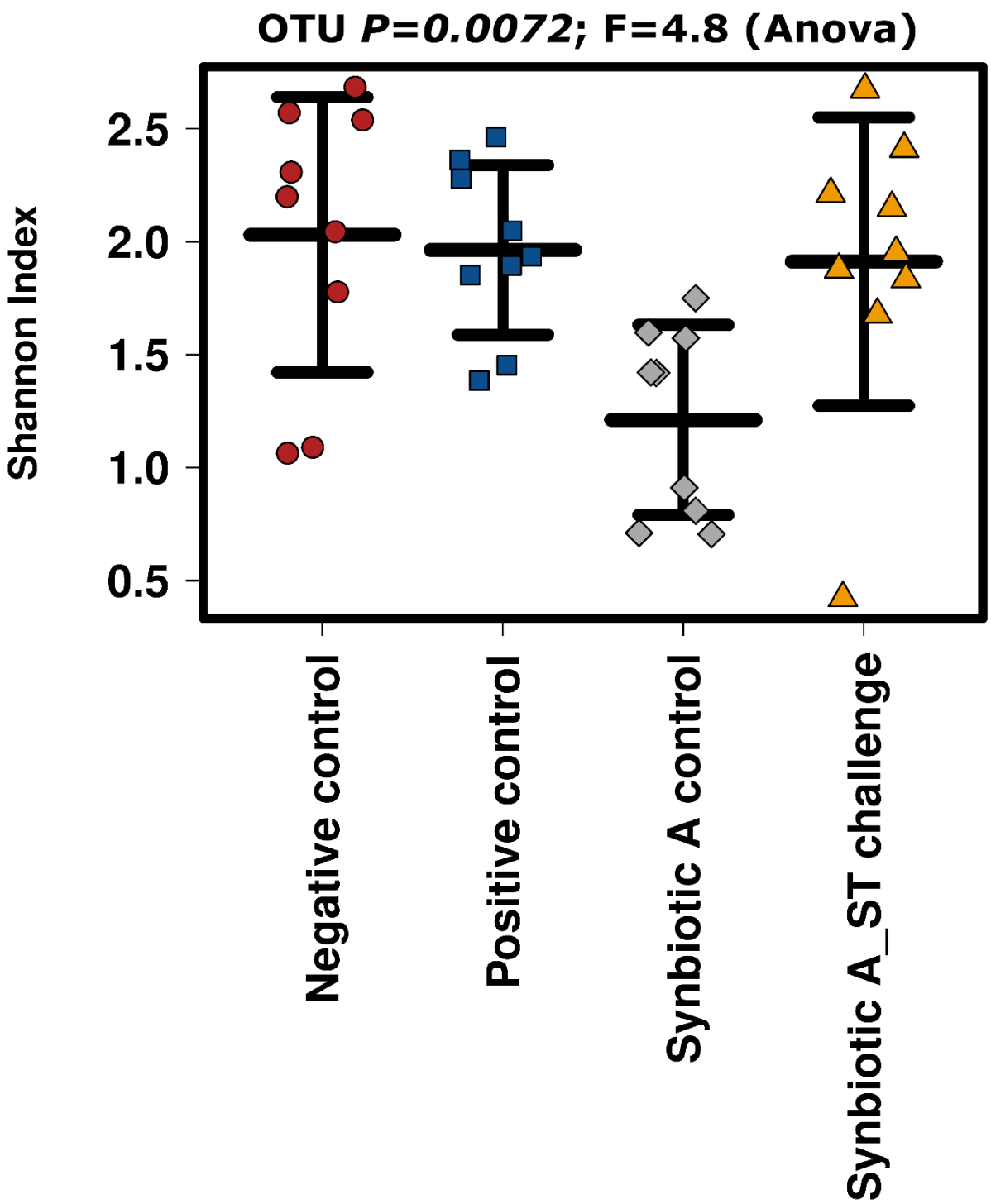


Figure 1-7 Diversity of microbial communities affected by synbiotic A (PoultryStar sol) supplementation and *Salmonella* Typhimurium challenge

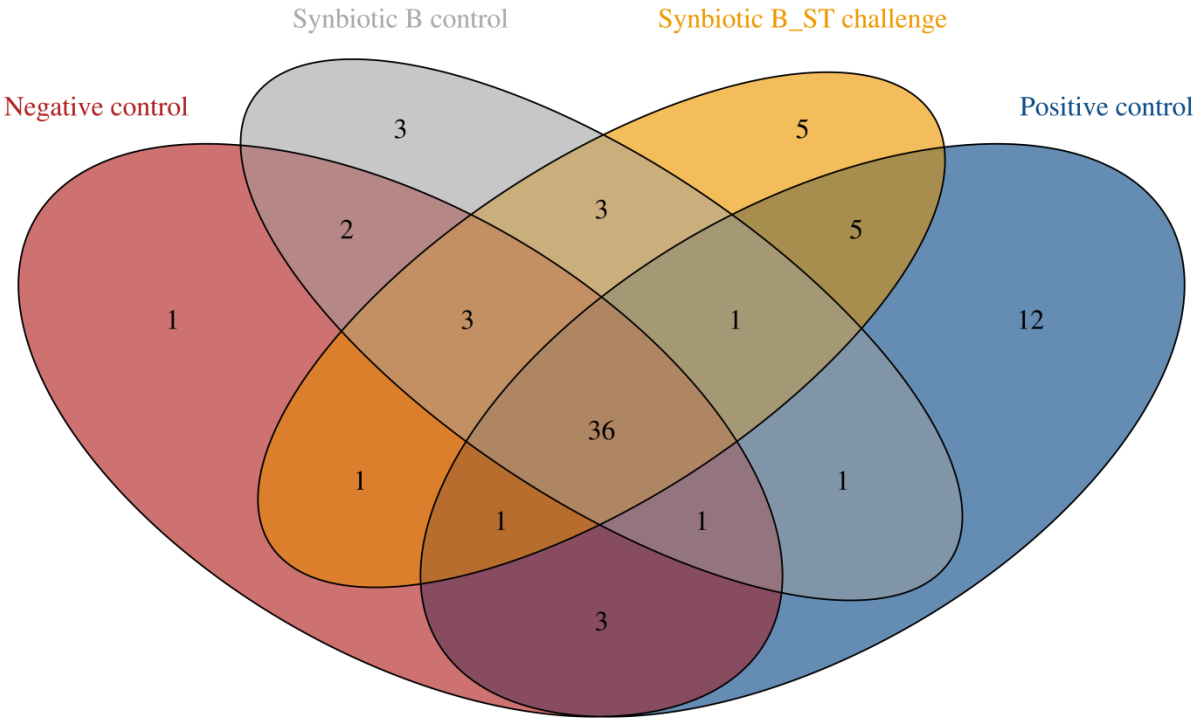


Figure 1-8 Caecal core microbiome (at OTU level) affected by synbiotic B (Synbiotic) supplementation and *Salmonella* Typhimurium challenge

