



Effects of microbiota 'seeding' on gut health and egg production

Final Project Report | AUGUST 2024

A report for Australian Eggs Limited

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Australian Eggs Limited Publication No INNO003

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ISBN 978-1-920835-82-8

Project Title: Effects of microbiota 'seeding' on gut health and egg production

Australian Eggs Limited Project Number INNO003

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Published in April 2025

Foreword

The productivity of a layer hen is influenced by several factors including stock genetics, effective disease control, feed composition, and management. Gut health principally influences general flock health and feed efficiency of layers, which can affect farm profitability and management. This project was conducted to investigate the use of gut 'seeding' as a potential layer hen management strategy to improve gut health. It has the potential to reduce the incidence of gut dysbiosis, reduce infections, and improve egg quality and productivity.

The aims of this project were to first compare the gut microbiota of high performing flocks with flocks with known production issues. Transport can induce physiological stress, which may induce dysbiosis in pullets rendering them vulnerable to colonisation by bacterial pathogens. To date, the effect transport has on the gut microbiota has not been investigated in layer hens. An additional goal of this project was to evaluate changes in the gut microbiota in response to transport. How 'seeding' the gut microbiota with a live *Lactobacillus* strain at different time points was also evaluated.

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.

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Acknowledgments

The authors would like to acknowledge Dr Nicky-Lee Willson, Dr Samiullah Khan, Siyuan Jia, Jiawei Zhang, and Stephanie Hewitt for their technical and animal husbandry support during this study. We would like to acknowledge all egg producers and field veterinarians for providing samples for this project. Australian Eggs Limited provided the funds that supported this project.

About the Authors

Dr Andrea McWhorter is a microbiologist specialising in poultry health and zoonotic foodborne pathogens. Her research also extends to understanding the transmission of foodborne pathogens from farms to the food supply chain, leading to human disease. Andrea collaborates closely with the egg and chicken meat industries to address the challenges of improving bird health and mitigating zoonotic bacteria in food.

Professor Rob Moore is a Professor at RMIT, Melbourne Australia. Professor Moore leads an active research group that publishes widely, secures funding from industry and government, and works with a wide network of collaborators. Over the last decade, the research in his laboratories has been largely industry supported by funding through the Australian Research Council Linkage Program, Poultry Cooperative Research Centre, Poultry Hub Australia, Australian Eggs, AgriFutures Australia, and various collaborations with commercial partners. He is currently working with several industry partners to commercialise various aspects of his work on Necrotic Enteritis and Spotty Liver Disease of chickens, vaccines, therapeutics, and diagnostics. It is anticipated that these commercial developments will deliver tools to the industry to allow the control and management of these important diseases.

Associate Prof Hao Van is a molecular microbiologist. She has led impactful industry related research, which has been recognised internationally and nationally. Her areas of expertise include gut microbiota characterisation and manipulation, identification of bacterial pathogens and deciphering their biology, and developing intervention strategies to combat bacterial diseases, including development of probiotics, antimicrobial compounds and vaccines.

Professor Kapil Chousalkar is Deputy Head of the School of Animal and Veterinary Sciences at the University of Adelaide. He leads the Food Safety and Poultry Health Research Group. Kapil has led several research projects over the years and supervised many students and scientists. He teaches poultry health and food safety in three degree programs, and offers his veterinary service to commercial egg and chicken meat producers in South Australia. Kapil also chairs the South Australian Poultry Liaison Group and sits on the South Australian Chicken Meat Council.

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Abbreviations

CFU	Colony Forming Units
DMEM	Dulbecco's Modified Eagle Media
g	g force (in relation to centrifugation)
g	Gram
h	Hour
MRS	DeMan, Rogosa, and Sharpe
mg	Milligram
min	Minute
mL	Millilitre
L	Litre
OTU	Operational Taxonomic Unit
µg	Microgram
µL	Microlitre

Executive Summary

Maintaining optimal gut health is crucial for the welfare and productivity of layer hens in egg production. This project evaluated the differences in the gut microbiota profile of healthy > 60-week-old layer flocks and those experiencing production issues. Results from this study demonstrated that the gut microbiota profiles were influenced by geographical location. Key bacterial genera were identified as potential drivers of differences between the flock production types.

‘Seeding’ the gut microbiota with live probiotic bacterial strains has the potential to positively influence the development of the gut microbiota profile. This project also evaluated the capacity of five *Lactobacillus* strains administered immediately after hatch to colonise and persist in the gastrointestinal tract. All five strains successfully colonised the gut microbiota of chicks and, while not significant, led to an increase in alpha-diversity of the gut microbiota. Inhibition of *Salmonella* invasion into the caecal epithelial was also observed. The greatest impact was observed for *Lactobacillus saerimneri*.

The effect of a single oral dose of *Lactobacillus saerimneri* at hatch was evaluated over 45 weeks. Culturable *Lactobacillus saerimneri* persisted for 20 weeks. After a stress event, culturable bacteria were observed again between 27 and 36 weeks. Administration enhanced alpha-diversity and modified the abundance of certain bacterial phyla compared with control birds. Administration of *Lactobacillus saerimneri* at point-of-lay resulted in colonisation of the gut with culturable bacteria observed for the entire 10-week experiment. Changes in the gut microbiota were observed; alpha-diversity decreased at 8 and 10 weeks after treatment.

This project also investigated whether transport of pullets from rearing to production farms affected the gut microbiota. Results showed significant shift in the composition of the gut microbiota following transport. Richness of bacterial species present in the gut microbiota was negatively impacted. Additional experiments were conducted to determine if administration of *Lactobacillus saerimneri* post-transport affected the gut microbiota. *Lactobacillus saerimneri* treatment affected the relative abundance of several bacterial species but over time was linked with a reduction in evenness.

Additionally, the project examined how *Lactobacillus saerimneri* administration at late lay (70 weeks) impacts the gut microbiota. Birds were obtained from a commercial free range farm and transported to experimental housing. Upon arrival, birds were administered a single, oral dose of *Lactobacillus saerimneri*. Results demonstrated that the change in diet, not *Lactobacillus saerimneri* administration, significantly affected the gut microbiota profile.

Overall Conclusions

- Geographical location influenced variation in gut microbiota among flocks older than 60 weeks of age.
- High-producing flocks and flocks that had experienced production issues from the same state exhibited similar gut microbiota profiles but show variability in several bacterial genera.
- ‘Seeding’ the gut with *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, or *Lactobacillus saerimneri* led to the colonisation of the chick gastrointestinal system and increased alpha-diversity of the gut microbiota.
- Using an *in vitro* everted sleeve model, administration of *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, and *Lactobacillus saerimneri* inhibited the invasion of *Salmonella* into the caecal epithelia.
- A single dose of *Lactobacillus saerimneri* at hatch induced persistent colonisation in the gastrointestinal system over a 45-week period.
- Age was a major driver in differences in the gut microbiota over 45 weeks.
- While not significant, pullets and birds in early lay that were ‘seeded’ with *Lactobacillus saerimneri* had higher average alpha-diversity compared with control birds.
- *Lactobacillus saerimneri* administration at point-of-lay, post-transport led to colonisation of the gut for the 10-week experiment. Alpha-diversity decreased in birds administered *Lactobacillus saerimneri*.
- Transport of pullets from rearing to production farms impacted the composition of the gut microbiota.
- A change in diet, not *Lactobacillus saerimneri* administration, significantly affected the gut microbiota profile in 70-week old layer hens.

1 Introduction

Eggs are a highly nutritious and cost-effective protein source that also provide multiple essential vitamins and minerals. In Australia, egg consumption has increased 19% over the past decade, with this upward trajectory projected to persist (Australian Eggs). To meet this demand, egg farmers manage approximately 22 million birds producing over 6.7 billion eggs each year (Australian Eggs). Thus, ensuring hen health is important to maintaining a consistent egg, high quality supply.

The gut microbiota is comprised of a diverse community of microorganisms. A fundamental aspect of bird health lies in the maintenance of a robust gut microbiota, which in conjunction with diet contributes to digestive efficiency, nutrient absorption, immune function, egg production, and general health (Khan et al. 2020). In addition, a healthy gut microbiota can offer protective effects against pathogenic bacteria (Wickramasuriya et al. 2022). The gut microbiota has emerged as a key area of research for the agricultural sector, with the aim of improving animal health and productivity. Poor gut health can lead to a myriad of health and management issues within poultry production, including dysbiosis, poor uniformity, increased susceptibility to infection, higher morbidity rates, and decreased egg production. As a result, strategies aimed at preserving and enhancing gut health have become a focal point.

As chickens age, the composition of the gut microbiota changes (Joat et al. 2021a). Other factors can also contribute to the composition of the microbiota. Production system has been found to play an important role in shaping the development and composition of the gut microbiota. Free range laying hens, for example, have been found to have a higher abundance of microbial genera involved in amino acid and glycan metabolic pathways compared with birds in caged housing system (Xu et al. 2016). Additionally, cage birds exhibit lower levels of the bacterial phylum *Bacteroidetes*, and higher levels of *Firmicutes* compared to free range birds (Xu et al. 2016). Rearing conditions, age, breed, and feed can also significantly influence the development and composition of the gut microbiota. Correlations have been observed between genotype, age, and body composition, with the prevalence of several microbial genera. Microbial genera like *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*, for example, tend to be more abundant in low body weight laying birds (Zhao et al. 2013).

In the first few days of life, the gut microbiota undergoes rapid development (Ballou et al. 2016; Cui et al. 2017). A recent study found that chick meconium, in general, lack microbiota (Joat et al. 2021a). Of the meconium samples positive for microbes, the microbiota was predominantly composed of bacteria from the *Firmicutes* phylum (Joat et al. 2021a). On the day of hatch, the caecal microbial community has been shown to consist of only five species, indicating a gut microbiota of very low complexity (Crhanova et al. 2011). Two weeks post-hatch, there is a substantial increase in *Ruminococcus* and *Firmicutes*, and a decrease in the representation of *Enterobacteriaceae* (Crhanova et al. 2011).

Recent studies have shown that exposure to a hen in a nest environment contributes to direct transmission of intestinal microbiota (Chen et al. 2020). In mammals, the maternal-to-offspring transfer or 'seeding' of bacteria has been shown to have an influence on the developing gut microbiota that can have significant health effects later in life. Commercial layer hen chicks, however, are typically hatched without direct contact with a hen, and acquire their microbiota through feed and the rearing environment. The introduction of feed supplements, such as probiotics or prebiotics, can influence the developing gut microbiota, promoting overall gut health and potentially improving productivity over the lifetime of a hen.

Probiotics have a rich history dating back centuries. The earliest records come from the ancient Greeks and Romans, indicating their consumption of fermented foods and beverages. The concept of using live microorganisms to confer health benefits gained momentum in the early 20th century. Since then, probiotics have been studied extensively and incorporated into feed and supplements. The present study investigated the 'seeding' approach of probiotic delivery, with the primary aim of shaping the establishment and maintenance of gut microbiota during rearing and at different stages of production to enhance gut health, egg production and egg quality. This project was comprised of six separate studies:

1. **Chapter 2** compared the gut microbiota of high-producing free-range layer hen flocks with flocks experiencing known production issues. The aim of this study was to determine if there was a difference in the gut microbiota profile between high and low producing flocks.
2. **Chapter 3** established a gut 'seeding' methodology using five different *Lactobacillus* probiotic strains. The colonisation capacity and persistence of these *Lactobacillus* strains was evaluated at both two and four weeks of age. Their effect on the developing microbiota was also investigated.
3. **Chapter 4** investigated the persistence of *Lactobacillus saerimneri* following a single 'seeding' dose. The effect of 'seeding' on the gut microbiota was characterised at 4, 10, 16, 22, and 45 weeks of age representing different stages in the layer hen production cycle.
4. **Chapter 5** evaluated the effect of transport on the gut microbiota of point-of-lay-pullets. Samples were collected prior to transport and then at 2, 6, 10, and 14 days following transport to determine how the gut microbiota is affected.
5. **Chapter 6** investigated how a single dose of *Lactobacillus saerimneri* administered at point-of-lay affected the gut microbiota.
6. **Chapter 7** investigated how a single dose of *Lactobacillus saerimneri* administered in late lay (65 weeks) could influence the gut microbiota. The gut microbiota in late lay hens often decreases in richness and evenness, which can contribute to changes in egg laying patterns as well as egg quality.

2 Gut microbiota variation between flocks with high production and production issues

2.1 Background

Egg production varies over the course of the lifetime of commercial layer hens and is marked by three distinct phases: the pullet stage, peak egg production, and late lay. Each phase is associated with unique physiological demands, hormonal fluctuations, and dietary requirements. Hens older than 60 weeks of age are generally considered to be in late lay and often experience a decrease in egg production and quality. Many flocks are depopulated between 68 and 75 weeks because of the drop in production and quality. The egg industry, however, aims to extend the production period to over 100 weeks to help meet the demand for eggs and reduce the number of flocks needed, ultimately improving industry sustainability. By optimising management practices, nutrition, and animal health during late lay, farmers can potentially enhance egg production, extending the productive lifetime of layer hens.

Age significantly influences changes in the gut microbiota. In late lay, the gut microbiota may be impacted not only by age, but also by hormonal fluctuations and altered metabolism. It is important to understand the variations in gut microbial composition during late lay to figure out how it can be improved. In this study, the gut microbiota of high producing flocks and flocks with production issues were evaluated to determine: 1) if there was a healthy 'profile' for birds in late lay; and 2) whether there were key indicators of production issues. The outcomes of this study were aimed at providing insights into strategies for prolonging egg production and maintaining egg quality in late lay.

2.2 Methods

2.2.1 Sample collection

Fresh caecal samples were collected from flocks over 65 weeks of age. Nineteen free range flocks were sampled from three states: South Australia (SA), Queensland (QLD), and Victoria (VIC). Based on producer data, ten flocks were classified as high production (PH) and nine were categorised as having production issues (PI) primarily because of health-related issues (Spotty Liver Disease or *Mycoplasma*) and reduced egg production. Twenty samples were collected from each flock from farm infrastructure. Samples were stored at -80°C until processed for DNA extraction.

DNA was purified from caecal contents using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Australia) according to the manufacturer instructions with minor modifications. Briefly, 180–220 mg caecal material was weighed into a 1.5 mL Safe Lock (Eppendorf, Australia) microcentrifuge tube. 750 µL InhibitEX Buffer and a mixture of acid washed beads with diameters of 106 µm and between 425 and 600 µm (Sigma Aldrich, Australia) was added to each tube. Caecal contents were homogenised with a BulletBlender (Next Advance, USA) at full speed for 5 minutes. Tubes were incubated at 95°C for 7 minutes and then spun at 20,000 x g for 1.5 minutes. 600 µL of the supernatant was added to a 2 mL Safe Lock (Eppendorf, Australia) microcentrifuge tube containing 25 µL proteinase and K. Buffer AL (600 µL) was then added to each tube. Tubes were incubated at 70°C for 10 minutes. Following incubation, 600 µL 100% ethanol (Sigma, Australia) was added to each tube. The lysate was then added to the QIAamp spin column placed in a collection tube. Columns were centrifuged at 20,000 x g for 1.5 minutes. This was repeated until all the lysate had been passed over the column. Columns were washed with buffer AW1 followed by AW2. DNA was eluted from the column using

100 µL buffer ATE. DNA quantity (ng/µL) and quality (260/280 ratio) was assessed using a NanoDrop Spectrophotometer (Thermo Fisher, Australia). Samples were sent to the Royal Melbourne Institute of Technology (RMIT, Victoria) for 16S rRNA sequencing.

2.2.2 Amplicon sequencing and analyses

The 16S rRNA gene (V3–V4 region) was amplified using the Q5 High Fidelity DNA Polymerase (New England Biolabs, USA) with dual indexing, variable spacer primers (338F: 5'-ACTCCTACGGGAGGCAGCAG-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3') (Fadrosh et al. 2014). PCR cycling conditions were 98°C for 1 min followed by 35 cycles of 98°C for 10 s, 49°C for 30 s, and 72°C for 30 seconds, and a final extension at 72°C for 10 min. Amplicon sequencing was performed using an Illumina MiSeq (2 x 300 bp). Illumina software was used to demultiplex sequencing data and the analysis was performed in Quantitative Insights into Microbial Ecology 2 (QIIME2). Quality filtering, denoising, and chimera removal were performed using Dada 2 as a QIIME2 plugin with all recommended parameters. Sequences were grouped into amplicon sequence variants (ASV). Microbial taxonomy was assigned using the SILVA v138.1 database. The ASV frequency table was uploaded into the online MicrobiomeAnalyst system for data normalisation using the cumulative sum scaling (CSS) option. Data were analysed using alpha-diversity (Chao1, Shannon, and Simpson indices), beta-diversity (PERMANOVA and ANOSIM), linear discriminant analysis effect size (LEfSe), and non-parametric tests in accordance with the software user guidelines.

Data are shown as the mean $\pm$ error of the mean for continuous variables. P-value < 0.05 was considered statistically significant.

2.3 Results

2.3.1 The gut microbiota of high and low-production flocks

The gut microbiota of high and low-production layer flocks was compared in this study. ANOSIM analysis revealed a significant difference in the beta-diversity of gut microbiota in high and low-production flocks ($R = 0.047$; Figure 2-1a). Beta-diversity measures are estimates of similarity or dissimilarity between bacterial populations between samples. It was interesting to note that the richness and evenness of microbial communities did not vary significantly between high and low-production flocks (Figure 2-1b,c,d).

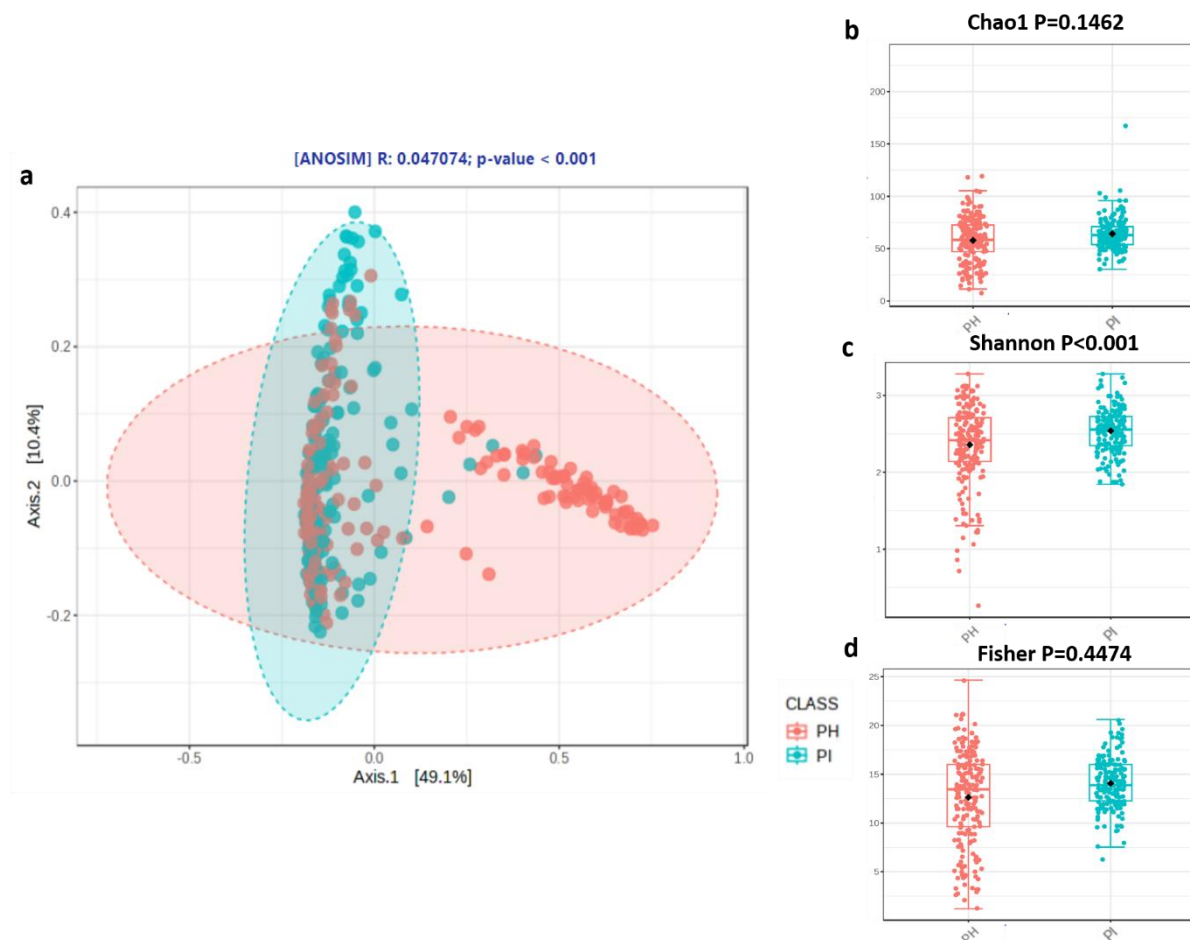


Figure 2-1 Alpha and beta-diversity of gut microbiota taxa between two groups: high-performance (PH) and with production issues (PI) at the genus level

(a) Principal coordinates analysis (PCoA) plot of Bray-Curtis distance displayed beta-diversity of gut microbiota between groups.

