Understanding the development of gut microbiota and effect of Salmonella Typhimurium infection on gut microbiota in layers

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Abstract

In the last two decades, the focus on chicken gut microbiota has increased exponentially due to its critical role in digestion, nutrient absorption, modulation of immunity, and protection against enteric pathogens. Thus, understanding its role in food-producing animals like chickens is essential because it can act as a source of zoonotic diseases. In chickens, the majority of the research in gut microbiota is focused on broilers and there was a need for detailed studies in layer chickens. In chapter 2, the development and composition of gut microbiota of hens housed in a single aged cage farm was studied from hatch to week 75. These hens were reared in cages from day old. The results showed that the richness of gut microbiota increased with the age and stabilized from a mid-lay phase (week 40). The gut microbiota was dominated by *Firmicutes* at an early age which was replaced by phylum *Bacteroidetes* at the later stages. The gut microbiota richness reduced significantly at week 18, after shifting the flock from the rear to the production shed. Flock's production performance was optimal till the end of the production cycle.

The layer birds are raised in different housing systems such as cage, free-range, and barn housing. Chapter 3 focused on understanding the gut microbiota development in three commercial layer flocks raised in different housing systems from hatch to end of production cycle i. e flock A (Free Range), flock B (Barn), and flock C (multi-age cage system). The taxonomic composition of gut microbiota in all three flocks was significantly different. All three flocks showed significant differences in the development pattern of gut microbiota. The richness of gut microbiota was significantly different between and within all three flocks. In flock A and B, the richness of gut microbiota increased with age. In flock C, the richness of gut microbiota increased up

to week 20 after which it remained relatively stable. The gut microbiota in flock A and B was dominated by phylum *Firmicutes* up to mid lay phase after which it was dominated by phylum Bacteroidetes. In flock C, phylum *Bacteroidetes* was dominant from an early lay phase. The richness of gut microbiota decreased at week 18 in two flocks (A and C) which could have been due to shifting to production shed, change in feed, transportation stress, the onset of lay.

Different enteric pathogens and stressors can disrupt the gut microbiota composition and significantly reduces the richness and diversity of gut microbiota. In chapter 4, the effect of *Salmonella* Typhimurium on gut microbiota was assessed. Further, this study focused on the effect of different potential stressors on *Salmonella* Typhimurium shedding and gut microbiota composition in pullets. The study showed persistent shedding of *Salmonella* Typhimurium in all the infected birds for up to week 8 post-infection. The *Salmonella* Typhimurium shedding increased significantly due to intramuscular injections and feed withdrawal. The intramuscular injections disrupted the gut microbiota composition leading to a significant reduction in abundance of phylum *Firmicutes* and increase in phylum *Proteobacteria* which could lead to dysbiosis in chickens.

This study produced robust data regarding gut microbiota development in layer chickens in different housing systems. In all 4 flocks, phylum Firmicutes dominated at an early age which was replaced by phylum Bacteroidetes in mid and late lay phases. The taxonomic composition of gut microbiota varied considerably between all 4 flocks. However, the production performance was high in all four flocks, which shows that different communities can produce similar productivity. Multiple variables were identified which could potentially change gut microbiota such as intramuscular injection, feed withdrawal, transportation, and the onset of lay. Typhimurium infection

at an early agecan disrupt the gut microbiota and reduce richness and diversity. Therefore, it is important to formulate interventions to reduce potential pathogen colonisation. This could be done by modifying diet using probiotics and prebiotics.

Thesis Declaration

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Name: Nitish Narendra Joat

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"It Always Seems Impossible Until It's Done – Nelson Mandela"

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List of Abbreviations

%	Percent
ºC	Degree Celsius
<	Less than
µL	microlitre
ach	acetylcholine
AECL	Australian Egg Corporation Limited
ANOVA	Analysis of variance
bp	Base pair
BPW	Buffered peptone water
BSA	Brilliance Salmonella agar
CC	Conventional cages
CFU	Colony forming unit
CI	Confidence interval
CORT	Corticosterone
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EC	Enriched colony cages
FCR	Feed conversion ratio
gm	Gram
h	Hour
min	Minute
mL	Millilitre
NE	Nor-epinephrine

nm	Nanometre
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Q-PCR	Quantitative Polymerase Chain Reaction
RDA	Redundancy analysis
RVS	Rappaport Vassiliadis soya peptone
Sec	Second
SPI	*Salmonella* Pathogenicity Island
spp	Species
TSR	Typhimurium- specific (genomic) region
XLD	Xylose lysine deoxycholate

List of Publications

Joat, N. N., Van, T. T. H., Stanley, D., Moore, R. J., Chousalkar, K. K. (2021) Temporal dynamics of Gut Microbiota in caged laying hens: A field observation from hatching to end of lay. *Applied Microbiology and Biotechnology*, 105 (11), 4719-4730.

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Williams, L., Joat, N. N., Khan, S., Chousalkar, K. K. Prevalence of *Campylobacter* in caged and free-range layer flocks: a longitudinal study. Clinical Research project report submitted by the DVM 1 student

Van, T. T. H., Joat, N. N., Stanley, D., Chousalkar, K. K., Moore, R. The gut microbiota in different tissue segements of laying hens (underprepration).

Peer reviewed symposium papers and presentation.

Joat, N. N. Chousalkar, K. K. (2018) Role of endocrine hormones in recrudescence of *Salmonella* Typhimurium in layers. 3 minute thesis competition. *The University of Adelaide*.

Joat, N. N., Moore, R. J., Stanley, D., Chousalkar, K. (2019) Does housing systems influence gut microbiota of pullets? *Australian Veterinary Poultry Association*.

Joat, N. N., Khan, S., Chousalkar, K. (2020) Role of corticosterone in recrudescence of *Salmonella* Typhimurium in Layers. 26th World Poultry Congress.*

*Could not travel because of COVID-19 pandemic.

N. Joat, T.T.H. Van, R. Moore, D. Stanley, K. Chousalkar. The development of gut microbiota in commercial layer flocks: multiple routes to high performance flocks. Finalist-Students awards, Australian Society for Microbiology, Adelaide, 2021.

Chapter 1 Literature Review

1.1 Introduction

The commercial egg layer industry is a leading animal food producing industry in the world. World egg production in 2017 reached 80.1 million metric tons, which is an increase of 25% from 2007 (PoultryTrends, 2019). The growth of the poultry sector is expected to increase as the human population will reach up to an estimated 9.6 billion in 2050, 70% of which settling in urban areas with estimated increased income by 2% a year (Mottet and Tempio, 2017). In the future, the demand for eggs could increase by 65% between 2005 and 2050, according to the projected data (Alexandratos and Bruinsma, 2012). In Australia, the annual egg production in June 2019 was 518.08 million dozen eggs, with a gross egg production value of AUD 828.2 million (AECL, 2019). Some of the key factors behind the exponential growth and profitability in the commercial layer industry are the low feed conversion ratio (FCR) and low space required per unit of proteins produced. Eggs are low cost highly nutritious animal food that contains protein, 13 different vitamins and minerals. Australia's per capita consumption of eggs has gone up to 247.1 in 2019 (AECL, 2019) from 226.8 in 2015 (AECL, 2015). In the commercial layer industry, maintaining low FCR with high egg production is essential, which requires feed with high energy and proteins. Naturally, the feed constitutes about 70% of the total production cost in layers. To balance the cost-economic ratio, it is essential to maintain high production throughout the life cycle, which requires intensive production systems. Birds raised in intensive production systems are prone to disease outbreaks, especially in geographical areas with changing climatic conditions (Diaz Carrasco, Casanova and Fernández Miyakawa, 2019). Thus, it is essential to maintain optimal health in birds.

The gut microbiota plays a crucial role in performing multiple functions that are essential for the host. It influences the host by regulating nutrient digestion, intestinal functions, and immune system modulation (Khan et al., 2020). These functions are accomplished in two ways- complex interaction between microbes and by using metabolites. These metabolites are produced by the member of the microbial community in the gut or by the host or obtained from the metabolism of the diet (Khan et al., 2020). The microbial metabolites involved in host-microbiota communication include tryptamine, short-chain fatty acids, indole and its derivatives, conjugated linoleic acids, and bile acids transformed by the gut microbiota (Khan et al., 2020). Thus, these functions are dependent on and can be altered by the change in gut microbiota composition. There are multiple host and environmental factors that can affect the gut microbiota composition in chickens.

Further, the gut microbiota richness and diversity have been correlated with chickens' overall health (Stanley, Hughes and Moore, 2014). The richness is defined as the number of operational taxonomic units present in the gut microbiota. Reduced richness has been correlated with diminished gut health (Valdes et al., 2018). The diversity of gut microbiota is an encompassing concept that also accounts for the amount and distribution of each bacterial species in the gut microbiota. The reduced diversity has been correlated with human diseases such as Inflammatory bowel disease, psoriatic arthritis and obesity (Valdes et al., 2018). Thus, the gut microbiota composition richness and diversity are important factors contributing to the host's health, and it is essential to understand factors affecting it. This review is focused on the effect of age, housing systems, diet, stress, *Salmonella* infection and stress-*Salmonella* dynamics on the gut microbiota in layer birds.

Salmonella Typhimurium is one of the most important zoonotic pathogens in Australia. In Australia, a total of 990 outbreaks were reported from 2001 to 2016 due to *Salmonella* spp. out of which 79% were foodborne or suspected foodborne transmissions (Ford et al., 2018). Most of these outbreaks (84%) were due to *Salmonella* Typhimurium (Ford et al., 2018). Among these foodborne transmissions, 50% of outbreaks of *Salmonella spp.* were associated with egg or egg-based products (Sauces, lightly cooked eggs, ice-cream, clustered etc.), out of which *Salmonella* Typhimurium was responsible for 95% of the outbreaks (Ford et al., 2018). However, in the USA (28%) (Jackson et al., 2013) and Canada (39%) (Bélanger et al., 2015), where eggs or egg products are the common vehicles for foodborne or suspected foodborne *Salmonella spp*. outbreaks, trends were different. In Australia, the predominant serotype was *Salmonella* Typhimurium (84%) compared to other countries where it was *Salmonella* Enteritidis (Jackson et al., 2013, Bélanger et al., 2015).

Further, it has been reported in earlier studies that *Salmonella* infection reduces the diversity and disrupts the gut microbiota in chickens (Mon et al., 2015, Khan and Chousalkar, 2020). Considering the zoonotic importance of *Salmonella* Typhimurium and reports suggesting its effects on gut microbiota composition in chickens, it is essential to investigate how *Salmonella* Typhimurium infection can affect the gut microbiota composition in chickens at an early age.

1.2 Gut microbiota in hens

Data regarding the establishment and development of gut microbiota is limited. Very few longitudinal studies focus on layer birds, as mentioned in the introduction (Videnska et al., 2014, Ngunjiri et al., 2019). These studies mainly describe the

development pattern of gut microbiota, which is an abundance of phyla *Proteobacteria* and *Firmicutes* at an early age, replacing phylum *Proteobacteria* with phylum *Bacteroidetes* with increasing age hens. Some cross-sectional studies conducted over a limited lifespan in layers support these findings (Callaway et al., 2009, Videnska et al., 2013). However, the composition of gut microbiota is not uniform and varies with studies.

Gut microbiota is a complex structure and is termed a forgotten organ by O'Hara and Shanahan (2006). The gut microbiota plays a crucial role in digestion, nutrient absorption immunity, gut health and physiology of a host (O'Hara and Shanahan, 2006, Rychlik, 2020). In layer birds, gut microbiota structure is complex (with a life span of 80 weeks) as compared to broilers birds (with an average life span of 35 to 42 days) (Khan et al., 2020). Naturally, the birds hatched in the nest acquires gut microbiota from the nest environment and the hens, which leads to the acquisition of diverse microbiota at an early age. However, gut microbiota development dynamics have changed with clean commercial practices such as hatching in incubators and fumigation. Most of the studies to understand gut microbiota were conducted in broilers could be mainly due to their shorter life span (Sun et al., 2018, Ocejo, Oporto and Hurtado, 2019, Stanley et al., 2013b, Stanley et al., 2013a). Few studies are focused on layers; however, none of them has studied the development of gut microbiota over the commercial lifespan of hens. Some of the earlier studies were focused on the part of the lifespan of egg layers or a specific segment of the gastrointestinal tract (GI) tract (Videnska et al., 2014, Ngunjiri et al., 2019).

Most of the available data is based on the analysis of caecum samples in hens. The data from Videnska et al. (2014) showed that gut microbiota development occurs in four stages. The first stage of microbiota occurs in the first week of a chick's life. In the

first few days, *Proteobacteria* dominated the gut microbiota profile by forming nearly 50% of the total microbial population. The dominant members of the phylum *Proteobacteria* were the family *Enterobacteriaceae* and genus *Escherichia*. The remaining part of the microbiota was made up of the phylum *Firmicutes* with the dominant family *Lachnospiraceae*. The second stage of gut microbiota development was between week 2 to week 4 of the bird's age. In this stage, the abundance of phylum *Proteobacteria* decreased significantly to less than 10% of the total gut microbiota, while phylum *Firmicutes* was dominant, forming 90% of the gut microbiota. The significant dominance of families *Lachnospiraceae* and *Ruminococcaceae* was observed in phylum *Firmicutes*. Stage three of the development was between 2-6 months of the chicken's age. This stage was characterised by a succession of phylum *Firmicutes* and its replacement by phylum *Bacteroidetes*. Another study supported these findings in which gut microbiota from 8-week-old hens showed the dominance of phyla *Firmicutes* and *Bacteroidetes* (Cui et al., 2017). By the age of 6-months, *Bacteroidetes* dominated the phylum, making 55% of total gut microbiota (Videnska et al., 2014). The abundance of minor phyla was less than 1% of total gut microbiota, including *Deinococcus-Thermus*, *Euryarchaeota* and *Fusobacteria*. In another study, caecal samples collected at week 18 of flock's age showed that phyla *Bacteroidetes* and *Firmicutes* dominated gut microbiota and the minor phyla included *Proteobacteria, Fusobacteria, Actinobacteria* and *Deferribacteres* (Nordentoft et al., 2011*)*. The gut microbiota of hens above seven months of age was the fourth and last stage of gut microbiota succession. In this stage, the microbiota was dominated by phyla *Firmicutes* and *Bacteroidetes*, each of them forming approximately half of the total gut microbiota (Videnska et al., 2014). These results can be supported by a recent study focusing on the gut microbiota composition in the late production phase. It showed

that the dominant phylum in the late lay phase was *Bacteroidetes,* followed by phylum *Firmicutes* (Adhikari et al., 2020). At stage 4, the phylum *Proteobacteria* reappeared at 34 weeks, forming around 5% of the microbiota. However, the dominating members of the phylum *Proteobacteria* were *Desulfovibrio* and *Succinivibrio* (Videnska et al., 2014). The abundance of *Desulfovibrio sp.* could be related to the increased abundance of phylum *Bacteroidetes*. Some members of phylum *Bacteroidetes* can release sulphate from sulphated chondroitin or mucin produced by host cells which can be reduced to H_2S by *Desulfovibrio sp.* (Rey et al., 2013).

In contrast, Zhao et al. (2013) reported gut microbiota composition showing *Firmicutes* (37%), *Proteobacteria* (33%), *Actinobacteria* (18%) and *Bacteroidetes* (10%) at the age of 35 weeks in breeder hens. This difference in microbiota could be due to differences in sample collection, i. e. for this study, faecal samples were collected at a single point compared to earlier studies in which cecal and ileal contents were collected over time the period. Further, in a separate study, the gut microbiota composition in hens at the age of 30 weeks showed a high abundance of phyla *Firmicutes* and *Bacteroidetes;* however, all five phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*) were present (Cui et al., 2017).

1.3 Taxonomic composition of gut microbiota

Various studies have described the gut microbiota in chickens at different taxonomic levels, such as phylum, family, and genus level (Videnska et al., 2014, Ngunjiri et al., 2019, Rychlik, 2020). The common phyla reported in the mentioned studies were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*. Therefore, it is vital to understand the functions and composition of these phyla.

Phylum *Proteobacteria* includes Gram-negative, non-spore-forming bacterial families such as *Desulfovibrio, Sutterrella, Parasutterella, Anaerobiospirillum* etc. *Proteobacteria* also includes pathogens like *Campylobacter, Escherichia, Helicobacter, Salmonella* and *Vibrio (Rychlik, 2020).* The phylum *Proteobacteria* has been associated with dysbiosis (Litvak et al., 2017). Further, increased *Proteobacteria* abundance has been correlated to gut inflammation in humans, including patients with inflammatory bowel disease and colorectal cancer (Litvak et al., 2017).

Firmicutes are one of the major phyla in chickens gut microbiota. Some of the important families include *Lachnospiraceae, Ruminococcaceae, Lactobacillaceae*, *Veillonellaceae* and *Erysipelotrichaceae (Rychlik, 2020)*. The analysis of caecal microbiota in layer chicken showed that *Lachnospiraceae* and *Ruminococcaceae* are dominant in gut microbiota at two weeks to 4 weeks, making 90% of the total gut microbiota. In the family *Lachnospiraceae*, genera *Balutia* and *Roseburia* were dominant, while in the family *Ruminococcaceae,* genus *Faecalibacterium* was dominant (Videnska et al., 2014). Phylum *Firmicutes* are mainly responsible for butyrate production (Walker et al., 2005, Duncan et al., 2007, Louis and Flint, 2007), which is the preferred substrate of intestinal epithelial cells (Roediger, 1982, Scheppach, 1994, Ahmad et al., 2000). Genus *Faecalibacterium* is also associated with a gain in body weight and improved FCR in broilers (Stanley et al., 2016). *Lactobacillus* is another important genus in phylum *Firmicutes.* Genus *Lactobacillus* is a commensal micro-organism and is considered beneficial for gut health and growth in chickens (Huang et al., 2004). Although earlier studies also indicated that not all *Lactobacillus* spp. are beneficial, some of which might be responsible for reduced lipid intake, loss of dietary energy and poor performance in chickens (De Boever et al., 2000, Stanley et al., 2012a).

Phylum *Bacteroidetes* includes families *Rikenellaceae, Bacteroidaceae, Provotellaceae and Porphyromonadaceae*. Members of phylum *Bacteroidetes* are associated with the production of propionate and acetate (Wrzosek et al., 2013, Yang et al., 2013), which are less preferred substrates for intestinal epithelial cells. Phylum *Bacteroidetes* and families *Lachnospiraceae* and *Ruminococcaceac (*phylum *Firmicutes)* together represent the unique gut microbiota profile in the caecum (Rychlik, 2020).

Phylum *Actinobacteria* is Gram-positive, strictly aerobic bacteria, including family *Coriobacterium* with genera *Olsenella* and *Collinsella* and family *Bifidobacteriaceae* with genus *Bifidobacterium* (Rychlik, 2020). Genus *Bifidobacterium* is associated with lactic acid production and improved gut health in chickens (Binda et al., 2018). Some of the minor phyla like *Fusobacterium* are opportunistic pathogens associated with colon cancer in humans; however, their role in chickens is yet not known (Kelly, Yang and Pei, 2018). Generally, the abundance of *Fusobacterium* above 5% indicates gut health problems in chickens (Rychlik, 2020).

This data shows that the gut microbiota composition was significantly different between all the studies. These differences can be attributed to multiple factors which are discussed ahead. Although gut microbiota composition was significantly different, some common patterns such as the dominance of *Bacteroidetes* in adult hens or reduced abundance of phylum *Proteobacteria* after week two were observed in all the studies. However, detailed studies focusing on understanding the pattern of gut microbiota from hatch to end production cycles are lacking. In the future, to positively modulate the gut microbiota for better production and gut health, it is essential to understand the baseline development of gut microbiota in field conditions.

1.4 Factors affecting gut microbiota in Hens

Multiple studies in chickens gut microbiota have revealed significant differences in the composition of gut microbiota (Videnska et al., 2014, Ngunjiri et al., 2019, Ocejo, Oporto and Hurtado, 2019). The majority of these studies were focused on broilers (Stanley et al., 2013b, Stanley, Hughes and Moore, 2014, Stanley et al., 2012a); however, some studies in layers also produced similar results (Videnska et al., 2014, Ngunjiri et al., 2019). Some of the significant variables in the above studies included different genetic sources, feed formulations, varied climatic conditions, and management protocols. Even in a controlled study, significant differences in the gut microbiota were observed (Stanley et al., 2013b). The gut microbiota in chickens can be affected by multiple hosts related (age, sex, genetics) and environmental (housing system, geological location and climatic conditions) factors which are discussed in an earlier study (Kers et al., 2018). This review focuses on the effect of age, housing systems, diet, stress, and *Salmonella* infection on the gut microbiota in hens.

1.4.1 Housing systems

Globally, the layers are housed in various housing systems, including Cage, Free-range, and Barn type. In the last two decades, multiple studies have been conducted to understand the impact of different housing systems on the welfare of hens (Meseret, 2016, Duncan, 2001, Ferrante, 2009, Janczak and Riber, 2015). The European Union in 1999 decided to phase out cage housing and replace it with alternate housing systems for the layer chickens (Appleby, 2003). Recently, New Zealand and Canada also announced to follow a similar policy (Hartcher and Jones, 2017). In Australia, the commercial layers are housed in different systems i. e. Cage (40%), Free-range (47%)

Barn (11%), and a small percentage of speciality eggs (2%) (AECL, 2019). Acquisition of gut microbiota is continuing process and starts from the birth of the host.

In mammals, gut microbiota acquisition starts from the foetal stage through the placenta (Aagaard et al., 2014). In avian species, the placenta does not exist, and thus the chances of transfer of maternal gut microbiota are less. However, an earlier study has indicated that embryonated eggs contain microbiota that might have originated from the laying hen (Ding et al., 2017). This was a stand-alone study, and more research is required to confirm these results. Chicks acquire microbiota from the nest environment post-hatching in a natural environment, which originates from maternal hen (Apajalahti, Kettunen and Graham, 2004). However, in the commercial setup, eggs are hatched in hatcheries and transported to the rearing farms. The hatcheries are cleaner and have a lower bacterial load. Thus hatcheries and rearing farms become the primary source of microbiota which initially colonises the chicks (Apajalahti, Kettunen and Graham, 2004). An earlier study compared gut microbiota development in slow-growing meat-type chickens reared in the cage and free-range system, which reported significant differences (Sun et al., 2018). The results showed that the phylum *Actinobacteria (*family *Coriobacteriaceae)* was more abundant in the free-range group than the cage group (Sun et al., 2018). Phylum *Actinobacteria* also includes genus like *Bifidobacterium*, which are responsible for better gut health (Pokusaeva, Fitzgerald and van Sinderen, 2011).