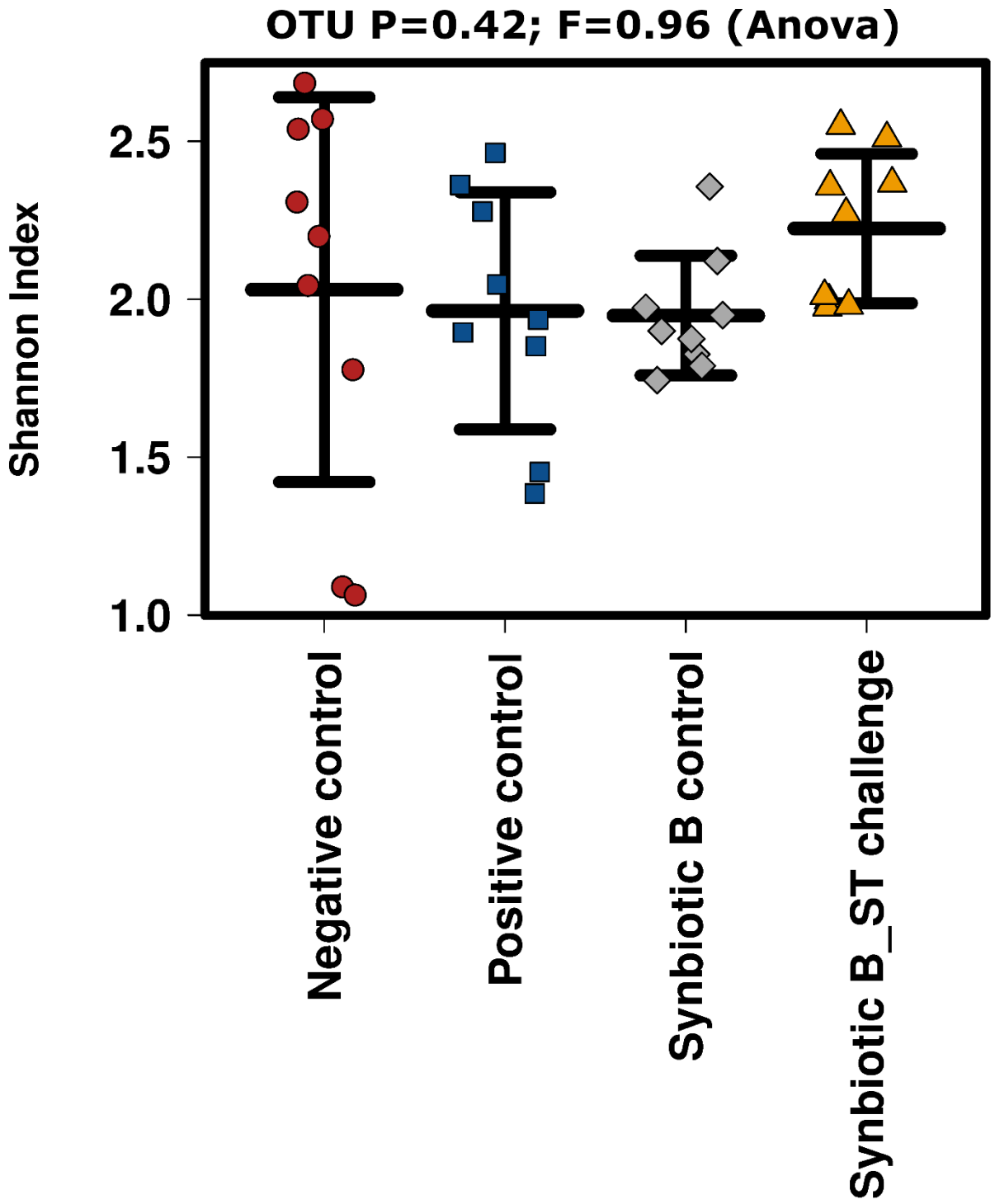


Figure 1-9 Diversity of microbial communities affected by synbiotic B (Synbiotic) supplementation and *Salmonella* Typhimurium challenge

Table 2-1 Faecal status of *Salmonella* Typhimurium challenged chicks and gross lesions observed in the caeca at different time-points of sampling

Sampling time-point (day)	Lesions observed	Birds affected (%)	Birds (%) positive for *Salmonella* Typhimurium
3	Partially filled caeca and mucous plug	33.33	100
5	Partially filled caeca	33.33	100
7	Partially filled caeca	66.66	100

Salmonella free birds were raised and challenged with *Salmonella* Typhimurium on day 8 post-hatch.
Faecal samples from the birds were collected at day 3, 5 and 7 post-infection for *Salmonella* detection through culture method.
The challenged birds were processed for caecal tissue collection at day 3, 5 and 7 post-infection for RNA sequencing.

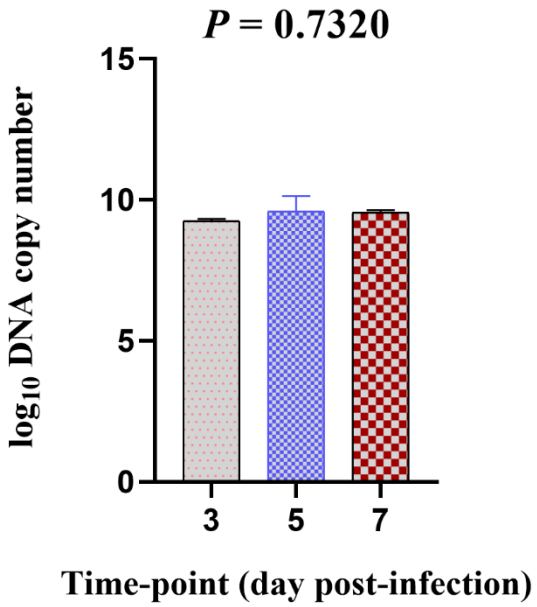


Figure 2-1 *Salmonella* Typhimurium load in caecal contents of the challenged birds

The mean load (log_{10} genomic DNA copy number/g of caecal contents) was quantified through qPCR.
There were 3 birds in each treatment group on days 3, 5 and 7 post-infection.
Values are mean ± standard deviation.

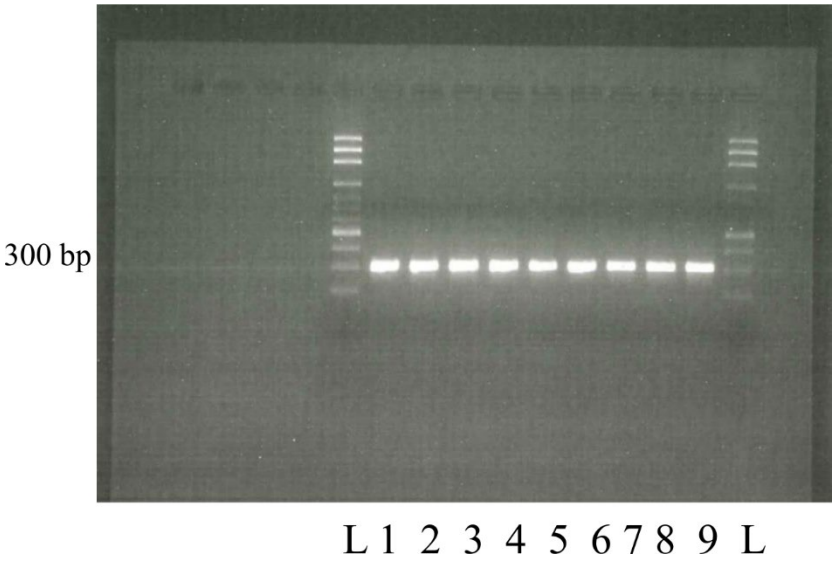
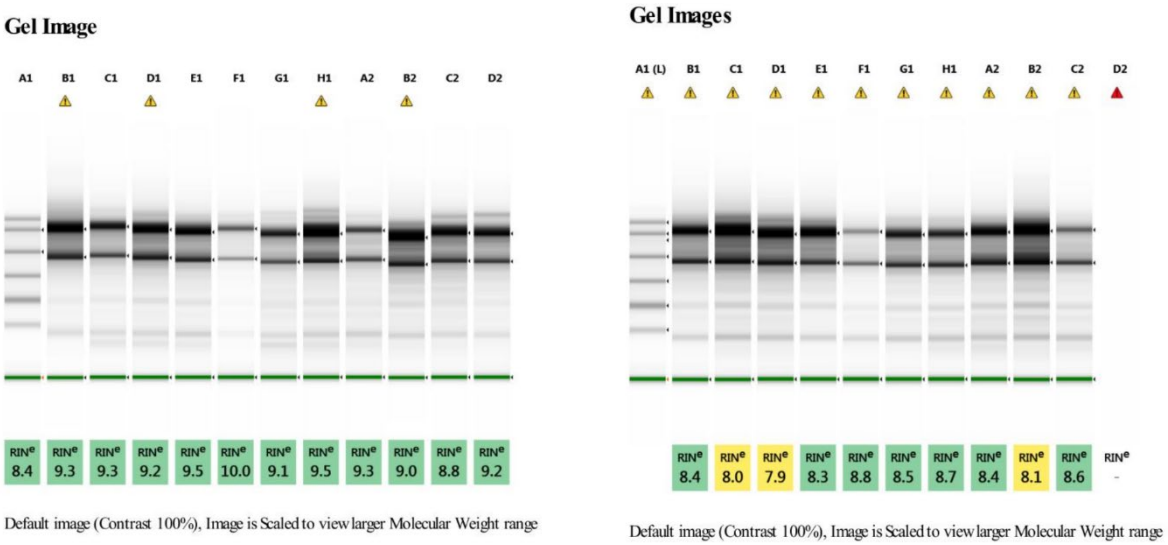


Figure 2-2 Expected size of amplicon bands on agarose gel from the *Salmonella* challenged birds

L. DNA marker.
Bands 1 to 9 indicate that the caecal contents of individual chicks collected on days 3, 5 and 7 p.i. were positive for *Salmonella* Typhimurium.
The qPCR result was confirmed by running the amplified products on 2% agarose gel.
All the samples from the negative control group gave Ct values after cycle 30 and did not show the appropriate band size on the agarose gel.