(b) Chao1.

(c) Shannon.

(d) Fisher alpha-diversity indices between PH and PI groups.

Colour of PH: Red; PI: Green.

2.3.2 Comparison of flocks within a single company (Company A)

To study whether the difference in the diversity of the gut microbiota was influenced at a flock level, we compared the gut microbiota of the flocks that were reared by the same company. The management, feeding and husbandry practices of flocks within this company were comparable. In addition to this, many flocks ($n = 9$) were sampled from this company. Five flocks were high-performing and four flocks had a low-production history.

At the genus level, there was a significant difference between the gut microbiota composition of high and low-production flocks (Figure 2-2a). Although most of the samples from both PH and PI groups were clustered together, some samples from high-production flocks separated from the cluster. This could indicate that the difference in the microbiota was driven by some birds from PH flocks. No significant difference was observed for Chao1 (Figure 2-2b) or Fisher (Figure 2-2d) between PH and PI groups. However, the Shannon diversity index was significantly different ($P < 0.05$) between PH and PI groups.

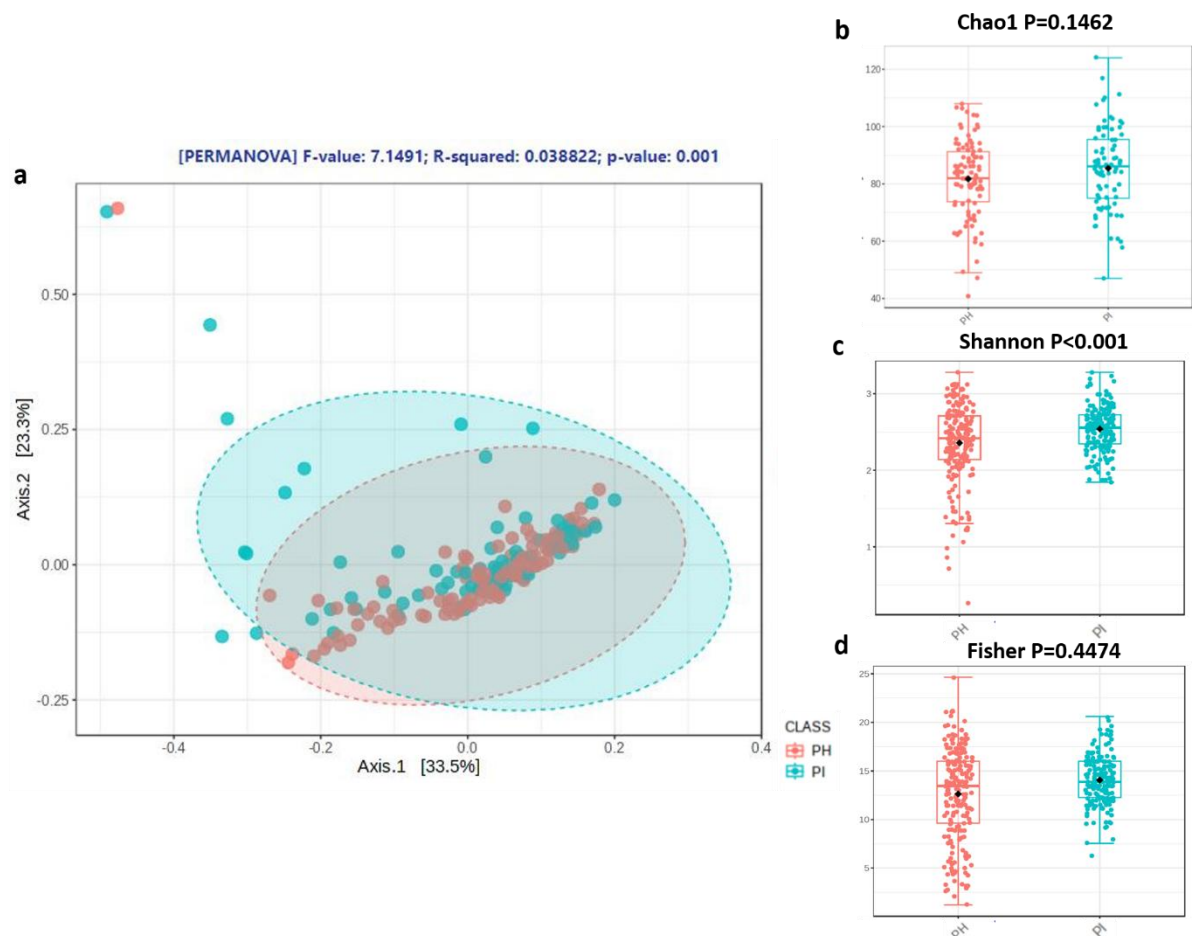


Figure 2-2 Beta and alpha-diversity analysis of gut microbiota taxa of two groups: high-performance (PH) and with production issues (PI)

(a) Beta-diversity: PERMANOVA using Bray Curtis, and alpha-diversity using (b) Chao1 (c), Shannon and (d) Fisher.

2.3.3 Abundance of taxa

In both high and low-production flocks, at the phylum level, *Bacteroidota* and *Firmicutes* (20.4% vs. 20.9%) were dominant phyla (Figure 2-3a). Overall, at the genus level, *Bacteroides* was dominant followed by *Phascolarctobacterium*, *Lactobacillus*, and *Prevotella* (Figure 2-3b).

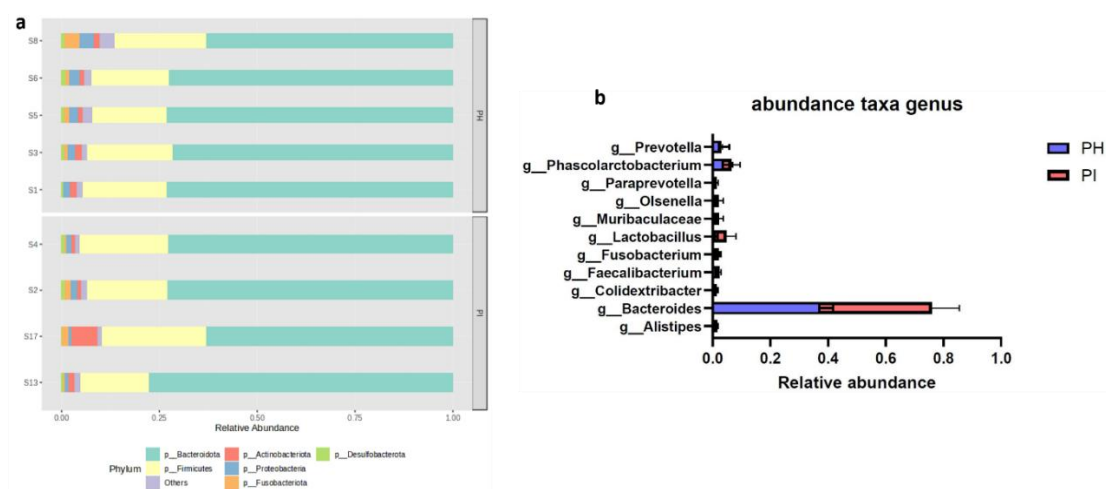


Figure 2-3 Comparison of microbial community composition at the phylum (a) and genus (b) levels for PH and PI

2.3.4 Linear discriminant analysis between PH and PI

The linear discriminant analysis (LDA) effect size (LEfSe) was performed to determine the differential abundance of genera between PH and PI (Figure 2-4). LEfSe determines the operational taxonomic units that are most likely to influence the differences between groups (high and low-production groups in this study) (Figure 2-4). In the group, *Prevotella*, *Phascolarctobacterium*, *Megamonas*, *Megasphaera*, *Faecalibacterium*, *Colidextribacter*, *Fusobacterium*, and *Treponema* (LDA=4.25) were highly abundant genera. On the other hand, in the PI group *Staphylococcus* and *Lactobacillus* were more abundant.

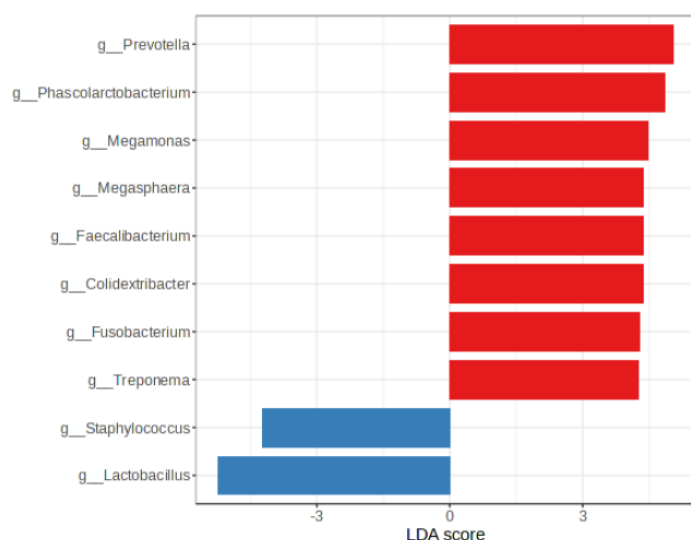


Figure 2-4 Differential abundance genera between PH and PI performed by LEfSe analysis

Genera were selected from the top 10 based on relative LDA scores (LDA=3.0).

Red: PH; Blue: PI.

2.4 Discussion

The gut microbiota plays an important role in the overall health and performance of poultry flocks. Recent studies have suggested that variations in gut microbiota composition could be linked with differences in egg production. In this study, the gut microbiota composition was compared between flocks with a history of high and low egg production.

The diversity of gut microbiota in poultry is vital for optimal gut health. A diverse microbiota enhances the breakdown and absorption of nutrients like proteins, vitamins and minerals from the diet (reviewed by Alagawany et al. 2021). Also, a wide range of enzymes is secreted by a diverse gut microbiota, which helps in the digestion of complex carbohydrates and fibres (Hou et al. 2020) for efficient feed utilisation. Additionally, a diverse microbiota can help competitively exclude harmful microorganisms, to maintain a healthy gut environment for layer hens (Ricke 2003). In this study, the diversity of gut microbiota exhibited significant differences between PH and PI flocks although the richness and evenness of gut microbiota was not significantly different. The results indicated that the number and the distribution of bacterial communities were not influenced by the high or low production of flocks. To test this further, we compared the flocks with a history of high and low production reared by the same company. However, the results were similar. Not surprisingly, geographical location was also found to contribute to diversity of the gut microbiota. To further evaluate the differences between flocks with low and high-production, more flocks from each geographical location would be needed.

The composition of gut microbiota is dynamic and influenced by flock age (Joat et al. 2021a). Our findings contrast with the previous findings by Wang et al. (2020) who found significant differences in the alpha and beta-diversity of high and low-production flocks. It could be deduced that although there was a global difference in the microbial diversity of high and low-production flocks, this difference could have been driven by individual flock management. We further investigated the abundance of taxa to understand if any dominant genus led to the differences in microbial diversity. Our findings on the dominance of *Bacteroides* in both PH and PI flocks are in agreement with Wang et al. (2020).

There were two dominant genera observed in PI flocks from company A, *Lactobacillus* and *Staphylococcus*. The abundance of *Lactobacillus* in PI groups was significantly higher compared with in PH in our study, which did not match the results in previous studies (Xu et al. 2022; Qiao et al. 2019). Although genus *Lactobacillus* has many species that have established probiotic properties, not all strains and or species are protective. The association between the dominance of *Lactobacillus* and poor egg production needs further investigation. *Staphylococcus* is an opportunistic pathogen and can cause staphylococcal infections in poultry. *Staphylococcus* is not always associated with clinical disease.

The observation of gut microbiota in PH and PI flocks gave the background information on factors that may or may not affect the gut microbiota composition.

3 *Lactobacillus* administration influences early microbiota development in chicks

3.1 Background

At hatch, the microbiota of chicks has low diversity. For the first few days after hatch, the microbiota is dominated primarily with the phylum *Firmicutes* (Joat et al. 2021a). Diversity increases over time and the relative abundance of different taxa changes. In a nest environment, the microbiota primarily comes from the hen, with faecal and skin bacteria from the nest contributing to the development of the gut microbiota (Chen et al. 2020). In commercial poultry systems, where nest rearing is not feasible, chicks are colonised by bacteria present in their feed and the environment (Stanley et al. 2013).

The development of the early gut microbiota plays a crucial role in shaping the profile later in life. Studies have shown that the bacterial species that colonise chicks can have both positive and negative effects. Positive outcomes include improved gut health, enhanced nutrient absorption, and overall better production performance. Negative outcomes, however, may include susceptibility to pathogen colonisation and compromised immune function.

In humans, the mother to child transfer (or 'seeding') of the microbiota is receiving considerable interest, and has been shown to have links to infant health and development (Stinson et al. 2017). Infants born through vaginal delivery exhibit a higher relative abundance of *Lactobacillus* and *Bacteroides* compared with infants delivered by caesarean section (Liu et al. 2023). The primary aim of this study was to determine the colonising capacity of five *Lactobacillus* species previously identified for their strong probiotic characteristics. *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, and *Lactobacillus saerimneri* were previously isolated from the caeca of healthy poultry and represent potential seeding strains for the chick gut microbiota. The aim was to identify the most effective species capable of colonising and shaping the gut microbiota while also preventing the colonisation of foodborne pathogens, such as *Salmonella*. By understanding the colonisation capacity of specific *Lactobacillus* species and pathogen prevention, this research contributes to the enhancement of the health and well-being of chicks in commercial poultry systems. An additional outcome is the establishment of a single shot 'seeding' method as a potential mechanism that can be used to shape the developing gut microbiota.

3.2 Methods

3.2.1 Bacteria

Isolates of *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, and *Lactobacillus saerimneri* were obtained from Rob Moore's research group (RMIT, Victoria). These isolates had previously been characterised and identified as potential probiotic candidates. All five isolates were naturally resistant to rifampicin.

Prior to administration to chicks, bacteria were cultured overnight in 25 mL MRS broth (DeMan, Rogosa, and Sharpe; Thermo Fisher, Australia). Overnight cultures were centrifuged at 5,000 x g (Eppendorf Model 5424) for 10 minutes and resuspended in an equal volume of sterile 0.9% saline. Bacteria were administered to chicks within 30 minutes of suspension in saline.

3.2.2 Animals and treatment

Fertile, Hy-Line Brown eggs (n = 120) were obtained from a commercial hatchery. Eggs were incubated at 37.5°C with 55% humidity until Day 18. Humidity was increased to 65% until hatching. One-hundred-eight chicks hatched. Once dry, chicks were transported to brooder cages within the layer hen shed at the Roseworthy Poultry Unit. Chicks were separated into six treatment groups summarised in Table 3-1. Before having access to food or water, a single dose of *Lactobacillus* suspension (0.3 mL) was orally administered to the chicks. Chicks in the control treatment group received an equal volume of saline.

Table 3-1 Probiotic treatment groups

Treatment	No. of chicks
Control	16
<i>Lactobacillus reuteri</i>	18
<i>Lactobacillus salivarius</i>	18
<i>Lactobacillus crispatus</i>	18
<i>Lactobacillus johnsonii</i>	19
<i>Lactobacillus saerimneri</i>	19

One week post-probiotic administration, cloacal swabs were collected and tested for the presence of each *Lactobacillus* strain. Two and four weeks post-probiotic treatment, 8 birds were humanely killed. Sections of the small intestine (duodenum, jejunum, and ileum), the caeca as well as caecal contents were collected for bacterial culture. A separate sample of caecal contents was collected for 16S rRNA sequencing.

For bacterial detection and quantification, tissue segments were placed into a 1.5 mL Safe Lock (Eppendorf, Australia) microcentrifuge tube containing sterile stainless-steel beads ranging in size from 0.2–2.0 mm (Next Advance, USA) and 0.5 mL sterile 0.9% saline. Tissues were homogenised using a BulletBlender (Next Advance, USA) at full speed for 5 minutes. Serial 10-fold dilutions of tissue homogenate were prepared in 0.9% saline and 100 µL of each dilution was spread plated onto MRS agar plates containing 10 µg/mL rifampicin (Sigma, Australia) and 0.002% bromophenol blue (Sigma, Australia). Plates were incubated under anaerobic conditions in a Coy chamber for 24 hours. Characteristic colonies of each *Lactobacillus* strain were enumerated. Bacterial species were confirmed by MALDI-TOF in the Roseworthy Animal Hospital Veterinary Pathology Laboratory, the University of Adelaide.

Chicks were fed *ad libitum* Lauke Mills Red Hen chick starter. All housing, animal husbandry, and experiments were performed according to animal ethics protocol approved by the University of Adelaide Animal Ethics Committee, S2023-030.

3.2.3 Everted sleeve *Salmonella* invasion assay

To investigate the capacity of *Salmonella* to invade the caecal epithelia following probiotic administration, caeca were collected from each chick at both two and four weeks. Caeca were carefully everted over the sterile handle of a swab. During the eversion, the caecal contents were collected and diluted 1:5 in Dulbecco's Modified Eagle Media (DMEM). Caecal contents and DMEM were vortexed to mix. Prior to experiments, a late log phase growth of *Salmonella* Typhimurium phage type 9 was prepared.

Briefly, *Salmonella* Typhimurium was resuscitated two days before the experiment from frozen stock on nutrient agar and incubated overnight at 37°C. A single colony was then added to 10 mL of Luria Bertani (LB) broth and cultured for 6–8 hours. 10 µL of this culture was then added to 25 mL of LB and incubated with shaking 150 rpm overnight. The optical density (OD) 600 nm was measured adjusted to and OD of 0.2 equivalent to 10⁸ colony forming units (CFU) per mL.

Salmonella Typhimurium was added to the DMEM caecal contents mixture at a concentration of 10⁸ CFU per mL. Everted caeca were added to the solution and incubated at 37°C for one hour. Caeca were washed two times in sterile phosphate buffered solution (PBS) and then incubated in 200 µg/mL gentamicin for 15 minutes to kill any bacteria that had attached but not invaded the caecal epithelia. Caeca were washed an additional two times and then transferred to 1.5 mL Safe Lock microcentrifuge tubes containing 0.2–2.0 mm stainless-steel beads and homogenised in a BulletBlender at full speed for 5 minutes. Serial 10-fold dilutions of the caecal homogenate were prepared in 0.9% saline. 100 µL of each dilution was plated onto xylose lysine deoxycholate (XLD) agar plates (Thermo Fisher, Australia). Plates were incubated at 37°C overnight. Characteristic *Salmonella* colonies (black centre with slightly red translucent zone) were enumerated. Data are presented at log₁₀ CFU *Salmonella* Typhimurium per gram tissue.

3.2.4 DNA purification

DNA was purified from caecal contents using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Australia) according to the manufacturer instructions with minor modifications. Briefly, 180–220 mg caecal material was weighed into a 1.5 mL Safe Lock (Eppendorf, Australia) microcentrifuge tube. 750 µL InhibitEX Buffer and a mixture of acid washed beads with diameters of 106 µm and between 425 and 600 µm (Sigma Aldrich, Australia) was added to each tube. Caecal contents were homogenised with a BulletBlender (Next Advance, USA) at full speed for 5 minutes. Tubes were incubated at 95°C for 7 minutes and then spun at 20,000 x g for 1.5 minutes. 600 µL of the supernatant was added to a 2 mL Safe Lock (Eppendorf, Australia) microcentrifuge tube containing 25 µL and proteinase K. Buffer AL (600 µL) was then added to each tube. Tubes were incubated at 70°C for 10 minutes. Following incubation, 600 µL 100% ethanol (Sigma, Australia) was added to each tube. The lysate was then added to the QIAamp spin column placed in a collection tube. Columns were centrifuged at 20,000 x g for 1.5 minutes. This was repeated until all the lysate had been passed over the column. Columns were washed with buffer AW1 followed by AW2. DNA was eluted from the column using 100 µL buffer ATE. DNA quantity (ng/µL) and quality (260/280 ratio) was assessed using a NanoDrop Spectrophotometer (Thermo Fisher, Australia). Samples were sent to the Royal Melbourne Institute for Technology (RMIT, Victoria) for 16S rRNA sequencing.