Furthermore, a study conducted in intensive farming and free-range farming in broiler production showed a significantly distant gut microbiota profile in broilers (Ocejo, Oporto and Hurtado, 2019). The study's primary finding showed that the gut microbiota profile was richer and more complex in free-range broilers. However, this difference cannot be associated only with housing systems because breeds of chickens were

different in both housing systems (intensive farming- Broiler (Ross-308), Free-range-Slow-growing chicken (Sasso-T451A) (Ocejo, Oporto and Hurtado, 2019). A study conducted in Dagu chickens observed the effect of the housing system in caecal microbiota composition. The authors concluded that Dagu chicken raised in the free-range system showed a higher abundance of bacteria associated with the glycan metabolic pathway (Xu et al., 2016). This study further concluded that the ratio of *Firmicutes/Bacteroidetes* is lower in Dagu chicken with an increased abundance of *Bacteroidetes (Xu et al., 2016)*. The study in broiler chickens (Ross) suggested that outdoor access enriches the abundance of *Bifidobacterium* in caeca and ileum (Gong et al., 2008). A recent study focused on the effect of housing types in the late lay phase in layer hens concluded that the housing systems significantly affect the gut microbiota composition in laying hens (Adhikari et al., 2020). In this study, laying hens housed in conventional cages (CC) and enriched colony cages (EC) were monitored for the gut microbiota composition. The EC hens showed a high abundance of *Proteobacteria* (genus *Campylobacter*) and higher alpha diversity than CC hens. Further, the study also showed significant differences in Beta diversity and functional differences in the gut microbiota. The study concluded that the housing system significantly affects the microbiota composition in chickens. Another study conducted in laying hens compared gut microbiota composition between caged and cage-free hens (Hubert et al., 2019). It concluded that the cage-free hens showed higher gut microbiota diversity than caged hens. However, these studies were conducted with controlled variables, which included the same genetic source, same feed, and controlled environment, which is impossible in field conditions. Further, in both studies, the hens were not reared from day old, affecting the gut microbiota structure. The major influencing factors in housing conditions also include litter and floor types (Diaz Carrasco, Casanova and Fernández

Miyakawa, 2019). Extensive research has been conducted on the effect of litter on gut microbiota in broilers due to the deep litter rearing systems (Diaz Carrasco, Casanova and Fernández Miyakawa, 2019). The birds on the floor continuously peck and ingest litter material which contributes toward the gut microbiota. Interns, the litter consist of faecal material from the birds, which constitutes microbiota in the litter (Pan and Yu, 2014). The choices of litter material affect the composition of gut microbiota (Wang et al., 2018). Further, reusing litter is a common method to reduce costs (Diaz Carrasco, Casanova and Fernández Miyakawa, 2019). This could be beneficial or harmful depending on the litter conditions. One of the earlier studies has indicated that reused litter could lead to an increased level of *Faecalibacterium prausnitzii*, a commensal butyrate-producing species (Wang, Lilburn and Yu, 2016). The butyrate is the preferred substrate of intestinal epithelial cells and important short-chain fatty acid (Roediger, 1982, Scheppach, 1994, Ahmad et al., 2000). However, if the litter is damp and moist, the chances of pathogen transmission could increase and lead to health risks (Cressman et al., 2010, Oakley et al., 2013). In the layer industry. rearing practices are different from broilers. The layer chicks reared on a floor (dirt or concrete) or in cages up to rearing and then transferred to the different production systems. There is no specific data on the effect of floor type in the layer birds; however, dirt floor raring can be compared to deep litter systems in broilers. A study in Shaoxing ducks showed significant variation in gut microbiota composition in plastic mesh floor, and litter floor raised ducks (Wang et al., 2018). In the group raised on plastic mesh, Firmicutes and *Bacteroidetes* were dominant phyla, while in the group raised on litter, *Proteobacteria* and *Bacteroidetes* were dominant. Phylum *Proteobacteria* contains many opportunistic pathogens. This could have happened because a litter floor raised group had to access a pond, which increased the chance of acquiring pathogenic

genera. Further, the study suggested that plastic mesh rearing could be more beneficial for the ducks (Wang et al., 2018). In another study, ducks reared in cages showed significantly lower gut microbiota diversity and richness than floor reared ducks (Zhu et al., 2020). In housing systems, stocking density is also considered an important factor as it can directly affect the flock's performance. It has been reported earlier that increased stocking density reduced body weight, body weight gain and increased feed intake in broilers and ducks (Guardia et al., 2011, Abo Ghanima et al., 2020). Further, it also reduced gut microbiota abundance in broilers and meat quality in ducks (Guardia et al., 2011, Abo Ghanima et al., 2020). Specific data regarding the effect of stocking density on layer performance and gut microbiota composition is not available; however, it can be assumed that increased stocking density can adversely affect the gut microbiota and performance of the birds.

Most of the above studies are focused on broilers, and there is a scarcity of data related to layer flocks. The studies conducted in layer flocks were controlled studies and did not mimic the field conditions. The housing systems are designed and built according to the field conditions and are significantly different. It is impossible to follow similar guidelines for housing systems throughout the industry. Thus, data obtained from the controlled studies has its limitations. To understand the effect of housing systems on gut microbiota, more field studies are required.

1.4.2 Age

Age is one of the important factor which affects the gut microbiota composition in chickens (Ngunjiri et al., 2019, Ocejo, Oporto and Hurtado, 2019). Earlier studies in broilers have shown age-dependent variation in the gut microbiota composition (Chambers and Gong, 2011, Stanley et al., 2013b). The longitudinal gut microbiota

studies in layers showed age-driven development (Videnska et al., 2014, Ngunjiri et al., 2019). These findings can be supported by different cross-sectional studies in layers (Callaway et al., 2009, Adhikari et al., 2020). However, it is important to note that the structure and composition of gut microbiota in each study were significantly different. Earlier studies also suggested that the gut microbiota profile in chickens differs with age and is associated with respected specific age groups (Ocejo, Oporto and Hurtado, 2019, Videnska et al., 2014). The details of age dependant development of gut microbiota have been explained in an earlier section (gut microbiota in hens). The general observation in the development of gut microbiota in layers suggests that the acquisition and development of gut microbiota were rapid at an early age which slows down over time (Ngunjiri et al., 2019). It also suggested that the gut microbiota become more resilient to change with age. In general, at an early age, phyla *Proteobacteria-Firmicutes* dominates the gut microbiota, while after 30 weeks of age, phyla *Firmicutes* and *Bacteroidetes* become dominan (Videnska et al., 2014). The exact mechanism of age driven gut microbiota is not yet understood. However, it could be related to the physiological functions of the host. At an early age, the high energy requirement for rapid growth could be related to the dominance of phylum *Firmicutes*. Phylum *Firmicutes* include families that can be correlated with butyrate production, which is the primary substrate for intestinal epithelial cells (Videnska et al., 2014). The high abundance of *Firmicutes* in the rear and early lay phase means higher butyrate production, that generates a high amount of energy. This energy is utilised for the overall growth and egg production. In the mid and late lay phase, the energy demand reduces as it is primarily required for production, and thus some proportion of *Firmicutes* get replaced by *Bacteroidetes*. The primary products of phylum *Bacteroidetes* are propionate and acetate, which are not the primary substrate of

intestinal epithelial cells (Videnska et al., 2014). Thus, the dominance of phylum *Bacteroidetes* could be related to sustenance in the mid and late lay stages.

1.4.3 Diet

Intestinal gut microbiota is a function of the diet itself because dietary ingredients also act as nutrients for bacterial growth (Gabriel et al., 2006). Diet is one of the most important part of the poultry production system owing to its importance in economics and its critical role in health and production. The cost of the feed accounts for approximately 70% of the total production cost. A good quality diet is essential for sound production and maintaining the overall health of the flock. The majority of the trials focused on diet and gut microbiota were conducted in broilers because of the shorter life span (Stanley et al., 2012a, Stanley et al., 2012b, Stanley et al., 2013a, Oakley et al., 2013). However, due to the longer life span (80 weeks), the physiological requirements of layers are different; thus, the diet of layers differs significantly. The layer diet used in the commercial industry is divided into different phases. The basic parameters considered are given in Tables 1& 2. (Leeson and Summers, 2009).

Earlier literature has reviewed different commercial products used to modulate the gut microbiota in layer birds (Khan et al., 2020, Chambers and Gong, 2011). These products include prebiotics, probiotics, immunostimulants, acidifiers and essential oils. Prebiotics are non-digestible foods or food products that are not digested by a host and help promote the growth and activity of gut microbiota (Chambers and Gong, 2011). It includes inulin, beta-glucans, galactooligosaccharides, xylooligosaccharides, fructooligosaccharides, pectin and resistant starch (Khan et al., 2020). The probiotics are the live organism that helps enhance gut health when administered in sufficient quantity (Chambers and Gong, 2011). Generally, probiotics include Gram-positive

bacteria, yeast, and moulds. Commonly used probiotics include *Streptococcus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, and *Bacillus* (Khan et al., 2020). The mechanism through which probiotics act is varied. They modulate the immune system, produce SCFA, reduce or prevent pathogen colonisation, and help maintain the gut's structure and integrity (Chambers and Gong, 2011, Khan et al., 2020).

Few studies are conducted in layers to understand the development of gut microbiota (Videnska et al., 2014, Ngunjiri et al., 2019, Xing et al., 2019). These studies used different diets and showed significant differences in composition and abundance of different taxa in gut microbiota. Although the basic feed parameters (Protein %, Fat %, Metabolic energy) remain the same in the commercial poultry industry, the actual feed ingredients and their proportion are different.

1.4.4 Stress

The concept of stress is a complex and multifaceted topic and is defined in different ways (Murray, Baber and South, 1996). According to Dhabhar and Mcewen (1997), "Stress is a constellation of events, consisting of stimulus that precipitates a reaction in the brain, which activates physiological response fight-or-flight systems in the body". There are three essential components in the stress mechanism in the host. First, stimulus, also called stressors, is responsible for triggering stress, for example, heat & cold stress, the onset of laying, feed change, vaccination, transport, etc. The second component is a reaction in the brain, i. e., stress perception, which is a stress mechanism triggered by stressors. It includes activation of multiples pathways in a host by secretion of different neurotransmitters and neuroendocrine hormones. The final component is a response to stress in which the host exhibits a fight or flight response. However, in commercial layers, the stress response can affect gut

microbiota and help shed enteric pathogens colonised in the gut (Holt et al., 1994, Zhu et al., 2019).

The evolution of stress response in animals was linked to safety mechanisms as it forms the basis for fight or flight response that is essential for survival (Dhabhar, 2009, Hughes et al., 2009). However, recent studies showed that the same stress response is responsible for providing a conducive environment to pathogens indirectly by suppressing the immune system and directly through stress hormones which trigger the shedding of pathogens from the host and adversely affect the gut microbiota structure (Lyte, 2004, Zhu et al., 2019).

1.4.4.1 Neuroendocrine Stress Pathway

The stressors activate the neuroendocrine pathway, which ultimately results in a stress response. The stress response is carried to the host organs by two major groups of stress hormones catecholamines and glucocorticoids (Verbrugghe et al., 2012). The catecholamines include epinephrine and norepinephrine, and glucocorticoids include corticosterone.

The secretion of catecholamines is regulated by the sympathetic nervous system (SNS). Under stress, the sympathetic nervous system activates preganglionic sympathetic nerve fibres in the adrenal medulla, which secretes acetylcholine (ach). The acetylcholine acts on the adrenal medulla, which secrets epinephrine in the bloodstream. Furthermore, sympathetic nerve fibres start secreting norepinephrine in lymphoid organs as sympathetic nerve fibres are closely associated with immune cells (Webster Marketon and Glaser, 2008, Yang and Glaser, 2002).

Also, stress activates the Hypothalamic-Pituitary-Adrenal axis (HPA). The paraventricular nucleus of the hypothalamus secretes corticotrophin-releasing factor

(Webster Marketon and Glaser, 2008). The corticotrophin-releasing factor binds with the corticotrophin-releasing factor subtype1 on the adrenal pituitary (Taché and Brunnhuber, 2008). Interns the adrenal pituitary releases the adrenocorticotropic releasing hormone in the bloodstream, which acts on the adrenal medulla leading to the secretion of glucocorticoids, i. e., corticosterone in birds. These glucocorticoids bind to glucocorticoid receptors in the bloodstream.

1.4.4.2 Effect of stress on gut microbiota

The layer chickens undergo multiple stressful events from hatch to the end of the production cycle. Some of the important stressors in layers are heat, the onset of lay, transportation, feed withdrawal, etc. These stressors have far-reaching effects on the gut microbiota, growth performance, physiology, immunity, intestinal morphology and overall health (Shi et al., 2019, Song et al., 2018, Xu et al., 2018). Multiple studies have illustrated these outcomes in broilers over the last few decades (Yang, He and Zheng, 2007, Quinteiro-Filho et al., 2010). These stressors are responsible for high mortality and weight loss in broiler birds (St-Pierre, Cobanov and Schnitkey, 2003, Cramer et al., 2018). The stressors could cause intestinal injuries that lead to intestinal barrier dysfunctions (Yang, He and Zheng, 2007). Further, stressors in poultry could lead to injury to the intestine and change normal intestinal microbiota, resulting in pathogen colonisation (Burkholder et al., 2008).

A study conducted in laying hens showed a significant reduction in phylum *Firmicutes* and increased phylum *Bacteroidetes* due to heat stress (Zhu et al., 2019). This altered abundance of gut microbiota could be related to obesity and liver diseases (Chen et al., 2011). These results were contradicted by another trial in laying hens where there was no significant difference in the abundance of phyla *Firmicutes* and *Bacteroidetes*.

(Xing et al., 2019). However, a study in Shaoxing ducks showed a significant increase in phylum *Firmicutes* after exposure to high temperatures (Tian et al., 2020). Further, a study in wild birds showed a decrease in members of phylum *Firmicutes* and a decrease in the abundance of pathogenic genera. It also suggested the possibility of beneficial effects of stress in reducing the risk of infection in the host (Noguera et al., 2018).

From the above studies, it can be concluded that stress is an important factor that affects the gut microbiota composition. However, the data regarding the effect of stress in layer birds from hatch to the end of the production cycle is not available. Further, studies in layer birds also show conflicting reports regarding the change in the composition of microbiota after exposure to stress (Xing et al., 2019, Zhu et al., 2019). Thus, it is essential to understand how the stressors will impact gut microbiota in layer hens. It is also important to know if the changes in gut microbiota composition due to stress are reversible. Because there is no data available on these aspects and further studies are required.

1.5 Gut microbiota and *Salmonella* interaction

As an enteric pathogen, the gut microbiota plays a critical role in the survival and colonisation of *Salmonella* in the gut. Gut microbiota (cecal content) from birds older than three weeks inoculated in day-old chicks conferred protection against subsequent *Salmonella* Enteritidis challenge. However, two-day-old chicks administered with cecal contents from 35-week-old hens post-*Salmonella* Enteritidis infection failed to protect chickens (Varmuzova et al., 2016). From this study, it could be concluded that the gut microbiota from chicken older than three weeks protected against *Salmonella* Enteritidis infection as compared to the gut microbiota from young chickens (1 week

of age), and pre-infection administration of cecal content provided protection compared to post-infection administration against *Salmonella* Enteritidis (Varmuzova et al., 2016). This might be because, after two weeks of age, the microbiota is dominated by phyla *Firmicutes* which are primarily responsible for producing short-chain fatty acids, mainly butyrate (Videnska et al., 2014, Polansky et al., 2016). The butyrate is essential for the energy metabolism in the host epithelial cell. They are known to suppress type III secretion systems in *Salmonella*, associated with cell invasion (Van Immerseel et al., 2003, Gantois et al., 2006).

On the other hand, *Salmonella* Enteritidis infection at an early age in layer chicks influenced the composition of gut microbiota (Mon et al., 2015). Infected chickens showed a significant reduction in microbial diversity (Mon et al., 2015). The family Enterobacteriaceae dominated the *microbiota* with a substantial shift in phyla structure, leading to the expansion of phylum *Proteobacteria* and reduction of *Firmicutes*. On the other hand, in the non-infected group, *Firmicutes* were dominant, followed by *Proteobacteria*. An earlier study in mice showed that *Salmonella* Typhimurium infection reduces *Clostridia* from gut microbiota using its virulence factors (Rivera-Chávez et al., 2016). These *Clostridia* are responsible for producing butyrate in the gut (Rivera-Chávez et al., 2016). The reduction in *Clostridia* increases the free-flowing oxygen through intestinal epithelium leading to the aerobic expansion of *Salmonella* Typhimurium in the gut lumen (Rivera-Chávez et al., 2016). *Salmonella* Typhimurium infection in mammals leads to colonisation in the gut leading to the inflammatory response by the host (Thiennimitr, Winter and Bäumler, 2012). As mentioned above, the type III secretion system helps *Salmonella* to invade intestinal cells triggering the immune response (neutrophils migration) (Thiennimitr, Winter and Bäumler, 2012). The migration of neutrophils kills bacteria invading tissue; however,

the luminal *Salmonella* Typhimurium thrives due to a sizeable fraction of gut microbiota (Thiennimitr, Winter and Bäumler, 2012).

Further, secretion of neutrophils in gut lumen during *Salmonella* Typhimurium infection is associated with the change in gut microbiota composition leading to depletion of *Bacteroidetes* and *Closteridiales* (Thiennimitr, Winter and Bäumler, 2012). Thus, a change in colonocyte metabolism due to *Salmonella* infection can change the gut microbiota composition (Litvak, Byndloss and Bäumler, 2018). A *Salmonella* Typhimurium challenge study conducted in 18-weeks old pullets showed disruption of gut microbiota in challenged groups. The abundance of genera such as *Faecalibacterium*, *Enorma*, *Blautia*, *Sellimonas* decreased significantly in *Salmonella* Typhimurium challenged group, causing dysbiosis in chickens (Khan and Chousalkar, 2020). This study also reported significantly lower alpha diversity of gut microbiota in the *Salmonella* Typhimurium infected group (Khan and Chousalkar, 2020).

In chickens, comparative analysis between microbial communities of the non-infected and infected group showed a significant reduction in the abundance of families *Lachnospiraceae* and *Rumminococcaceae* in the infected group (Mon et al., 2015). These families are primely associated with SCFA and specifically butyric acid production (Videnska et al., 2014). It has been reported that butyric acid suppresses the invasive gene expression (SPI-I) in *Salmonella (Gantois et al., 2006, Van Immerseel et al., 2005)*. Earlier studies showed the use of butyric acid reduced the colonisation and shedding of *Salmonella* in chickens (Van Immerseel et al., 2005, Fernández-Rubio et al., 2009). The family *Lachnospiraceae* also showed an inverse relationship with family *Enterobacteriaceae* in gut microbiota (Mon et al., 2015), contrary to an earlier study that observed only minor modifications in chicken gut microbiota without any reduction in family *Lachnospiraceae* (Videnska et *al., 2013).*

This can be attributed to different study designs, different ages of infection and different sampling points. The commercial layer chickens do not receive gut microbiota from adults like other species due to hatching in a clean hatchery environment (Crhanova et al., 2011). Thus, newly hatched chicks have significantly less microbiota which opens up the avenue for microbial colonisation in the gut. So, the primary source of the gut microbiota could be the diet and environment to which chicks are initially exposed, including a hatchery, transport, and rearing farm. (Apajalahti, Kettunen and Graham, 2004, Crhanova et al., 2011). The susceptibility to pathogen colonisation at an early age is very high, and the pathogens colonised at an early age affects the overall development of gut microbiota (Mon et al., 2015).

The reduction of the butyric acid-producing member in gut microbiota due to *Salmonella* infection and the role of butyric acid in conferring the protection against *Salmonella* indicates the complex nature of gut microbiota relationship between *Salmonella* colonisation and shedding. However, the studies focusing on gut microbiota and *Salmonella* dynamics are limited to broilers due to its direct effect on FCR and weight gain (Zhao et al., 2013, Stanley et al., 2013a, Rinttilä and Apajalahti, 2013, Meng et al., 2014, Schokker et al., 2015). The studies in layer chicks have been predominantly focused on early age and ultimately failed to provide scientific data on long term effects associated with persistent shedding of *Salmonella,* which currently is the concern for the egg industry (Mon et al., 2015).

The above studies provide evidence of interaction between *Salmonella* infection and gut microbiota. It can also be concluded that salmonella infection disrupts the normal gut microbiota composition to colonise the GIT, especially in chicks. The disruption of gut microbiota at an early age could impact gut health and overall health in chickens. In Australia, *Salmonella* Typhimurium is an important food -safety concern (Moffatt et

al., 2016). Thus, it is essential to understand the impact of *Salmonella* Typhimurium infection on gut microbiota composition at an early age in chickens.

1.6 Understanding the stress and *Salmonella* Recrudescence

The earlier studies have established the effect of stress and *Salmonella* infection on gut microbiota. However, research in the last two decades has highlighted the interaction between stress hormones and pathogens. The effects of stress hormones on the host immune system have been extensively investigated and are primarily associated with its impact on the course of the infection. However, earlier research indicates that stress hormones affect pathogens and alter host-pathogen interaction outcomes (Lyte, 2004). The study of stress, stress hormones and their interaction with microbes are termed endocrinal microbiology (Lyte, 2004). The endocrinal microbiology is an intersection of microbiology and neuroendocrine physiology (Lyte, 2004). Earlier research in endocrinal microbiology suggests that bacteria in the host can exploit the neuroendocrine alteration due to stress to its advantage as a growth signal or pathogenic processes (Lyte, 2004, Freestone et al., 2008). Animals are exposed to different types of stresses, such as food withdrawal, transport, temperature fluctuations linked to increased persistent shedding of foodborne pathogens, disease susceptibility, and carcass contamination with foodborne pathogens (Burkholder et al., 2008, Rostagno, 2009).