A **B**

Figure 2-3 Quality of total RNA tested in TapeStation 2200

A RNA extracted from the caeca of chickens challenged with *Salmonella* Typhimurium and sampled at day 3, 5 and 7 post-infection. In the image, A1 is DNA ladder, B1-D2 are individual RNA samples.
B RNA extracted from the caeca of control group. A1 is DNA ladder, while B1 to C2 are individual RNA samples.
The quality of RNA was tested following the protocol of RNA Screen Tape (Agilent Technologies, Australia).

Table 2-2 RNA sequencing reads quality generated in the study

Treatment	Sample	Total reads	Number of reads mapped to genome	Percentage of reads mapped to genome	Number of reads mapped to one feature	Percentage of reads mapped to one feature	Number of mapped reads not mapped to any feature	Percentage of total reads that mapped to the genome but not to any known features
***Salmonella* challenged**	D3_a	36263105	35164816	96.97%	18174408	50.12%	16929919	46.69%
	D3_b	33527487	31955623	95.31%	15939205	47.54%	15939713	47.54%
	D3_c	33859285	32843643	97.00%	15846275	46.80%	16911827	49.95%
	D5_a	24399097	23457002	96.14%	13025067	53.38%	10389852	42.58%
	D5_b	23233692	22175990	95.45%	11294682	48.61%	10838143	46.65%
	D5_c	21753577	20710695	95.21%	10923221	50.21%	9735189	44.75%
	D7_a	24180602	23084347	95.47%	11373300	47.03%	11659949	48.22%
	D7_b	24381304	23173334	95.05%	13803208	56.61%	9331245	38.27%
	D7_c	27703127	26343521	95.09%	13240034	47.79%	13041865	47.08%
Control	D3_a	39624489	38309269	96.68%	19593768	49.45%	18606352	46.96%
	D3_b	37375542	36080068	96.53%	18507895	49.52%	17453069	46.70%
	D3_c	36135438	34799111	96.30%	18444459	51.04%	16241612	44.95%
	D5_a	41205550	39716297	96.39%	20260109	49.17%	19338552	46.93%
	D5_b	38196540	29871626	78.21%	17457348	45.70%	12381178	32.41%
	D5_c	37280413	35289121	94.66%	20233099	54.27%	14991722	40.21%
	D7_a	36827852	35465860	96.30%	21011112	57.05%	14384618	39.06%
	D7_b	34589602	33471131	96.77%	18253990	52.77%	15156960	43.82%
	D7_c	36880780	35694732	96.78%	18232297	49.44%	17366118	47.09%

D3, 5 and 7 refer to day of sampling after *Salmonella* Typhimurium challenge, while a, b and c refer to biological replicates in each sampling time-point.

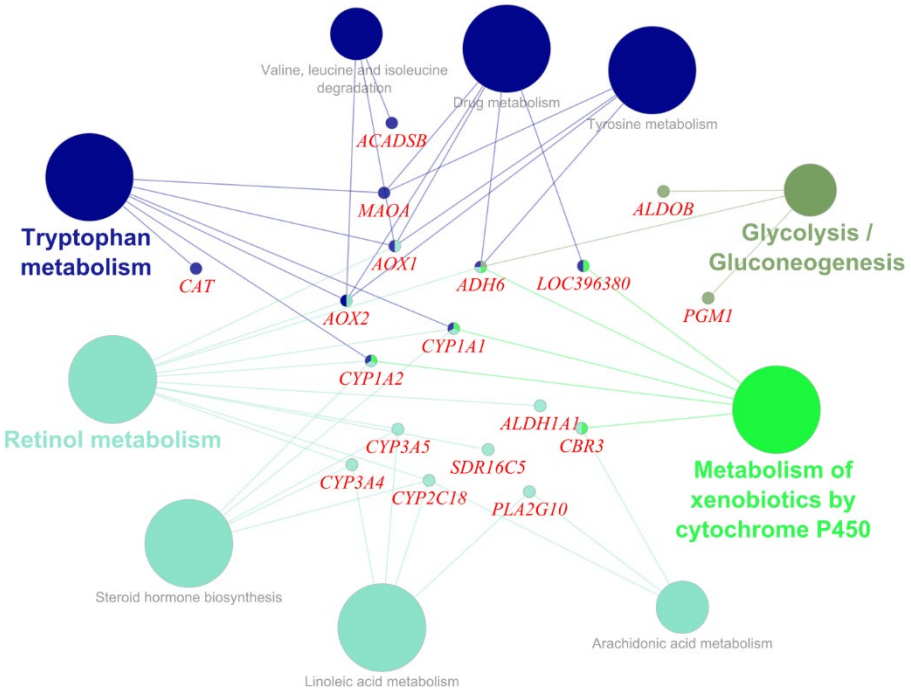


Figure 2-4 Enriched KEGG pathway terms and their associated genes obtained from the mapping of down-regulated genes at day 3 *Salmonella* Typhimurium post-infection

In ClueGO, pathways only significantly (p-value < 0.05) enriched were visualised.
Size of the nodes indicates the number of associated genes affected by *Salmonella* Typhimurium challenge.