3.2.5 Amplicon sequencing and analyses

The 16S rRNA gene (V3–V4 region) was amplified using the Q5 High Fidelity DNA Polymerase (New England Biolabs, USA) with dual indexing, variable spacer primers (338F: 5'-ACTCCTACGGGAGGCAGCAG-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3') (Fadrosh et al. 2014). PCR cycling conditions were 98°C for 1 min followed by 35 cycles of 98°C for 10 s, 49°C for 30 s, and 72°C for 30 seconds, and a final extension at 72°C for 10 min. Amplicon sequencing was performed using an Illumina MiSeq (2 x 300 bp). Illumina software was used to demultiplex sequencing data and the analysis was performed in Quantitative Insights into Microbial Ecology 2 (QIIME2). Quality filtering, denoising, and chimera removal were performed using Dada 2 as a QIIME2 plugin with all recommended parameters. Sequences were grouped into amplicon sequence variants (ASV). Microbial taxonomy was assigned using the SILVA v138.1 database. The ASV frequency table was uploaded into the online MicrobiomeAnalyst system for data normalisation using the cumulative sum scaling (CSS)

option. Data were rarefied to normalise sequencing depth, and were analysed using alpha-diversity (Chao1, Shannon, and Simpson indices), beta-diversity (PERMANOVA and ANOSIM), linear discriminant analysis effect size (LEfSe), and non-parametric tests in accordance with the software user guidelines (Dhariwal et al. 2017).

3.2.6 Statistical analyses

Data obtained from *Lactobacillus* and *Salmonella* enumeration from tissue homogenates were analysed using GraphPad Prism (version 10.2.2). Data were tested for normalcy using a Shapiro-Wilk test. Normally distributed data were analysed using an analysis of variance (ANOVA). When a significant result was found, a Tukey's multiple comparisons test was used to determine which groups differed significantly from each other. Non-normally distributed data were analysed using a Kruskal-Wallis with a Dunn's comparison test. A p-value of less than 0.05 was considered significant.

3.3 Results

3.3.1 Lactobacillus culture

The colonisation capacity of the five *Lactobacillus* species was evaluated when chicks were two and four weeks of age. Data are presented as log₁₀ colony forming units (CFU) per gram tissue standard error of the mean. At two weeks, *L. reuteri*, *L. salivarius*, *L. crispatus*, *L. johnsonii*, and *L. saerimneri* all colonised segments of the small intestine and the caeca (Figure 3-1). In all segments of the intestine, *L. reuteri*, *L. crispatus*, *L. johnsonii*, and *L. saerimneri* exhibited the highest mean loads and differed significantly from the control treatment (p 0.01). The mean load of *L. salivarius* was, in general, lower than all other *Lactobacillus* treatment groups and only differed significantly from the control treatment in the ileum (p 0.05).

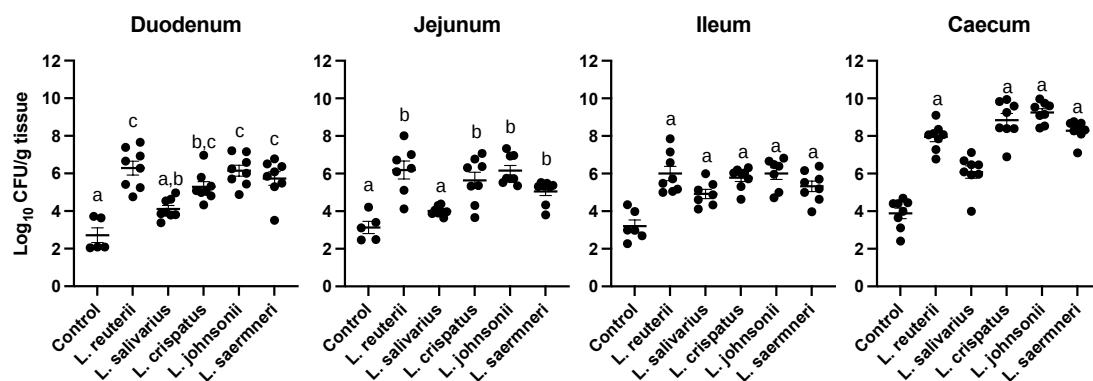


Figure 3-1 *Lactobacillus* colonisation of the two-week-old intestine

L. reuteri, *L. salivarius*, *L. crispatus*, *L. johnsonii*, and *L. saerimneri* were all able to colonise segments of the small intestine and caeca.

Treatments with the same letter do not differ significantly.

At four weeks of age, the mean load of *L. reuteri*, *L. salivarius*, and *L. crispatus* decreased substantially in all segments of the intestine (Figure 3-2). The mean bacterial load for the *L. johnsonii* and *L. saerimneri* treatment groups, however, were not significantly lower than samples collected at two weeks. In the small intestine segments, the mean loads of *L. johnsonii* and *L. saerimneri* were significantly higher than the other *Lactobacillus* treatment groups (p 0.01). In the caeca, *L. crispatus*, *L. johnsonii*, and *L. saerimneri* exhibited the highest mean loads and differed significantly from *L. reuteri*, *L. salivarius*, and control treatment groups (p 0.01).

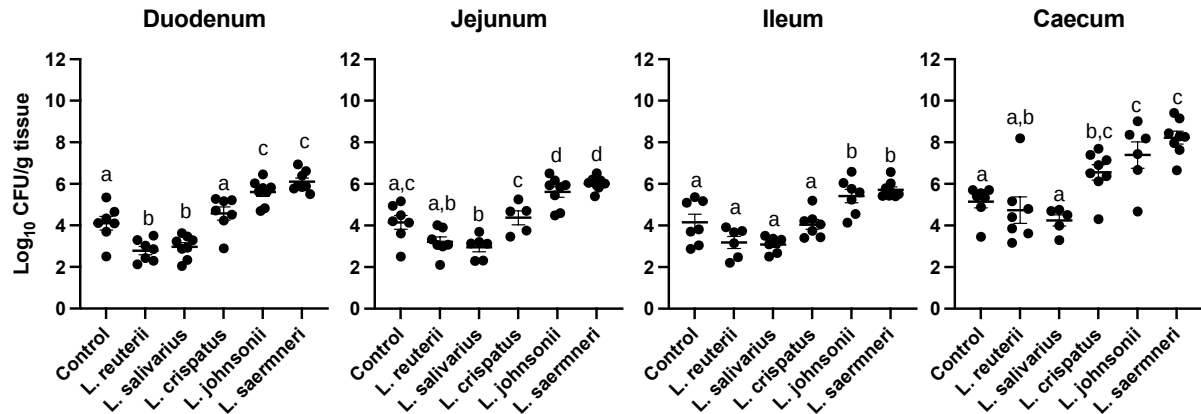


Figure 3-2 *Lactobacillus* colonisation of four-week-old intestine

Colonisation of segments of the small intestine and caecal were reduced for *L. reuteri*, *L. salivarius*, and *L. crispatus* but not *L. johnsonii*, and *L. saerimneri*.

Treatments with the same letter do not differ significantly.

3.3.2 *Salmonella* invasion of caecal epithelia

Control of *Salmonella* Typhimurium is important to the layer hen industry. The ability of *L. reuteri*, *L. salivarius*, *L. crispatus*, *L. johnsonii*, and *L. saerimneri* to prevent invasion of *Salmonella* into the caecal epithelium was evaluated using an everted sleeve model (Figure 3-3). Data are presented as log₁₀ CFU *Salmonella* per gram of tissue. The *Salmonella* Typhimurium load in the control group was significantly higher ($p < 0.01$) than all *Lactobacillus* treatment groups, demonstrating the capacity of these species to inhibit pathogen colonisation.

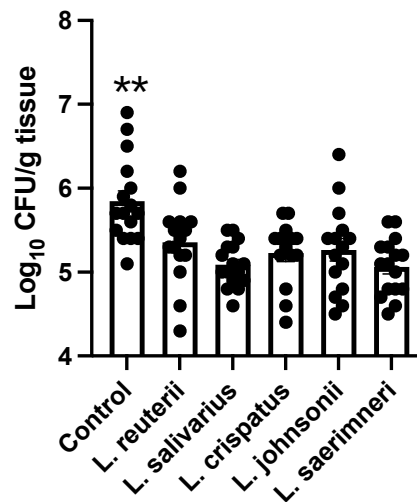


Figure 3-3 *Salmonella* invasion of the caecal epithelia

Everted caecal were co-incubated in a 1:5 mixture of DMEM and caecal contents with 10^8 CFU/mL *Salmonella* Typhimurium.

Data are presented as the log₁₀ CFU/mL *Salmonella* Typhimurium.

** denotes significance ($p < 0.01$).

3.3.3 Bacterial abundance

The caecal microbiota was profiled by sequencing the V3–V4 region of the 16S rRNA gene at Weeks 2 and 4. Eight caecal samples were sequenced from each group. Sequencing produced a total number of 867,132 reads after quality trimming and chimera removal. An average of 10,201 reads were generated for each sample from a total of 85 samples. Overall, *Firmicutes* was the dominant bacterial

phyla observed in all treatment groups with abundance ranging between 92.8 and 97.6% (Figure 3-4). The phylum, *Actinobacteria*, was also present in all treatment groups but the abundance was low ranging between 2.4 and 5.94%. *Proteobacteria* was found only in the *L. crispatus* (1.26%) and *L. reuteri* (0.06%) treatment groups.

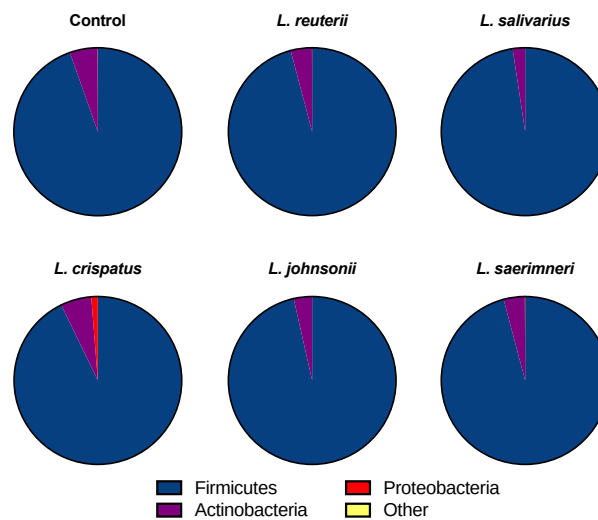


Figure 3-4 Per cent abundance of bacterial taxa

The microbiota of 2 and 4-week-old birds (combined) are dominated by *Firmicutes*.

3.3.4 Alpha and beta-diversity are influenced by age

Due to the low diversity of bacterial phyla present in the microbiota, all remaining analyses are performed using Genus as the taxonomy level. Irrespective of treatment, species richness (Chao1) of the caecal microbiota was significantly greater in 4-week-old chicks ($p < 0.001$) (Figure 3-5). The Shannon (species richness and evenness) and Simpson indices (dominance and evenness) did not differ between the two age groups.

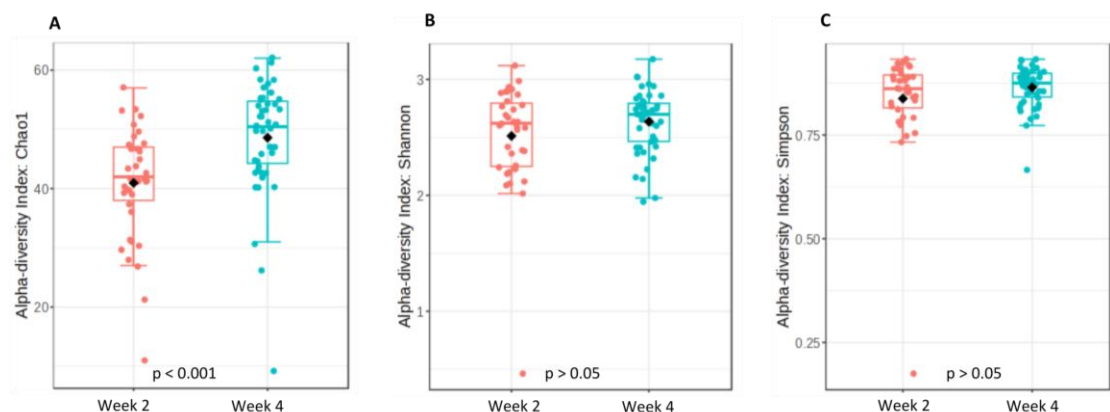


Figure 3-5 Alpha-diversity is influenced by age

Alpha-diversity was evaluated using (A) Chao1, (B) Shannon, and (C) Simpson indices. A significant difference was observed for species richness (Chao1, A).

PERMANOVA showed that bacterial species composition also significantly (F-value: 9.67; R-squared: 0.10435; p-value: 0.001) differed between two- and four-week-old chicks (Figure 3-6).

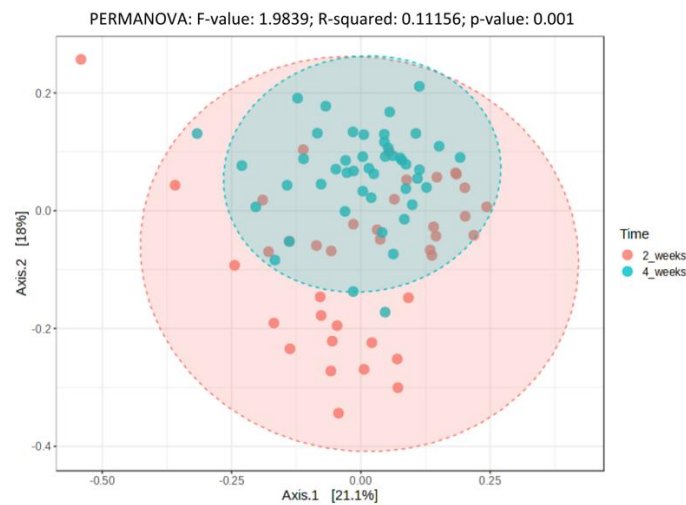


Figure 3-6 Age affects beta-diversity

3.3.5 Effect of *Lactobacillus* treatment on alpha and beta-diversity

The effect of *Lactobacillus* treatment on the chick caecal microbiota was evaluated at Weeks 2 and 4. To evaluate the species richness, diversity, and evenness Chao1, Shannon, and Simpson Index analyses were conducted. At Week 2, alpha-diversity index values were higher in *Lactobacillus* treatment groups but the difference was not significant (Figure 3-7). No significant difference was detected between treatment groups at Week 4 (Figure 3-7).

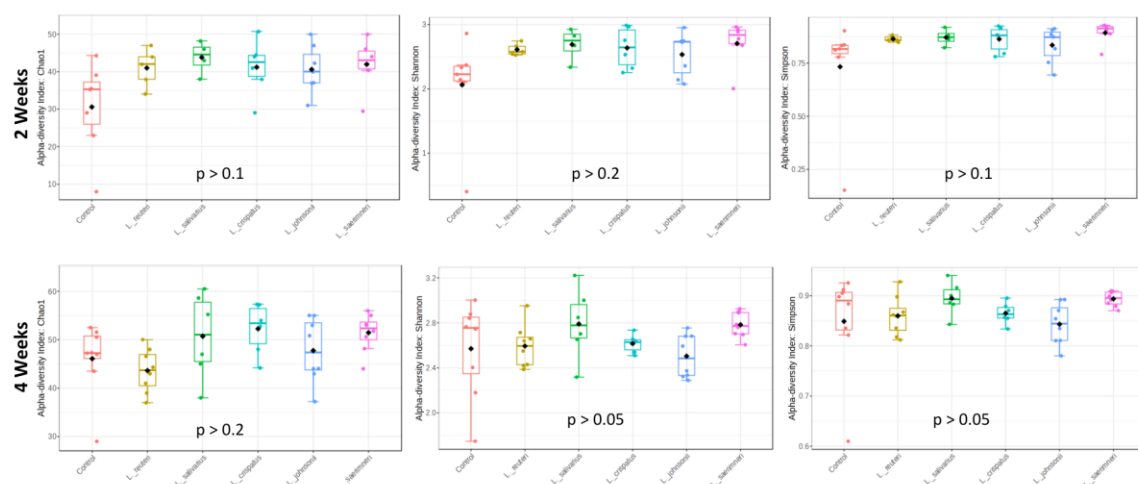


Figure 3-7 Alpha-diversity at 2 and 4 weeks of age

Beta-diversity differed significantly between Weeks 2 and 4 ($p < 0.01$, data shown). At Week 2, there was a significant effect of treatment on beta-diversity ($p = 0.05$). Control sample beta-diversity was significantly different from *Lactobacillus reuteri* and *Lactobacillus salivarius* treatment groups ($p < 0.05$). Beta-diversity of the *Lactobacillus reuteri* treatment differed from *Lactobacillus salivarius*, *Lactobacillus crispatus*, and *Lactobacillus saerimneri* groups ($p < 0.05$, FDR < 0.05). Similarly, beta-diversity for the *Lactobacillus salivarius* treatment differed significantly from *Lactobacillus crispatus*,

Lactobacillus johnsonii, and *Lactobacillus saerimneri* groups ($p < 0.05$, FDR < 0.05).

At Week 4, a significant effect of treatment was also observed ($p = 0.001$). Community structure in the microbiota for control birds differed significantly from the *Lactobacillus salivarius* and *Lactobacillus reuteri* treatment groups (Figure 3-8). Both the *Lactobacillus reuteri* and *Lactobacillus salivarius* treatment groups differed significantly from all other treatments ($p < 0.05$, FDR < 0.05).

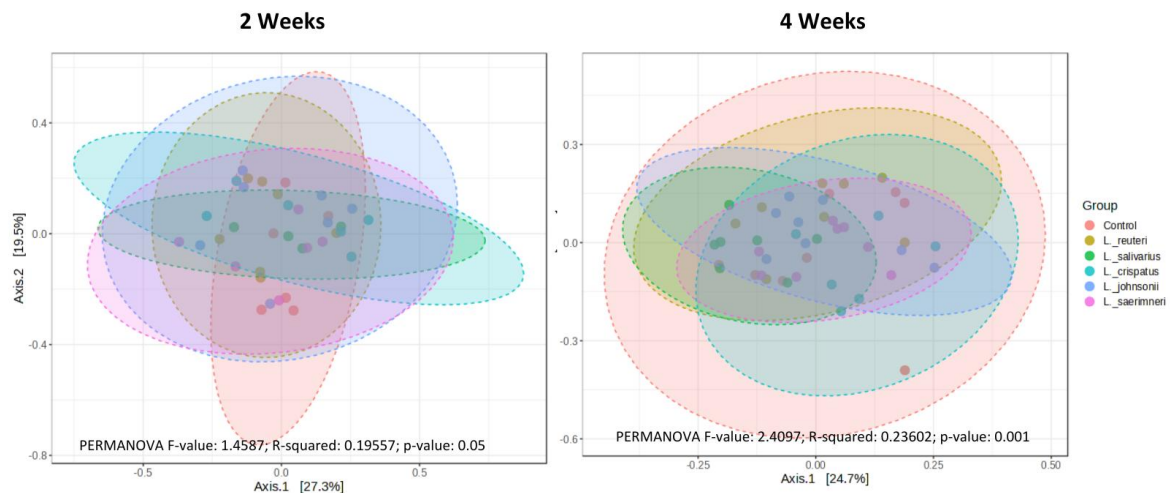


Figure 3-8 Beta-diversity at 2 and 4 weeks of age

Beta-diversity was determined by the distance method Bray Curtis index with pairwise PERMANOVA. Time and treatment resulted in significant variance in beta-diversity.

3.3.6 Effects of treatment on caecal microbiota

A linear discriminant analysis effect size (LEfSe) analysis was performed on the caecal microbiota. LEfSe identifies the driving force behind differences between treatments. Here, a LEfSe was performed to evaluate the differences in the relative abundance of different bacterial genera represented in each treatment group. At Week 2, significant differences (FDA < 0.05) in the relative abundance of three bacterial genera were observed including *Sellimonas*, CAG 56 (*Firmicutes* bacterium), and *Clostridia* UGC014 (Figure 3-9).