In vitro research showed that norepinephrine (NE) can promote growth and increase motility in *Salmonella* Typhimurium, which was attributed to the role of NE in supplying iron to *Salmonella* Typhimurium using catecholamine specific iron transport systems (Bearson and Bearson, 2008, Bearson et al., 2008, Methner et al., 2008). The chickens pre-treated with NE and infected with *Salmonella* Enteritidis showed a

significant increase in caecal colonisation and systemic spread over the liver compared to non-treated chickens (Methner et al., 2008). In chickens, multiple stress factors are involved, for example, heat stress or feed withdrawal. These stressors may increase the chances of attachment and colonisation of *Salmonella* Enteritidis in chickens (Burkholder et al., 2008). The stress associated with feed withdrawal and transport has been shown to increase the caecal carriage of *Salmonella* in broilers (Rigby and Pettit, 1980, Bolder and Mulder, 1983). Furthermore, the susceptibility of *Salmonella* infection increases with feed and water withdrawal stress (Bierer and Eleazer, 1965). The stress due to feed withdrawal resulted in an increase in crop contamination by *Salmonella* before slaughter (Holt et al., 1994, Line et al., 1997, Ramirez et al., 1997, Corrier et al., 1999). Studies previously showed increased intestinal colonisation and the systemic spread of *Salmonella Typhimurium* in mice pre-treated with NE (Kuriyama, Machida and Suzuki, 1996, Methner et al., 2008). These findings correlated with the results of earlier studies establishing that stress causes increased frequency and persistence of *Salmonella* infection (Previte et al., 1970, Previte, Alden and Egbert, 1973).

Further, in an in vivo trial, pigs infected with NE-treated *Salmonella* Typhimurium resulted in increased replication of bacteria in various tissues (Toscano et al., 2007). In earlier studies, it was noted that the infection of *Salmonella* Typhimurium in pigs could remain undetected, leading to continuous shedding due to stress caused by transportation or feed withdrawal (Boyen et al., 2008, Isaacson et al., 1999, Martín-Peláez et al., 2009). The effect of feed withdrawal showed that the intestinal load of *Salmonella* Typhimurium increases on feed withdrawal (24 hours) in pigs which correlated with serum cortisol levels. Further, research confirmed the findings when pigs injected with dexamethasone demonstrated stress-induced shedding

(Verbrugghe et al., 2011). In vitro trials conducted to understand the shedding mechanism of *Salmonella* Typhimurium revealed that the replication of *Salmonella* Typhimurium was driven by cortisol, not by epinephrine or norepinephrine, in porcine macrophages (Verbrugghe et al., 2011). Also, the transcriptomic analysis showed that cortisol does not directly affect the gene expressions or growth of *Salmonella* Typhimurium in an enriched medium which implied that the intracellular proliferation of *Salmonella* Typhimurium could be driven by the effect of cortisol on macrophages (Verbrugghe et al., 2011). The replication of *Salmonella* Typhimurium in the pigs is driven by cortisol induced cytoskeletal rearrangement of Salmonella containing macrophages (Verbrugghe et al., 2016). This results in the formation of *Salmonella* Containing Vacuoles (SCV), a replicative niche (Verbrugghe et al., 2016). The bacterial replication in SCVs ultimately leads to the formation of SCV containing a single bacterium (Verbrugghe et al., 2016). These results were further confirmed by a proteomic and transcriptomic analysis which showed increased expression of cytoskeletal associated proteins (tubulin beta chain (TUBB2A), F-actin capping protein subunit 2 (CAPZB), thymosin beta-4 (TMSB4X), actin-related protein 3B (SCT3), and tropomyosin 3 (TPM3) in *Salmonella* infected macrophages (Verbrugghe et al., 2016). This cortisol induced proliferation of bacteria in macrophages was driven by the *scsA* gene of *Salmonella* Typhimurium. When *scsA* deleted mutant, infected macrophages were exposed to cortisol; the proliferation effect was abolished (Verbrugghe et al., 2016).

The data from these research studies show that stress can directly affect the colonisation and replication of *Salmonella* in a host. Till now, no specific studies have been conducted in chickens to understand the effect of stress on *Salmonella* infection in chickens. Stress and *Salmonella* infection can alter the gut microbiota composition,

which has been explained earlier in this review. However, stress with increased *Salmonella* count (because of its direct interaction with *Salmonella)* could pose a more significant threat to gut microbiota composition. Thus, it is vital to understand the combined impact of stress and *Salmonella* Typhimurium colonisation on gut microbiota in chickens.

1.7 Aims and Objective

The gut microbiota plays an important role in multiple physiological processes in the host. In the last two decades, multiple studies have highlighted different functions of gut microbiota that are essential for the host (Diaz Carrasco, Casanova and Fernández Miyakawa, 2019, Rychlik, 2020). Because of its role in gut health and production, its importance in food-producing animals like layers has been recognised. However, most studies investigating different aspects of gut microbiota are conducted in broiler chickens due to their shorter life span (Stanley et al., 2012b, Stanley et al., 2012a, Stanley et al., 2013a). In layers, the production period, management, physiological functions are markedly different from broilers. Further, parameters like age, sex, housing, diet are also significantly different in layer birds. Thus, the development and composition of gut microbiota can be significantly different.

The commercial layer birds are reared in different housing systems. These flocks can have birds from different genetic origins with different feed, vaccination schedules, and managemental practices. These variables could affect the development of gut microbiota in chickens. Most of the studies conducted earlier were controlled, and the data from the controlled studies have limited application. Therefore, it is important to understand the gut microbiota development in field conditions. Although the data generated from the field study will show the impact of multiple variables mentioned

above, the accuracy and applicability of the data generated in field trials will be higher than in controlled studies. Thus, it is essential to study the gut microbiota of layer hens reared in different housing systems in the commercial setup.

The life span in layers from hatch to end of the production cycle is 80 weeks. During this, the layers experience stress because of different stressors, for example, vaccination, transport from rear to production, feed withdrawal, the onset of lay, heat stress etc. Earlier studies have described the impact of stress on gut microbiota in broiler and layer at a single time point (Zhu et al., 2019, Shi et al., 2019). Therefore, it is essential to understand the effect of multiple stress events on gut microbiota composition in layers. Enteric pathogens like *Salmonella* Typhimurium infection at an early age or point of lay can affect the gut microbiota adversely. It is also known that, under stress, a host can communicate with pathogens through stress hormones and help to replicate them. Stress and *Salmonella* infection could pose a more significant threat to the gut microbiota composition in layers which has not been investigated yet. Considering the different perspectives and gaps highlighted in the literature, the main objectives of this thesis are-

1. To understand the development of gut microbiota in commercial flocks raised in cage housing systems from hatch to end of the production cycle.
2. To investigate the development of gut microbiota in commercial layer flocks raised in free-range and barn housing systems.
3. To study the effect of intramuscular injection, feed withdrawal and *Salmonella* Typhimurium infection on gut microbiota in pullets.

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Table 1.1 Dietary specifications for brown egg pullets.

Parameters	Starter (0-6 weeks	Grower (6 to10 weeks)	Developer (10-16 weeks)	Pre-lay (16 to 18 weeks)
Crude Protein (%)	20	18	15.5	16.0
Metabolizable Energy (kcal/kg)	2900	2850	2800	2850
Calcium (%)	1	0.95	0.90	2.25
Available Phosphorus (%)	0.5	0.42	0.38	0.42

Table 1.2 Dietary specifications for brow egg layer birds.

Parameters	Layer-phase 1 (18-32 weeks)	Layer-phase 2 (32-45 weeks)	Layer-phase 3 (45-60 weeks)	Layer-phase 4 (60-70 weeks)
Crude Proteins (%)	20	19	17.5	16
Metabolizable energy (Kcal/kg)	2900	2875	2850	2800
Calcium (%)	4.2	4.4	4.5	4.6
Available Phosphorus (%)	0.50	0.43	0.38	0.33

Chapter 2 Temporal dynamics of gut microbiota in caged laying hens: A field observation from hatching to end of lay

Statement of Authorship

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Contribution to the Paper	Contributed to study design, animal trials, sample processing, data analysis and drafting of manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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2.1 Abstract

Gut health has major implications for the general health of food-producing animals such as the layer birds used in the egg industry. In order to modulate gut microbiota for the benefit of gut health, an understanding of the dynamics and details of the development of gut microbiota is critical. The present study investigated the phylogenetic composition of the gut microbiota of a commercial layer flock raised in cages from hatch to the end of the production cycle. This study also aimed to understand the establishment and development of gut microbiota in layer chickens. Results showed that the faecal microbiota was dominated by phyla *Firmicutes* and *Proteobacteria* in the rearing phase, but *Bacteroidetes* in mid lay and late lay phase. The gut microbiota composition changes significantly during the transfer of the flock from the rearing to the production shed. The richness and diversity of gut microbiota increased after week 6 of the flocks' age and stabilised in the mid and late lay phase. The overall dynamics of gut microbiota development was similar to that reported in earlier studies, but the phylogenetic composition at the phylum and family level was different. The production stage of the birds is one of the important factors in the development of gut microbiota. This study has contributed to a better understanding of baseline gut microbiota development over the complete life cycles in layer chickens and will help to develop strategies to improve gut health.

Keywords- Gut Microbiota, 16S rRNA gene sequencing, Pullets, Laying hens, Gut health, Layers, Gut microbiota, *Firmicutes, Proteobacteria*

Key Points:

- Faecal microbiota of caged hens was dominated by phyla *Firmicutes* and *Proteobacteria* in the rearing phase.
- The gut microbiota composition changed significantly during the transfer of the flock from the rearing to the production shed.
- The richness and diversity of gut microbiota increased after week 6 of the flocks age and stabilised in the mid and late lay phase.

2.2 Introduction

In the last few decades, the poultry sector has emerged as a global livestock industry leader as poultry products are one of the major consumed food products in the world (Mottet and Tempio 2017). This can be credited to the improved management practices and continued research to enhance the production standards. Globally, egg production and consumption have increased over the years (Mottet and Tempio 2017; PoultryTrends 2019). The per capita egg consumption in Australia has increased over the years and was 247.1 eggs in 2019 (AECL 2019). In Australia, even though the majority of commercial eggs are produced in free-range systems, 40% of the eggs are still produced in the cage production system (AECL 2019). Many supermarkets in Australia impose standards which require the cage eggs producing flocks to be raised in cages from day old. Careful management of flock health is necessary to maximise performance and production.

The gut microbiota has emerged as one of the key factors that influence the productivity and health of poultry (Khan et al. 2020). Gut microbiota is actively involved in the development of the immune system, can confer protection from pathogen infection via competitive exclusion and production of antimicrobial compounds, it

supplies micronutrients, amino acids and short-chain fatty acids, and influences the development of intestinal epithelium (Khan et al. 2020).

The negative impact of antibiotic use in animal production, such as increased antibiotic resistance in pathogens and antibiotic residuals in products and the environment, has prompted the investigation of alternative strategies to maintain animal gut health and productivity while reducing the reliance on antibiotics. There has been a research focus on the identification, development, and testing of feed supplements such as probiotics, prebiotics, and organic acids (Alagawany et al. 2018; Donoghue 2003). In Australia, antibiotic growth promotants are not used in layer diets.

A number of studies have investigated layer gut microbiota composition at single sampling times (Callaway et al. 2009; Ngunjiri et al. 2019), but there is little or no information available in the scientific literature on the temporal studies on gut microbiota overtime/age in commercially raised hens in field conditions (Videnska et al. 2014; Videnska et al. 2013a). To manipulate feed supplements for optimal performance and maintain gut health, it is important to understand the baseline composition and development of the gut microbiota over time/age in layers. The majority of the earlier research work in poultry is focused on broilers (Oakley and Kogut 2016; Stanley et al. 2013a; Stanley et al. 2013b). However, layers have a longer lifecycle, different genetics, different management protocols, and different diets than broilers. Further, on a commercial egg layer farm, all the birds are females, in contrast to broiler flocks, and gut microbiota can be influenced by sex hormones (Lumpkins et al. 2008). Thus, the extensive data generated from broiler studies are not necessarily relevant for layers due to these significant differences. The studies on layer microbiota that have been published are limited to a specific age or focused on caecal microbiota or the specific tissue segments (Callaway et al. 2009; Nordentoft et al. 2011; Videnska

et al. 2014; Videnska et al. 2013b). Although the caecum is an important organ in the chicken gut, the other parts of the gut also have unique physiological functions and are essential for sound gut health.

This study investigated the development of gut microbiota in commercial layer chickens from the day old to the end of the commercial life span.

2.3 Materials and methods

2.3.1 Farm details

The commercial layer flock selected for this study was reared in cages from day 1. There were 38,500 Hy-line birds in the flock. The flock was reared as per the Hy-line management guidelines. The birds were transferred to the production shed at 16 weeks of age. The distance between the rearing and production farm is 188 km (Two hrs of road travel). The feeding protocol was Starter (0-6 weeks), Grower (7-12 weeks), Developer (13-15 weeks), Pre-lay (16-17 weeks), and Lay (18 weeks onwards). Wheat-Soyabean-Canola was the basic feed ingredient. The flock did not receive any feed supplements throughout the life span. The flock was vaccinated for Infectious Bronchitis, Infectious Laryngotracheitis, Newcastle Disease, Avian Encephalomyelitis, Fowl Cholera, Egg Drop Syndrome, and Marek's Disease. A hundred birds from the flock were weighed every week, and mortalities were monitored from hatch to end of the production cycle.

2.3.2 Sample collection

Faecal samples (n=30) were collected using cloacal swabs at each timepoint in 1.5 ml sterile microcentrifuge tubes and stored at -20°C for further processing. The samples were collected from 30 random birds from the flock at days 2, 5, 10, 20, then weeks 6,

12, 18, 20, 22, 28, 34, 40, 46, 52, 58, 64, 70, 75 of the flock's age. A total of 540 samples were collected for this study.

2.3.3 DNA extraction-

The extraction of DNA from faecal samples (n=540) was performed using QIAamp Fast DNA Stool Mini kit (Qiagen, USA). A modified protocol (Knudsen et al. 2016), based on the manufacturer's recommended procedure, was further modified to optimize the procedure for this study. Briefly, 20 mg of faecal sample was vortexed following the addition of 1 ml preheated InhibitEx buffer in 1.5 ml sterile micro-centrifuge tubes. The samples were homogenized at top speed for 5 minutes (Bullet Blender, Next Advance, USA) after adding 390 mg beads (Sigma glass beads, acid washed, 450-600 (180 mg) µm & 106 µm (210 mg)). The samples were then incubated at 94°C for 7 minutes, followed by cooling on ice for 30 seconds. The supernatant was collected after centrifugation (2 minutes for 20000 x g) and processed according to the modified QIAamp Fast DNA Stool Mini Kit Protocol (Knudsen et al. 2016). The DNA was eluted in 100 µl ATE buffer. The samples were stored at -20°C.

2.3.4 16S rRNA gene sequencing

The V3-V4 region of 16S rRNA genes were amplified, and the Illumina MiSeq system (2 x 300 bp) was used to perform sequencing using dual indexing variable spacer (Fadrosh et al. 2014). QIIME 1.9.1 was used to demultiplex the sequences and analyse trim for quality control (Caporaso et al. 2010). Operational taxonomic unit (OTU) were picked using the UCLUST algorithm. Pintail was used to identify and inspect chimeric sequences (Ashelford et al. 2005). The taxonomy was initially assigned using the GreenGenes database 13.8, and then the NCBI 16S database was

interrogated using BLASTn on representative sequences for additional taxonomic analysis (DeSantis et al. 2006). The OTU table was filtered to remove low abundance OTUs that represented less than 0.01% of the sequence output, and square root transformed, and Total Sum Normalized prior to analysis.

2.3.5 Statistical analysis

The production data provided by farms were analysed and visualised in MS-Excel. To understand microbial community structure, the OTU table was analysed by Calypso software (Zakrzewski et al. 2016). The richness (alpha diversity) and abundance of gut microbiota community were analysed using one- and two-way ANOVA. Beta diversity was analysed using ANOSIM (Zakrzewski et al. 2016). Principle coordinate analysis was used to investigate the variation in the gut microbiota at different time points. In this study, all the samples with less than 1000 sequences were removed from the analysis. The minimum reads per sample in this analysis were 1906.

2.4 Results

2.4.1 Flock performance

In the present study, we collected flock data regarding mortality, body weight gain and weekly egg production to evaluate the flock performance. The overall mortality in the flock (rear to production) was significantly lower (0.035%) ($P < 0.0001$) compared to standard mortality (1.77%) expected for Hy-line Brown chickens (Hy-line management guide, Nov. 2018). The weekly egg production in the present flock was higher ($P = 0.9250$) than the expected production of Hy-Line Brown chickens (Figure 2.1a). The body weight gain in this flock was high during the rear as compared to standard body

weight gain but followed the standard body weight gain according to average expectation from Hy-Line Brown chickens (Figure 2.1b).

This study generated a large set of data from 540 cloacal swab samples, which provided a detailed characterization of the establishment and development of the phylogenetic composition of the gut microbiota throughout the life span of the commercial layer flock. The results were considered, and data were analysed with respect to and the different phases of the production cycle; Phase 1 was the rearing phase and included data from day 2 to week 18; Phase 2 was the early lay phase and included data from week 20 to week 40; Phase 3, mid-lay phase, included data from week 40 to week 60; and Phase 4, late lay phase, included data from week 60 to week 75 of flock age.

2.4.2 The richness and diversity of gut microbiota

Richness is defined as the number of operational taxonomic units (OTUs) present in the gut microbiota. In this study, the richness of the gut microbiota significantly (P= 6.6e-109, ANOVA) increased with age and was significantly different at each time point (Figure 2.2). The richness increased significantly (P=3.6e-26, ANOVA) at week 12 compared to all sampling time points during the rear. At week 18, after birds were moved from rearing farm to production farm, the richness of gut microbiota decreased. In the early lay phase, the richness of gut microbiota was low at weeks 20 and 22, after which it increased until week 40. The richness of gut microbiota stabilized in mid and late lay phases and showed less variation as compared to rearing and early lay phase.

The gut microbiota composition was significantly different and changed with the age of the flock. The principal coordinate analysis of rearing, early, mid and late phase

showed the effect of age on gut microbiota composition (Figure 2.3). The statistical analysis (ANOSIM) concluded significant age-dependent dissimilarity in gut microbiota composition in this flock (R=0.521, P= 0.001).

2.4.3 Composition of gut microbiota during rear

In the rearing phase, the gut microbiota was dominated by phylum *Firmicutes* except for days 2 and 42, where phylum *Proteobacteria* was dominant. In phylum *Firmicutes,* the dominant family was *Lactobacillaceae*. The abundance of *Lactobacillaceae* was highest at day 5 and lowest at week 12 during the rearing period. The abundance of *Peptostreptococcaceae* increased significantly (P= 5.8e-06, ANOVA) at week 12. The abundance of *Ruminococcaceae* increased significantly (P= 2.5e-08, ANOVA) at day 10 compared to earlier time points. In phylum *Proteobacteria*, the family *Enterobacteriaceae* was the dominant taxa throughout the rearing phase.

The abundance of phylum *Actinobacteria* in gut microbiota increased significantly (P = 2.8e-12, ANOVA) at day 10 of the flock's age. The dominant family in the phylum *Actinobacteria* was *Bifidobacteriaceae*. The abundance of phylum *Actinobacteria* was highest at week 12 and lowest at day 42. The highest abundance of phylum *Bacteroidetes* (family *Rikenellaceae*) and phylum *Fusobacteria* (family *Fusobacteriaceae)* was noted at week 12 (Figure 2.4). The diversity of the gut microbiota during the rear was highest at week 12.

2.4.4 The gut microbiota composition after the shift from rear to production

The gut microbiota composition changed significantly at week 18, after the transfer of birds from the rearing to the production farm. At week 18, the abundance of phyla *Bacteroidetes and Proteobacteria* decreased, and the abundance of phyla

Actinobacteria and *Fusobacteria* increased compared to week 12; however, the changes in abundance were not statistically significant. Although the abundance of phyla did not change significantly, the composition of different phyla showed significant variation at the family level at week 18 compared to week 12. In phylum *Firmicutes*, the abundance of family *Lactobacillaceae* decreased significantly (P= 0.012, ANOVA) at week 18 compared to week 12. *Peptostreptococcace* was the most abundant family in phylum *Firmicutes* at week 18. In phylum *Bacteroidetes*, at week 12, the dominant family was *Rikenellaceae*, while at week 18, the dominant family was *Bacteroidaceae* (Figure 2.5a). The dominant family in phylum *Actinobacteria* was *Bifidobacteriaceae* at week 12, but dominance switched at week 18 to *Coriobacteriaceae*. In the phylum *Proteobacteria*, *Enterobacteriaceae* was the dominant family at both weeks 12 and week 18. The abundance of family *Alcaligenaceae* increased significantly (P= 0.0019, ANOVA) at week 18. The principal coordinates analysis (Figure 2.5b) at week 12 and week 18 showed the differences in the gut microbiota composition, and the richness (Figure 2.5c) of the gut microbiota decreased significantly at week 18 (P=0.0097, ANOVA). The beta diversity analysis (ANOSIM) showed distinct differentiation (R= 0.132, P=0.003) in gut microbiota composition at week 12 and week 18 in the flock (Figure 2.5d).

2.4.5 Composition of gut microbiota in early lay

The gut microbiota composition in the early lay phase showed temporal variation in the relative abundance of taxa. The phylum *Firmicutes* were dominant in early lay with the highest abundance at week 22 and the lowest abundance at week 28 of flock's age. In phylum *Firmicutes*, *Lactobacillaceae* was a dominant family throughout the early lay phase, and the abundance of families *Ruminococcaceae* and

Lachnospiraceae was high at week 34 of the flock's age. Phylum *Bacteroidetes* replaced phylum *Proteobacteria* as the second most abundant phylum after week 20 of the flock's age. In phylum *Bacteroidetes*, family *Bacteroidaceae* was the most abundant family except at week 28, where family *Paraprevotellaceae* was the dominant taxon. The highest abundance of phylum *Bacteroidetes* was recorded at week 28 and phylum *Fusobacteria* at week 34 of the flock's age. In phylum *Proteobacteria*, the abundance of family *Enterobacteriaceae* decreased significantly (P = 2.6e-05, ANOVA) with the flock age and was replaced by family *Desulfovibrionaceae* and family *Alcaligenaceae*. The abundance of phylum *Actinobacteria* was high at week 20 of the flock's age compared to other time points in the early lay phase. (Figure 2.6).

2.4.6 Composition of gut microbiota in mid lay

In the mid lay phase, the relative abundance of phylum *Firmicutes* decreased gradually from week 46 to week 58, while phylum *Bacteroidetes* increased in abundance over the same time frame. In phylum *Firmicutes*, the abundance of family *Lactobacillaceae* was the highest at week 46, then decreased significantly (P = 0.0096, ANOVA) to week 58, while the abundance of family *Ruminococcaceae* increased with the age of the flock. In phylum *Bacteroidetes*, the family *Bacteroidaceae* was the dominant genus throughout the mid lay phase. The abundance of *Fusobacteria* increased significantly (P = 5.5e-09, ANOVA) with the age of the flock in mid lay, and the abundance of phylum *Proteobacteria* was less throughout the mid lay phase, dominated by family *Alcaligenaceae* (Figure 2.7).