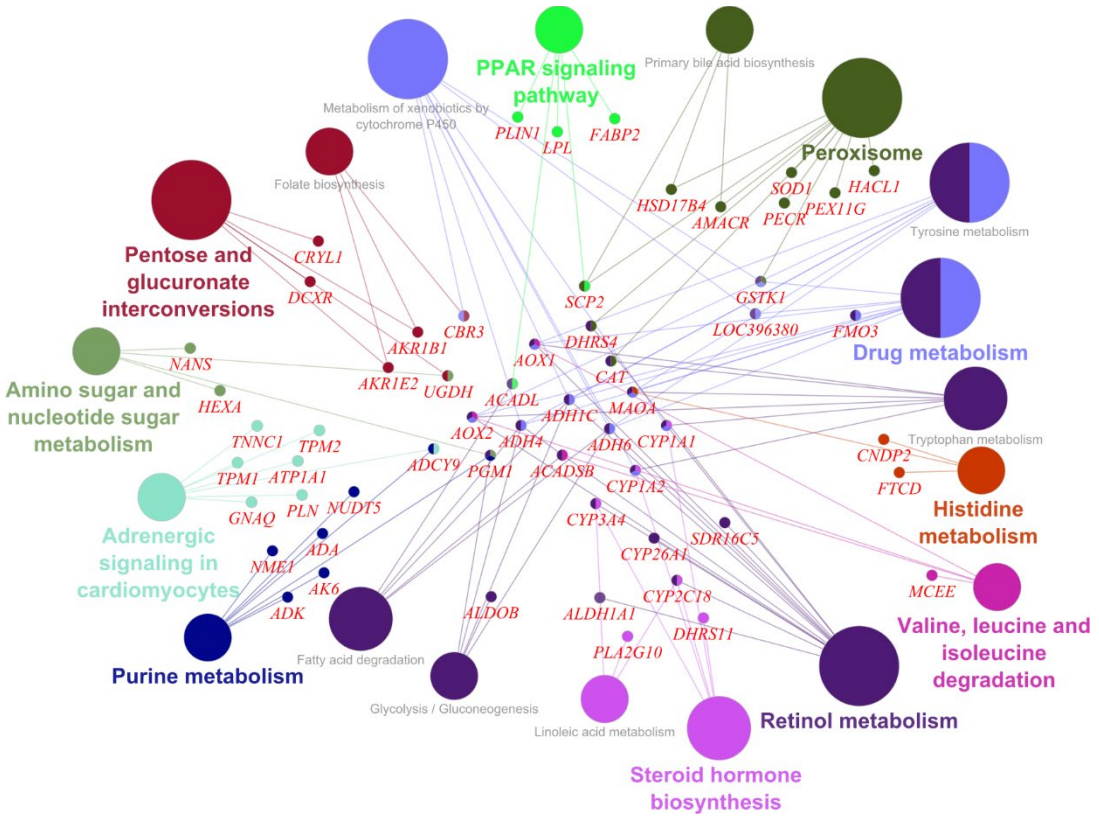


Figure 2-5 Enriched KEGG pathway terms and their associated genes obtained from the mapping of down-regulated genes on day 5 *Salmonella* Typhimurium post-infection

In ClueGO, pathways only significantly (p-value < 0.05) enriched were visualised.
Size of the nodes indicates the number of associated genes affected by *Salmonella* Typhimurium challenge.

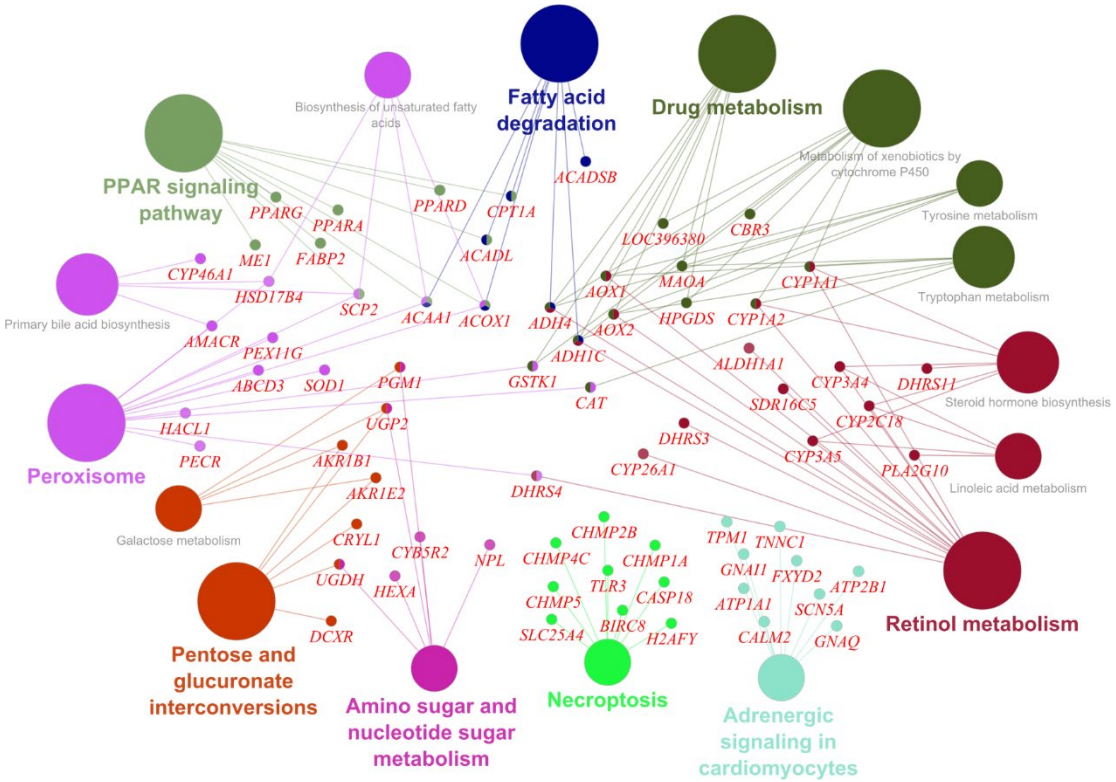


Figure 2-6 Enriched KEGG pathway terms and their associated genes obtained from the mapping of down-regulated genes on day 7 *Salmonella* Typhimurium post-infection

In ClueGO, pathways only significantly (p-value < 0.05) enriched were visualised.
Size of the nodes indicates the number of associated genes affected by *Salmonella* Typhimurium challenge.

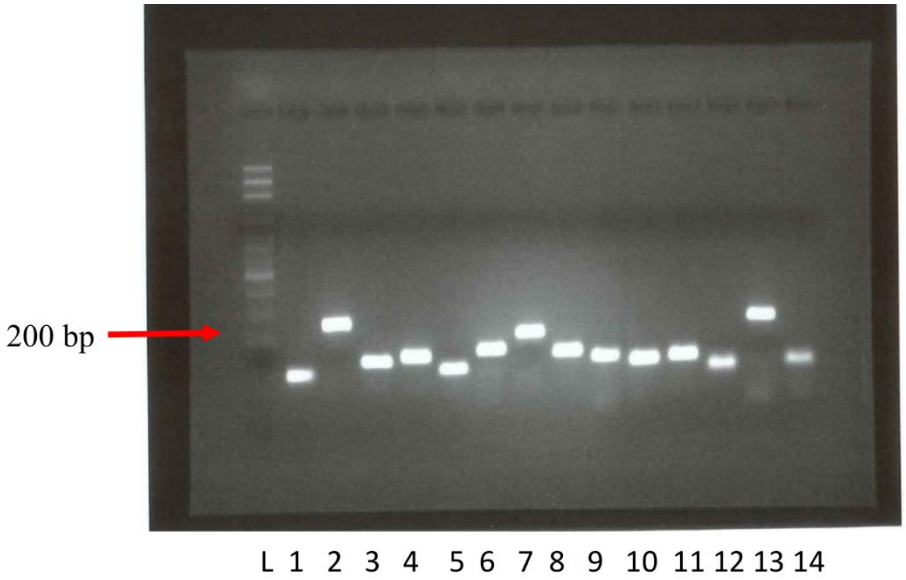


Figure 2-7 Size of gene fragments showing the specificity of candidate target and reference genes used for the validation of RNA sequencing data

L DNA ladder
1 *IL8L2* (76 bp)
2 *ACOD1* (239 bp)
3 *MMP7* (107 bp)
4 *FABP2* (122 bp)
5 *EXFABP* (78 bp)
6 *IL6* (131 bp)
7 *IL4I1* (183 bp)
8 *CALB1* (118 bp)
9 *ALB* (102 bp)
10 *TBP* (88 bp)
11 *YWHAZ* (94 bp)
12 *NOS2* (66 bp)
13 *CCLL4* (213 bp)
14 *MYL1* (75 bp).