At Week 4, 11 bacterial genera differed significantly (FDA < 0.05) across the six different treatment groups, including *Lactobacillus*, *Subdoligranulum*, *Paludicola*, *Oscillibacter*, *Colidextribacter*, CAG 56, *Blautia*, *Streptococcus*, the *Eubacteriumcopro-stanoligenes* group, *Enterococcus*, and an uncultured genus (Figure 3-9).

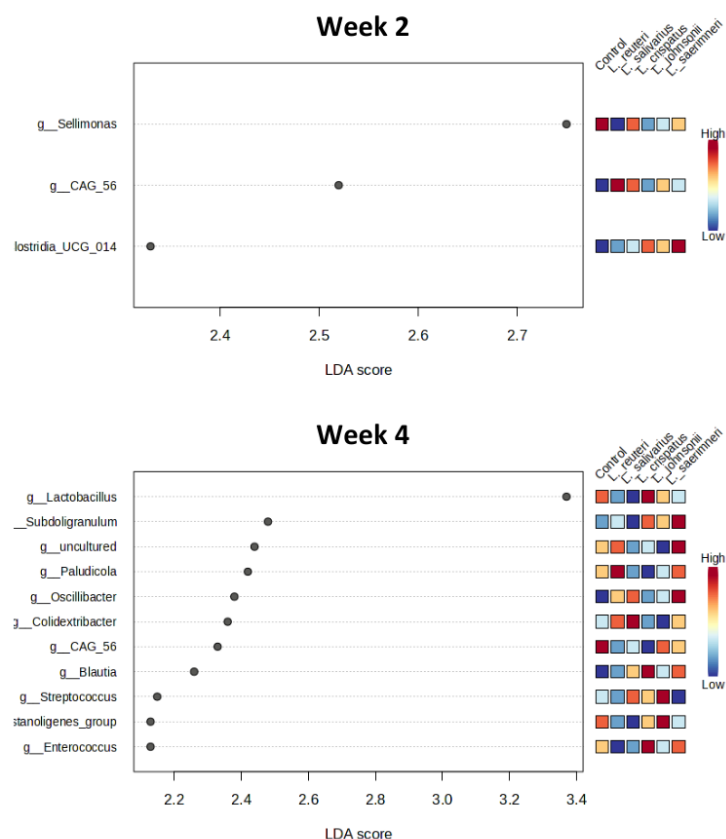


Figure 3-9 Effects of *Lactobacillus* treatment on caecal microbiota

Differences in the relative abundance of bacterial genera were observed at both Weeks 2 and 4.

3.4 Discussion

The maternal-to-offspring transfer of microbes serves as an important source of colonisation for the development of a normal healthy gut. The ultra-cleanliness of commercial hatcheries means that chicks hatch into an environment of low microbial load. The absence of hens in the hatchery environment means that there is little to no maternal microbiota contributed to the early formation of the chick gut microbiota. Thus, chicks predominantly acquire their gut microbiota from the feed and environment (Broom & Kogu 2018; Stanley et al. 2014), which has the potential to affect the long-term gut microbiota profile. The use of probiotics can circumvent this. The practice of ‘seeding’ the gut microbiota with live bacterial strains is becoming of interest to agriculture. Administration of probiotics early in the development of the gut microbiota has the potential to have long-lasting impacts. In this study, five potential ‘seeding’ *Lactobacillus* strains were tested for their ability to colonise and persist in the gut of chicks and how they affected the composition of the developing gut microbiota.

Lactobacillus reuteri, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, and *Lactobacillus saerimneri* all exhibited the capacity to colonise the gastrointestinal system of chicks. Bacterial loads of *Lactobacillus reuteri*, *Lactobacillus salivarius*, and *Lactobacillus crispatus* decreased at Week 4 but *Lactobacillus johnsonii*, and *Lactobacillus saerimneri* loads remained constant. Limited research has been conducted on the colonisation of *Lactobacillus* strains in chicks.

In the present study, differences were observed in the combined overall data between two and four weeks of age. The community structure and richness of bacterial species were significantly different between the two age groups. Other studies have demonstrated that at hatch, there is low diversity

and there is considerable change in the gut microbiota over the first few weeks of life. The gut microbiota of chicks is dominated by *Firmicutes* in the first month of life. The results obtained in the present study are consistent with this.

Significant differences were observed in the bacterial communities between control and *Lactobacillus* treated birds. While not significant, Chao1 Index, Shannon Index, and Simpson Index all had higher mean values for *Lactobacillus* administered birds at two weeks compared with control birds. This suggested that treatment may have impacted bacterial species and richness. Supplementation with a *Bacillus subtilis* probiotic also impacted the species diversity of the gut microbiota (Khan & Chousalkar 2021).

An important attribute of a probiotic bacterial species is the ability to protect the host from invasion and colonisation of pathogenic species (Nair et al. 2017). In the present study, we evaluated the capacity of *Lactobacillus* administration to prevent *Salmonella* invasion of the caecal epithelia. In these experiments, a key feature is that we combined caecal material collected from the chicks with the media. This meant that components of the caecal material, such as lactic acids from the *Lactobacillus* strains and other bactericidal components would be present. All *Lactobacillus* strains inhibited the invasion of *Salmonella* into the caecal epithelia indicating their presence in the caeca is beneficial for controlling this important zoonotic bacterial pathogen. Similar *Salmonella* inhibitory effects have been obtained from other *in vitro* (Salehizadeh et al. 2020; Hai et al. 2021) and *in vivo* (Muyyarikkandy & Amalaradjou 2017) studies with other strains of *Lactobacillus*.

Considering all aspects of colonisation, inhibition of pathogen invasion, and effects on the developing gut microbiota for all remaining experiments, *Lactobacillus saerimneri* was used as the single shot seeding probiotic.

4 Effect of ‘seeding’ of a live *Lactobacillus* strain on the development of the gut microbiota and its persistence (0 to 45 weeks)

4.1 Background

Data collected in Chapter 3 demonstrated that a single dose of a live *Lactobacillus* strain could colonise the gastrointestinal system of chicks and persist in the gut for 4 weeks. Co-incubation of *Salmonella* Typhimurium with the caecal contents of birds administered each of the five *Lactobacillus* species also significantly inhibited invasion of *Salmonella* into the epithelium of the caeca. While small, a shift in the microbial communities in the caeca was also observed between control and *Lactobacillus* treated birds. Differences in bacterial richness, diversity and evenness, while not significant, were all increased in birds administered the *Lactobacillus*.

Of the five *Lactobacillus* strains, titres of both *Lactobacillus saerimneri* and *Lactobacillus johnsonii* remained high. *Lactobacillus saerimneri* also exhibited the best inhibition of *Salmonella*. To date, *Lactobacillus saerimneri* has not previously been evaluated as a probiotic *in vivo*. *In vitro*, however, this species has shown strong probiotic characteristics and inhibitory properties against other avian pathogens (Ma et al. 2023). The next step in evaluating the administration of a single dose of live *Lactobacillus* was to evaluate its persistence over an extended period and characterise the impacts on the gut microbiota.

In humans and other birds, ‘seeding’ of the gut microbiota at an early age influences how the mature gut microbiota develops (Chen et al. 2020; Liu et al. 2023; Stinson et al. 2017). In this experiment, a single dose of *Lactobacillus saerimneri* was administered to chicks at hatch. The persistence of *Lactobacillus saerimneri* was monitored using standard bacteriological culture methods. The composition of the gut microbiota changes significantly with bird age. Thus, the effect of *Lactobacillus* ‘seeding’ on the gut microbiota was also evaluated at four different ages: chicks (4 weeks), pullet (Weeks 10 and 16), early lay (Week 22) and mid lay (Week 45).

4.2 Methods

4.2.1 Bacteria

Based on outcomes from Chapter 3, the *Lactobacillus saerimneri* isolate was selected to evaluate the effects of ‘seeding’ the gut microbiota over a 45-week period. As in Chapter 3, *Lactobacillus saerimneri* were cultured overnight in 25 mL MRS broth (DeMan, Rogosa, and Sharpe; Thermo Fisher, Australia). Overnight cultures were centrifuged at 5,000 x g (Eppendorf Model 5424) for 10 minutes and resuspended in an equal volume of sterile 0.9% saline.

4.2.2 Seeding experiment

Fertile, Hy-Line Brown eggs (n = 100) were obtained from a commercial company. Eggs were incubated at 37.5°C with 55% humidity until Day 18. Humidity was increased to 65% until hatching. Ninety chicks hatched. Once dry, chicks were transported to brooder cages (15 birds per cage within the layer hen shed at the Roseworthy Poultry Unit). Chicks were separated into two treatment groups, control and *Lactobacillus saerimneri*. Before having access to food or water, a single dose of

Lactobacillus suspension (0.3 mL) was orally administered to the chicks. Birds were transitioned from brooder cages to layer hen cages at 15 weeks of age. The Roseworthy layer hen shed is comprised of two rows of cages each with two runs of 45 cages (total 90 cages per row). Each row of cages has its own manure belt. The rows are separated by a walkway that is approximately 2 metres wide. The shed has cross ventilation. Control birds were housed closest to the fans and *Lactobacillus* treated birds were closest to the vents. Treatment groups were separated by the walkway and one run of cages. Chicks were fed Lauke Mills Red Hen chick starter. Pullets received Barastoc Pullet Grower and were transitioned onto Barastoc Champion Layer Hen feed at 16 weeks of age.

When birds were 4, 10, 16, 22, and 45 weeks of age, 7 birds in each treatment group were humanely killed. Caecal contents were collected from each bird to evaluate the effect of gut microbiota seeding over time using 16S rRNA sequencing.

The presence of culturable *Lactobacillus saerimneri* was evaluated in freshly collected caecal material fortnightly over the course of the entire 45-week experiment. Prior to caecal material collection, each manure belt was run. Freezer paper was placed below each bird. Caecal contents were collected from the freezer paper using a sterile swab. The *Lactobacillus saerimneri* isolate used in these experiments was naturally resistant to rifampicin facilitating culture isolation of the seeding strain from caecal samples. Swabs were used to prepare the primary inoculate on MRS agar plates containing 10 µg/mL rifampicin (Sigma, Australia) and 0.002% bromophenol blue (Sigma, Australia). Secondary streaking was performed using a disposable 10 µL microbiological loop. Plates were incubated under anaerobic conditions in a Coy chamber for 24 hours. Samples were scored as either positive or negative for characteristic colonies of the seeding *Lactobacillus saerimneri* isolate.

4.2.3 Egg quality assessment

Egg quality parameters were also assessed to evaluate the potential effects of ‘seeding’ the gut microbiota. Egg weight, width, and length measurements were collected. Albumen and yolk height, yolk diameter as well as albumen pH, yolk colour, and shell thickness were also measured. Albumen height and egg mass were used to calculate Haugh units for each egg.

4.2.4 DNA purification, amplicon sequencing and statistical analyses

DNA purification, amplicon sequencing, and statistical analyses were performed as described in Chapter 2.

4.3 Results

4.3.1 Culture isolation of *Lactobacillus saerimneri* from caecal samples

Between 2 and 20 weeks of age, *Lactobacillus saerimneri* was routinely cultured from freshly voided caecal samples collected from birds (Figure 4-1). The proportion of *Lactobacillus saerimneri* positive birds was highest at Week 10 but steadily declined. Between Weeks 22 and 26, *Lactobacillus saerimneri* was not isolated. At the end of Week 26, there was a stress event (influx of birds and increased numbers of staff). Following this, a re-emergence of *Lactobacillus saerimneri* was observed from Weeks 27 through 36. Birds were culture negative for *Lactobacillus saerimneri* from Weeks 38 to 44. It should be noted that *Lactobacillus saerimneri* was isolated from a small proportion (5-10%) of control birds over the course of the experiment.

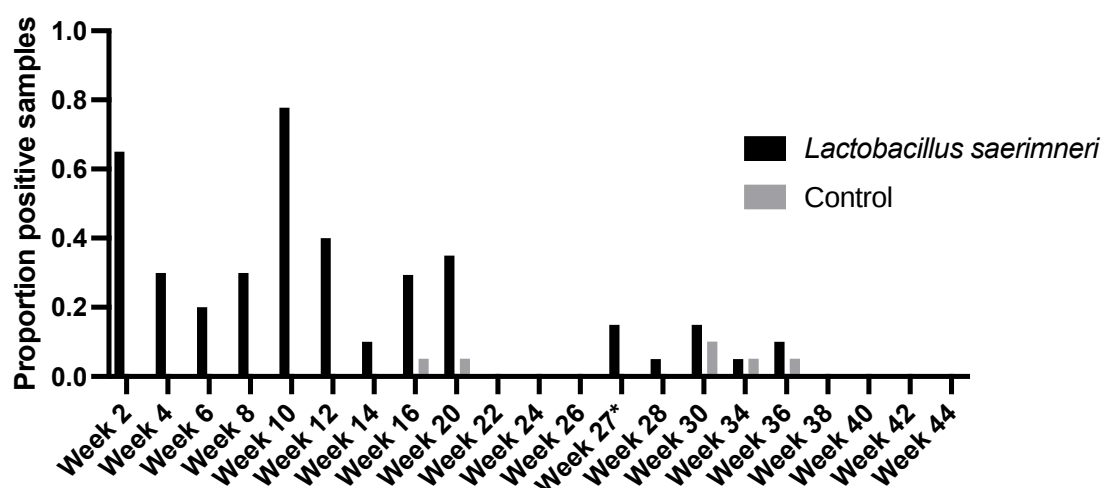


Figure 4-1 Culture isolation of *Lactobacillus saerimneri*

Freshly voided caecal samples were collected and tested for the presence of *Lactobacillus saerimneri* using culture methods.

Samples were scored as either positive or negative.

Data are expressed as the proportion of positive samples.

* Designates a stress event in the layer hen shed.

4.3.2 Effects of microbiota seeding on egg quality

No significant difference was observed in the onset of lay between the control and *Lactobacillus saerimneri* treatment groups. Egg quality was also measured and no difference between treatment groups was observed (data not shown).

4.3.3 *Lactobacillus saerimneri* seeding affects the bacterial phyla in the gut microbiota

Twenty caecal samples were collected from the control and *Lactobacillus saerimneri* treatment groups at weeks 4, 10, 16, 22 and 45 of age. The caecal microbiota was profiled by sequencing the V3–V4 region of the 16S rRNA gene. Sequencing produced a total number of 1,463,062 reads after quality trimming and chimera removal. An average of 16,135 reads were generated for each sample from a total of 316 samples. MicrobiomeAnalyst was used to analyse the sequencing data. A low variance filter was applied removing 10% of reads (546 features) based on standard deviation. Cumulative sum scaling was performed to correct for differences in sampling depth. Over the 10-week experiment, *Bacteroidetes* and *Firmicutes* were the dominant phyla in both control and *L. saerimneri* pullets (Figure 4-2). The relative abundance of phyla varied as a consequence of both age and treatment.

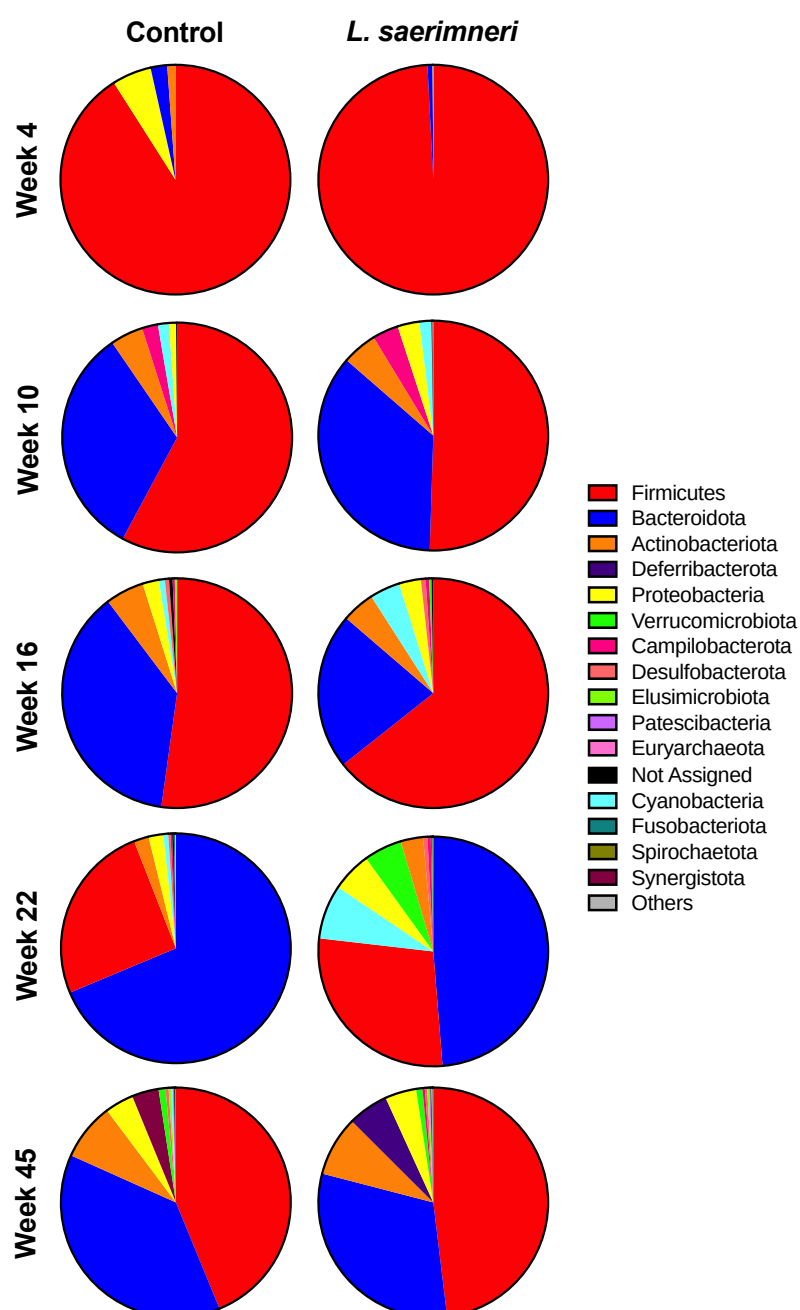


Figure 4-2 Abundance of bacterial phyla present in the gut microbiota at different ages

The relative abundance of bacteria phyla varies between birds in the control and *Lactobacillus saerimneri* treatment groups.

4.3.4 Composition of the gut microbiota is changes with bird age

Patterns of bacterial community composition were evaluated using beta-diversity analyses with age as the factor. Both PERMANOVA (Figure 4-3) and ANOISM (data not shown) revealed that there was a strong effect of age on diversity at both the phylum ($p = 0.001$, R-squared = 0.71669) (Figure 4-3A) and genus ($p = 0.001$, R-squared = 0.47897) taxonomic levels (Figure 4-3B). At the phylum level, a significant difference in community composition was observed between 4-week-old chicks and all other age groups. No significant difference in bacterial phyla was observed between 22- and 45-week-old birds. At the genus level, age has a more profound effect on the caecal microbiota community. A significant shift occurs between Weeks 4 and 10 ($p = 0.001$). Another shift was observed between the pullet stage (Weeks 10 and 16) and early lay (Week 22) and again at Week 44.

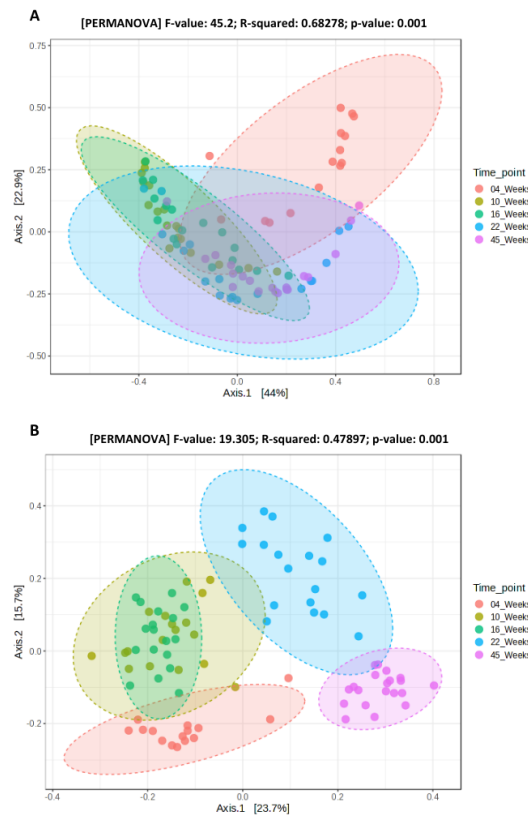


Figure 4-3 Community structure of gut microbiota is affected by age

Bacteria phyla (A) and genus (B) are influenced by bird age.