2.4.7 Composition of gut microbiota in late lay

In the late lay phase, *Firmicutes* was the dominant phylum at week 64 but was replaced as the dominant phylum by *Bacteroidetes* at weeks 70 and 75. In phylum *Firmicutes*, the family *Peptostreptococcaceae* was dominant at week 64, and the family *Lactobacillaceae* was dominant at week 70 and 75. The abundance of family *Ruminococcace* was significantly higher (P= 0.00012, ANOVA) at week 70 and 75 than at week 64. In phylum *Bacteroidetes,* the family *Bacteroidaceae* was dominant throughout the late lay phase. In the late lay phase, the abundance of phyla *Proteobacteria* (P=1.2e-07, ANOVA) and *Fusobacteria* (P=0.02, ANOVA) decreased significantly after week 64 of the flock's age. In phylum *Proteobacteria*, the family *Enterobacteriaceae* was dominant at week 64. The abundance of family *Desulfovibrionaceae* (P=1.4e-05, ANOVA) increased significantly after week 64 of the flock's age (Figure 2.8).

Overall, the abundance of phylum *Firmicutes* was relatively high up to mid lay phase, after which it decreased. The relative abundance of phylum *Proteobacteria* was high in the rearing phase, while the relative abundance of phylum *Bacteroidetes* was higher in early, mid, and late lay phases. The phylum *Actinobacteria* was first noted on day 10 of the flock's age. The highest abundance of phylum *Actinobacteria* was on week 18 of the flock's age. The abundance of *Actinobacteria* reduced significantly at week 58, and throughout the late lay phase. The abundance of phylum *Fusobacteria* was highest at week 18. Further, abundance was high in early lay at week 34, mid lay at week 58 and in late lay at week 64 of the flock's age (Supplementary Figure S1).

2.5 Discussion

2.5.1 Development pattern of gut microbiota depends on age, production stage, feed, and management of the flock

The objective of this research was to characterize the development of gut microbiota over the commercial life span of layer chickens raised in a typical cage production system. The gut microbiota composition changed with the flock's age showing distinct variation at each time point. There is evidence that higher levels of richness and diversity of intestinal microbiota are correlated with positive health outcomes (Stanley et al. 2014). A number of previous studies in broiler chickens have revealed positive correlations between productivity and microbiota richness (Stanley et al. 2012; Yan et al. 2017). However, some studies have found the reverse, with better performance correlated with lower richness (Siegerstetter et al. 2017). In our study, we found that microbiota richness was positively correlated to the production of the flock. However, more studies are required to understand the exact impact of gut microbiota richness on production performance in layers. The significant reduction in the richness of gut microbiota observed after shifting the flock from the rearing shed to the production shed could represent a period of vulnerability in which the changing microbiota makes the birds susceptible to colonization by pathogens as previous studies in humans have demonstrated that reduced richness has been correlated with disease conditions, although this is yet to be demonstrated in chickens (Manichanh et al. 2006; Ott et al. 2004). The relative stabilization of gut microbiota richness in mid and late lay may be attributed to the management system. In this study, the flock was reared in an all-in-all-out system. In the all-in-all-out system, the complete flock is transferred to the shed at once and culled at once. The birds of the same age receive the same diet and have the same management protocol. Thus, after transporting to the production shed, the

birds acclimatize to the environment in the production shed, which is similar till the end of the production cycle. These factors could have contributed to the relative stabilization of the richness in the mid and late lay phases, which could indicate that the flock can be productively maintained even after 80 weeks of age. In this study, the weekly egg production at week 75 was above expected production from the Hy-Line Brown birds. Generally, layer flocks are culled after 80 weeks due to lowered production; however, considering the above information, if the flocks are reared for a longer period, it will positively impact the economic output. The pattern of gut microbiota development in this study partially agrees with the results from earlier studies i. e. the overall pattern of gut microbiota development and shifts in gut microbiota were similar however the composition and relative abundance of phyla was significantly different in our study (Ngunjiri et al. 2019; Videnska et al. 2014). However, the composition of diet and feeding protocol was according to the breed of the birds, which were different in both studies.

The phylogenetic composition of the gut microbiota and how it changed as the birds aged was, in many respects, consistent with that observed in other layer studies that have reported microbiota at single or a few time points. In an earlier study in layers, the gut microbiota at week one in pullets was dominated by phylum *Proteobacteria* family *Enterobacteriaceae* (Videnska et al. 2014). In this study, on day 2 of the flock's age, the gut microbiota was dominated by phylum *Proteobacteria*, family *Enterobacteriaceae;* however, from day 5 onwards, phylum *Firmicutes* was dominant in our study. This might be due to the sampling protocol of an earlier study that sampled the flock only once in week 1 and also used caecal samples. Another study in local chicken breeds in China reported the dominance of phylum *Proteobacteria* on day one and dominance of phylum *Firmicutes* on day 3 of the bird's age in the control

group, which supported our results (Liu et al. 2018). The colonization of intestines by phylum *Proteobacteria* (family *Enterobacteriaceae*) at day 2 in our study and at an early age in other studies could be due to the lower microbial diversity immediately after hatching. Family *Enterobacteriaceae* includes pathogens like *Salmonella, Shigella, E. coli, Klebsiella,* which pose a serious threat to the host (Mon et al. 2015). Earlier studies in mice concluded that gut microbiota harbouring a higher proportion of commensal *E. coli* is more likely to be susceptible to *Salmonella enterica* infection (Stecher et al. 2010). Indeed, a similar bacterial population could attract members of closely related bacterial species by providing a more favourable gut environment. Videnska et al. (2014) also reported that from week 2 to week 4, the gut microbiota was dominated by families *Lachnospiraceae* and *Ruminococcaceae* in phylum *Firmicutes*. Our study found that from day 5 onwards, phylum *Firmicutes* dominated the community, with *Lactobacillaceae* as the dominant family. There are conflicting reports available regarding the role of family *Lactobacillaceae* in chickens. Some of the earlier studies conducted in broiler chickens have shown the adverse effects of some members of *Lactobacillaceae* on weight gain in the birds (Johnson et al. 2018; Stanley et al. 2016). Studies in layer birds have also indicated poor feed conversion correlated with *Lactobacilli* abundance in adult hens (Nahashon et al. 1996). However, studies also indicated that members of family *Lactobacillaceae* are associated with improved gut health, intestinal microbiology and productive performance (De Cesare et al. 2017; Forte et al. 2018). Data from earlier studies also indicate that the *Lactobacillus* supplementation positively correlated with egg size and egg quality (Mahdavi et al. 2005). In this study, we analysed the gut microbiota up to the family level, so it would be challenging to conclude the exact effect of a specific member from the family *Lactobacillaceae* in this trial. Considering this flock's egg production

performance and feed efficiency, we can assume that the abundance of family *Lactobacillaceae* was beneficial for the birds. This study reported a significant abundance of family *Ruminococcaceae* at day 10 of the flock's; however, it was not the dominant family as reported in an earlier study by Videnska et al. (2014). The high abundance of phylum *Firmicutes* has been correlated with rapid weight gain in birds in the rear phase.

Rapid weight gain in early life requires efficient nutrient absorption and butyrate is the most preferred energy substrate of intestinal epithelial cells (Ahmad et al. 2000; Roediger 1982; Scheppach 1994; Videnska et al. 2014). Phylum *Firmicutes* represents the main butyrate producer in gut microbiota (Duncan et al. 2007; Louis and Flint 2007; Walker et al. 2005). *Bifidobacteriaceae* was the most abundant family in phylum *Actinobacteria* until week 12. The *Bifidobacteriaceae* family has been associated with short-chain fatty acid (SCFA's) production and improved gut health (Parada Venegas et al. 2019). One of the important characteristics of *Bifidobacteriaceae* is its fructose-6-phosphate phosphoketolases pathway (F6PPK) for energy production. By using the F6PPK pathway, family *Bifidobacteriaceae* can yield 2.5 ATP molecules compared to 2 ATP molecules generated by regular fermentative glycolysis due to lactic acid bacteria (Egan and Van Sinderen 2018). Therefore, the abundance of phylum *Actinobacteria* in the early lay phase may indicate higher energy production within the microbiota at this stage of bird growth

2.5.2 Significant differences in the gut microbiota composition at week 18.

A substantial change in gut microbiota composition was observed at week 18 compared to the previous time point. Between these two time points after the flock was shifted from rearing to production shed. The birds experience several stresses

and changes in this period, such as transportation stress, stress due to onset of sexual maturity, change in feed, climate, environment and management (Kers et al. 2018). This combination of factors disrupted the gut microbiota, including a substantial drop in microbiota richness. More controlled trials are required to understand the effect of individual factors on gut microbiota. At week 18, we noted that the abundance of phylum *Fusobacteria* increased. The phylum contains known pathogenic genera. We also observed a significant increase in the abundance of known pathogenic family *Peptostreptococcaceae* from the *Firmicutes* phylum. It has been reported that, at week 18, after transport, pathogenic bacteria such as *Salmonella* tend to colonize in the gut in layer birds (Gole et al. 2014). Clean production sheds can result in the reduction of the pathogen load in the environment, thereby reducing the chances of acquisition and colonization of pathogenic genera, contributing to better gut health. Thus, cleaning status and protocols for production shed biosecurity and management become critical points to reduce the pathogen load and colonization of pathogens in birds.

2.5.3 Gut microbiota composition changed significantly with the age and production stage in the flock

The results from the early, mid, and late lay phase of this study are in partial agreement with earlier studies that reported the succession of phylum *Proteobacteria* by *Bacteroidetes* and *Firmicutes-Bacteroidetes* as dominating phyla in laying hens and the particular dominance of phylum *Bacteroidetes* in the gut microbiota of 75 weeks old hens (Callaway et al. 2009; Videnska et al. 2014). However, in our study, the abundance of phylum *Bacteroidetes* was significantly higher. This could be attributed to different food or the fact that Callaway et al. (2009) analysed caecal samples

compared to our study, where we focused on cloacal swabs. Further, it has been reported that the *Bacteroidetes* can metabolize complex polysaccharides (Stanley et al. 2013a). Increased abundance of phylum *Bacteroidetes* at late production can be correlated with increased production of substrates acetate and propionate, which are not the primary choices of a substrate in the intestine for fermentation (Videnska et al. 2014; Wrzosek et al. 2013; Yang et al. 2013). Some of the *Bacteroidetes* members are also capable of releasing sulphate in the intestine, which can be effectively utilized by members of the family *Desulfovibrionaceae* which is part of the phylum *Proteobacteria* to produce H_2S (Benjdia et al. 2011; Rey et al. 2013; Yang et al. 2013). It has been reported earlier that H_2S plays key roles in physiological processes at lower concentrations; however, at higher concentrations, it is toxic and can lead to pathological conditions in the colon, such as inflammatory bowel disease (Guo et al. 2016). The biological roles of H_2S identified in rats intestines are relaxation of ileal smooth muscles, increased colonic secretions and protection of intestines from ischemic- reperfusion injury (Guo et al. 2016). The accumulating data from this and previous studies on layer gut microbiota will help design future intervention strategies to modulate gut microbiota for improved production and gut health.

In conclusion, increased knowledge of microbiota establishment and maturation over time in a field setting has been produced, with the long-term goal of formulating strategies to modulate microbiota and establish consistent healthy gut microbiota in chickens. This will contribute towards improved production and health. In this study, we noted significant changes in gut microbiota composition at each time point, indicating that age has an important influence on gut microbiota composition. The gut microbiota composition changed significantly at week 18, which is a critical time point in a flock's life as birds experience multiple stressors, such as shifting from rear to

production, the onset of lay, and change in feed. Due to these stressors, the richness of gut microbiota decreased, which could be the possible reason for colonisation of pathogenic bacteria such as phylum *Fusobacteria* and family *Peptostreptococcaceae* in the gut. Thus, it is essential to develop strategies to minimise the disruption of gut microbiota at critical time points in production cycles due to stressors. The dominance of *Firmicutes* at an early age aligns with the high energy requirement for rapid weight gain, and its replacement with *Bacteroidetes* at later ages could be supportive of ongoing sustenance and production. The stabilized richness of gut microbiota composition can be co-related to feed efficiency in layers; however, detailed studies are needed to assess the effect of richness on egg production and possibilities of extending life cycles in layer birds. It could be concluded that the change in gut microbiota composition is not focused on a single genus or phylum but takes place throughout the gut microbiota. Thus, in future, it will be interesting to explore the use of consortia of bacteria tailored for particular stages of bird growth, rather than single probiotic isolates, to enhance production and to maintain gut health.

2.6 Declarations

Ethics approval and consent to participate

The experimental setup was approved by the University of Adelaide, Animal Ethics Committee under Approval Number No. S-2018-015. The protocol was carried out in accordance with the guidelines -specified in Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition 2013.

Consent for publications

All authors have approved the submission of the manuscript.

Availability of data and materials

The 16S rRNA sequence data are available from the MG RAST database under the accession number mgl832560.

Competing interests

The authors declare that they have no competing financial interests.

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Authors' contributions

NJ, HV, RM, DS, KC contributed to the study design, animal trials, samples processing and critical revision of the manuscript. NJ and KC collected samples from animals, NJ processed the samples for DNA extraction, HV and RM performed 16S rRNA gene sequencing, DS and NJ analysed the data, NJ drafted the manuscript. All authors approved the manuscript for publication.

Conflict of interest

The Authors declare that they have no conflict of interest.

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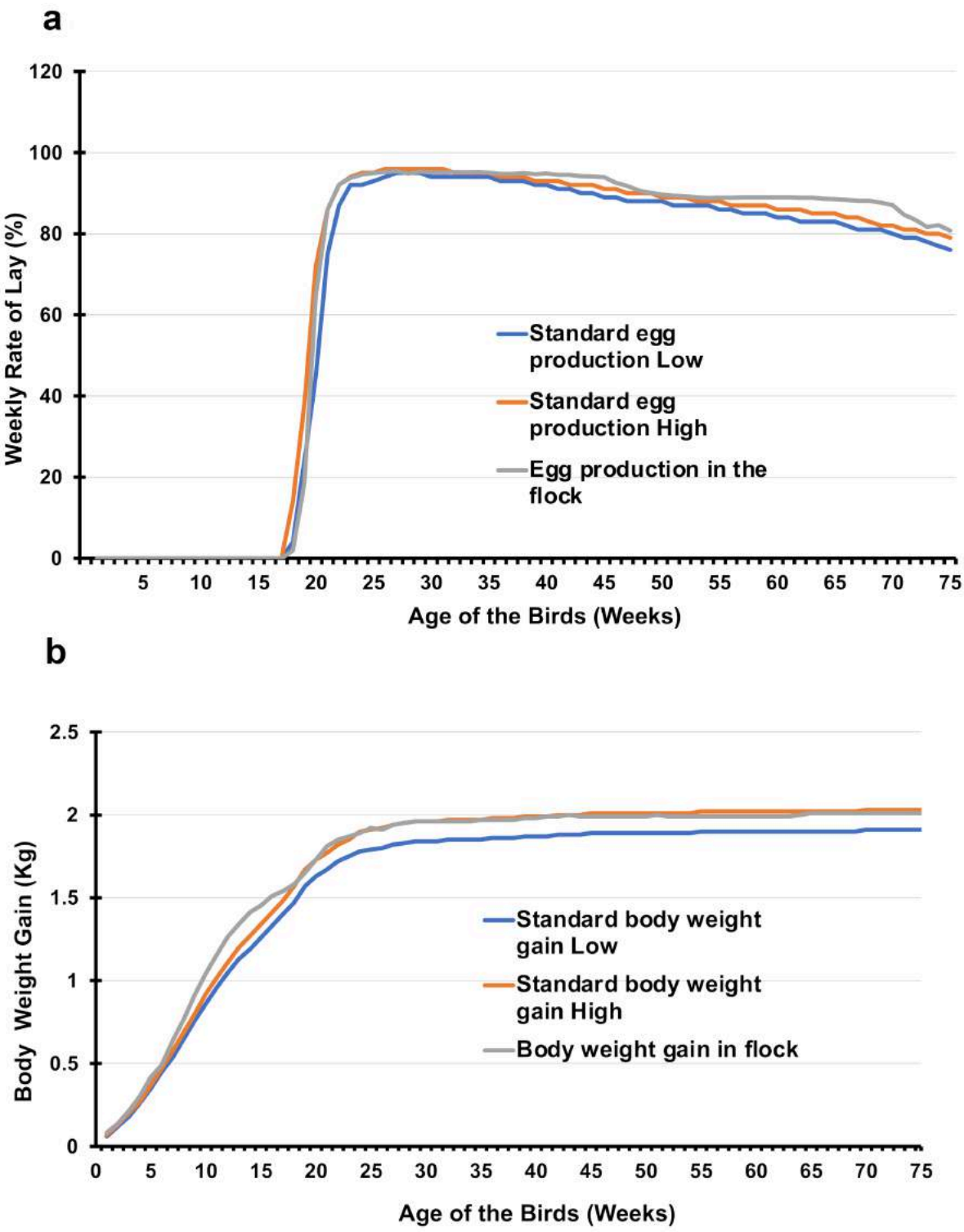


Figure 2.1 Comparison of weekly rate of lay in the trial flock with the expected weekly rate of lay in Hy-Line chickens (2.1a). Comparison of body weight gain in trial flock with the expected weight gain in Hy-Line chickens (2.1b). In both the graphs the range of expected values (highest and lowest) are plotted against the flock sampled in this trial.

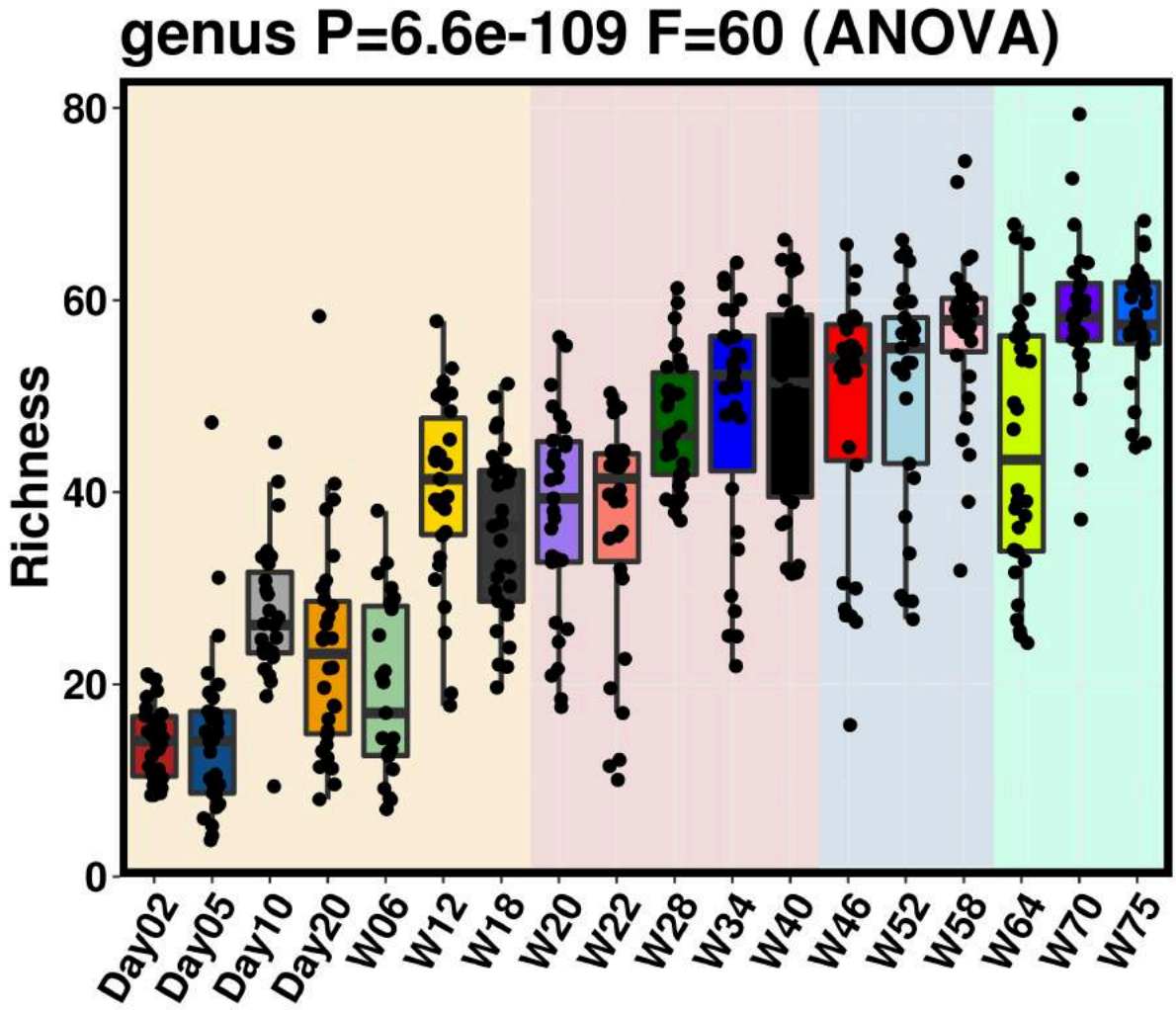


Figure 2.2 The dot plot graph showing richness of gut microbiota in the flock from hatch to week 75 of the flocks age, significant differences in gut microbiota richness during rear and various production stages. In the boxplot, boxes including median are showing the average richness median richness of gut microbiota while the upper whisker shows samples with higher richness and lower whisker showing the samples with lower richness. The back dot represents the individual samples collected at that time point. It also highlights the individual variation between the samples collected at the same time point.