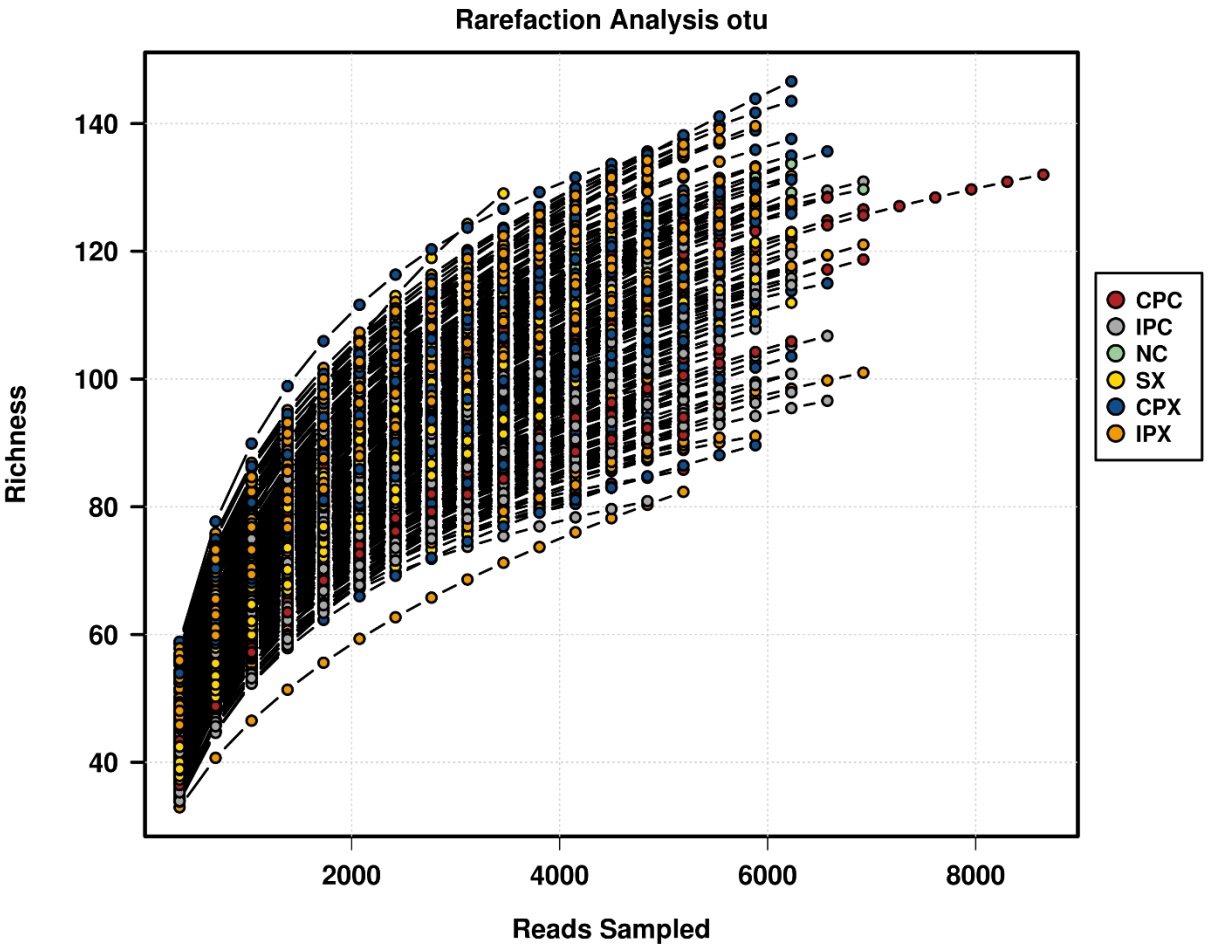


Figure 4-1 Rarefaction analysis of OTUs showing the quality of the reads generated from DNA obtained from chicken faeces

The flattening curves towards right show that the underlying microbial communities were well covered by the sequenced data.

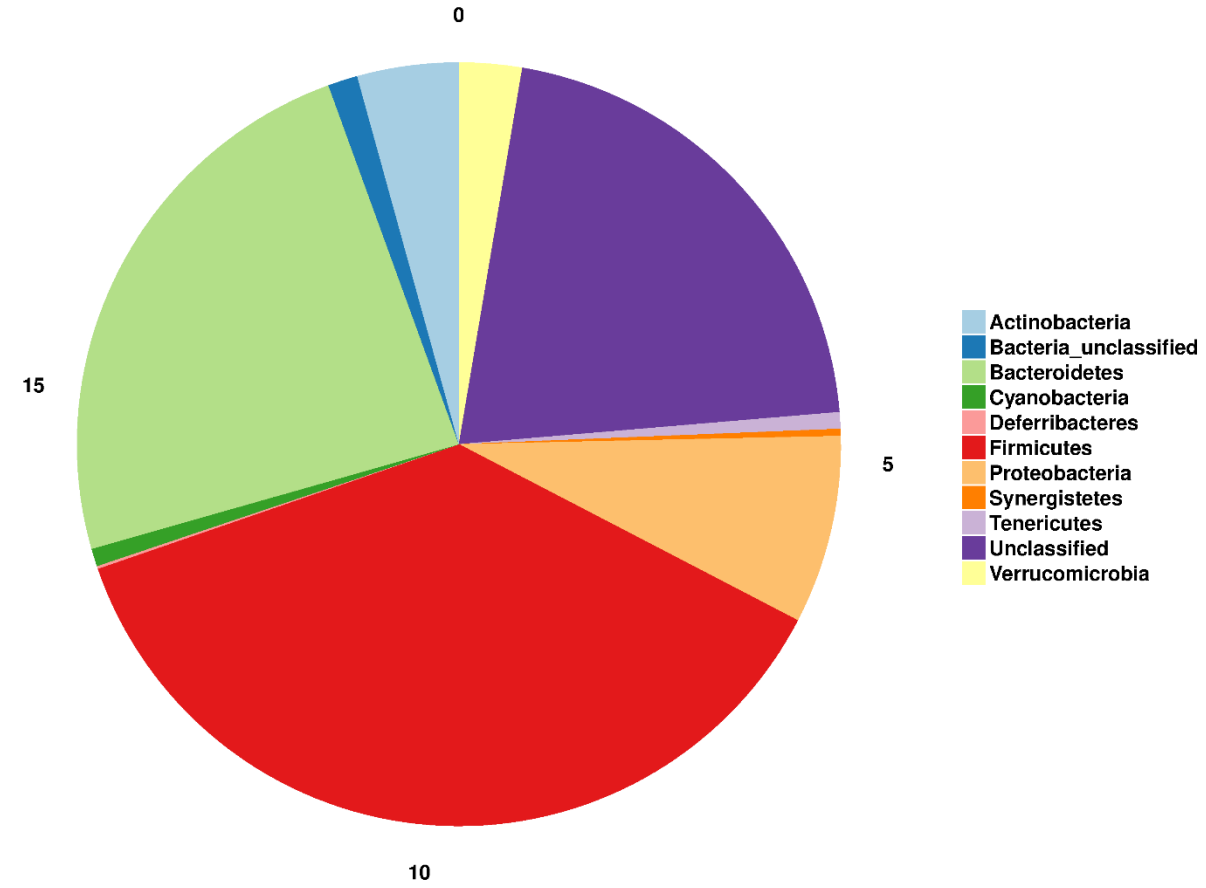


Figure 4-2 Abundance of microbial communities at phylum level in faeces

Data for all the treatment groups were mapped in Calypso software to get the abundance of different phyla.