Gut microbiota richness (Chao1 Index) increased over the course of the experiment at both the bacterial phylum (Figure 4-4A) and genus (Figure 4-4D) taxonomic levels, demonstrating a significant effect of bird age ($p < 0.001$). At the phylum level, the Shannon (Figure 4-4B) and Simpson (Figure 4-4C) indices also increased with age, suggesting both a rise in the number of bacterial phyla present in the gut microbiota and greater evenness in their representation within each sample. Similar results were observed for bacterial genera in the gut microbiota (Figure 4-4E,F).

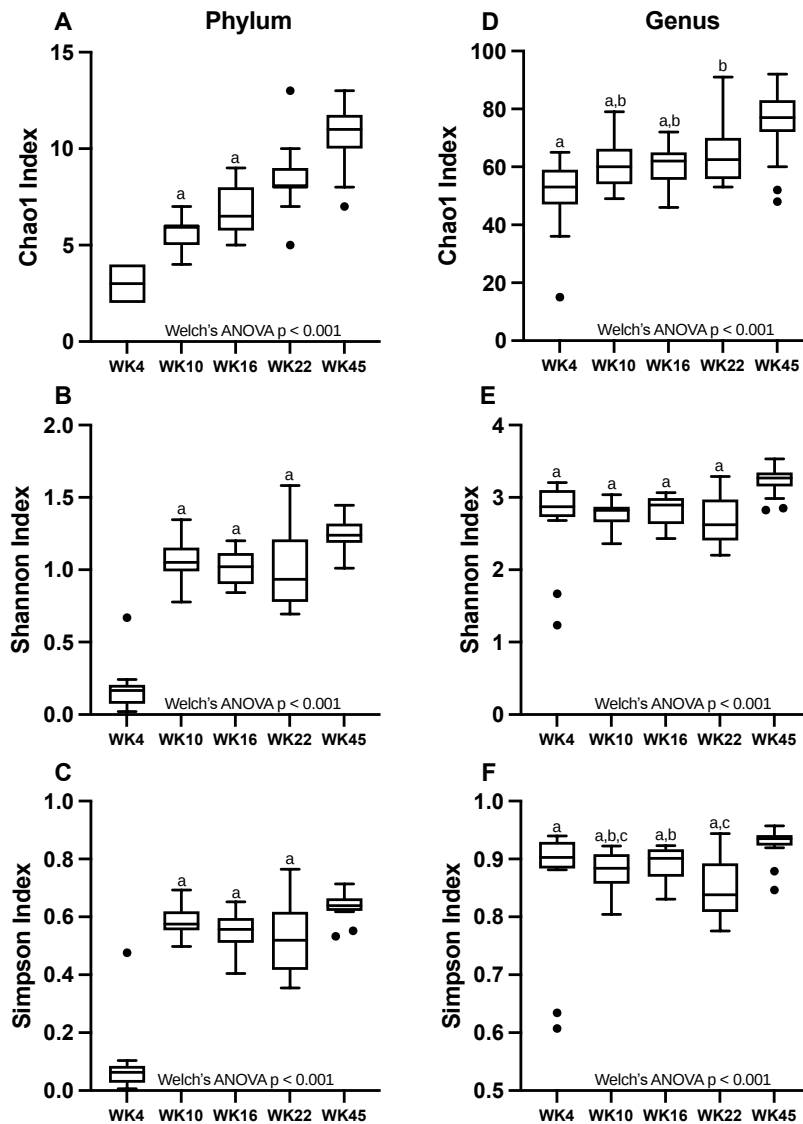


Figure 4-4 Bacterial species richness and evenness are influenced by bird age

Chao1 Index revealed a significant effect of age on species richness at both the (A) phylum and (D) genus taxonomic level. Shannon (B) and Simpson (C) indices evaluating bacterial richness, dominance and evenness show a significant change in richness after 4 weeks of age and again at 45 weeks of age.

At the genus level, Shannon (E) and Simpson (F) exhibit similar patterns across time indicating evenness of species in the gut microbiota at different ages.

Treatments designated with the same letter do not differ significantly.

4.3.5 Composition of the gut microbiota is influenced by *Lactobacillus saerimneri*

Patterns of bacterial community composition were evaluated using beta-diversity analyses with treatment as the factor (control versus *Lactobacillus saerimneri* administration). Both PERMANOVA (Figure 4-5) and ANOISM (data not shown) revealed that there was a strong effect of age on diversity at both the phylum ($p = 0.001$, $R\text{-squared} = 0.7677$) (Figure 4-5A) and genus ($p = 0.001$, $R\text{-squared} = 0.53497$) taxonomic levels (Figure 4-5B). Treatments cluster with age groups. At both the phylum and genus taxonomic levels, significant differences in community composition were observed between control and *Lactobacillus saerimneri* treatment groups at 4 weeks and again at 22 weeks ($p < 0.05$).

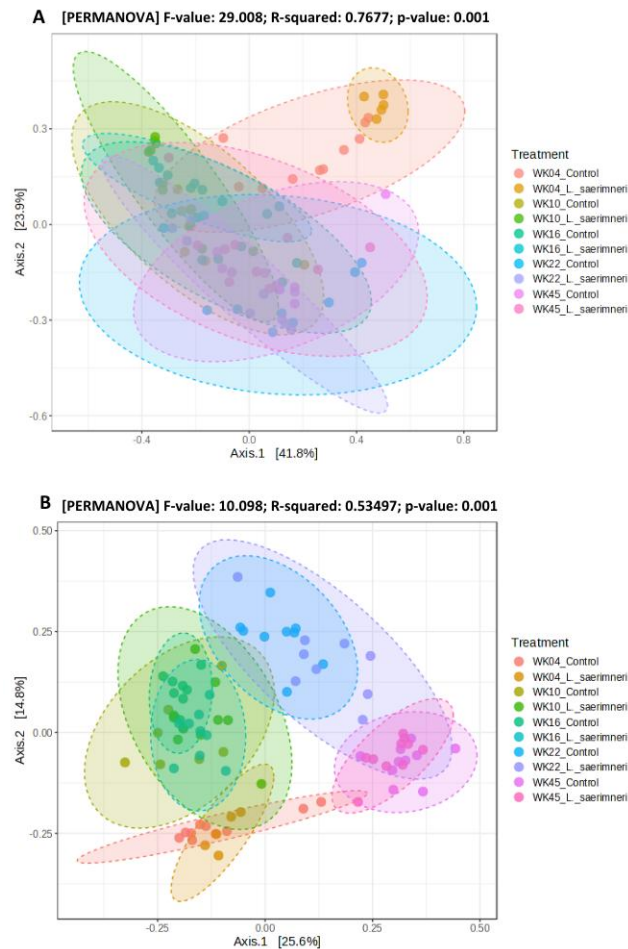


Figure 4-5 Community structure of gut microbiota following *Lactobacillus saerimneri* administration

The composition of bacteria phyla (A) and genus (B) are influenced by bird age; treatments cluster with bird age.

Significant ($p < 0.01$) differences in composition of the gut microbiota were observed between treatment groups at 4 and 22 weeks of age at both taxonomic levels.

4.3.6 Richness and evenness of the gut microbiota is influenced by *Lactobacillus saerimneri*

The effect of *Lactobacillus saerimneri* treatment on the caecal microbiota was evaluated. To evaluate the species richness, diversity, and evenness Chao1, Shannon, and Simpson Index analyses were conducted for separate production phases (chick, pullet, early lay, and mid lay) (Figure 4-6). Richness (relative abundance of bacterial species) as evaluated using a Chao1 index did not differ between treatment groups over the course of the experiments. At 22 weeks, the mean Shannon index value observed in the *Lactobacillus saerimneri* treatment group was significantly higher than control birds ($p = 0.04$). No significant difference was observed for other sampling time points. No significant differences were observed between treatment groups for the Simpson Index analyses. It should be noted, however, that the mean values obtained for the *Lactobacillus saerimneri* treatment group for both the Shannon and Simpson indices were higher than the control group at Weeks 4, 10, 16 and 22. These results suggest that *Lactobacillus saerimneri* administration was having a positive effect on the evenness of the gut microbiota.

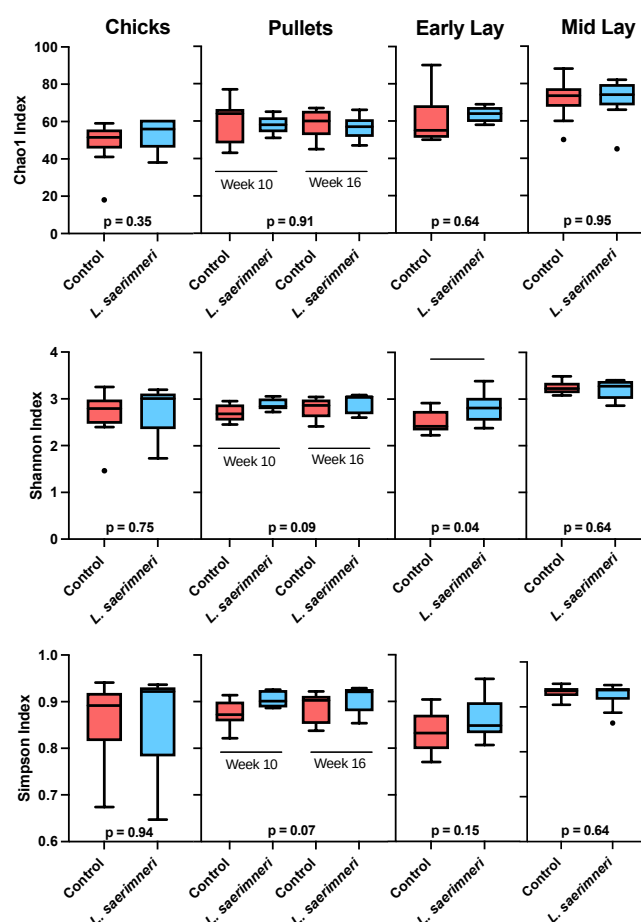


Figure 4-6 Richness and evenness of the gut microbiota following *Lactobacillus saerimneri* administration

No significant effect on gut microbiota richness (Chao1) was observed over the 45-week trial.

Shannon and Simpson indices evaluating bacterial richness, dominance and evenness show a significant change in richness at 22 weeks of age.

4.4 Discussion

The gut microbiota changes substantially as birds age (Joat et al. 2023; Joat et al. 2021a). In Chapter 3, we demonstrated that a single dose administration of *Lactobacillus* resulted in the colonisation of the gastrointestinal system. Persistence of bacteria was observed for four weeks and had an impact on the community structure and evenness of organisms present in the gut. Here we demonstrated that a single dose of *Lactobacillus saerimneri* remained detectable by bacteriological culture methods over a 20-week period. Bacteria were not culturable for several weeks but then reemerged between 27 and 36 weeks of age following a stress event. Changes in stress hormones can induce replication of some bacterial species resident in the gastrointestinal tract (Verbrugghe et al. 2011) but it is not known whether this also occurs for *Lactobacillus* species.

Egg quality parameters were measured over the 45 weeks trial. No effect on quality was observed between control and *Lactobacillus saerimneri* treated birds. The gut microbiota can influence egg quality, in terms of calcium and vitamin D absorption. Throughout the study, birds were fed a high-quality diet, which was supplemented with shell grit. This likely contributed to the maintenance of egg quality and had more of a dominant effect than probiotic administration.

Over the 45-week trial, the bacterial community composition of the gut microbiota was most influenced by age. These results are consistent with previous studies in caged and free range layer hens (Joat et al. 2023; Joat et al. 2021a). No significant difference in bacterial species richness was observed. The mean Shannon and Simpson values, while not significant, were higher in *Lactobacillus saerimneri* treated birds between 10 and 22 weeks of age. These results suggest that *Lactobacillus* administration impacted the evenness of species in the gut microbiota. The relative abundance of bacterial phyla was impacted by probiotic treatment, suggesting that *Lactobacillus saerimneri* has the potential to influence the gut microbiota over extended periods of time. This is one of the only studies to have evaluated the persistence of *Lactobacillus* probiotic strain following a single dose over such a long period and evaluate the ongoing effects on the gut microbiota.

5 Effect of transport on the gut microbiota of point-of-lay pullets

5.1 Background

Transport from rearing to egg production farms is a stressful process for point-of-lay pullets. During transportation, birds may have limited access to feed and/or water and can be exposed to suboptimal environmental conditions. Handling before and after transport can also be distressful for birds. Following transport, adjusting to a new environment and feed can also induce stress in some birds. Recent studies have demonstrated that indirect effects of transport stress including inappetence, as well as heat, and handling can impact the gut microbiota of hens (Joat et al. 2021b; U. Bello et al. 2018; Zhu et al. 2019).

Stress can also have direct impacts on bacteria in the gut. In broilers, for example, transport from farm to processing plants has been linked with increases in *Campylobacter* loads in the gastrointestinal contents (Stern et al. 1995; Whyte et al. 2001). To date, however, there have been limited studies investigating the direct effects of transport on the gut microbiota of point-of-lay pullets and how it changes as they establish in a new environment. In this study, caecal samples were collected from point-of-lay pullets prior to transport and again at Days 2, 6, 10 and 14 following transport to characterise changes in the gut microbiota.

5.2 Methods

5.2.1 Sample collection

Twenty freshly voided caecal samples were collected from shed infrastructure 4–5 days prior to transport, and again at Days 2, 6, 10 and 14 after transport. Caecal samples were kept on ice during transport back to the lab and frozen at -80°C until thawed for DNA purification. Samples were collected from four different flocks from two different production companies.

5.2.2 DNA purification, amplicon sequencing, and statistical analyses

DNA purification, amplicon sequencing, and statistical analyses were performed as described in Chapter 3.

5.3 Results

5.3.1 Beta-diversity of the gut microbiota is affected by transport.

The effect of transport on caecal microbiota was profiled by sequencing the V3–V4 region of the 16S rRNA gene. Samples were collected from four flocks. Flock A was transported the greatest distance from the pullet rearing to the production farm. Flocks B, C and D were part of the same commercial egg production company but were housed on different properties. Flocks B and D were reared on the same property but in different sheds. It should be noted that the diet of all flocks was changed at least one week after transport.

Twenty caecal samples were collected at each time point. The samples were collected several days prior to transport (pre-transport) and at Days 2, 6, 10, and 14 post-transport. Sequencing produced a

total number of 5,546,645 reads after quality trimming and chimera removal. An average of 14,149 reads were generated for each sample from a total of 392 samples.

Beta-diversity provides a measure of the degree that samples (or groups) differ from each other, and was analysed using a principal coordinate analysis (PCoA). The farther apart groups of data are placed in a PCoA plot (Figures 5-1 and 5-2), the more dissimilar they are. Data for all farms were combined and analysed to evaluate whether there was an effect of transport on the beta-diversity or composition of gut microbiota. A significant effect of time was observed (PERMANOVA F-value = 22.565, R-squared = 0.18912, $p = 0.001$). The composition of the gut microbiota of the pre-transport samples differed significantly from all other time points ($p = 0.001$, FDR = 0.00167) (Figure 5-1A). Day 2 differed from Day 6 ($p = 0.002$, FDR = 0.0028), as well as Day 10 and 14 ($p = 0.001$, FDR = 0.00167). Day 6 was significantly different from Day 14 ($p = 0.002$, FDR = 0.0028). The gut microbiota composition at Day 10 was also significantly different from Day 14 post-transport samples ($p = 0.014$, FDR = 0.0156).

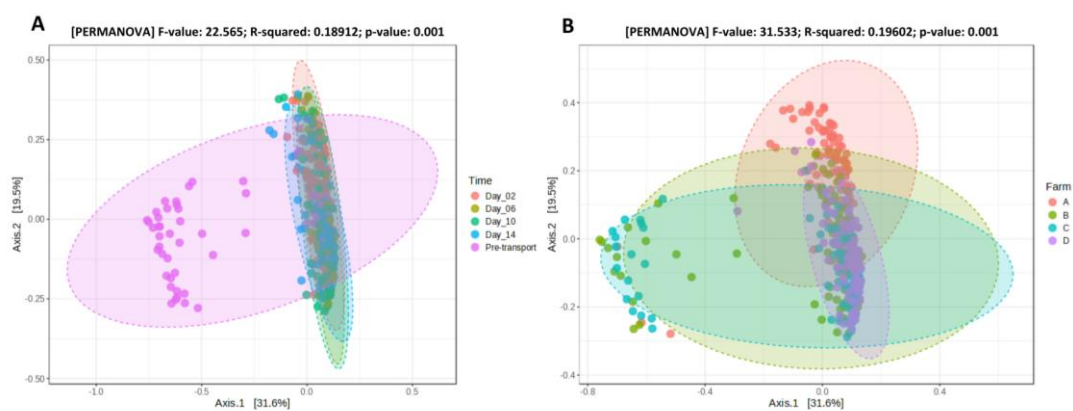


Figure 5-1 Composition of the gut microbiota

Pre and post-transport samples differed significantly in Beta-diversity (A).
The gut composition also varied significantly between farms (B).

Beta-diversity was also evaluated for each farm to determine whether there were differences between the four flocks included in this study. Composition of the gut microbiota differed significantly between the four farms (PERMANOVA, F-value: 31.533, R-squared = 0.19602, $p = 0.001$) (Figure 5-1B). Thus, to evaluate the effect of transport on the composition of the gut microbiota the data were separated and analysed for each farm (Figure 5-2).

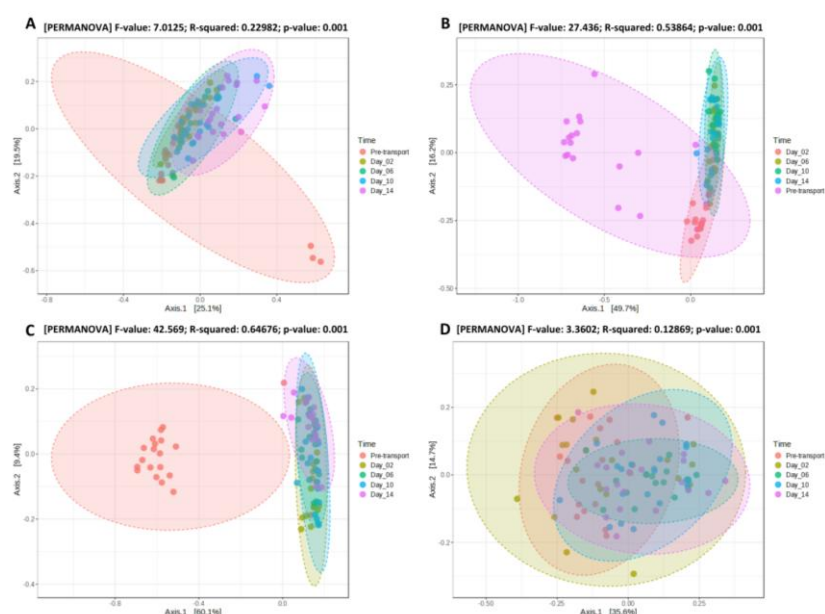


Figure 5-2 Composition of the gut microbiota changes over time

Beta-diversity was compared for Farm A (A), Farm B (B), Farm C (C), and Farm D (D) for pre-transport and post-transport samples.

For all farms, a significant difference in gut composition was observed for pre-transport samples.

Day 2 and Day 6 samples also exhibited significantly different gut microbiota composition, but no difference was observed for samples collected on Days 10 and 14.

The composition of the gut microbiota of pre-transport samples for Farm A was significantly different from all other time points ($p = 0.001$, FDR = 0.00125) (Figure 5-2A). Day 2 samples differed from Day 6 ($p = 0.01$, FDR = 0.011), Day 10 ($p = 0.001$, FDR = 0.00125), and Day 14 ($p = 0.001$, FDR = 0.00125). Day 6 was different from Day 10 ($p = 0.039$, FDR = 0.039) and Day 14 ($p = 0.001$, FDR = 0.00125). Day 10 also differed from Day 14 ($p = 0.001$, FDR = 0.00125).

Overall, these results show that the transition from rearing farm to production farm can have significant impacts on the composition of the gut microbiota and that it continues to shift over time.

5.3.2 The influence of transport on the alpha-diversity of the gut microbiota

The alpha-diversity of the gut microbiota is measured as the observed richness (or total number of taxa) and evenness (relative abundance of taxa) of a given sample. Different indices are used to evaluate one or more of these aspects of the gut microbiota. A Chao1 index was used to evaluate the effect of transport on the species richness in the gut microbiota of birds. Pre-transport samples collected from Farms B and C had significantly lower species richness compared with post-transport samples. This was not observed for Farms A and D. For Farm A, Day 2 had the highest species diversity and Day 14 the lowest. No significant difference was observed between samples for Farm D.