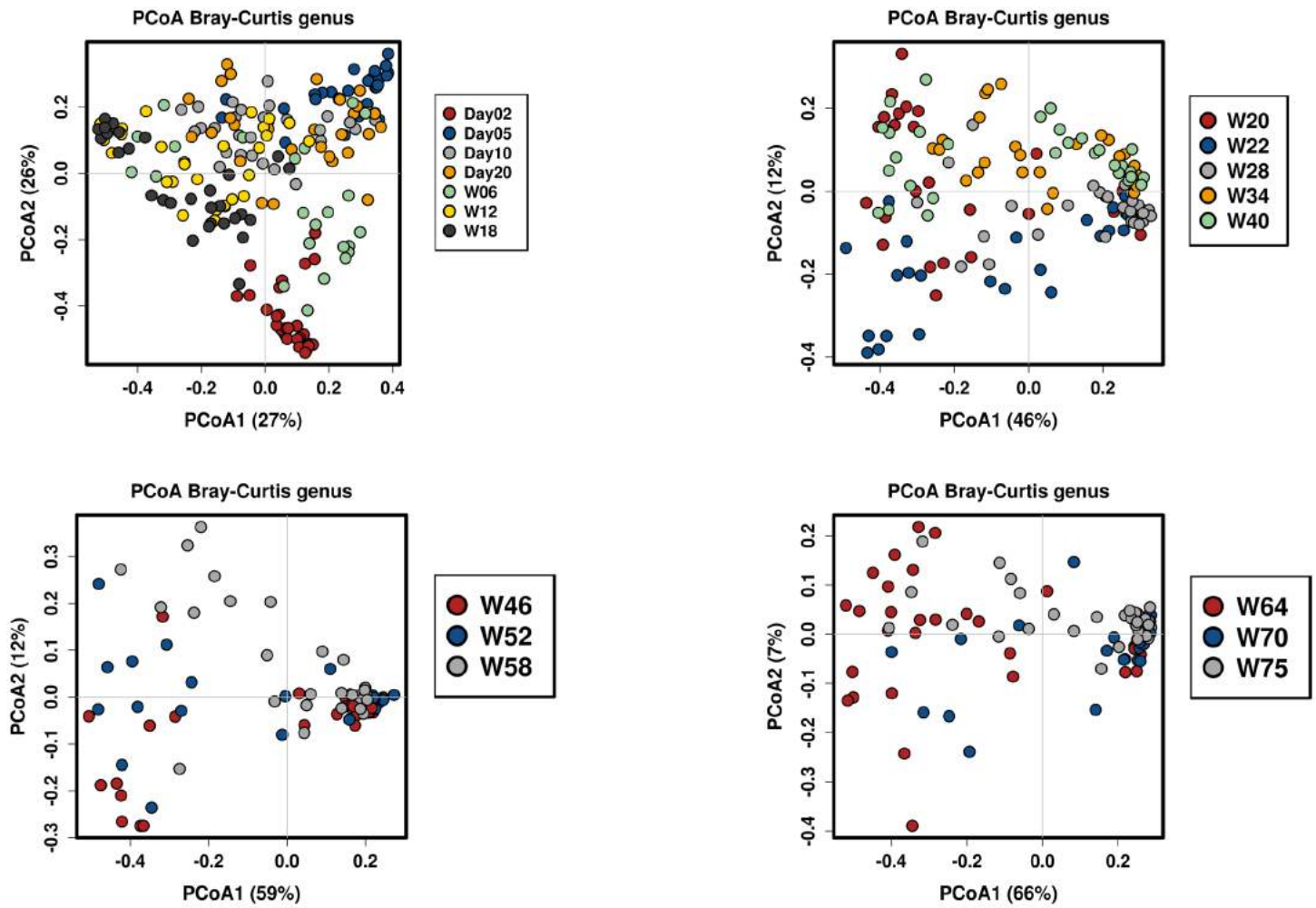


Figure 2.3 The principal coordinates analysis of gut microbiota composition from day 2 to week 75 of the flocks age showing the effect of age and production stage on the gut microbiota composition in the flock. In the figures, each dot represents the individual sample collected per bird and different colours of dots denote the specific time point of the sample collection.

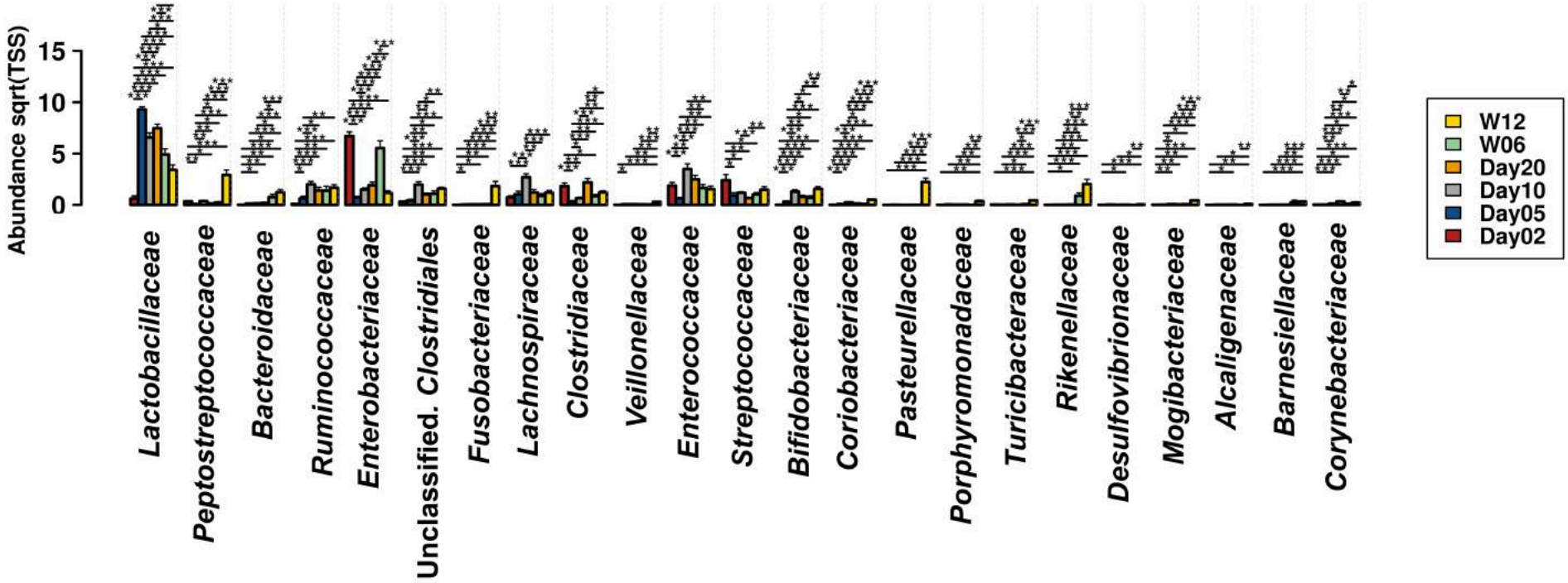


Figure 2.4 Comparative analysis of significant differences (P<0.05) in microbial community abundance at family level in rearing phase (up to week 12) in the flock. The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colors of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.

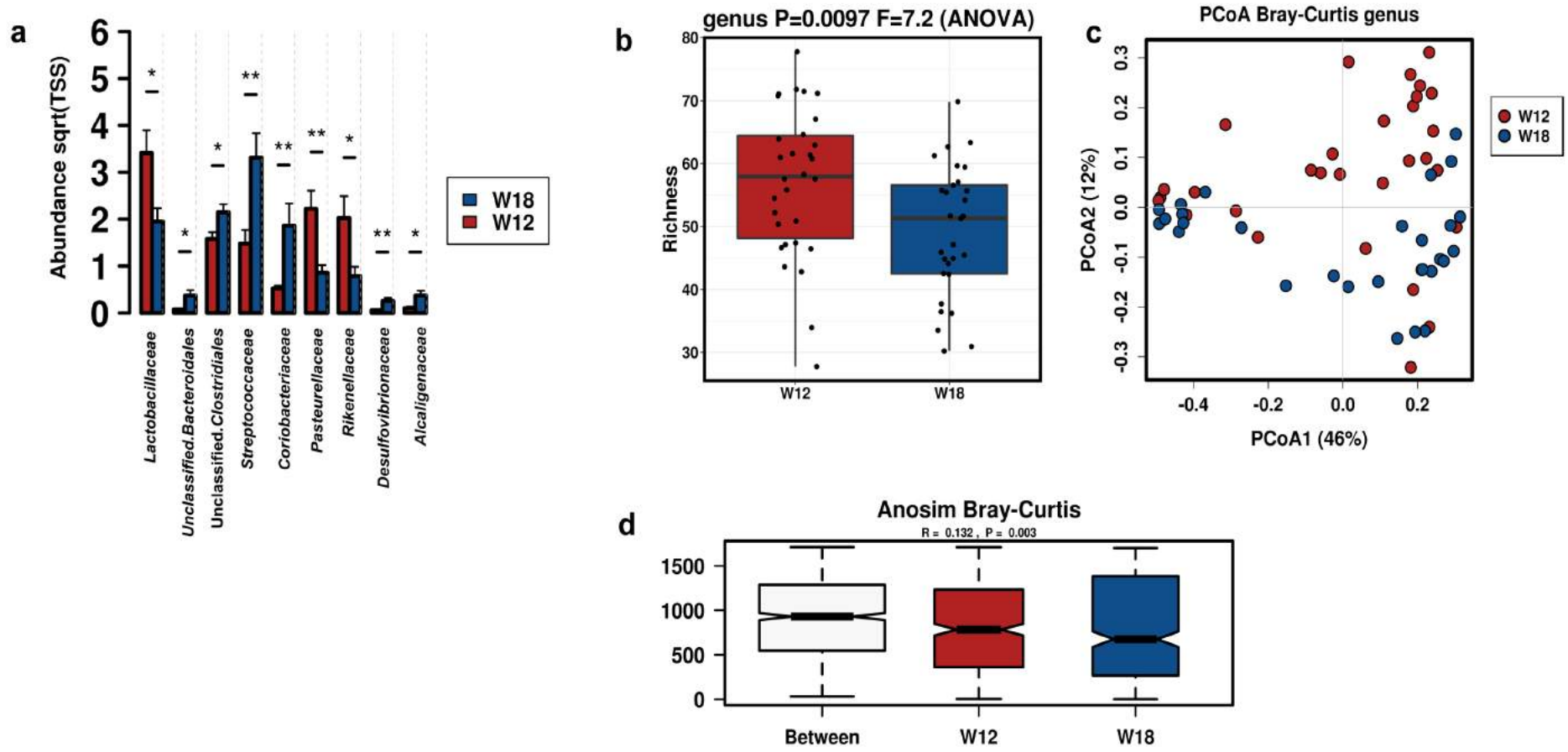


Figure 2.5 Comparative analysis of abundance of families (P <0.05) showing differences in gut microbiota composition before (week 12) and after (week 18) shifting of flock from rearing to production shed at phylum level (2.5a). Richness (2.5b) and Principal coordinates analysis (2.5c) showing significant differences in the gut microbiota composition at week 12 and week 18 of the flock's age. Beta diversity analysis using ANOSIM showing significant dissimilarities in the gut microbiota composition at week 12 and week 18 of flock age (2.5d).

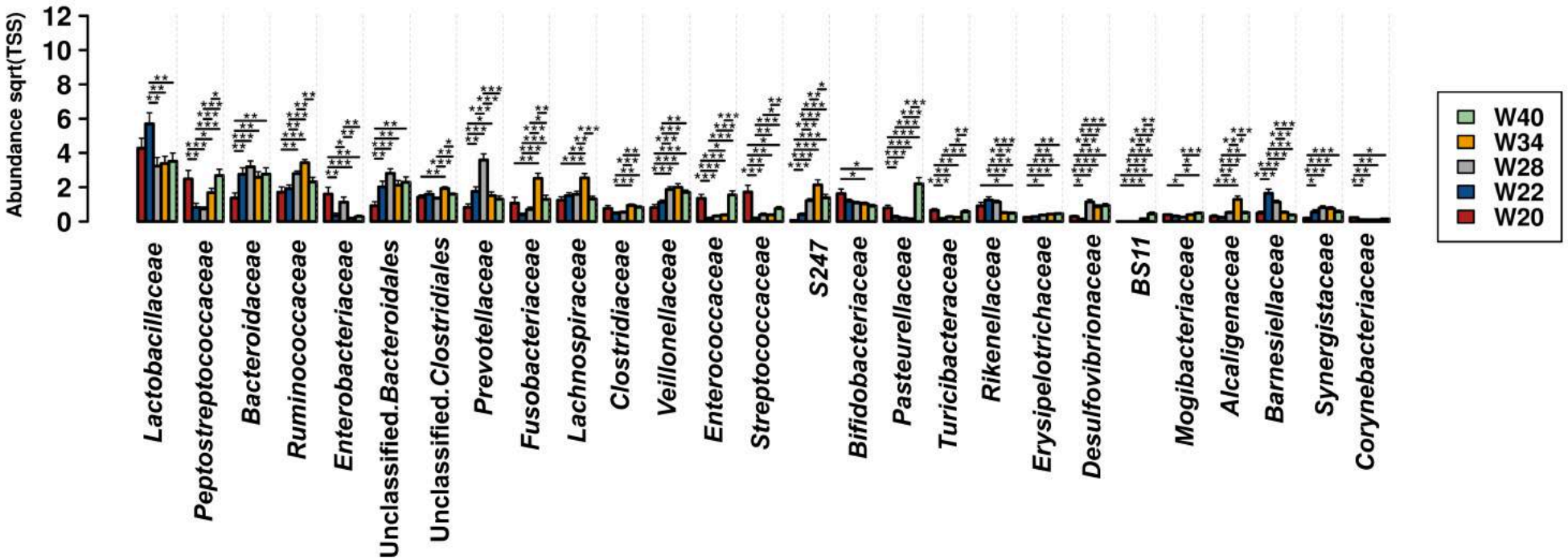


Figure 2.6 Comparative analysis of significant differences (P<0.05) in microbial community abundance at family level in early lay phase (week 20 to week 40) in the flock. The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colours of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.

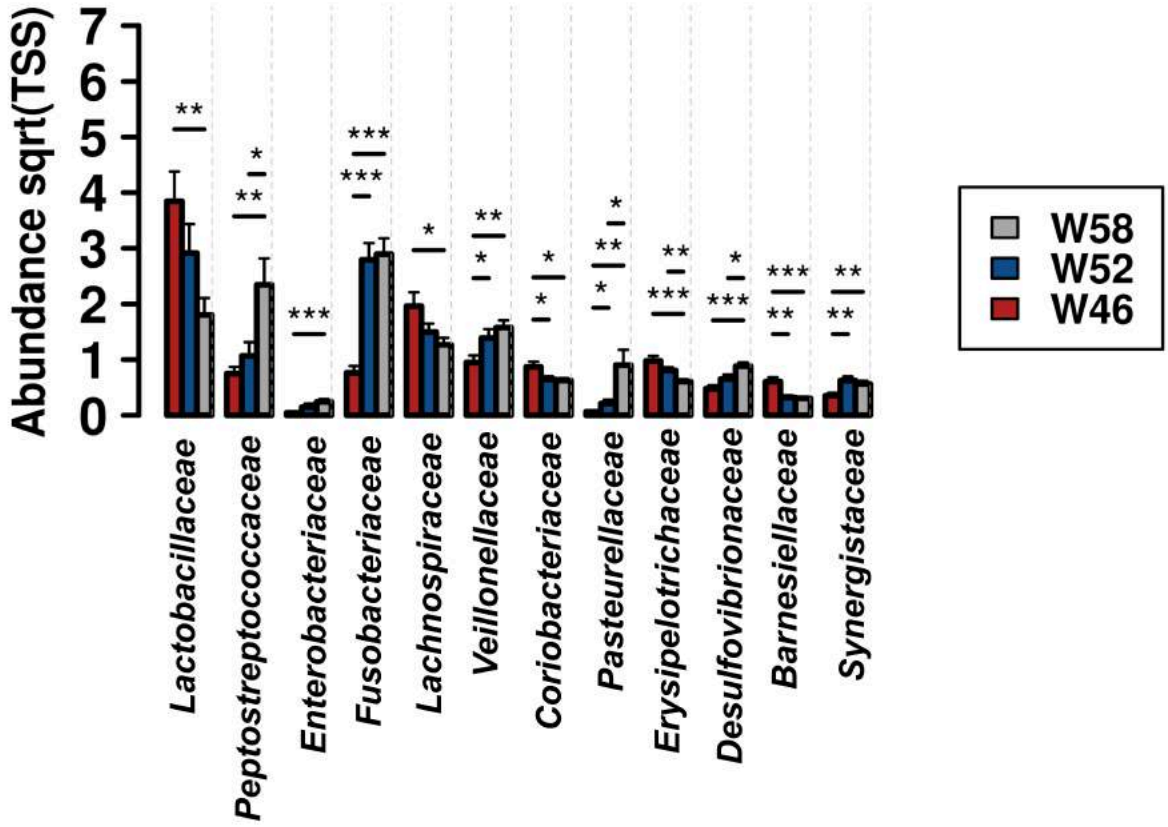


Figure 2.7 Comparative analysis of significant differences (P<0.05) in microbial community abundance at family level in mid lay phase (week 40 to week 60) in the flock. The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colours of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.

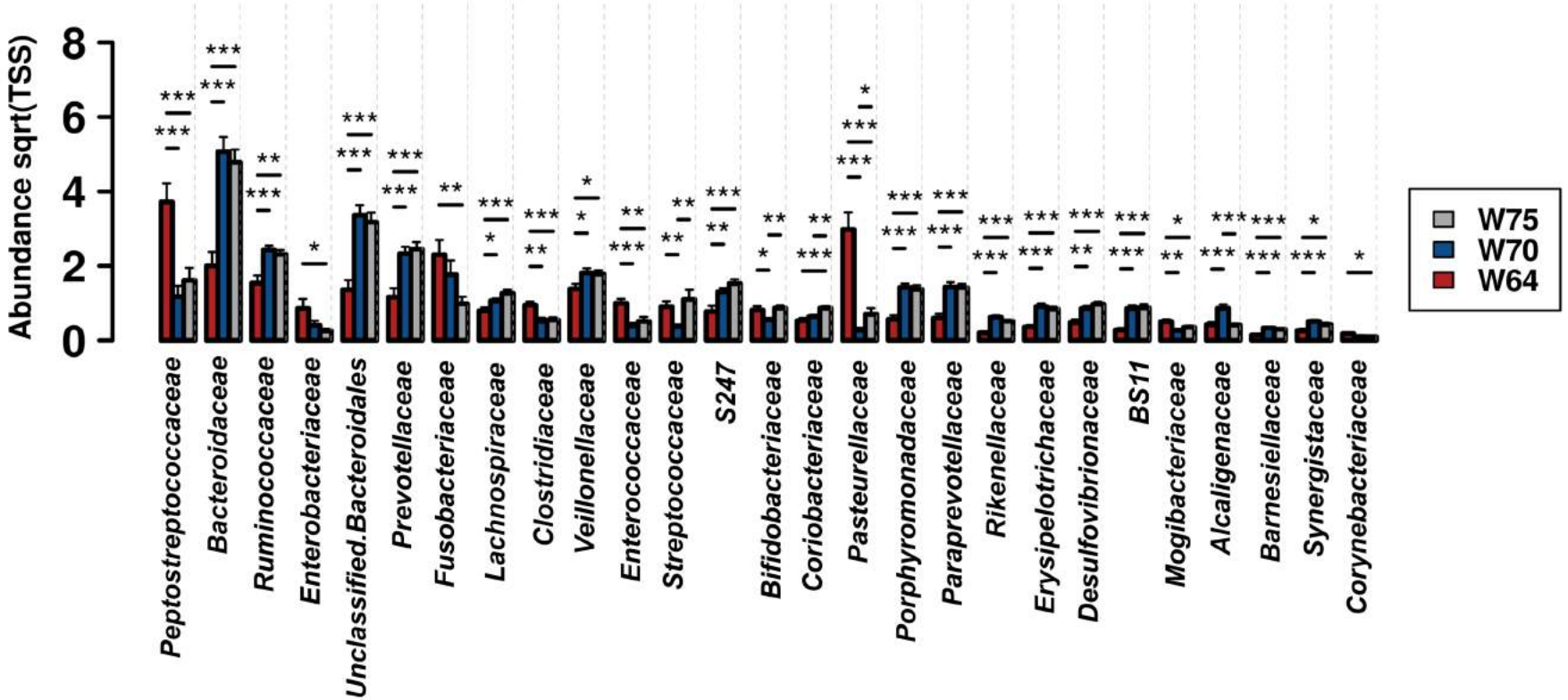
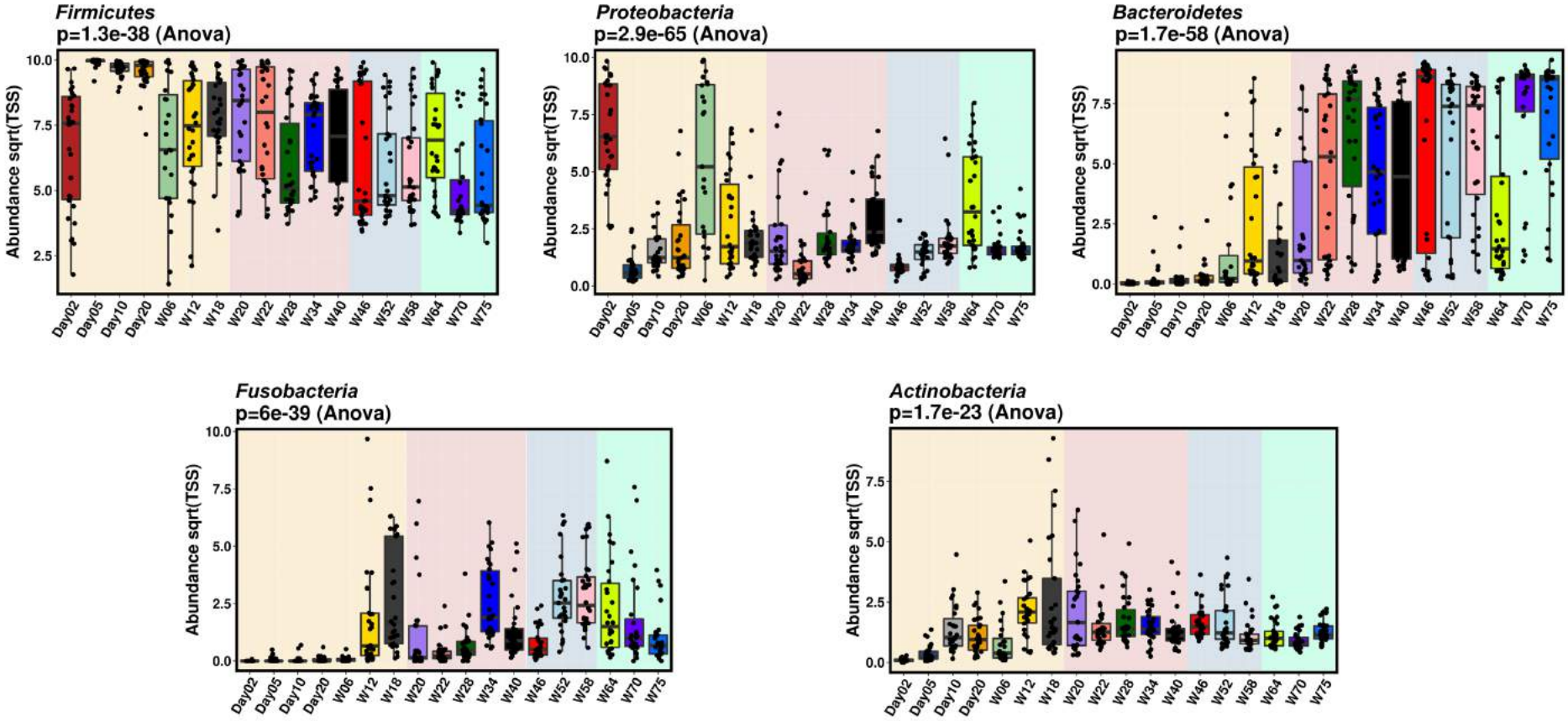


Figure 2.8 Comparative analysis of significant differences (P<0.05) in microbial community abundance at family level in late lay phase (week 60 to week 75) in the flock. The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colours of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.