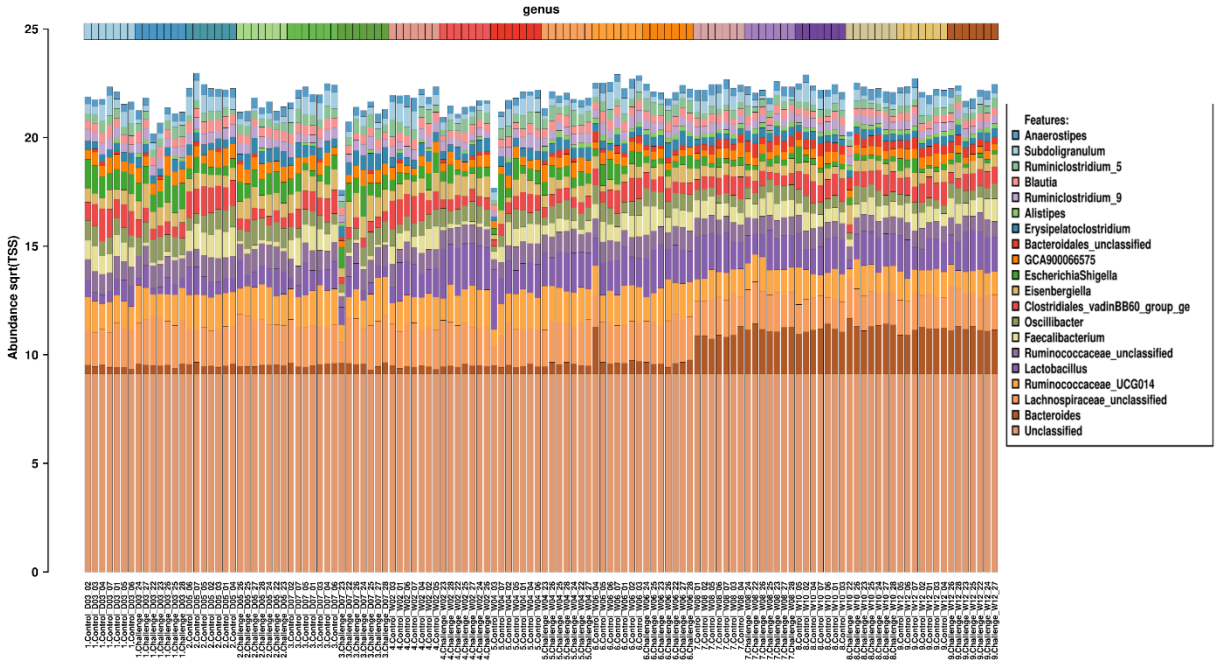


Figure 4-3 Abundance of microbial communities at genera level in faecal samples of individual chickens in the negative control and *Salmonella* challenged chickens sampled at different time-points (days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12 post-challenge)

The genus bar is based on sampling time-points post-challenge.

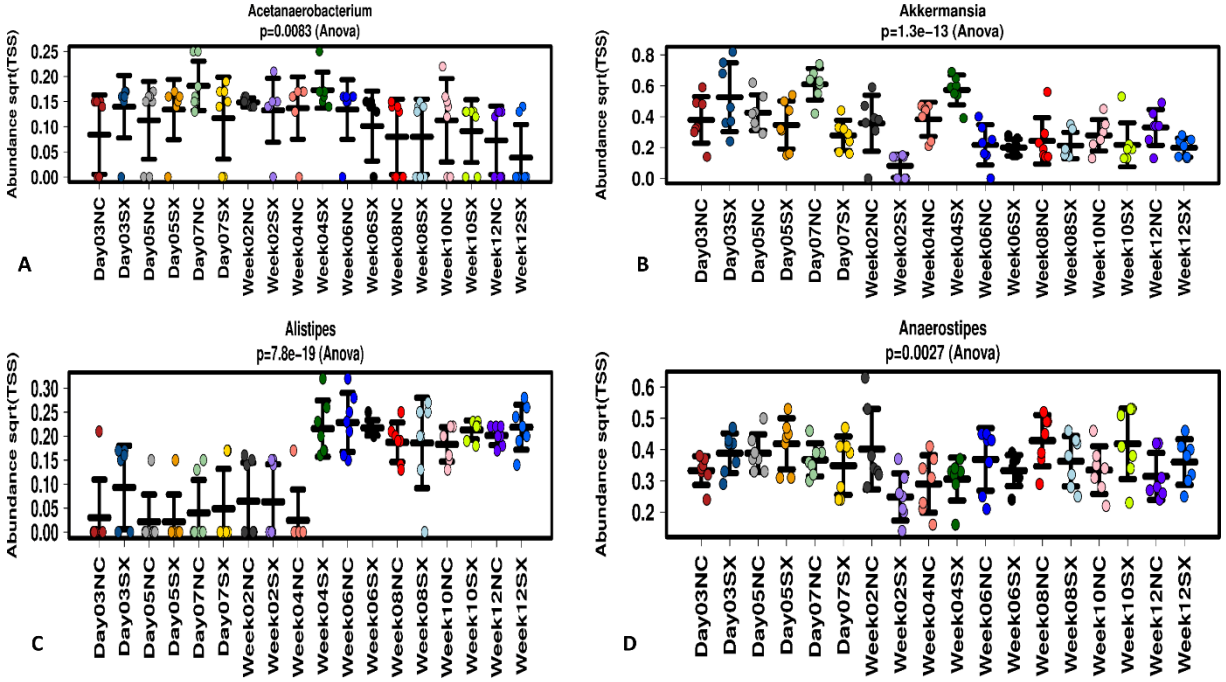


Figure 4-4 Faecal microbial abundance affected by *Salmonella* Typhimurium challenge at different sampling time-points in laying chickens

Panel labels (a-d) show the effect of *Salmonella* on individual bacterial genera.

NC is negative control, SX is *Salmonella* challenged.

Data from the faecal samples collected at days 3, 5, and 7, and week 2, 4, 6, 8, 10 and 12 post-challenge were used for comparison between the two treatment groups (NC and SX).

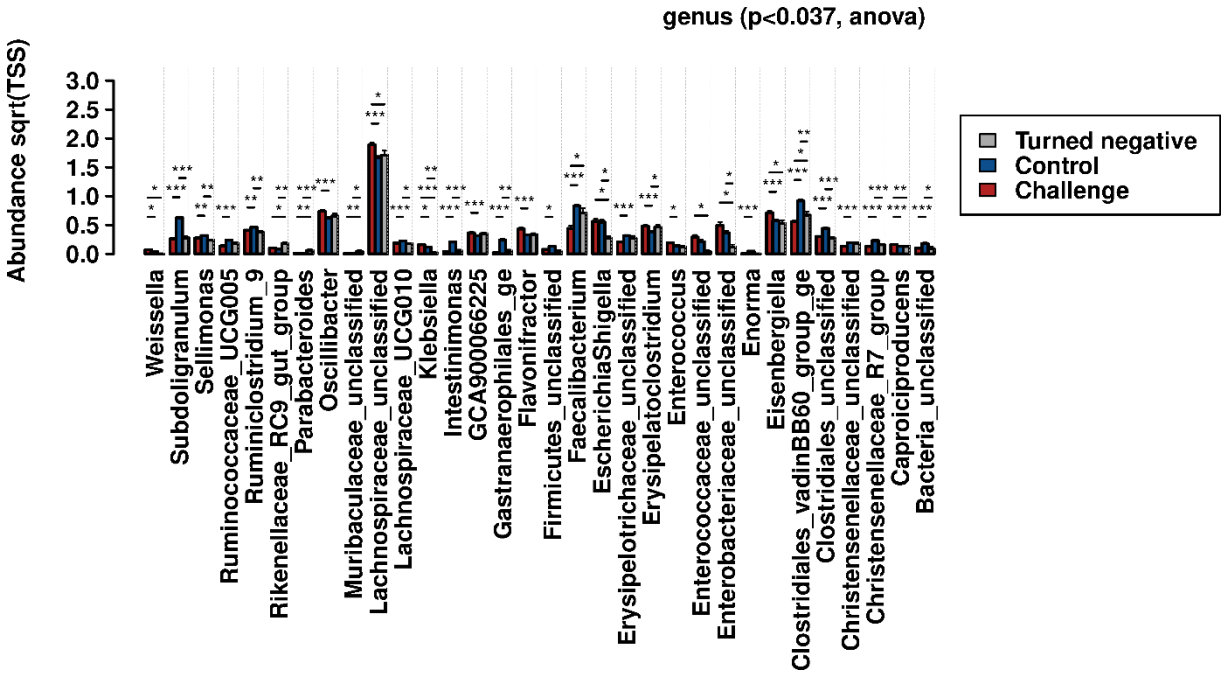


Figure 4-5 Microbial genera abundance of *Salmonella* turned negative chickens

The abundance level of the *Salmonella* turned negative chickens (n = 2) was compared with consistently *Salmonella* shedding chickens (n = 5) and negative control groups (n = 7).