Species richness and evenness were evaluated using a Shannon Index. For Farm A, the Shannon index was significantly lower for pre-transport compared with Day 2 ($p < 0.01$) but no difference was observed between other time points. Farm B, pre-transport samples had the lowest Shannon index but were significantly different from Day 2, 6 and 14 ($p < 0.05$). Farm C pre-transport samples were significantly lower than all post-transport time points. For Farm D, the mean Shannon index was highest for pre-transport samples but only significantly different from Day 10.

A third alpha-diversity index was used to evaluate species dominance and evenness. Simpson index values for pre-transport samples collected from Farm A were lower on average compared with post-

transport samples, but were significantly different only at Days 2, 10 and 14 ($p < 0.01$). Farm B samples collected two days after transport exhibited significantly higher Simpson index values compared with all other time points ($p < 0.001$). Farm C also had a trend of increased Simpson values, but this was not significant. Pre-transport samples were significantly different from only Days 6 and 10 ($p < 0.05$). For Farm D, Simpson values from pre- and post-transport samples did not differ significantly.

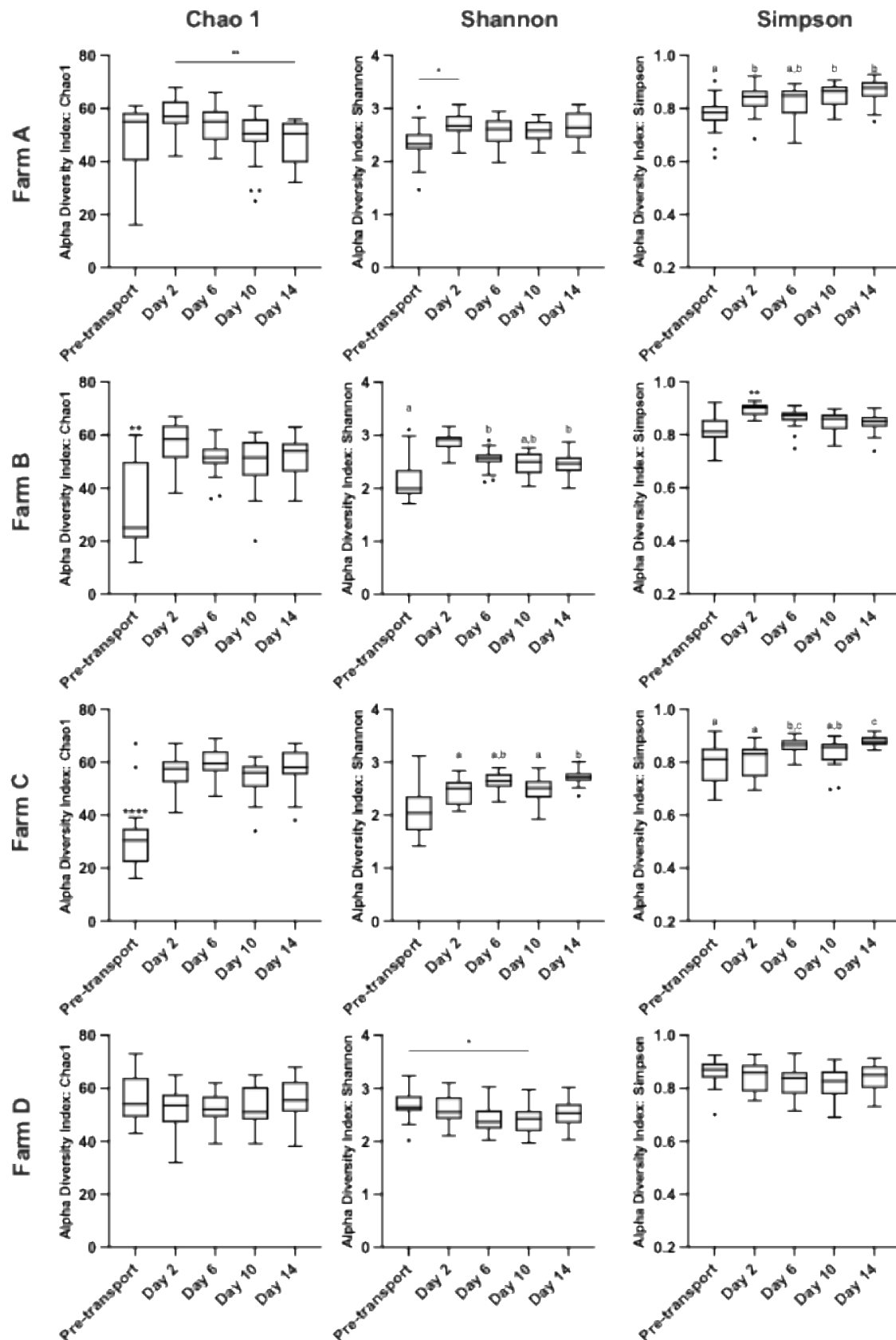


Figure 5-3 Alpha-diversity indices pre- and post-transport for Farms A, B, C and D

Treatment groups with the same letter do not differ significantly from each other. P-values < 0.01 are denoted by *, ** indicates a p-value < 0.001, and **** represents a p-value < 0.0001.

5.3.3 Movement from rearing to production farms influences gut microbiota composition.

Linear discriminant analysis effect size (LEfSe) analysis determines which taxonomic units are responsible for differences between groups. Farms A, B and C all had 15 different bacterial species whose abundance was significantly changed over the sampling period. Farm D had 13. It should be noted that each of the farms sampled had only a few common bacterial species that exhibited changes in overall abundance. *Bacteroides* was modulated in all but the pattern of relative abundance was not the same for each farm at each time point. *Lactobacillus* was high in pre-transport samples for Farms B and C, and then decreased in subsequent post-transport samples. For Farms A and D, however, *Lactobacillus* was low at pre-transport. Post-transport, the abundance of *Lactobacillus* varied over time for Farm A and D samples. *Turicibacter* was high in all pre-transport samples but dropped over time in post-transport samples in all flocks. *Faecalibacterium* was low pre-transport in Farms A and C, but absent in B and D. Post-transport, the abundance of *Faecalibacterium* increased over time. The relative abundance of *Olsenella* was low prior to transport for Farm A and D but increased post-transport.

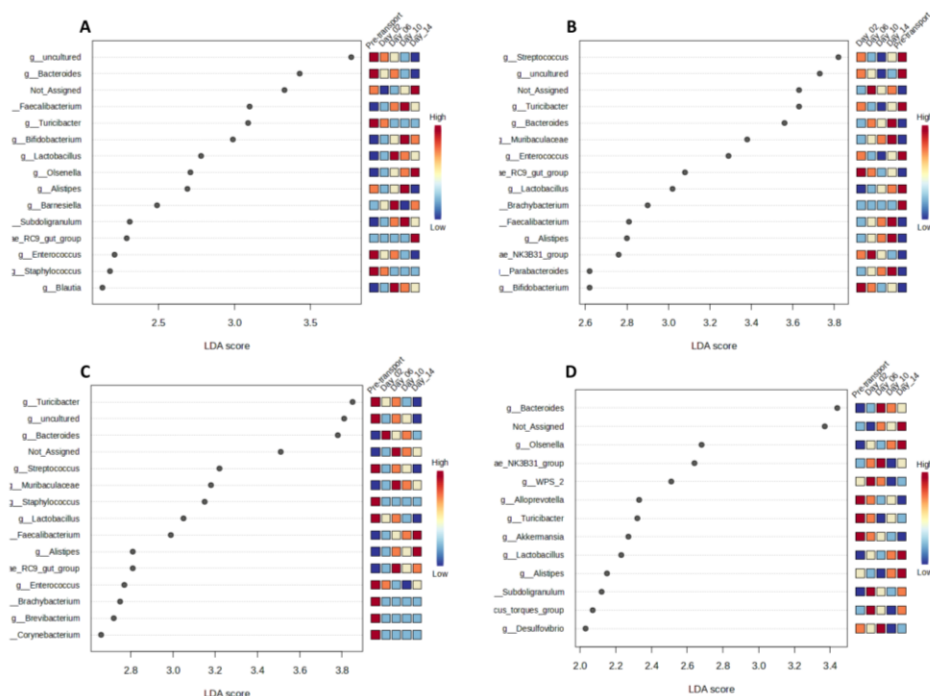


Figure 5-4 Composition of the gut microbiota changes over time

5.4 Discussion

It has previously been shown that gut microbiota diversity increases from hatch and continues to increase over 12 weeks (Joat et al. 2023). This study also demonstrated that, following transport, flocks experienced a drop in alpha-diversity, suggesting that an impact on the gut microbiota could be linked with movement, or change in farm environment (Joat et al. 2023). Transport is a significant event during the production life of a flock, involving extra handling, transport conditions, and ultimately a new environment. Following transport, birds often decrease their food and water consumption (Cheng & Jefferson 2008). Changes in feed can affect the diversity and composition of the gut microbiota (Xing et al. 2019; Joat et al. 2021a).

While the present study did not test stress directly, understanding the implications that the point of transport has on the gut microbiota is important. We observed a significant shift in the composition of the gut microbiota between pre-transport and all post-transport samples. Post-transport, significant changes in the gut microbiota composition were also observed. Changes in housing type could be one explanation for this, as the diet remained the same. All flocks were reared on the floor with shavings, and were moved to free range farms with sheds containing slat flooring. Chickens constantly peck at their environment, and the shavings on the floor would contain faeces, feed, dander, and other dust that they would ingest. In production sheds with slats, the bar would have recently been decontaminated, resulting in a cleaner environment than in the rearing shed. This would lead to a reduction in the environmental contribution to the gut microbiota. For example, *Staphylococcus* was overall higher in pre-transport samples compared to post-transport samples. *Staphylococcus* species are a highly abundant component of the skin microbiota and likely a component of dander released into the environment. A reduction in dander in the environment likely contributes to its reduced abundance in the gut microbiota post-transport.

Several bacterial species exhibited changes in the relative abundance, but which species were affected varied between flocks. This variability could be attributed to the different environmental conditions experienced by each flock. Conducting repetitive sampling of multiple flocks reared under identical conditions and then transported to the same shed repeatedly could offer a more comprehensive understanding of the effects of transport on flocks. By correlating these findings with changes in corticosterone levels in birds, the direct effect of transport-induced physiological stress on gut microbiota composition could be evaluated. This integrated approach can provide valuable data on the interplay between environmental stressors, gut microbiota dynamics, and stress hormone levels in birds, contributing to a more holistic understanding of the impact of transport on avian health and welfare.

Most pullets are transported from rearing to production farms when they are between 14 and 16 weeks of age. This is a critical period for birds as they are also experiencing physiological changes associated with the onset of lay (Mumma et al. 2006). These combined events can render the birds immunocompromised, and they are then at risk for being susceptible to both avian specific as well as zoonotic pathogens. Further study is needed to evaluate whether these changes in the gut microbiota create a favourable environment for the colonisation of pathogens or permit them to replicate. Understanding the dynamics of the gut microbiota in response to transport-related stress and its potential impact on pathogen colonisation is crucial for ensuring the health and well-being of the flock. Investigating how alterations in the gut microbiota composition may influence the susceptibility of birds to pathogen colonisation can provide valuable insights for developing strategies to mitigate the risks associated with transport-induced stress.

6 Administration of a live, *Lactobacillus* probiotic at point-of-lay influences the gut microbiota

6.1 Background

Commercial layer pullets are typically transported to egg production farms between 16 and 18 weeks of age, just prior to the onset of lay. The process of handling and transporting birds can induce stress, leading to negative physiological impacts (Cheng & Jefferson 2008) including disruption of the gut microbiota (Joat et al. 2021a). Gut microbiota disruption, or dysbiosis, creates a period of vulnerability where an unhealthy microbiota profile may develop, potentially affecting the long-term health and productivity of layer hens. Additionally, the onset of lay can also impact hen physiology. This period is often associated with immunosuppression, stress, and further impacts on the gut microbiota. It is crucial to consider these factors when managing the transition of pullets to egg production farms.

The use of probiotics can have a positive impact on the gut microbiota (Khan et al. 2020), and administration of commercial probiotics prior to onset of lay has been shown to reduce mortality and improve productivity over time (Shini et al. 2013). Commercial probiotics manufactured for poultry often include strains of *Lactobacillus*, *Bacillus*, *Bifidobacterium*, and *Streptococcus* but multiple doses or continuous use are often required, representing a significant cost to the industry.

The aim of this experiment was to determine if a single oral dose of a live *Lactobacillus* strain positively affected the bacterial species richness, abundance, and evenness in the microbiota of pullets post-transport.

6.2 Methods

6.2.1 Study design

Forty, 15-week-old Hy-Line Brown pullets were obtained from a commercial rearing farm. Upon arrival at the University of Adelaide Roseworthy Campus Layer hen shed, birds were randomly allocated to either control or *Lactobacillus saerimneri* probiotic treatment groups. Twenty birds were in each treatment. Birds in the probiotic treatment group were given 1 mL of a *Lactobacillus saerimneri* orally. The *Lactobacillus* suspension was prepared as described in Chapter 3, Section 3.2.1. Control birds received 1 mL saline orally. Following probiotic administration, birds had *ad libitum* access to feed (Barastoc Champion Layer Hen Feed) and water.

Fresh faeces were collected from the transport cages to determine the presence of rifampicin resistant bacteria. Samples were streaked in duplicate onto MRS-rifampicin agar plates (Chapter 3, Section 3.2.2). Given that the faeces were collected directly from transport cages and exposed to the environment for several hours, they were not included in the microbiota assessment.

Caecal samples were collected on Days 2, 6 and 10, and Weeks 2, 4, 6, 8 and 10 post-probiotic administration. A portion of the sample was used for bacterial culture to detect the presence of the *Lactobacillus saerimneri* strain over the course of the experiment. A second portion was collected and immediately frozen at -80°C for 16S rRNA sequencing. DNA purification, amplicon sequencing, and data analyses were performed as described in Chapter 3, Section 3.2.5.

6.3 Results

6.3.1 Culture of *Lactobacillus saerimneri* from caecal samples

Faecal samples collected from transport cages were all negative for rifampicin resistant bacterial species. Forty 15-week-old HyLine Brown pullets were randomly allocated into the control and *Lactobacillus saerimneri* treatment groups (n = 20 per treatment). Following probiotic administration, fresh caecal samples were collected at Days 2, 6 and 10, and Weeks 2, 4, 6, 8 and 10 to evaluate the culture presence of the probiotic *L. saerimneri* strain. On Day 2, 72% (18/25) of pullets were positive for *L. saerimneri*. The percentage of positive birds increased to 80% (20/25) on Day 6 (Figure 6-1). Pullets culture positive for *L. saerimneri* decreased starting from Day 10 but the proportion of positive birds stabilised between Weeks 6 and 10. One control bird at Weeks 6 and 8 and two at Week 10 were culture positive for rifampicin resistant *L. saerimneri*.

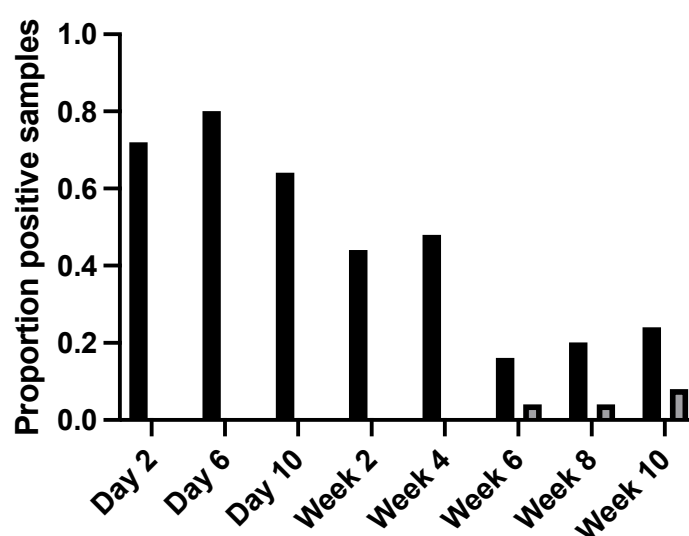


Figure 6-1 Probiotic *Lactobacillus saerimneri* strain is culturable from caecal material

Freshly voided caecal samples were collected from control and *L. saerimneri* treatments to evaluate the presence of the probiotic bacterial strain using bacteriological culture methods.

6.3.2 Microbiota community structure differs between treatment groups

The caecal microbiota was profiled by sequencing the V3–V4 region of the 16S rRNA gene at 8 time points (Days 2, 6 and 10, and Weeks 2, 4, 6, 8 and 10). Twenty caecal samples were sequenced from each group except Day 4 control (n = 17) and Day 8 control (n = 19). Samples were excluded because of quality issues. Sequencing produced a total number of 8,315,026 reads after quality trimming and chimera removal. An average of 26,313 reads were generated for each sample from a total of 316 samples. Over the 10-week experiment, *Bacteroidota*, *Actinobacteriota*, and *Firmicutes* were the dominant phyla in both control and *L. saerimneri* pullets (Figure 6-2). The greatest variation in abundance was observed in the phylum *Bacteroidota*.

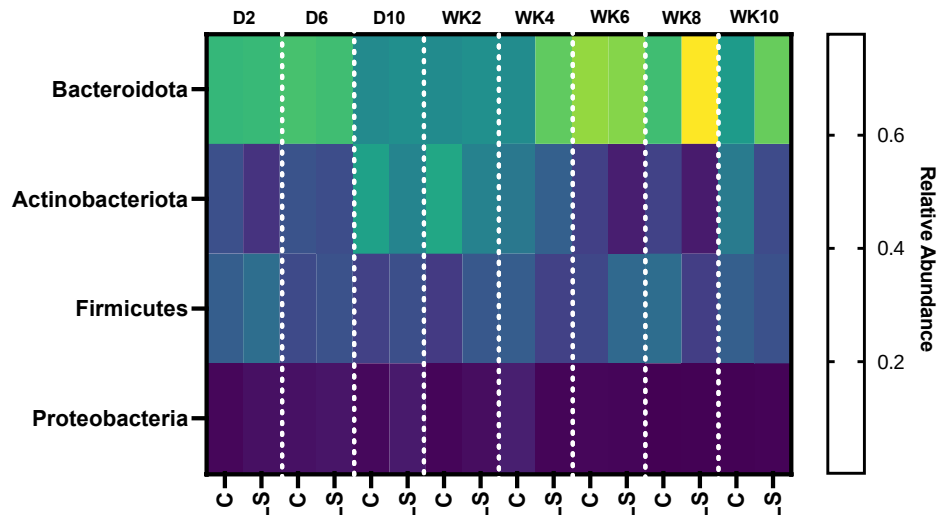


Figure 6-2 Relative abundance of bacterial phyla in the gut microbiota

The abundance of bacterial phyla varied between control (C) and *L. saerimneri* (LS) treatment groups over the 10-week experiment.

6.3.3 *Lactobacillus saerimneri* administration affects microbiota alpha and beta-diversity

Community structure similarly (beta-diversity) between control and *Lactobacillus* treated birds was assessed over the course of the 10-week experiment. A significant effect of time point was detected ($p < 0.05$). To evaluate the differences between treatments, data from each time point were analysed separately. Significant differences in beta-diversity were observed between the control and *Lactobacillus saerimneri* treatment groups at Weeks 2, 4, 6, 8, and 10 (Figure 6-3).