Supplementary Figure 2.1 Relative abundance of phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria* and *Actinobacteria* from day 2 to week 75 of flock's age. In the boxplot, boxes including median are showing the average median abundance of gut microbiota while the upper whisker shows samples with higher abundance and lower whisker showing the samples with lower abundance. The back dot represents the individual samples collected at that time point. It also highlights the individual variation between the samples collected at the same time point.

Chapter 3 The development of gut microbiota in commercial layer flocks.

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Nitish Narendra Joat		
Contribution to the Paper	Contributed to study design, animal trials, sample collection, DNA extraction, data analysis and drafting of manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	24/04/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Thi Thu Hao Van		
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Contribution to the Paper	Contributed to study design, animal trials, 16S rRNA gene sequencing, critical revision and editing of manuscript.		
Signature		Date	02/03/2021

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Contribution to the Paper	Contributed to study design, sample collection, critical revision and editing of manuscript.		
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3.1 Abstract

Gut health has major implications for the health and productivity of laying hens. It is important to understand the typical composition and temporal development of the gut microbiota in healthy layers, as it plays a significant role in the birds' health. The present investigation aimed to study the temporal development and phylogenetic composition of the gut microbiota of three commercially raised layer flocks, from hatch to end of the production cycle. The flocks were reared in different housing systems (A-free range, B-barn and C- multi-age cage). All three flocks had different feeding, vaccination and management protocols. *Firmicutes* and *Proteobacteria* dominated at an early age. The richness of gut microbiota increased up to week 70 in flock A. In flock B, the richness was plateaued at week 6 and showed an increase at week 52, while in flock C, the richness plateaued at week 6 and remained the same except at week 18 and week 75 where it reduced. The faecal microbiota developed gradually during the rearing phase and then underwent a significant change in composition after the shift to the production farms. The microbiota composition was significantly different between the flocks, and shifts in gut microbiota composition occurred at a different age. In flock A, the *Bacteroidetes* became dominant in the late lay phase, while in flock B, the shift to *Bacteroidetes* occurred in the mid lay phase. The gut microbiota composition flock C showed a completely different picture with *Firmicutes* as the dominant phylum in the late lay phase. Flock A had an outbreak of Spotty Liver disease caused by the *Campylobacter hepaticus* during the early lay phase (week 34). Flock A showed a shift to phylum Bacteroidetes at week 34 and a significant reduction in richness at week 40 which was not observed in flock B and C. Each flock (A, B and C) had a distinctive pattern of faecal microbiota development. Although the different flocks reached an equivalent microbiota richness level, the exact composition of gut

microbiota at phylum and genus level in each flock was significantly different. The birds' age was a vital factor in gut microbiota composition, with less complex structure in young birds and increasing complexity in older birds. In this study, all three flocks showed optimal production performance despite significantly different gut microbiota compositions. Management and major events like transportation and onset of lay could disrupt gut microbiota and lead to the reduction in richness. Such disruptions can make birds susceptible to pathogen colonisation and dysbiosis, which can adversely affect the production performance in layers. Understanding the development of gut microbiota under commercial field conditions and identifying the time points where gut microbiota is most vulnerable to disruption will help plan intervention strategies to improve microbiota stability and enhance gut health and production performance of the birds.

Keywords: Chicken microbiota, Faecal microbiota, Firmicute, Housing condition, Proteobacteria.

3.2 Introduction-

The gut microbiota is defined as the collection of all microbes found in the gastrointestinal tract. The microbiota establishes a symbiotic association with the host and has been shown to influence host physiology. It plays a critical role in the development and training of the immune system, intestinal health and physiology, nutrient release, and absorption in the host [1]. Food animals are an important protein source for the growing human population, and efficient production is critical to minimising the ecological impact of the industries [2]. Thus, understanding the structure of the typical healthy gut microbiota and how it can be maintained and positively manipulated is likely to be an essential avenue for enhancing bird productivity and health.

Egg and chicken meat industries constitute a large and growing proportion among the food-producing animal industries because of their production efficiency and competitive pricing. For poultry, most microbiota research has mainly been focused on broilers [3,4]. The meat-producing birds represent a large sector of the poultry industry, and broiler studies are less laborious due to their short commercial life span. Previous research has shown that birds from the same parent stock (i.e. similar genetics), from the same hatchery, raised in the same housing conditions, and on the same feed had highly variable gut microbiota [3]. The authors hypothesised that this variation in gut microbiota could be attributed to random colonisation with environmental bacteria in the absence of maternally derived bacteria in the very clean hatcheries that are typical within the industry [3]. A comparative study conducted in slow-growing chickens of different breeds raised in a cage and free-range systems showed that the composition and diversity of the gut microbiota might be influenced by the different housing systems [5]. Other studies have also shown that housing

systems can play a key role in the establishment of gut microbiota in birds [6-10]. However, it is important to note that these studies were conducted in the controlled environments of experimental/research animal facilities, which are different from field conditions.

Laying hens are genetically different, have different feed and housing, and have a much longer production lifespan than broilers. The dynamics of gut microbiota establishment and development in layers are expected to be markedly different from broilers [11]. In laying hens, the housing systems are substantially different (Cage, Free-range, Barn) from broilers raised on deep litter systems. In the last few decades, multiple studies focusing on the effect of housing systems on poultry welfare were published [12-15]. Further, in 1999 the European Union issued a directive to phase out battery cages and shift layer production to alternate housing systems [16]. More countries, like New Zealand and Canada, have announced plans to phase out cage systems in the future [17]. In Australia, the majority of eggs are produced in free-range (47%) and cage systems (40%), with a small percentage of barn production systems (11%) and 2% of speciality eggs (Australian Eggs, Annual Report 2019). The pullet rearing practices in Australia are variable across the egg industry. Generally, the flocks are raised on the floor until birds approach the point of lay and then shifted to free-range, barn, or cage production systems. Earlier studies in layer flocks focused on single flocks [18,19]. Some studies recently analysed the impact of the environment on gut microbiota in layers [8-10]. However, these studies were performed on hens during their early production. Previous studies compared the effects of cage-free vs caged housing systems [8], conventional cages vs enriched colony cages [9], and indoor housing vs outdoor housing [10] on gut microbiota. These studies investigated how gut microbiota composition was influenced by a few variables while attempting to reduce

variability in other factors (e. g., same feed, same management and environment). The controlled environments of experimental facilities do not entirely represent the field relevant practices followed by commercial farms. This study investigated the temporal development and structure of faecal microbiotas of three commercial layer flocks, reared in barn, free-range, and cage housing systems, from hatching to the end of the production cycle.

3.3 Results:

In this study, a total of 1650 samples were collected from three flocks. The results are presented in rearing (Day 2, 5, 10, 20, week 6 and 12), early lay (week 20, 22, 28, 34, and 40), mid lay (week 46, 52, and 60) and late lay (week 64, 70, and 75) production phase. The production performance in all three flocks was optimal except in flock at week 34. Flock A was diagnosed with spotty liver disease caused by *Campylobacter hepaticus*, which reduced egg production by 20%. This also caused a significant reduction in richness and shift in gut microbiota composition, this was observed only in flock A. These results are explained in detail in respective sections.

3.3.1 Richness, age and farm dependant variation in gut microbiota

The richness of gut microbiota was significantly different (*P* =4e-261, ANOVA) between the flocks and increased with the age of birds in all three flocks (Fig.3.1). However, there were significant differences in the development pattern of gut microbiota in the flocks. In flock A, gut microbiota richness tended to increase throughout the 70 weeks, but with significant drops in diversity at 18 and 40 weeks (*P* =2.9e-11, ANOVA). In flock B, diversity plateaued at six weeks and then showed a

modest increase after 52 weeks. In flock C, diversity plateaued at six weeks and was maintained at a similar level throughout, except for a significant dip at 18 weeks (*P* =6.8e-06, ANOVA) and at the final time point (75 weeks). Flock A and C showed a significant reduction in the richness of gut microbiota at week 18 as compared to week 12 of the flock's age. In comparison, flock B did not show a significant difference in gut microbiota richness between week 12 and week 18 of the flock's age.

The beta diversity analysis of the gut microbiota by ANOSIM showed significant dissimilarities (*P* = 0.001, R= 0.068) in the gut microbiota composition between all three flocks (Fig 3.2A). The beta diversity analysis of gut microbiota by ANOSIM showed significant effects of age on the gut microbiota composition in all three flocks (Supplementary Fig. 3.1). The RDA+ analysis also showed significant differences in gut microbiota composition between the groups (Fig 3.2B) and a significant effect of age on the gut microbiota composition within all three flocks (Supplementary Fig. 3.2).

The comparative analysis between three flocks showed significant differences (*P* <0.05) in gut microbiota composition at the phylum level (Fig 3). Flock B showed a significantly higher abundance of phylum *Proteobacteria* while flock A showed a significantly higher abundance of phylum *Firmicutes*. In flock C, the abundance of phyla *Fusobacteria, Bacteroidetes* and *Actinobacteria* was significantly higher. All the three flocks showed significant differences in the abundance at the phylum level from hatch to the end of the production cycle (Supplementary Fig. 3.3).

3.3.2 Meconium samples follow a similar trend in all three flocks

Ninety meconium samples were collected to characterise the at-hatch gut microbiota in pullets. The majority of samples had no amplifiable bacterial DNA. Of the 90

samples, 16S rRNA gene amplicons could only be generated from 12 samples, indicating that most samples had very low or no bacteria. The phylum Firmicutes dominated the microbiota composition in the meconium samples in all three flocks. The abundance was the highest in flock C. The dominant family within the *Firmicutes* was the *Clostridiaceae,* across all three flocks. The second most abundant phylum in meconium samples was *Proteobacteria* (family *Enterobacteriaceae*) (Supplementary Fig. 3.4). The abundance of phylum *Proteobacteria* was lowest in flock C. Low levels of *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* were present in all flocks.

3.3.3 Development of gut microbiota during the rear

The analysis of gut microbiota between the three flocks during the rearing phase (up to week 12) showed significant differences (P <0.05, ANOVA) in gut microbiota composition. Flock B showed a significantly higher (P <0.05, ANOVA) abundance of phylum *Proteobacteria*. In comparison, the abundance of phylum *Firmicutes* was significantly lower (P <0.05, ANOVA) as compared to flock A and C (Fig. 3.4A). The abundance of phylum *Bacteroidetes* was significantly (P <0.05, ANOVA) different in all three flocks (Supplementary Fig. 3.5). In flock A, the gut microbiota at day 2 was dominated by the phylum *Proteobacteria*, while from day five up to week 12, the *Firmicutes* was the dominant phylum (Fig 3.4A). *Enterobacteriaceae* was the dominant family within the *Proteobacteria* phylum, and the *Lactobacilleae* was the most dominant family in the *Firmicutes* until day 20 (Fig. 3.4A). A similar pattern continued up to week 6 in flock A. However, the flock showed significant diversity in phylum *Firmicutes* at week 12. The dominant family at week 12 in phylum *Firmicutes* was *Peptostreptococcaceae* replacing *Lactobacillaceae*, while *Enterobacteriaceae* remained the dominant *Proteobacteria* family in flock A (Fig. 3.4A). In flock C,

increasing diversity was observed at week 6 in the *Firmicutes* phylum, where the family *Peptostreptococcaceae* was the second most abundant following *Lactobacillaceae.* The trend continued at week 12, where the family *Peptostreptococcaceae* became dominant, followed by *Lactobacillaceae*. A major shift was observed in phylum *Proteobacteria* at week six, where *Helicobacteraceae* replaced *Enterobacteriaceae;* however, at week 12, the abundance of phylum *Proteobacteria* reduced significantly, making phylum *Bacteroidetes* the second most abundant phylum (Fig. 3.4C).

In flock B, the gut microbiota followed a different development pattern; phylum *Proteobacteria* was dominant on days 2, 10, and 20. On day 5, weeks 6, and 12, phylum *Firmicutes* was dominant. However, *Enterobacteriaceae* was the dominant family in the phylum Proteobacteria until week 12 (Fig.3.4B). In contrast, in the phylum *Firmicutes*, *Lactobacillaceae* was the dominant family until week 12 of the flock's age, similar to flock A and C. At week 12, the abundance of family *Peptostreptococccceae* and genus *Turicibactor* in phylum *Firmicutes* increased significantly in flocks A and B (Fig. 3.4).

3.3.4 Changes in microbiota composition following the transfer of birds to production farms

At week 16, the birds were transported to production houses. Analysis of samples taken at week 18 revealed that the gut microbiota composition in each flock underwent major changes compared to that observed at week 12 of the flock's age, the last sampling point before transfer. In all three flocks (A, B, C), at week 18, the *Firmicutes* was the dominant phylum; however, at the family level, the *Lactobacillaceae* was the most abundant in flock A while in flock B, and flock C, the most abundant family was *Peptostreptococcaceae*. In flock C, the abundance of genus *Turicibacter* was high.

Phylum *Fusobacteria* was the second most abundant at week 18 in flock A and B, while in flock C, the second most abundant phylum was *Actinobacteria* (Fig. 3.5A).

The gut microbiota richness showed significant differences (*P* = 8.7e-12, ANOVA) between week 12 and week 18 of the flock's age in flock A and C (Fig. 3.5B). The statistical analysis (ANOSIM) (Fig. 3.5C) revealed that the within-group dissimilarity of gut microbiota increased significantly at week 18 of the flock's age across all three flocks (R= 0.509, *P* = 0.001). The RDA+ analysis also showed significant time and flock dependant variation in gut microbiota composition (Supplementary Fig.3.6).

3.3.5 Development of gut microbiota in early lay phase

In the early lay phase, phylum *Firmicutes* was dominant in flocks A and B throughout, the only exception at week 34 in flock A where *Bacteroidetes* was the dominant phylum. However, the composition of phylum *Firmicutes* differed in both the flocks (Fig. 3.6A&B). In flock A, *Peptostreptococcaceae* was the dominant family, followed by the family *Lactobacillaceae* throughout the early lay phase (Fig. 3.6A). The significant differences in the abundance of family *Turicibacteriaceae* (*P* =7.8e-10, ANOVA) and *Ruminococcaceae* (*P* =3.1e-11, ANOVA) were noted at different time points in flock A (Fig. 3.6A). In flock B, phylum *Firmicutes* was dominated by the family *Lactobaciliaceae* except at week 34, where *Peptostreptococcaceae* was dominant (Fig. 3.6B). The abundance of phylum *Bacteroidetes* varied with time points in flocks A and B. In flock A, *Bacteroidaceae* was the dominant family in the early lay phase, while in flock B, it was dominant up to week 22, after which it was replaced by the family *Prevotellaceae*. The significant abundance of phyla *Proteobacteria* and *Actinobacteria* were also noted at different time points in flocks A and B (Fig. 3.6A&B).

In the early lay phase in flock C phylum, *Bacteroidetes* was the dominant phylum for up to week 28 of the flock's age which was replaced by phylum *Firmicutes* at week 34 and week 40. The dominant family in phylum *Bacteroidetes* was *Bacteroidaceae*, followed by *Prevotellaceae* throughout the early lay phase. In phylum *Firmicutes*, the abundance of family *Lactobacillaceae* was highest at week 40 of the flock's age in the early lay phase. The significant abundance of family *Ruminococcaceae* (*P* =1.5e-05, ANOVA) was observed throughout the early lay phase in phylum *Firmicutes*. The abundance of phylum *Proteobacteria* was highest at week 20, which decreased significantly (*P* =8.8e-17, ANOVA) with age (Fig. 3.6C).

3.3.6 Development of gut microbiota in mid lay phase

In flock A, *Firmicutes* was the dominant phylum in gut microbiota in the mid lay phase, followed by *Bacteroidetes*. In phylum *Firmicutes*, *Lactobacillaceae* was the most abundant family till age 52 weeks, while at week 58 of the flock's age, the abundance of *Petostreptococcaceae* was higher. In phylum *Bacteroidetes, Bacteroidaceae* was the most dominant family throughout the mid lay phase, followed by the family *Paraprevotellaceae*. The abundance of phylum *Fusobacteria* was significantly (*P* =0.002, ANOVA) high at week 58 of the flock's age (Fig. 3.7A).

In flock B and C, phylum *Firmicutes* was dominant at weeks 46 and 52 of the flock's age, replaced by phylum *Bacteroidetes* at week 58 of the flock's age. In phylum *Firmicutes*, the abundance of family *Lactobacillaceae* decreased from week 46 until week 58 of the flock's age. An abundance of family *Ruminococcaceae* increased with age in flock B (*P* =5.2e-05, ANOVA) and flock C (*P* =0.0012, ANOVA). In phylum *Bacteroidetes, Bacteroidaceae* was the dominant family in flock C at all three-time points; however, in flock B, it was dominant only at week 52 and then replaced by the

family *Paraprovotellaceae.* The abundance of phylum *Fusobacteria* increased significantly (*P* =0.042, ANOVA) with age in flock C and was noted at weeks 52 and 58 in flock B (Fig. 3.7B&C).

3.3.7 Development of gut microbiota in the late lay phase

The samples(weeks 64 & 70) were collected from Flocks A and B at 64 and 70 weeks to understand the microbiota composition in the late lay phase. In flock A and B, phylum *Bacteroidetes* was dominant, followed by phylum *Firmicutes*. In flock A, phylum *Bacteroidetes* was dominated by family *Bacteroidaceae* while in flock B, it was dominated by family *Paraprevotellaceae* at both time points. In phylum *Firmicutes*, *Peptostreptococcaceae* was the most abundant family in flock A. In flock B, family *Ruminococcaeceae* was most abundant at week 64, replaced by family *Peptostreptococcace* at week 70. In both the flocks, a high abundance of family *Ruminococcaeceae* was noted at week 64 and week 70 of the flock's age. The abundance of phylum *Fusobacterium* was significantly high at week 70 of the flock's age in flock A (*P* =0.0079, ANOVA) and flock B (*P* =0.043, ANOVA) (Fig. 3.8A&B).

In flock C, three samplings (week 64, 70, 75) were conducted in the late lay phase. The abundance of phylum *Bacteroidetes* was highest at week 64 and later replaced by phylum *Firmicutes* at weeks 70 and 75. In phylum *Firmicutes*, the family *Peptostreptococcaceae* was most abundant at week 64 and 70, while at week 75, the abundance of the family *Lactobacillaceae* was highest. In phylum *Bacteroidetes*, family *Bacteroidaceae* was most abundant at weeks 64 and 70 of the flock's age. However, at week 75 abundance of *Paraprevotellaceae* was high. The abundance of phylum *Fusobacteria* was highest at week 64, which decreased significantly (*P* = 0.0081, ANOVA) with the flock's age (Fig. 3.8C).

3.3.8 PICRUSt analysis in three flocks

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States was used to identify significantly different pathways predicted to be encoded by the microbiota of each flock (P <0.05, ANOVA). For the pathway investigation, all samples from each flock were considered together. Further investigation of this data could be undertaken to study the temporal shifts in microbiota function. The focus was directed on potential pathways that might impact production performance. Pathways including Amino acid metabolism (P = 0.00064), Biotin metabolism (P =2.8e-09), Carbohydrate metabolism (P =2.7e-15), Energy metabolism (P =4.2e-05), Lipid metabolism (P = 4.2e-15), Mineral absorption (P =6.8e-06), Protein digestion and absorption (P =4e-08), Thiamine metabolism (P =8.4e-06), Vitamin B6 metabolism (P =2.9e-08) were significantly different in abundance in the three flocks. Pathways, including Riboflavin metabolism (P =0.08), Fatty acid metabolism (P =0.14), Calcium signalling (P =0.06), did not show significant differences between the flocks (Supplementary Fig. 3.7).

3.4 Discussion

The three flocks included in this study had egg production according to the breed standard (apart from a brief dip in flock A production due to a Spotty Liver Disease outbreak) yet had significantly different microbiota development and composition patterns. These significant differences in the gut microbiota composition and beta diversity between different flocks could be due to multiple factors such as feed, age, or housing systems [11]. Previous studies have reported high variability of gut microbiota within and between the studies [3,4]. The gut microbiota richness in flocks A, B and C increased with age. The high richness of gut microbiota has been associated with

improved gut health and improved productivity [20]. Further, the richer microbiotas are generally considered an indicator of better gut health as rich populations tend to be more robust in resisting and recovering from perturbations and have a broader metabolic potential [21,22].