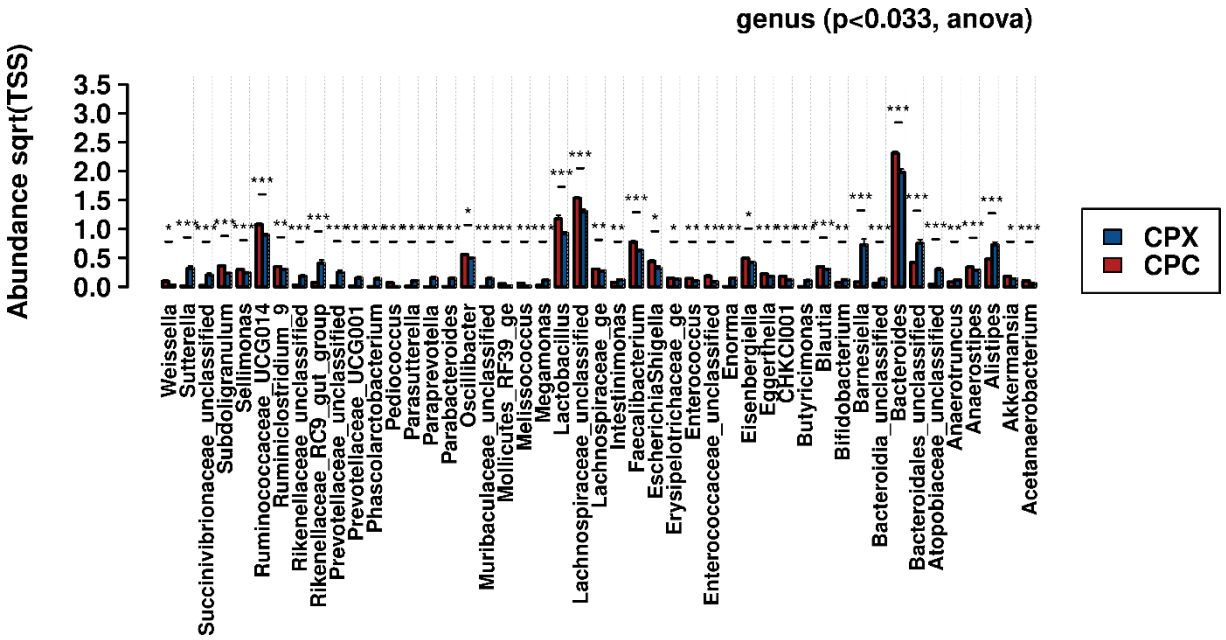


Figure 4-6 Microbial genera abundance affected by *Salmonella* Typhimurium challenge and continuous supplementation of probiotic

The microbial abundance at genera level of the continuous supplemented probiotic control (CPC) group was compared with the continuous supplemented probiotic and *Salmonella* Typhimurium challenged (CPX) group.
Data from the faecal samples collected at nine different sampling time-points (days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12) post-challenge were analysed for comparison between the two treatment groups (CPC and CPX).

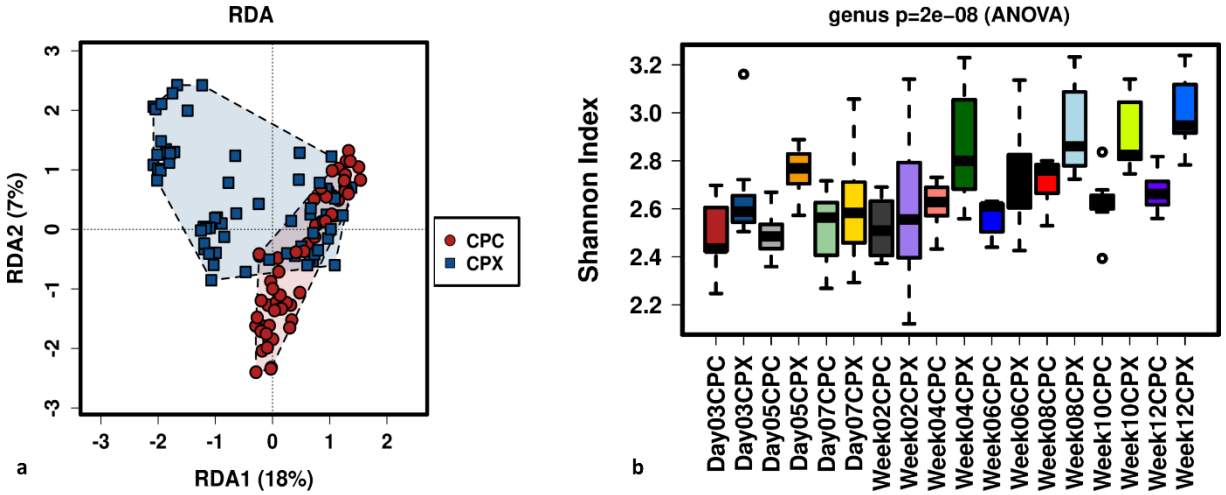


Figure 4-7 Microbial community composition and diversity affected by *Salmonella* Typhimurium and continuous supplementation of probiotic

(a) Microbial community composition between the continuous supplemented probiotic control (CPC) and the continuous supplemented probiotic and *Salmonella* Typhimurium challenged (CPX) groups.
(b) Microbial diversity between the CPC and CPX at different time-points (days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12) post-challenge.

Data from the faecal samples collected at days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12 post-challenge were used for the comparison between the two treatment (CPC and CPX) groups.

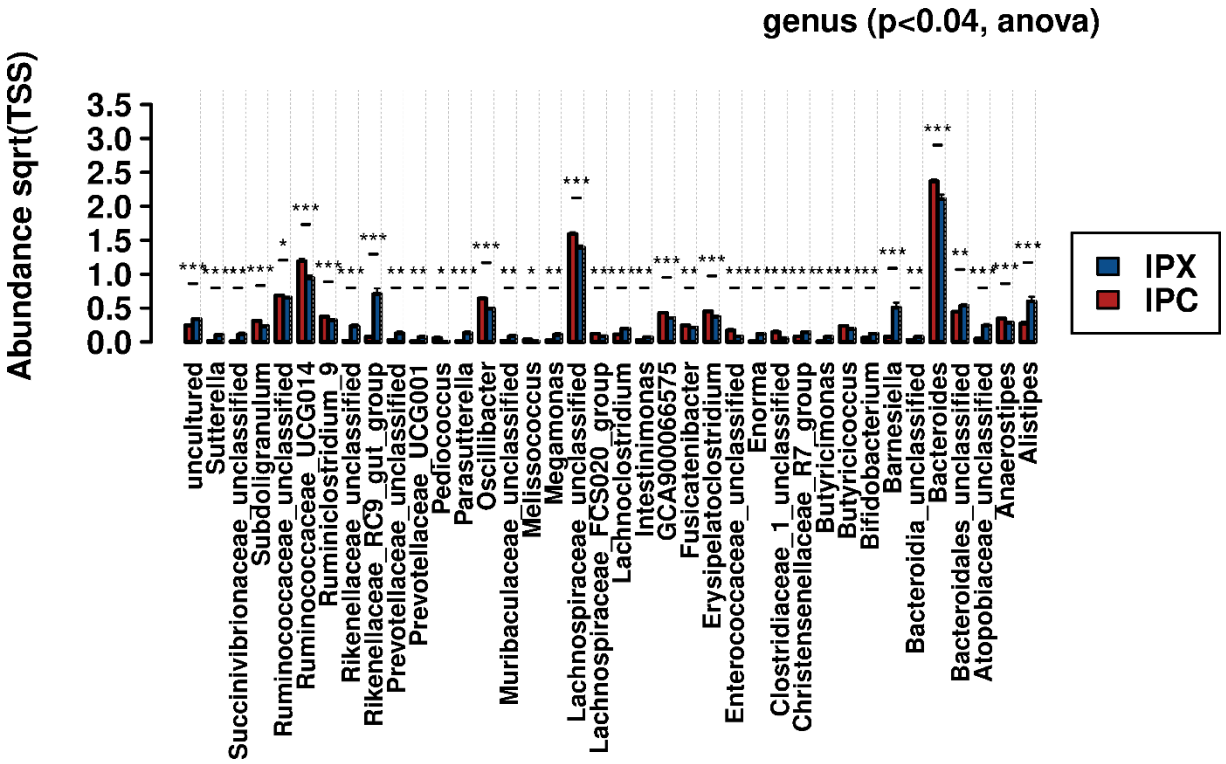


Figure 4-8 Microbial abundance of individual genera affected by *Salmonella* Typhimurium challenge and intermittent supplementation of probiotic

The microbial abundance at genera level of the intermittent supplemented probiotic control (IPC) group was compared with the intermittent supplemented probiotic and *Salmonella* Typhimurium challenged (IPX) group.
Data from the faecal samples collected at nine different sampling time-points (days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12) post-challenge were analysed for comparison between the two treatment groups (IPC and IPX).