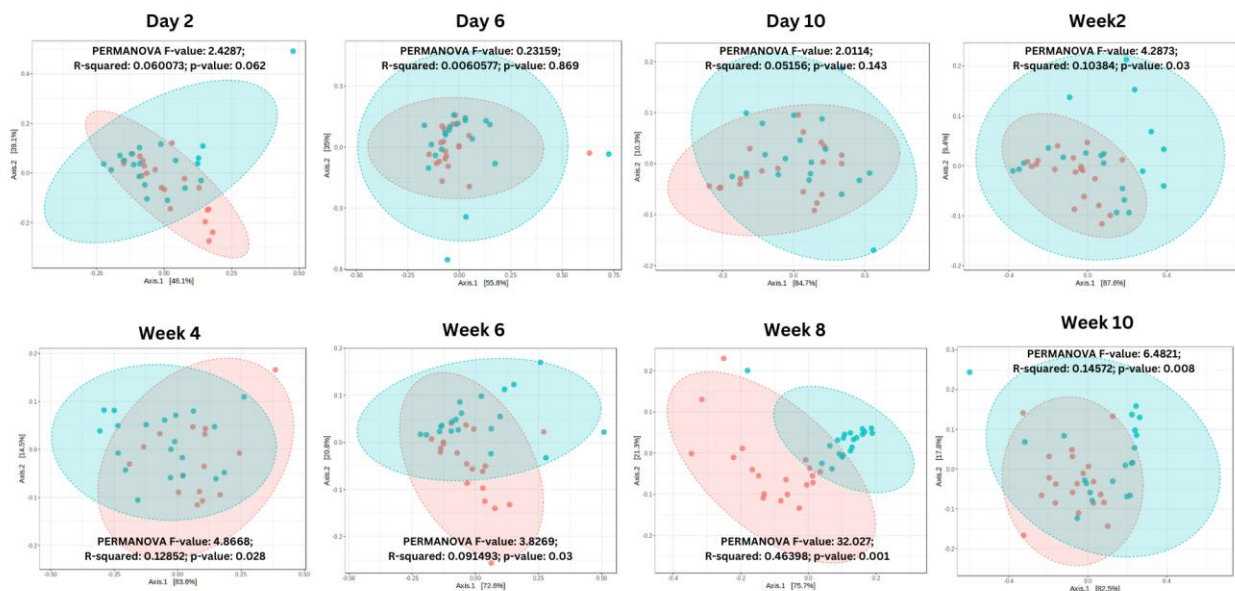


Figure 6-3 Beta-diversity is influenced by *Lactobacillus saerimneri* administration

Beta-diversity of the gut microbiota between control (red) and *Lactobacillus saerimneri* (green) treated birds was evaluated at Days 2, 6 and 10, and Weeks 2, 4, 6, 8 and 10 using the Bray Curtis index at the phyla level.

Each point in the Principal Coordinate Analysis (PCoA) represents an individual sample.

Significant differences between control and *Lactobacillus* treated birds were observed at Weeks 2, 4, 6, 8 and 10 post-treatment.

To evaluate factors driving the differences in community structure, alpha-diversity was first evaluated at the phylum taxonomic level using three tests, Chao1, Shannon Index, Simpson Index. Chao1 analysis was performed to evaluate the effect of a single shot of *Lactobacillus saerimneri* on gut microbiota species richness (total number of species) post-transport (Figure 6-4A). At each time point, no significant difference in the richness of bacterial phyla was observed between the control and *Lactobacillus saerimneri* treatment groups.

Shannon Index is a measurement of species abundance and evenness. Greater diversity is associated with a higher value. Four weeks post-administration of *Lactobacillus saerimneri*, the Shannon Index values observed for birds administered *Lactobacillus saerimneri* were lower compared with control birds, but the difference was not significant. At Weeks 6, 8 and 10 post-administration, Shannon Index values for *Lactobacillus saerimneri* birds were significantly lower than birds in the control group ($p < 0.001$, FDR < 0.05) (Figure 6-4B).

Simpson Index of diversity is a measure of species dominance and evenness. At Weeks 4 and 6 post-treatment, *Lactobacillus saerimneri* treated birds exhibited an overall greater diversity compared with control birds but the difference was not significant. Significant differences between control and *Lactobacillus saerimneri* treated birds were observed at both Weeks 8 and 10 post-administration ($p < 0.001$, FDR < 0.05) (Figure 6-4C).

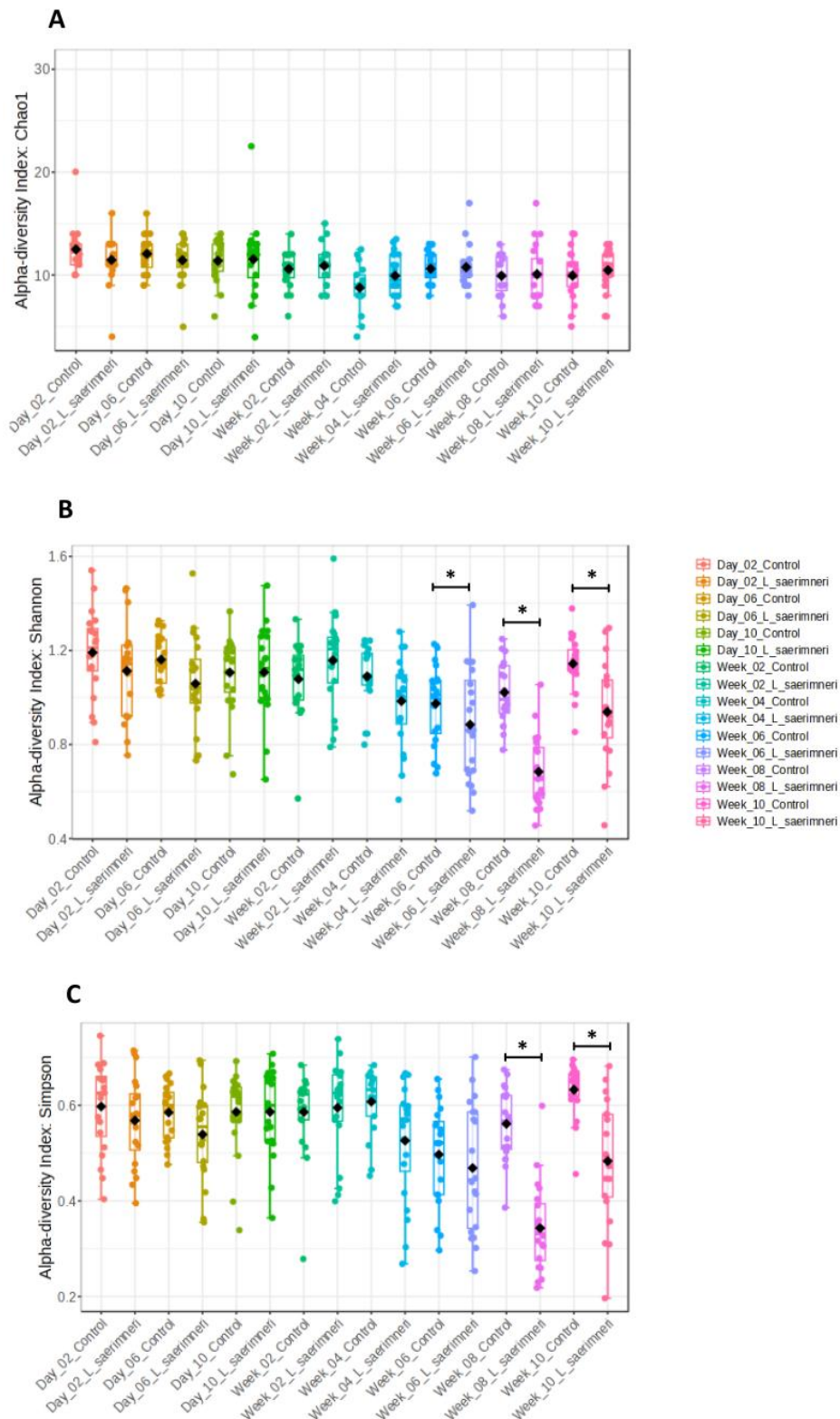


Figure 6-4 Alpha-diversity is influenced by *Lactobacillus saerimneri* administration

The alpha-diversity of the gut microbiota between control and *Lactobacillus saerimneri* treated birds was evaluated at Days 2, 6 and 10, and Weeks 2, 4, 6, 8 and 10, using (A) Chao1, (B) Shannon, and (C) Simpson indices. Significant differences between control and *Lactobacillus* treated birds were observed at Weeks 8 and 10 (Shannon diversity index, $p < 0.001$, FDR < 0.05), and Weeks 6, 8 and 10 (Simpson, $p < 0.001$, FDR < 0.05).

To evaluate whether differences in alpha-diversity were driven by taxonomic level, Chao1, Shannon Index, and Simpson index analyses were also performed using genus. Similar results were observed in

the alpha-diversity of bacterial genus (Figure 6-5A,B,C). A significant effect of richness was observed across all treatments, but this was driven by time (data not shown). At Week 8 post-administration, Shannon Index values for *Lactobacillus saerimneri* birds were significantly lower than birds in the control group ($p < 0.001$, FDR < 0.05) (Figure 6-5B). Significantly lower Simpson Index values were also observed between control and *Lactobacillus saerimneri* treated birds at both Weeks 8 and 10 post-administration ($p < 0.001$, FDR < 0.05) (Figure 6-5C).

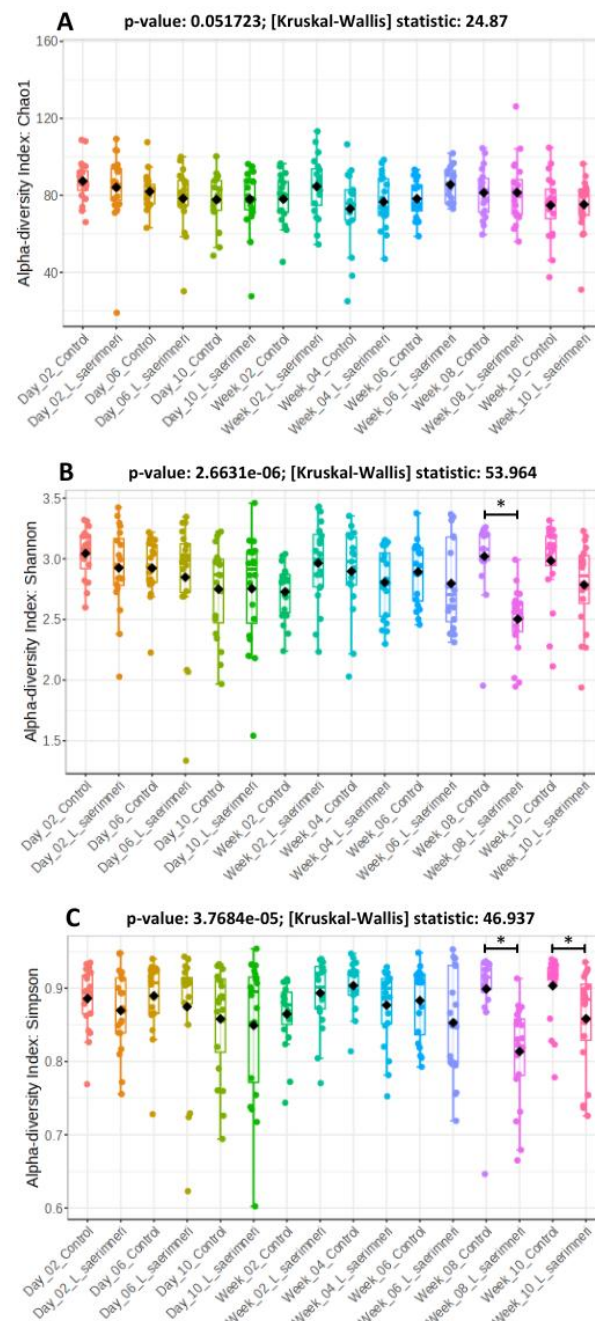


Figure 6-5 Alpha-diversity at the genus taxonomic level is influenced by *Lactobacillus saerimneri* administration

The alpha-diversity of the gut microbiota between control and *Lactobacillus saerimneri* treated birds was evaluated at Days 2, 6 and 10, and Weeks 2, 4, 6, 8 and 10 using (A) Chao1, (B) Shannon, and (C) Simpson indices.

Significant differences between control and *Lactobacillus* treated birds were observed at Week 8 (Shannon diversity index, $p < 0.001$, FDR < 0.05) and Weeks 8 and 10 (Simpson, $p < 0.001$, FDR < 0.05).

6.3.4 Effects of *Lactobacillus saerimneri* treatment on caecal microbiota

A linear discriminant analysis effect size (LEfSe) analysis was performed on the caecal microbiota to evaluate the differences in the relative abundance of different bacterial phyla represented in each treatment group. Thirteen bacteria phyla including *Bacteroidota*, *Actinobacteriota*, *Firmicutes*, *Proteobacteria*, *Fusobacteriota*, *Verrucomicrobiota*, *Desulfobacterota*, *Patescibacteria*, *Synergistota*, *Cyanobacteria*, *Elusimicrobiota*, *Euryarchaeota*, and *Camprilobacterota* exhibited significant relative abundance across all treatments (FDR < 0.05) (Figure 6-6). At all time points, differences in relative abundance were observed between control and *Lactobacillus saerimneri* treatment groups. Notably, at Weeks 8 and 10, the relative abundance of *Actinobacteriota* and *Firmicutes* were lower in the *Lactobacillus* treatment group relative to the control.

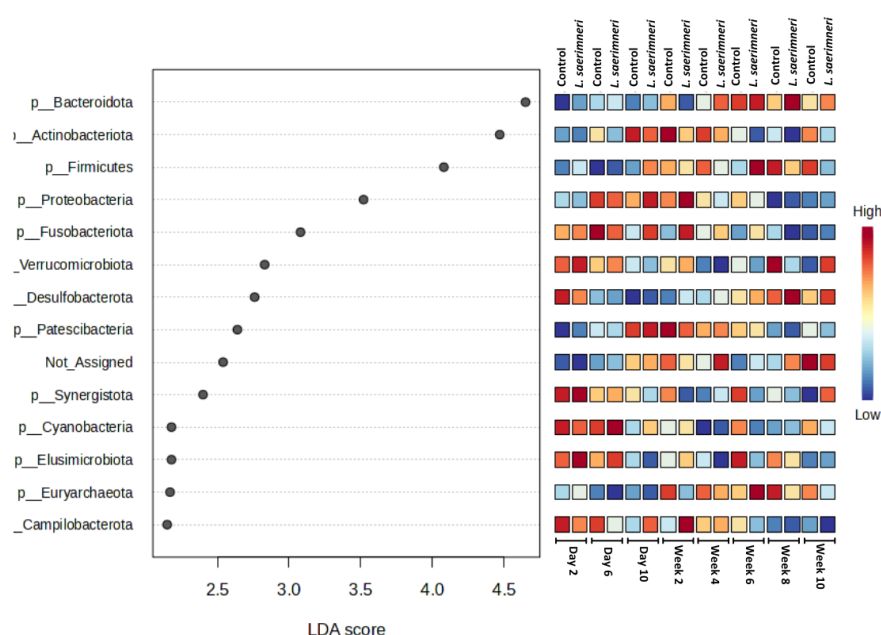


Figure 6-6 Effects of *Lactobacillus* treatment on caecal microbiota

Differences in the relative abundance of bacterial genera were observed over the 10-week trial.

Treatment with *Lactobacillus saerimneri* resulted in significant differences in the abundance of different bacterial phyla.

6.4 Discussion

Transport is stressful for pullets can impact the gut microbiota (Joat et al. 2021a). The primary aim of this study was to evaluate whether a single dose of *Lactobacillus saerimneri* could colonise an established gut microbiota and whether it impacted the profile of the gut microbiota. Birds arrived at the Roseworthy layer hen shed and were administered a dose of *Lactobacillus saerimneri* as they were removed from the transport crates. Following administration, birds were permitted water and feed unrestricted. The *Lactobacillus saerimneri* strain used as a probiotic in this study was detected by culture over the course of the entire experiment. The highest proportion of positive birds were observed early at Days 2, 6 and 10 post-administration. Few investigations have tested the effects of probiotic supplementation to minimise the effect of transport stress. Broilers administered a commercial *Bacillus subtilis* probiotic exhibited lower levels of corticosterone (Negm et al. 2023) but this study did not evaluate the effects on the microbiota.

Egg laying was recorded for all birds and egg quality scores were also measured. There was no difference in the onset of lay between the birds and no difference in the egg quality. As in Chapter 4, birds received a high-quality feed and shell grit. Feed likely has a more dominant effect on egg quality.

The community structure of the gut microbiota differed significantly between probiotic and control birds at Weeks 2, 4, 6 and 8 following probiotic administration, suggesting that there was an impact on the species types represented in each group. No significant difference was observed in overall species richness (Chao1), but Shannon and Simpson indices indicated that there was an overall decrease in evenness of the gut microbiota in birds that received the *Lactobacillus* probiotic. This effect, however, was not observed until 8 and 10 weeks after birds had received the dose. Few studies have investigated the impacts that transport has on layer hen stress (U. Bello et al. 2018) and the gut microbiota (Joat et al. 2021a).

Transport can have direct and indirect effects, which may have stronger impacts on the gut microbiota. Inappetence has been shown to occur following transport, which may have a greater impact than stress itself (U. Bello et al. 2018). Additionally, stress can trigger some bacteria to replicate, which may impact gut richness and evenness. Further studies need to be conducted to evaluate the impacts of transport stress on birds and how it impacts their gut health and egg production.

7 Effect of a live *Lactobacillus* probiotic on the gut microbiota of late lay hens

7.1 Background

The composition of the gut microbiota changes substantially as birds age (Callaway et al. 2009; Videnska et al. 2014; Ngunjiri et al. 2019; Joat et al. 2021a). The bacterial phyla *Firmicutes*, for example, has the highest abundance at an early age but *Bacteroides* become more dominant in mid to late lay (Joat et al. 2021a; Callaway et al. 2009). Gut microbiota richness also increases post-hatch as birds mature, but after 40 weeks of age a reduction in richness has been described (Joat et al. 2023). A diverse gut microbiota is more robust and has a stronger capacity to resist and recover from infection (Stanley et al. 2014).

Worldwide, the egg laying industry is striving to extend the duration of egg production. Currently, depopulation of layer flocks occurs between 68 and 75 weeks of age after 50–58 weeks of lay. As hens age, they can develop health issues that can impact productivity. A major impact is a decrease in egg quality. Birds older than 60 weeks of age exhibit a decrease in egg quality, particularly shell quality related to issues with calcium and vitamin D regulation (Saunders-Blades & Korver 2015). Composition of the gut microbiota can influence calcium absorption in the intestine (Chaplin et al. 2016). The relationship between the gut microbiota and overall health in late lay could play a key role in maintaining egg quality and production for longer periods. The relative abundance of the bacterial genera, *Lactobacillus* and *Fourneirella*, in the caecal content for example has been shown to have a positive impact on egg quality (Jin et al. 2024).

The present study investigated how the administration of a single dose of *Lactobacillus saerimneri* impacted the gut microbiota of 65-week-old layer hens. The primary aim of the study was to determine if the gut microbiota could be positively impacted, potentially offering a solution to some of the health challenges faced by hens in late lay.

7.2 Methods

7.2.1 Study design and animals

Forty, 70-week-old Hy-Line Brown free range laying hens were obtained from a commercial producer. Birds were transported to the University of Adelaide's Roseworthy Campus layer hen shed and were divided into two treatment groups, control and *Lactobacillus saerimneri*. Prior to placement, birds in the probiotic treatment group were given a single 1 mL oral dose of an overnight culture of *Lactobacillus saerimneri*. Control birds received 1 mL saline. *Lactobacillus saerimneri* was prepared as described in Chapter 3, Section 3.2.1. Birds were given water and Barastoc Champion layer *ad libitum*. Shell grit was added to the feed every other day. All housing, animal husbandry, and experiments were performed according to animal ethics protocol approved by the University of Adelaide Animal Ethics Committee, S2023-030.

Fresh caecal samples were collected from birds upon arrival, prior to treatment. Additional caecal samples were collected from all birds on Days 4 and 7 post-treatment and at Weeks 2, 3 and 4. Samples were collected for testing for the presence of *Lactobacillus saerimneri* by culture (Chapter 4, Section 4.2.2). Additional caecal material was immediately frozen at -80°C until needed for DNA purification.

7.2.2 DNA purification, 16S rRNA sequencing and analyses

DNA purification, amplicon sequencing, and data analyses were performed as described in Chapter 3, Section 3.2.5.

7.3 Results

7.3.1 Culture of *Lactobacillus saerimneri* from caecal samples

Faecal samples collected from birds upon arrival all tested negative for any rifampicin resistance bacterial strains.

At Day 4 post-administration of live *Lactobacillus saerimneri*, 3/20 (15%) were culture positive for *Lactobacillus saerimneri*. At all other time points, *Lactobacillus saerimneri* was not cultured from caecal samples.

7.3.2 Community structure of the gut microbiota

The caecal microbiota was profiled by sequencing the V3–V4 region of the 16S rRNA gene at 6 time points (arrival, Days 4 and 7, and Weeks 2, 3 and 4). Twenty caecal samples were sequenced from each group except arrival samples (n = 10). Sequencing produced a total number of 4,069,719 reads after quality trimming and chimera removal. An average of 20,554 reads were generated for each sample from a total of 210 samples.