A reduction in the richness of gut microbiota at week 18 was observed in flocks A and C. This could be attributed to the stress caused by the transport of birds from rearing to production housing. Surprisingly, no reduction in gut microbiota richness was noted in flock B that was transported over a distance of 80 km from the rear to the production shed. The reasons behind this observation are unknown; however, this highlights the necessity for detailed studies to understand how stressors such as transportation, the onset of lay, and feed change affect the gut microbiota. The significant decrease in gut microbiota richness at week 40 in flock A coincides with a Spotty Liver Disease (SLD) outbreak (caused by *Campylobacter hepaticus*). *C. hepaticus* is known to colonise in the small intestine and caeca of infected birds [23]. This drop-in gut microbiota richness could be because of the SLD outbreak. Gut microbiota in flock A also showed a significant shift towards phylum Bacteroidetes at week 34 coupled with a 20% decline in egg production. The adverse impact of disease conditions on egg production in layer birds is well known; however, the impact of *Campylobacter hepaticus* infection on gut microbiota has not been well documented. However, *Campylobacter jejuni* infection in chickens leads to the reduction of richness of gut microbiota[24]. Further, the immune response to *Campylobacter jejuni* in layers has been known to be significantly influenced by feed composition[24]. Flock A was treated for Campylobacter hepaticus infection with chlortetracycline for two weeks through the water. Earlier literature has showed that chlortetracycline treatment reduced richness/alpha diversity and altered gut microbiota composition in pigs[25]. Therefore,

the significant reduction in richness and shift in gut microbiota composition could have been caused due to chlortetracycline treatment. Depending upon the observations and literature available, it is essential to know how Campylobacter hepaticus infection impacts the gut microbiota? What is the impact of antibiotics used to treat the various infection on gut microbiota in chickens? To investigate these questions, further controlled studies are required. In flock A, the richness of gut microbiota increased up to week 70, while in flock B, richness plateaued between week 6 to week 46, after which it showed an increase. These findings suggest that the gut microbiota development was distinct in different flocks, supported by the earlier study [3].

In flock C, richness was significantly increased after week 20. This could be attributed to the multi-age cage production system where the experimental flock was shifted at week 16. It could be suggested that after shifting to production, young hens may have acquired the microbiota from the shed environment [11]. Interestingly, in flock C, the richness of microbiota decreased at week 75, coinciding with introducing a new flock into the multi-age shed. The reduction in the richness could have been caused by the stress effect of the introduction of new birds or could result from an ageing effect; however, further studies are needed to understand the richness pattern in multi-age cage flocks.

It is well known in mammals that infants are seeded with maternal gut microbiota [26,27]. However, the embryonated eggs in birds are not connected to the hen by the umbilical cord or placenta. Despite this, it has been suggested that there may be some acquisition of gut microbiota from the hen, however, this hypothesis is still being tested [28,29]. Multiple studies reported that the post-hatch environment is the primary source of microbiota acquisition [4,11]. Contemplating the clean hatchery practices used today for egg incubation, it can be speculated that microbiota in meconium samples is

(embryonic gut microbiota) acquired during egg formation in the hen. However, the low number of sequenced samples (12/90) indicates that the majority of the hatched chicks had little or no bacteria present within the gut.

Further, the results cannot be compared with any of the earlier studies as no specific studies focused on microbiota in meconium samples. It was observed that the microbiota was established from day two onwards and continued to develop over time. An earlier study has also suggested age as a driving factor for gut microbiota development [18], which agrees with results from our study showing age dependant development of gut microbiota. It is assumed that the early microbiota is acquired from the environment [3]. Influencing the stochastic process of early microbiota acquisition by introducing standardised and structured primary microbiota exposure (known beneficial genera) may provide a more consistent and predictable gut microbiota development. It could be achieved by seeding the birds with feed supplements such as probiotics or prebiotics at an early age (such as *in ovo* or immediately upon placement on a farm at day old) which might be beneficial [30,31].

An earlier study found that the gut microbiota was dominated by phylum *Proteobacteria* (family *Enterobacteriace*ae in phase 1), phylum *Firmicutes* in phase 2 and the succession of phylum *Firmicutes* to phylum *Bacteroidetes* in phase 3 [19]. In our study, all the flocks (A, B, C) showed a high abundance of phylum *Proteobacteria* on day 2; however, by day five, the *Firmicutes* had become the dominant phylum. The high abundance of *Firmicutes* at an early age can be related to the high energy demand as the growth rate at an early age is rapid [19]. This is in contrast with the earlier study where phylum *Proteobacteria* was dominant throughout the first week [19]. This difference might be attributed to the different sampling frequency/time in the current study compared to the previous study [19]. In the earlier study, in phase 2 (2-4 weeks),

gut microbiota was dominated by phylum *Firmicutes* which correlated with our results; however, at the family level, the dominant families in earlier studies were *Lachnospiraceae* and *Ruminococcaceae*, which are thought to be the main SCFA producers is in contrast with our results where family *Lactobacillaceae* was dominant. Family *Lactobacillaceae* from the phylum *Firmicutes* produces lactic acid, a short-chain fatty acid (SCFA) and can be converted to other SCFAs, such as butyrate [32]. Butyrate is the preferred energy substrate of intestinal epithelial cells [33,34]. Many *Lactobacillus* strains have been used as probiotics in chickens [35,36]. A previous study in broiler chickens demonstrated beneficial effects such as reducing pathogenic species and increased short-chain fatty acid-producing bacteria after administration of *Lactobacillus* strains after hatch [37]. However, it is essential to note that not all *Lactobacillus* are beneficial for health, and some studies indicated some *Lactobacillus* spp. are related to poor growth performance in broiler chicken [38,39]. Some *Lactobacillus* spp. reduce lipid intake and cause dietary energy losses in broilers [40]. Given the limited understanding of *Lactobacillus* spp. in layers, further studies focused on the role of *Lactobacillus* spp. in layers reared in different housing systems is essential. Thus, the high abundance of phylum *Firmicutes* may support rapid growth at an early age. Further, in the earlier study, phylum *Firmicutes* was succeeded by phylum *Bacteroidetes* between week 4 to week 26 of flocks age. Phylum *Bacteroidetes* accounted for 55% of the total gut microbiota [19], which was not recorded in flock A and B except at week 34 in flock A. At the same time, *C. hepaticus* outbreak was noted in flock A, which is known to colonise in the intestinal tract [23], which has been discussed in detail earlier. In flock C, phylum *Bacteroidetes* became the second dominant phylum at week 12; however, it succeeded phylum *Proteobacteria* and *Actinobacteria* instead of phylum *Firmicutes*, as reported in the earlier study [19]. The high abundance of

phylum *Bacteroidetes* in flock C during the early lay phase might be attributed to the multi-age farming system. Flock C was shifted to a multi-age production system at week 16, which housed older hens from different flocks. The gut microbiota of the older hens is dominated by the phylum *Bacteroidetes* [41]. It has also been reported that the environment has a significant impact on gut microbiota development[11]. This might have caused a high abundance of phylum *Bacteroidetes* in the gut microbiota at an early age in flock C. Nordentoft et al. [42] analysed gut microbiota of 18-week-old hens and found that the most abundant phyla were *Firmicutes*, and *Bacteroidetes,* followed by a lower abundance of phyla *Actinobacteri*a, *Proteobacteria* and *Fusobacteria*. In the present study, the gut microbiota at week 18 was dominated by phylum *Firmicutes,* and the abundance of other phyla fluctuated in each flock.

The dominance of *Firmicutes* in the mid lay phase in flock A and B was contrary to the earlier findings where *Firmicutes* and *Bacteroidetes* formed approximately half the gut microbiota [19]. However, the dominance of phylum *Bacteroidetes* noted in the late lay phase in flock A and B was recorded in the earlier study [41]. In layers, development at an early age and then egg production would require a high amount of energy. Phylum *Bacteroidetes* is responsible for the digestion of indigestible carbohydrates, including cellulose and resistant starch [4]. The propionate and acetate are the primary fermentation products of phylum *Bacteroidetes* [43,44]. From this data, it could be hypothesised that the high abundance of *Firmicutes* in the rearing and early lay phase is related to the physiological energy requirement of growth and egg production. In contrast, during mid and late lay, the host energy requirement is comparatively less and thus, to maintain the energy balance, phylum *Bacteroidetes* succeeds phylum *Firmicutes*. Thus, the gut microbiota might be modulated in response to the changing host energy use. Although the exact mechanism of the gut microbiota host

communication is not clear, previous research has indicated a role of host molecules like miRNA (miR-199a-5p, miR-1226), hormones (Insulin, Estradiol, Norepinephrine), cytokines (IL-1ß, TNF- alpha), host molecules (NLRP6) and metabolic signalling pathways (FXR signalling agonist (GW4064) and antagonist (Gly-MCA), Fucose) in communication with the resident and pathogenic microbiota [45].

The comparison with other studies indicates that flocks do not closely follow the same trends in gut microbiota development. The abundance of each phylum and the families within each phylum changed according to time and studies [18,19]. Comparison of microbiota composition across different studies can be difficult because of fundamental differences such as housing, genetics, feed and environmental influence [11]. It has also been documented that comparisons of microbiota analysis between different studies can be challenging as a range of different data acquisition and analysis methods are used. Any such differences can influence the outcomes and how they are reported [46,47]. In the current investigation, all the data acquisition and analysis methods were standardised and consistent across the whole study. It is assumed that the differences seen between flocks have a biological origin and are not caused by technical variations.

The change in gut microbiota at week 18 possibly occurred due to physiological changes such as the onset of sexual maturity, change in housing systems, change in feed and transportation of birds. The transition from rearing to production is a point of particular vulnerability for layer flocks when their health can be compromised by several pathogens such as *Salmonella* and *C hepaticus* (SLD) [48,49]. Efforts to minimise the gut microbiota disruption after transport may lead to beneficial health and productivity outcomes.

The gut microbiota analysis showed a varying abundance of beneficial genera at different time points in all three flocks. The abundance of the genus *Faecalibacterium* was highest in flock B and C. *Faecalibacterium*, a gram-positive anaerobe, is a common inhabitant of gut microbiota and produces butyrate and other short-chain fatty acids by fermenting dietary fibres [50]. It is also associated with improved FCRs and body weight gain in birds and may have a role in directing the development of the immune system [51]. The abundance of *Bifidobacterium* was also high in flocks B and C. *Bifidobacterium* is associated with the production of lactic acid as a primary product of glucose fermentation and has been reported to improve gut health [52].

The gut microbiota analysis also identified potentially pathogenic genera in all three flocks. These genera include opportunistic pathogens, notably, *Gallibacterium anatis* [53], genus *Streptococcus* [54], and *Enterococcus* [54]. In this study, the abundance of genera *Enterococcus*, *Fusobacterium*, *Galibacterium*, and *Streptococcus* were higher in pullets reared on a dirt floor (flocks A and B). This could be attributed to the complexity of cleaning and disinfecting dirt floors. The increased abundance of opportunistic pathogens like *Gallibacterium* and *Fusobacterium* at week 18 may be linked to the stress caused by transportation and the onset of sexual maturity. Further studies are needed to investigate this hypothesis.

Lastly, the PICRUSt analysis showed significant differences in multiple potential functions between the three flocks. These results can be correlated with significant differences in the microbiota in the flocks. Pathways like fatty acid metabolism and calcium signalling did not show any significant differences between the flocks even after the significant differences were observed in gut microbiota. It could be speculated that significant differences in gut microbiota composition and its potential function do not affect the production performance in commercial layers.

3.5 Conclusions

This study focused on gut microbiota development in three commercials layer flocks to establish information about composition, development, and variability. The taxonomic composition of gut microbiota varied considerably between flocks. It is clear that there is no single ideal microbiota for high productivity flocks; different communities can produce similar productivity outcomes for the host. Some birds had bacteria in meconium, but in most birds, the gut microbiota established immediately after hatch and continued to develop over time. Irrespective of the housing system, there was a major rearrangement in the gut microbiota composition of birds after transport from the rear to the production shed but the richness of gut microbiota reduced only in flocks A and C. This demonstrates the necessity for detailed studies to understand the effect of different stressors such as transportation, the onset of lay and change in feed on gut microbiota. The reduction in richness may increase the susceptibility of the flock to pathogens. The observation highlights a critical point of vulnerability for flock health and indicates the potential value of formulating strategies to modify diet (providing prebiotic, probiotics, vitamins) at critical stress points in the life cycle of laying hens to reduce the risk of gut pathogens. Flock A showed a reduction in egg production, significant decline in richness and shift in the gut microbiota composition, which coincides with *Campylobacter hepaticus* infection. This shows the need for more understanding of the impact of *Campylobacter hepaticus* infection and antibiotic treatment on gut microbiota of adult laying hens. The overall abundance of known opportunistic pathogens was higher in flocks reared on dirt flooring than on concrete floors. This indicates that to reduce the risk of gut infection, preference should be given to pullet rearing on a concrete floor.

3.6 Materials and Methods

3.6.1 Farms

Three commercial layers flocks were selected for this study. The selection of the farms was based on the willingness of the farmers to participate in the study and distance from the research laboratory. A and B flocks were reared on dirt floors from day-old and transferred to free-range and barn housing production systems, respectively. In flock A, a Spotty Liver Disease outbreak (caused by *Campylobacter hepaticus* infection) was noted at the age of 34 weeks, resulting in a drop in egg production (20%), which recovered in the next two weeks. The flock was treated with chlortetracycline through water for 1 week. Flock C was reared on a concrete floor from day old and transferred to a multi-age cage production system. All three flocks were transported to production houses at the age of 16 weeks. The birds in all three flocks belonged to the same breed (Hyline) and originated from the same hatchery. All three flocks were reared at different locations but in the same season. Flock A was vaccinated for Infectious bronchitis virus, Coccidia, Infectious laryngotracheitis virus, Newcastle disease virus, Fowl Pox, Avian encephalomyelitis virus, Fowl cholera, Egg drop syndrome, Marek's disease virus. Flock B and flock C were vaccinated against all the above diseases except Coccidia and Fowl Pox.

All three flocks received wheat and soya-based diet. The details regarding the feeding are listed in Table 3.1. The details of each flock are listed in Table 3.2.

3.6.2 Sample collection

Meconium samples (n=30 each) were collected from all three flocks by scrapping hatching trays in sterile sample containers from each flock at the hatchery (TechnoPlas- P10065SU). Faecal samples (n=30) were collected by cloacal swabbing and resuspended in 500 µL Phosphate Buffer Saline from each flock at days 0, 2, 5, 10, 20, week 6, 12, 18, 20, 22, 28, 34, 40, 46, 52, 58, 64, 70, of the flock's age for flock A and B (n=30×2×18) while for the flock C, additional sampling was conducted at week 75. All samples were transported on ice from the hatchery or farm to the laboratory and stored at -20°C until processed for DNA extraction.

3.6.3 Animal Ethics Statement

All the animal work was conducted according to national and international animal welfare guidelines. The experiment was approved by the Animal Ethics Committee, University of Adelaide (Approval No. S-2018-015).

3.6.4 DNA extraction

DNA was extracted and purified from meconium samples and cloacal swabs (n=1650) using the QIAamp Fast DNA Stool Mini kit (Qiagen, USA) using a modified protocol [55], which was further modified for this study. Briefly, 200 mg of faecal sample (semi-solid faecal slurry which was suspended in PBS using cloacal swabs) was vortexed after adding 1 mL of preheated (70°C) InhibitEx buffer (Qiagen, USA- 19593). The samples were homogenised after the addition of 390 mg glass beads (Sigma glass beads, acid washed, 450-600 µm (180 mg) &106 µm (210 mg) using a Bullet Blender (Next Advance, United States) for 5 minutes. The samples were incubated on ice for 30 seconds before incubating in a 95°C heat block for 7 minutes. The samples were

centrifuged for 2 minutes, and the supernatant was collected and then further processed according to the modified QIAamp Fast DNA Stool Mini kit protocol [55]. The DNA was eluted in 100 μL of ATE buffer and stored at -20°C.

3.6.5 16S rRNA gene sequencing

The 16S V3-V4 region was amplified with Q5 high fidelity DNA polymerase (New England Biolabs), using dual indexing, variable spacer primers, and amplicon sequencing was performed using an Illumina MiSeq system (2x300bp) [56]. Initial sequence processing, including demultiplexing, joining, quality trimming and OTU picking, were done with QIIME 1.9.1 software [57]. The dataset was inspected for chimeric sequences using Pintail [58]. OTUs were picked using the UCLUST algorithm (Edger 2010) using a 97% sequence identity setting. GreenGenes database 13.8 was used for the provisional taxonomy assignment [59]. The additional taxonomic analysis was done on representative sequences using Blast against the NCBI 16S database with inspection of individual sequence hits and alignments. Data were further analysed and visualised with the online analysis package Calypso [60], using the OTU biom table, filtered to remove low abundance OTUs (less than 0.01%), and square root transformed and total sum normalised.

3.6.6 Statistical analysis

The microbiota analysis and visualisation were performed using Calypso software [60]. Alpha diversity analysis of gut microbiota was performed using various indices, but only the representative Richness index is presented. ANOVA and ANOSIM were used to test similarities in beta diversity between groups. Redundancy analysis was

performed to estimate the effect of farm and age on the gut microbiota. The potential function of gut microbiota was analysed using ANOVA on PICRUSt generated functional predictions [61].

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3.7 Declarations

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Authors' contributions

NJ, HV, RM, DS, KC contributed to the study design, animal trials, samples processing and critical revision of the manuscript. NJ and KC collected samples from animals, NJ processed the samples for DNA extraction, HV and RM performed 16S rRNA gene sequencing, DS and NJ analysed the data, NJ drafted the manuscript. All authors

approved the manuscript for publication. KC, RM and DS obtained funding for the work.

Ethics approval and consent to participate

The experimental setup was approved by the University of Adelaide, Animal Ethics Committee under Approval Number No. S-2018-015. The protocol was carried out in accordance with the guidelines -specified in Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition 2013.

Consent for publications

All authors have approved the submission of the manuscript.

Availability of data and materials

The 16S rRNA sequence data are available from the MG RAST database under the accession number Mglxxx (pending)

Conflict of interests

The authors declare that they have no conflict of interests.

Funding

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Table 3.1 Feeding details of each flock.

Farms	Feed source	Feed supplements	Age of the birds at which specific feed provided				
			Starter	Grower	Developer	Pre-Lay	Lay
A	Feed Mill A	No	0-6 weeks	7-12 weeks	12-14 weeks	15-17 weeks	18 weeks onwards
B	Feed Mill A	No	0-6 weeks	7-12 weeks	12-16 weeks		16 weeks onwards
C	Feed Mill A	No	0-6 weeks	7-12 weeks	12-16 weeks		16 weeks onwards

Table 3.2 Details of each flock

Farms	Housing	Bird density	Average bodyweight @ week 15	Mortality during rear	Transportation distance from rearing to production farm
A	On the floor from day old	30 Kg/m^2	1.405 kg	1.8%	11.9 Km
B	On the floor from day old	30 Kg/m^2	1.315 kg	1.2%	79.7 Km
C	On the floor from day old	30 Kg/m^2	1.3 kg	1.1%	Same farm

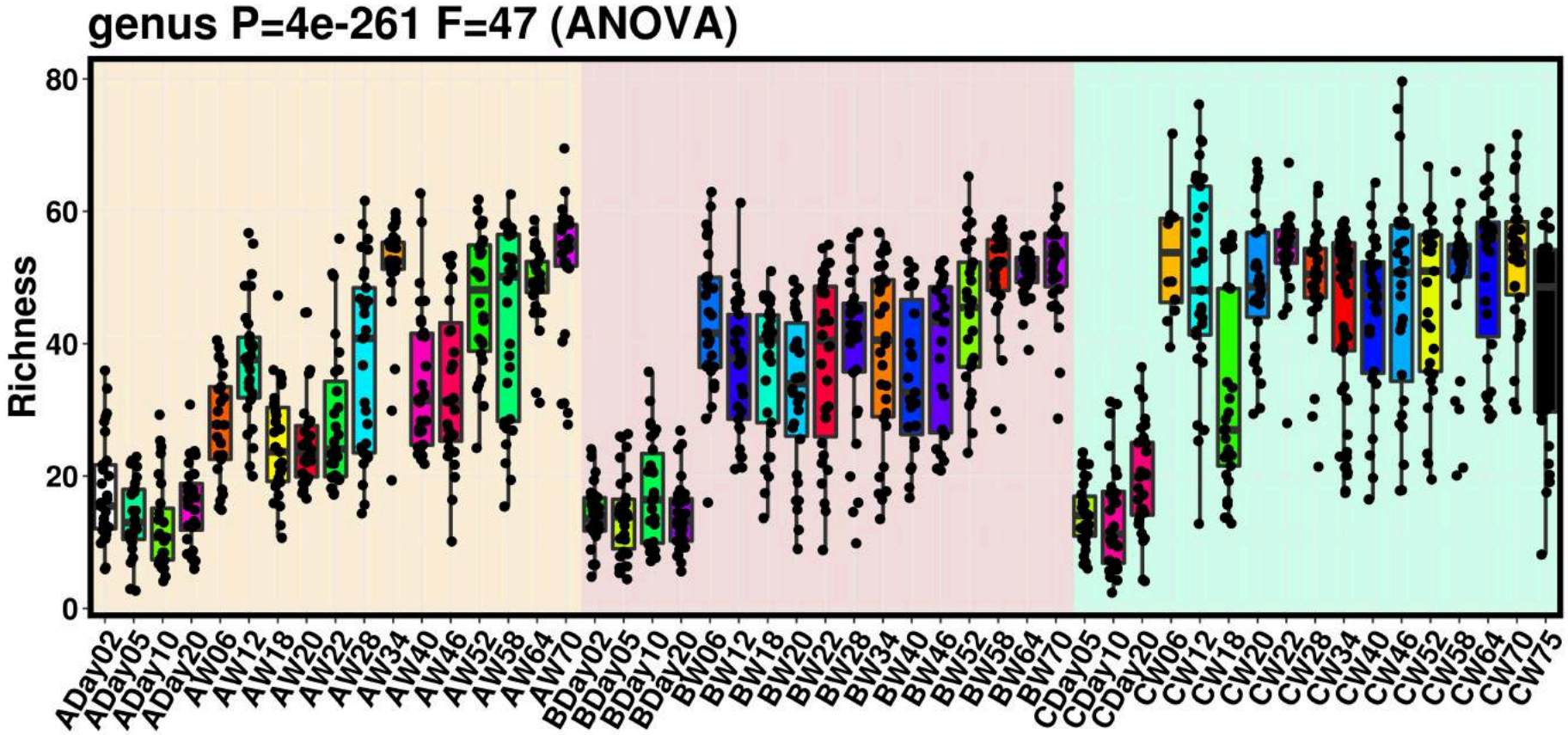


Figure 3.1: The comparative analysis of richness of gut microbiota in flock A, B, and C from hatch to the end of the production cycle. In the boxplot, boxes including median are showing the average richness median richness of gut microbiota while the upper whisker shows samples with higher richness and lower whisker showing the samples with lower richness. The back dot represents the individual samples collected at that time point. It also highlights the individual variation between the samples collected at the same time point.