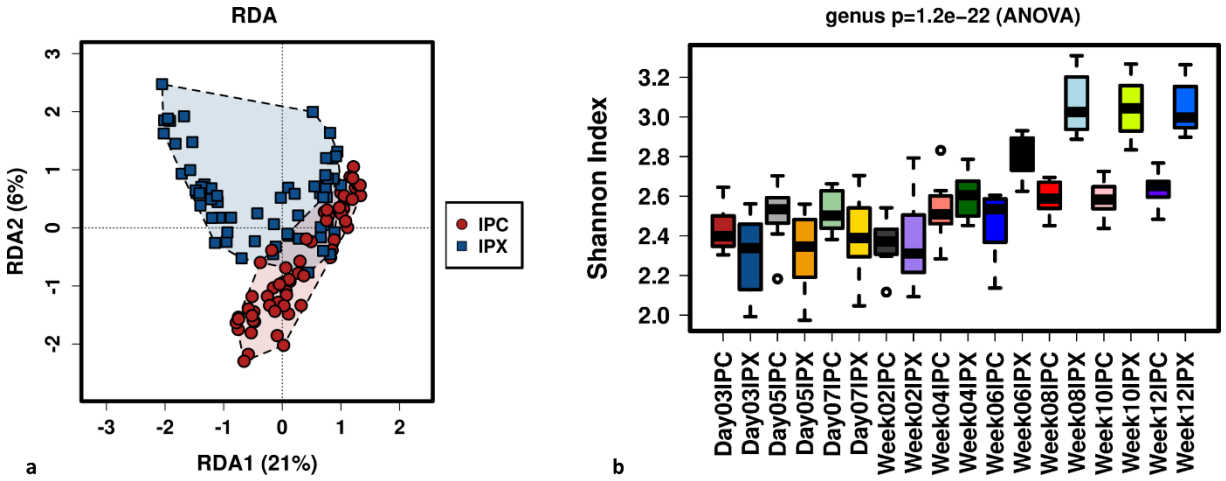


Figure 4-9 Microbial community composition and diversity affected by *Salmonella* Typhimurium and intermittent supplementation of probiotic

(a) Microbial community composition between the intermittent supplemented probiotic control (IPC) and the intermittent supplemented probiotic and *Salmonella* Typhimurium challenged group (IPX).
(b) Microbial diversity between the IPC and IPX at different time-points (days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12) post-challenge.

Data from the faecal samples collected at days 3, 5 and 7, and weeks 2, 4, 6, 8, 10 and 12 post-challenge were analysed for comparison between the two treatment groups (IPC and IPX).

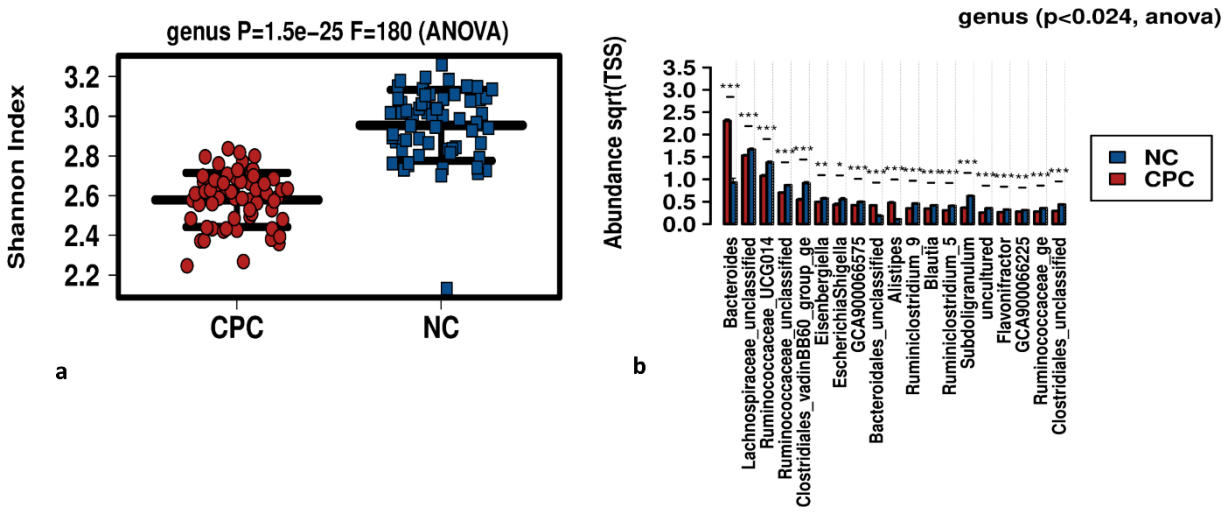


Figure 4-10 Microbiota diversity and abundance of microbial genera affected by continuous supplementation of probiotic

(a) Overall diversity of faecal microbiota.

(b) Abundance of faecal microbial genera.

For determining the effects of the probiotic on the diversity of gut microbiota and abundance levels of individual microbial genera, the negative control (NC) group was compared with the continuous supplemented probiotic (CPC) group (excluding *Salmonella* Typhimurium challenge).

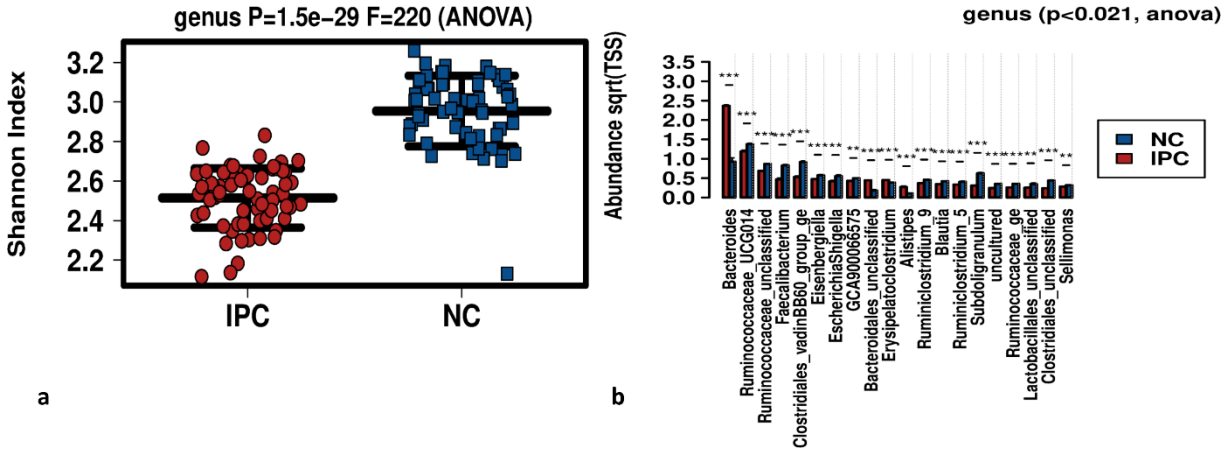


Figure 4-11 Microbiota diversity and abundance of microbial genera affected by intermittent supplementation of probiotic

(a) Overall diversity of faecal microbiota.

(b) Abundance of faecal microbial genera.

For determining the effects of the probiotic on the diversity of gut microbiota and abundance levels of individual microbial genera, the negative control (NC) group was compared with the intermittent supplemented probiotic (CPC) group (excluding *Salmonella* Typhimurium challenge).