Arrival samples exhibited the greatest variation in the community structure of the microbiota (Figure 7-1). Overall, the microbial diversity in the arrival samples was low and dominated by the phyla *Firmicutes* and *Fusobacteriota*. Samples collected at all other time points exhibited higher diversity, and the dominant phyla were *Bacteroidota* and *Firmicutes*. *Actinobacteriota* was absent in arrival samples but present in all other samples. The relative abundance of *Fusobacteriota* decreased in samples collected at Day 4, and this reduction was maintained throughout the duration of the experiment.

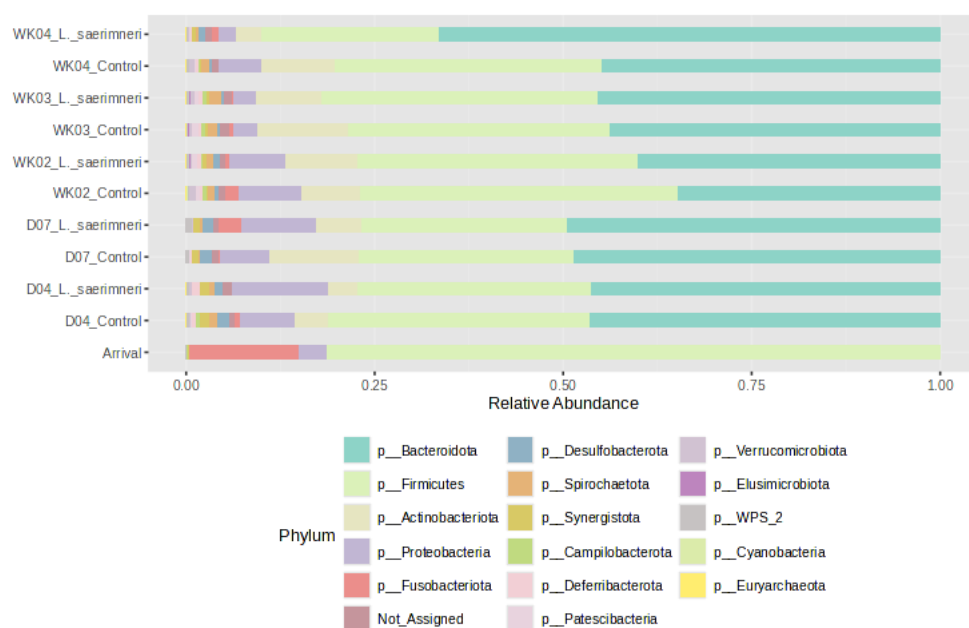


Figure 7-1 Relative abundance of bacterial phyla in the gut microbiota

The abundance of bacterial phyla varied between samples collected upon bird arrival and all subsequent collection time points.

7.3.3 *Lactobacillus saerimneri* administration affects microbiota alpha and beta-diversity

Community structure (beta-diversity) was evaluated between treatments, arrival, control and *Lactobacillus saerimneri*. A significant effect of treatment was detected (PERMANOVA, F-value 23.246, R-squared: 0.19252, $p = 0.001$) (Figure 7-2A). Pairwise PERMANOVA revealed that arrival samples differed significantly from both control and *Lactobacillus saerimneri* treatment groups ($p = 0.001$, FDR = 0.0015). Samples were reanalysed to determine whether there was an effect of time point on beta-diversity (Figure 7-2B). A significant effect of time was observed (PERMANOVA, F-value 12.411, R-squared 0.24426, $p = 0.001$). Arrival samples differed significantly from all other time points (Pairwise PERMANOVA $p = 0.001$, FDR = 0.0016667). Similar results were obtained when control and *Lactobacillus saerimneri* samples were separated at each time point. Beta-diversity of arrival samples differed significantly from all other treatments ($p = 0.001$, FDR = 0.0028947) (Figure 7-2C). No difference was detected between control and *Lactobacillus saerimneri* treatment groups.

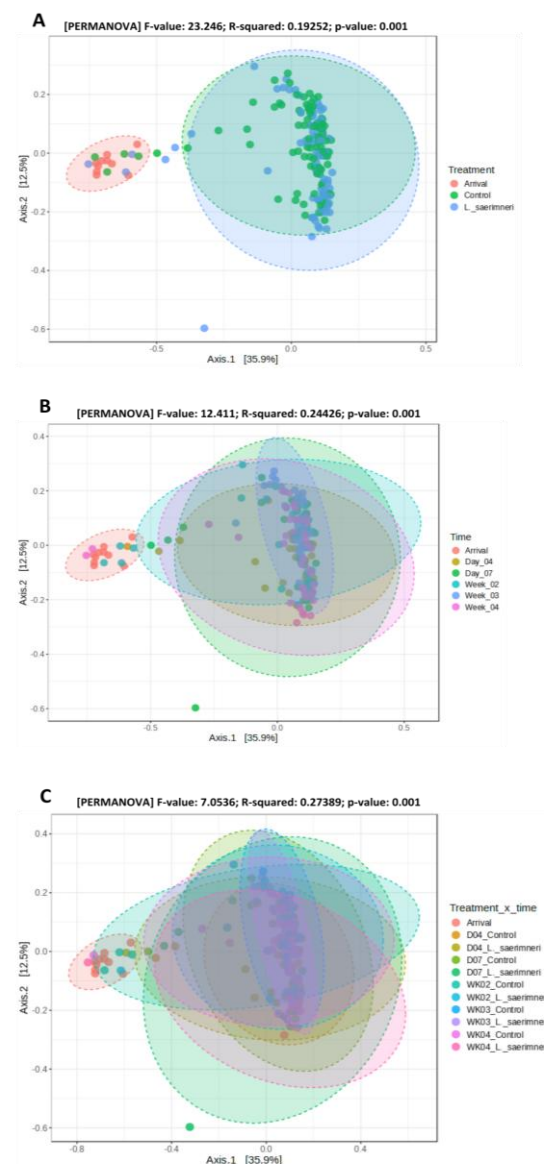


Figure 7-2 Beta-diversity is influenced by time

Beta-diversity of the gut microbiota between arrival, control, and *Lactobacillus saerimneri* treated birds was evaluated using the Bray Curtis index at the phyla level.

Each point in the Principle Coordinate Analysis (PCoA) represents an individual sample.

Significant differences between arrival and all other treatments were observed.

To evaluate factors driving the differences in community structure, alpha-diversity was first evaluated at the genus taxonomic level using three tests, Chao1, Shannon Index, and Simpson Index. Chao1 analysis was performed to evaluate the effect of a single shot of *Lactobacillus saerimneri* on gut microbiota species richness (total number of species). A significant effect of treatment was observed ($p < 0.0001$). Arrival samples exhibited significantly lower species richness compared with the control and *Lactobacillus saerimneri* treatment groups (Figure 7-3A).

Shannon Index is a measurement of species abundance and evenness. Greater diversity is associated with a higher value. Shannon Index values observed for arrival samples were significantly lower than both control and *Lactobacillus saerimneri* ($p < 0.0001$) (Figure 7-3B). Species dominance and evenness were evaluated using the Simpson Index of diversity. As with Chao1 and Shannon diversity analyses, Simpson diversity was significantly lower in arrival samples compared with control and *Lactobacillus saerimneri* treatment groups ($p < 0.001$) (Figure 7-3C).

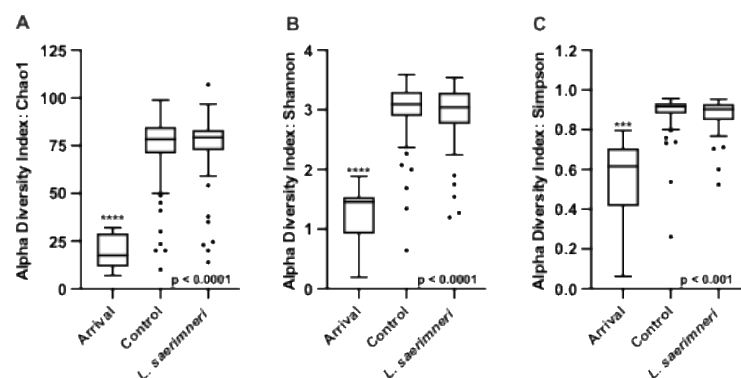


Figure 7-3 Alpha-diversity at the genus taxonomic level is influenced by time

The alpha-diversity of the gut microbiota between arrival, control, and *Lactobacillus saerimneri* treated birds was evaluated using (A) Chao1, (B) Shannon, and (C) Simpson indices.

Upon arrival at the Roseworthy Layer Hen Shed, birds were divided into control and *Lactobacillus saerimneri* treatment groups. A single dose of *Lactobacillus saerimneri* was administered prior to hens being placed into cages. Control birds were given a dose of saline. Alpha-diversity of samples was also analysed after separating treatment groups (control and *Lactobacillus saerimneri*) to determine whether *Lactobacillus saerimneri* administration affected species richness and abundance. No significant difference in total species abundance (Chao1) (Figure 7-4A), species richness/evenness (Shannon) (Figure 7-4B), or dominance/evenness (Simpson) (Figure 7-4B) was observed between control and *Lactobacillus saerimneri* treatment groups. Differences in alpha-diversity indices were driven by the arrival samples.

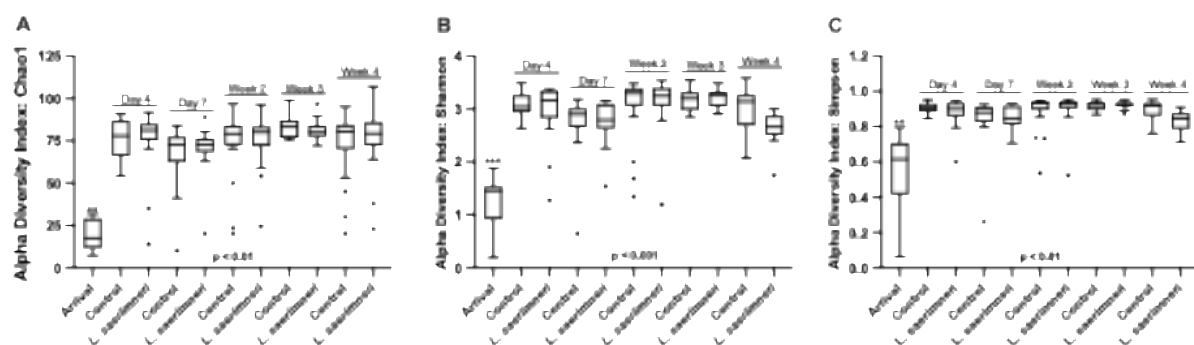


Figure 7-4 Alpha-diversity does not differ between control and *Lactobacillus saerimneri* treatment groups

The alpha-diversity of the gut microbiota between arrival, control, and *Lactobacillus saerimneri* treated birds was evaluated using (A) Chao1, (B) Shannon, and (C) Simpson indices over the experimental time course.

7.3.4 Effects of *Lactobacillus saerimneri* treatment on caecal microbiota

A linear discriminant analysis effect size (LEfSe) analysis was performed on the caecal microbiota to evaluate the differences in the relative abundance of different bacterial phyla represented in each treatment group. Thirteen bacterial phyla including *Bacteroidota*, *Actinobacteriota*, *Firmicutes*, *Proteobacteria*, *Fusobacteriota*, *Verrucomicrobiota*, *Desulfobacterota*, *Patescibacteria*, *Synergistota*, *Cyanobacteria*, *Elusimicrobiota*, *Euryarchaeota*, and *Camprilobacterota* exhibited significant relative abundance across all treatments ($p < 0.01$, FDR < 0.05) (Figure 7-5). Notably, the relative abundance of *Bacteroidota* was low while *Firmicutes* and *Fusobacteriota* were high in arrival samples.

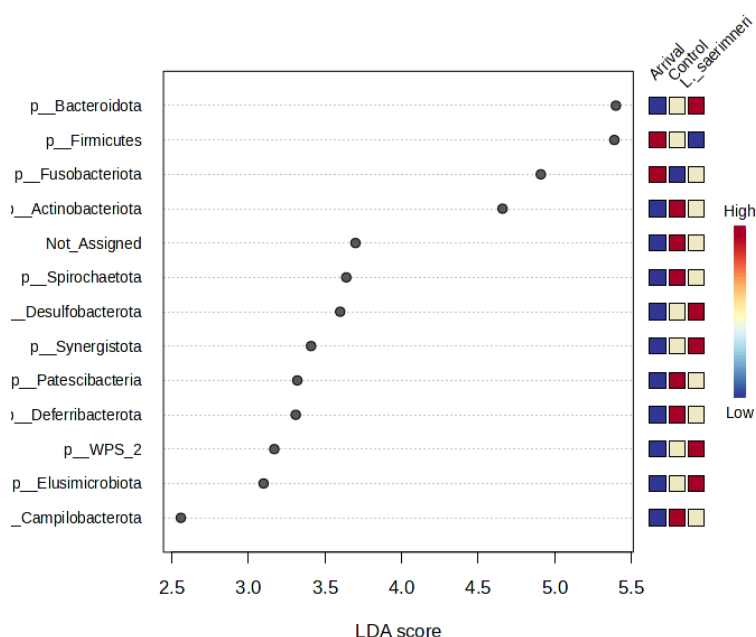


Figure 7-5 Effects of *Lactobacillus* treatment on caecal microbiota

Differences in the relative abundance of bacterial genera were observed between arrival and experimental treatment samples.

Treatment with *Lactobacillus saerimneri* did not result significant differences in the abundance of different bacterial phyla.

7.4 Discussion

As hens age, the diversity of the gut microbiota decreases, which can affect bird health and egg production. The present study evaluated whether a single dose of *Lactobacillus saerimneri* could impact the gut microbiota profile in 70-week-old hens. Birds were administered either saline or saline containing a fresh *Lactobacillus saerimneri* culture. Fresh caecal samples were collected when birds arrived at the research shed and again at Days 4 and 7, and Weeks 2, 3 and 4 post-treatment.

Rifampicin resistant *Lactobacillus saerimneri* were only detected in caecal samples collected at Day 4 post-treatment. Based on earlier experiments, this outcome was unexpected. In Chapter 4, a proportion of chicks given *Lactobacillus saerimneri* at hatch were culture positive for 20 weeks. A re-emergence of culturable bacteria in these birds was observed between 27 and 36 weeks but the proportion of positive birds was less. Similarly, in Chapter 6, caecal samples collected from a portion of the pullets administered *Lactobacillus saerimneri* were culture positive for the probiotic strains over the course of the 10-week trial. The poor colonisation of *Lactobacillus saerimneri* in 70-week-old hens suggests probiotic administration in older birds has less of an effect on the gut microbiota compared to younger birds. Bacterial species in the gut compete for resources and space. The niche that *Lactobacillus saerimneri* might have occupied may have been dominated by established, resident

species. According to the niche exclusion principle, species that utilise the same energy resources should not be able to cohabit the same space (Hardin 1960).

A significant change in the gut microbiota profile was observed between arrival samples compared with all other samples. Diet is the primary change between the point of arrival and time points during the experiment. The types of non-digestible carbohydrates, for example, may have differed substantially in the feed given to birds on the commercial farm compared with what was provided during the experiment. In humans, changing the major non-digestible carbohydrate from wheat bran to resistant starch in diets delivering the same intake of protein, fat and total carbohydrate, led to rapid shifts in the bacterial species composition of the gut microbiota over only a few days (Walker et al. 2011). *Lactobacillus saerimneri* administration did not lead to changes in the gut microbiota profile. Alpha and beta-diversity analyses revealed that the diversity in the gut microbiota did not differ between control and *Lactobacillus saerimneri* treatment groups.

Data obtained from this experiment demonstrate that diet is potentially a key factor shaping the gut microbiota of older birds. It should be noted that the dominance of *Firmicutes* in the arrival samples differed from the gut microbiota profiles of 60 + week-old flocks in Chapter 2. In a study of caged hens, *Firmicutes* was the dominant phyla in the gut microbiota of 64-week-old birds but *Bacteroidetes* became dominant after 70 weeks of age (Joat et al. 2021a). This differs in free range birds where *Bacteroidetes* is the dominant phyla in older birds (Joat et al. 2023). Management practices, especially feed composition, for older birds is likely a dominant force influencing the gut microbiota profile. Further research is needed to confirm this hypothesis and formulate feed that favours gut microbiota diversity.

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9 Plain English Summary

Project Title:	Effects of microbiota ‘seeding’ on gut health and egg production
Australian Eggs Limited Project No	INNO003
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Objectives	This project had six major objectives: 1. Compare the gut microbiota of flocks with high and low production. 2. Establish ‘seeding’ methods trialling five <i>Lactobacillus</i> strains and establishing their colonisation and persistence in the gastrointestinal tract of chicks. 3. Determine the persistence of a ‘seeding’ <i>Lactobacillus</i> strain from hatch till mid lay, and characterise its impacts at different production stages. 4. Investigate the impact of transport on the gut microbiota of point-of-lay pullets. 5. Determine how <i>Lactobacillus</i> administration post-transport can impact the gut of point-of-lay pullets. 6. Explore the effects of a <i>Lactobacillus</i> strain administered in late lay on the gut microbiota.
Background	Maintaining optimal gut health is crucial for layer hens. This project compared the gut microbiota of healthy > 60-week-old hens facing production issues. Results showed geographical influence on the microbiota. Certain bacteria genera were identified as key drivers of production differences. Introducing live probiotic strains could positively impact the gut microbiota development. Five <i>Lactobacillus</i> strains administered post-hatch successfully colonised the gut microbiota of chicks, increasing alpha-diversity. <i>Lactobacillus saerimneri</i> showed promise as a single dose, live probiotic strain having impacts on the gut microbiota and persisting in the gut for over 20 weeks post-administration.
Outcomes and Implications	1. Geographical location influenced variation in gut microbiota among flocks older than 60 weeks of age. The diversity of the microbial community in flocks with production issues was lower when high and low-production flocks within the same company were compared. The

lower microbial diversity has been linked with microbial imbalance or dysbiosis, which could be favourable for opportunistic pathogens.

2. 'Seeding' the gut at hatch with *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, or *Lactobacillus saerimneri* led to the early colonisation of the chick gastrointestinal system, and increased alpha-diversity of the gut microbiota. This indicated the importance of the timing for the probiotic administration. Administration of *Lactobacillus* strains immediately after hatch resulted in a higher level of colonisation.
3. *In vitro* studies indicated that a single time point administration of *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, and *Lactobacillus saerimneri* inhibited the invasion of *Salmonella* into the caecal epithelia. *In vivo* trials are necessary to investigate whether the *Lactobacillus* strains tested in the study can reduce the load of pathogenic *Salmonella* serovars.
4. A single dose of *Lactobacillus saerimneri* at hatch induced persistent colonisation in the gastrointestinal system over 45 weeks. This finding is significant for the industry, and paves the way for further research into investigating whether single time point administration of *Lactobacillus* strains at hatch results in long-term inhibition of gut pathogens. Currently, the industry spends significant resources on continuous feeding of probiotic supplements throughout the lifespan of birds.
5. While not significant, pullets and birds in early lay that were 'seeded' with *Lactobacillus saerimneri* had higher average alpha-diversity compared with control birds. Further, *Lactobacillus saerimneri* administration at point-of-lay, post-transport led to colonisation of the gut for the 10-week experiment. Alpha-diversity decreased in birds administered *Lactobacillus saerimneri*. Many egg producers adopt the strategic use of probiotics that include feeding probiotic supplements before or after transport. Findings from this study suggest that administration of *Lactobacillus* strains tested in this study, at early lay or post-transport did not have a significant impact on gut microbiota.
6. The transport of pullets from rearing to production resulted in negative impacts on gut microbiota in terms of richness. Further studies are required to investigate whether a single time point administration of *Lactobacillus* strains tested in this study can maintain the richness and diversity of gut microbiota after transport from rearing to production.
7. A change in diet, not *Lactobacillus saerimneri* administration, significantly affected the gut microbiota profile in 70-week-old layer hens. In addition to supplementing *Lactobacillus* strains in older birds, a careful feed formulation is required.
8. In this study, several pen trials were conducted to orally administer *Lactobacillus* spp. Once birds are placed on farm, *Lactobacillus* spp. or other probiotics are fed to birds either via feed or through water. This project highlighted the importance of administration of *Lactobacillus* spp. immediately after hatch. Further work is necessary to investigate the delivery methods for the uniform and less laborious

	administration of viable probiotics in chicks in the hatchery or during transport.
Key Words	Probiotic, <i>Lactobacillus</i> , gut health, transport, late lay, gut microbiota, egg quality
Publications	Pending