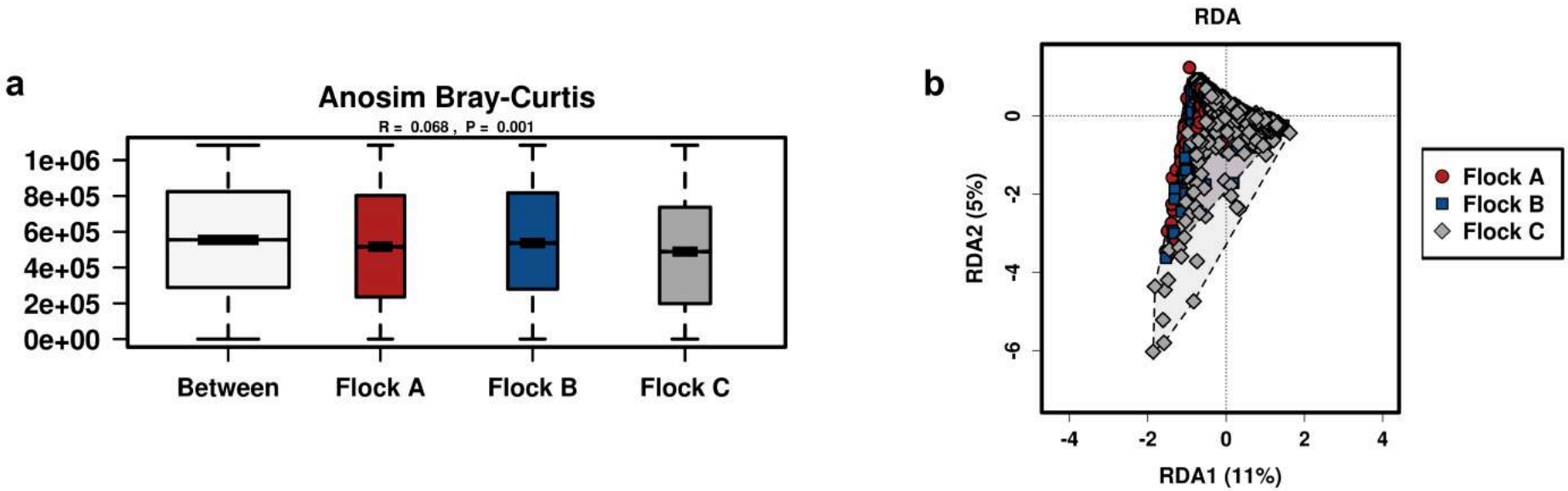


Figure 3.2: Beta diversity analysis of gut microbiota using ANOSIM between flock A, B and C from hatch to end of production cycle (3.2A). The white box represents the dissimilarity in gut microbiota between the three flocks while colored boxes represent the dissimilarity in gut microbiota within the flock. Redundancy analysis (RDA+) of gut microbiota in flocks A, B, and C (3.2B). Each square represents the individual sample and different colors denote the individual flocks in this study.

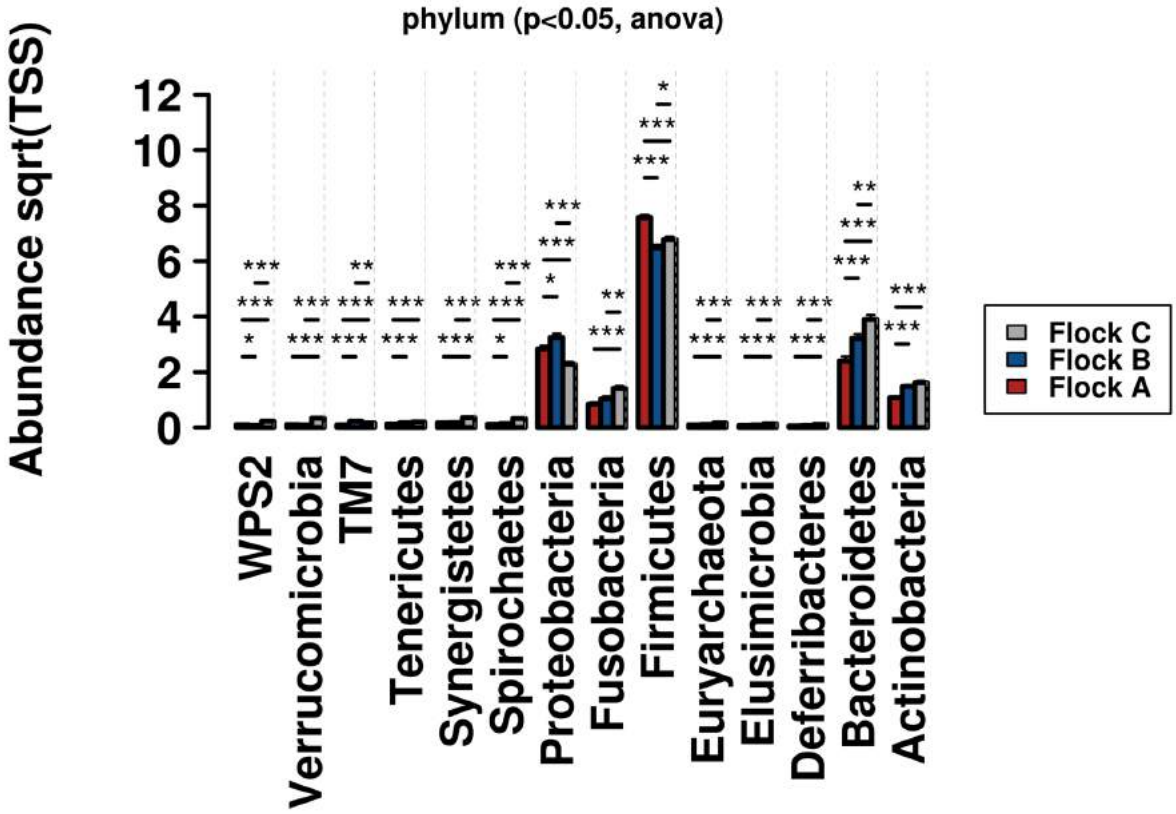


Figure 3.3: Analysis of significant differences (P <0.05) in abundance of gut microbiota at phylum level in flocks A, B and C from hatch to end of production cycle. The bar charts show the square root abundance of different phylum in three flocks denoted by three different colors. The asterisks show the significant differences in the three flocks at the phylum level.

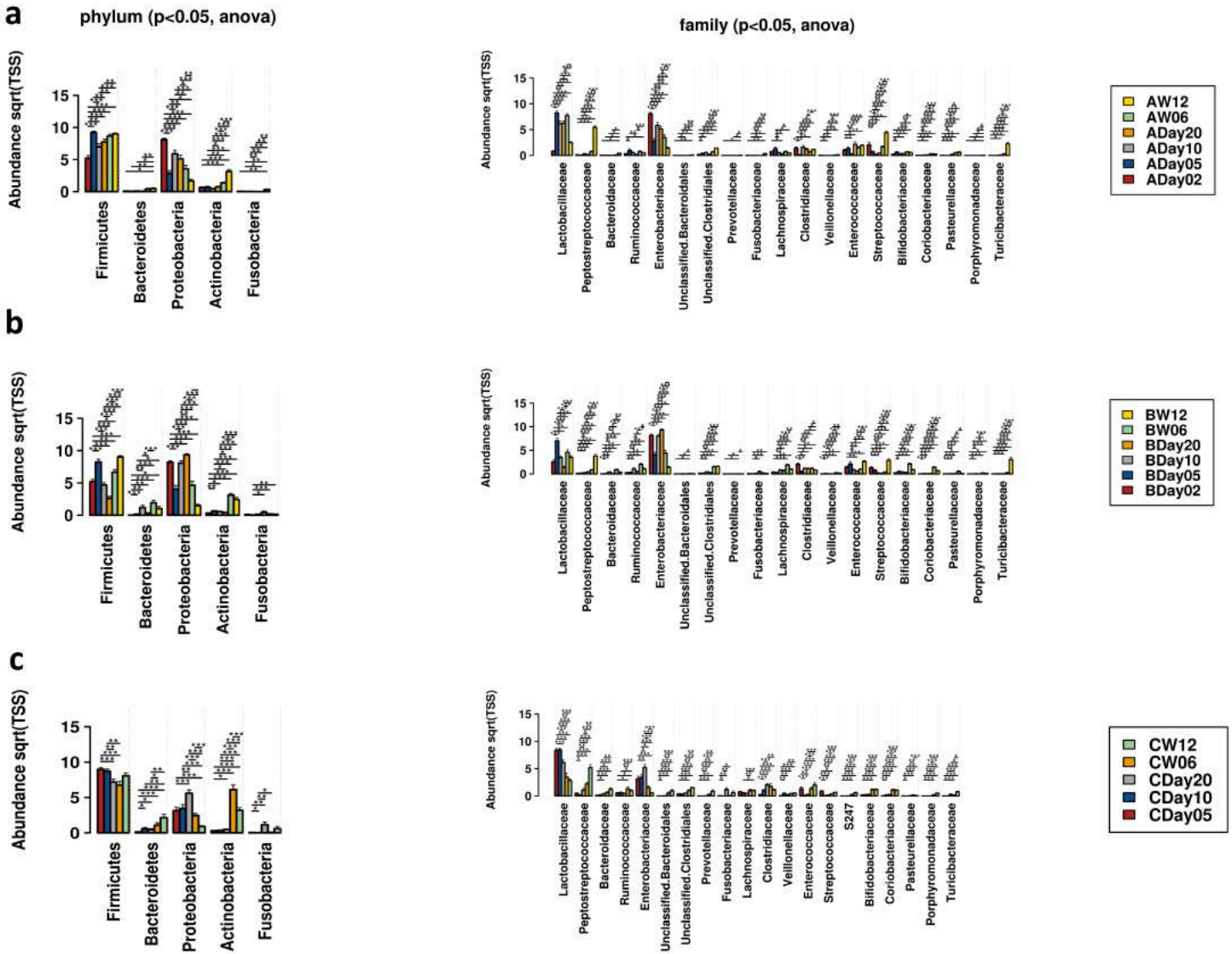


Figure 3.4: Comparative analysis of significant differences (*P* <0.05) in the abundance of gut microbiota at phylum level and family level in flocks A (a), B (b), and C (c) in the rearing phase (up to week 12). The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colors of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.

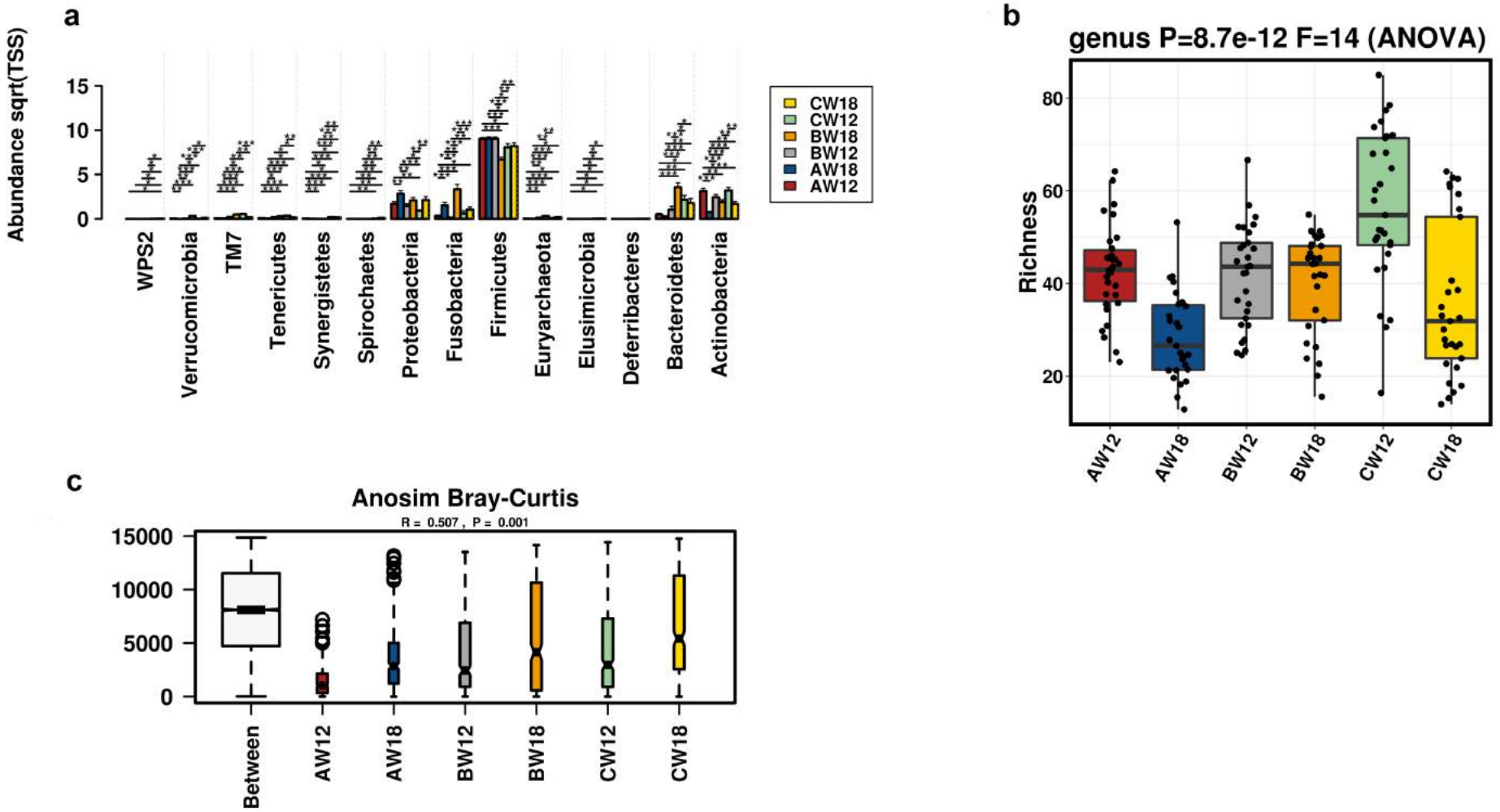


Figure 3.5: Comparative analysis of significant differences (*P* <0.05) in the gut microbiota composition at family level between week 12 and week 18 of the flocks age (Figure 3.5 A). Significant differences (*P* = 8.7e-12) in the richness of gut microbiota between week 12 and week 18 (Figure 3.5 B) and beta diversity analysis of gut microbiota between week 12 and week 18 in flocks A, B and C (Figure 3.5 C).

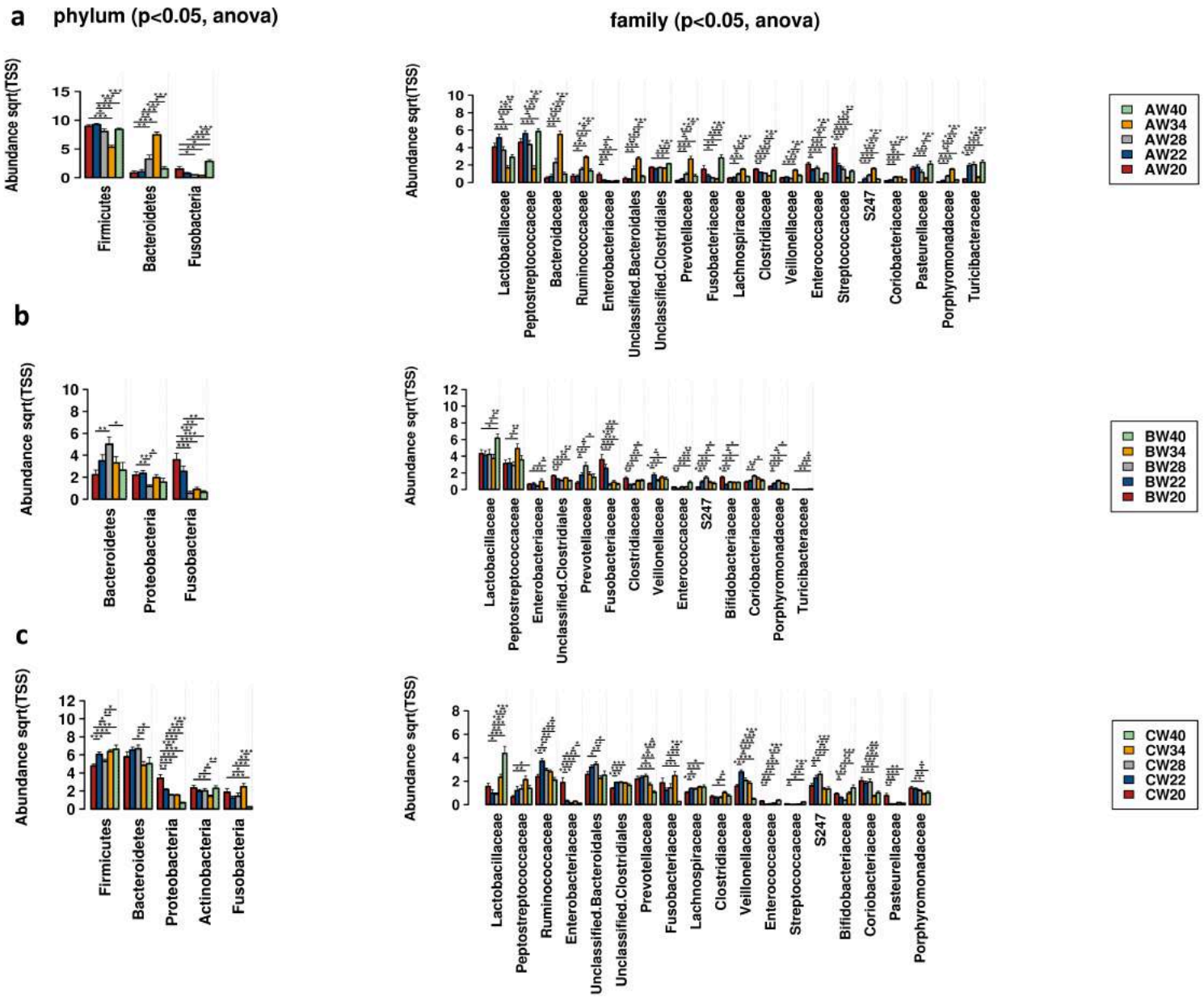


Figure 3.6: Comparative analysis of significant differences *(P* <0.05) in the abundance of gut microbiota at phylum level and family level in flocks A (a), B (b), and C (c) in the early lay phase (week 20 to week 40). The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colors of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.

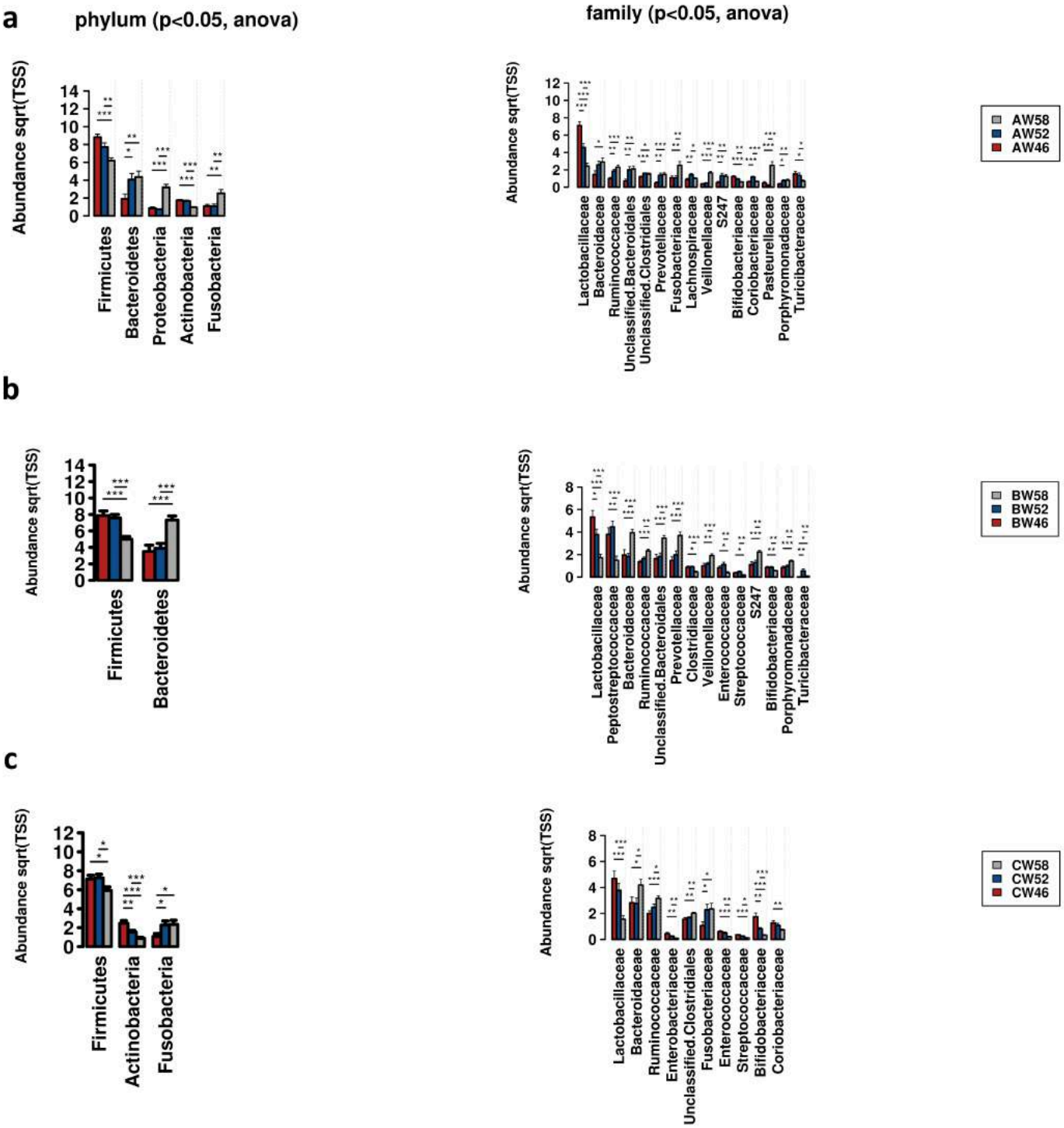


Figure 3.7: Comparative analysis of significant differences (*P* <0.05) in the abundance of gut microbiota at phylum level and family level in flocks A (a), B (b), and C (c) in mid lay phase (week 40 to week 60). The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colors of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.

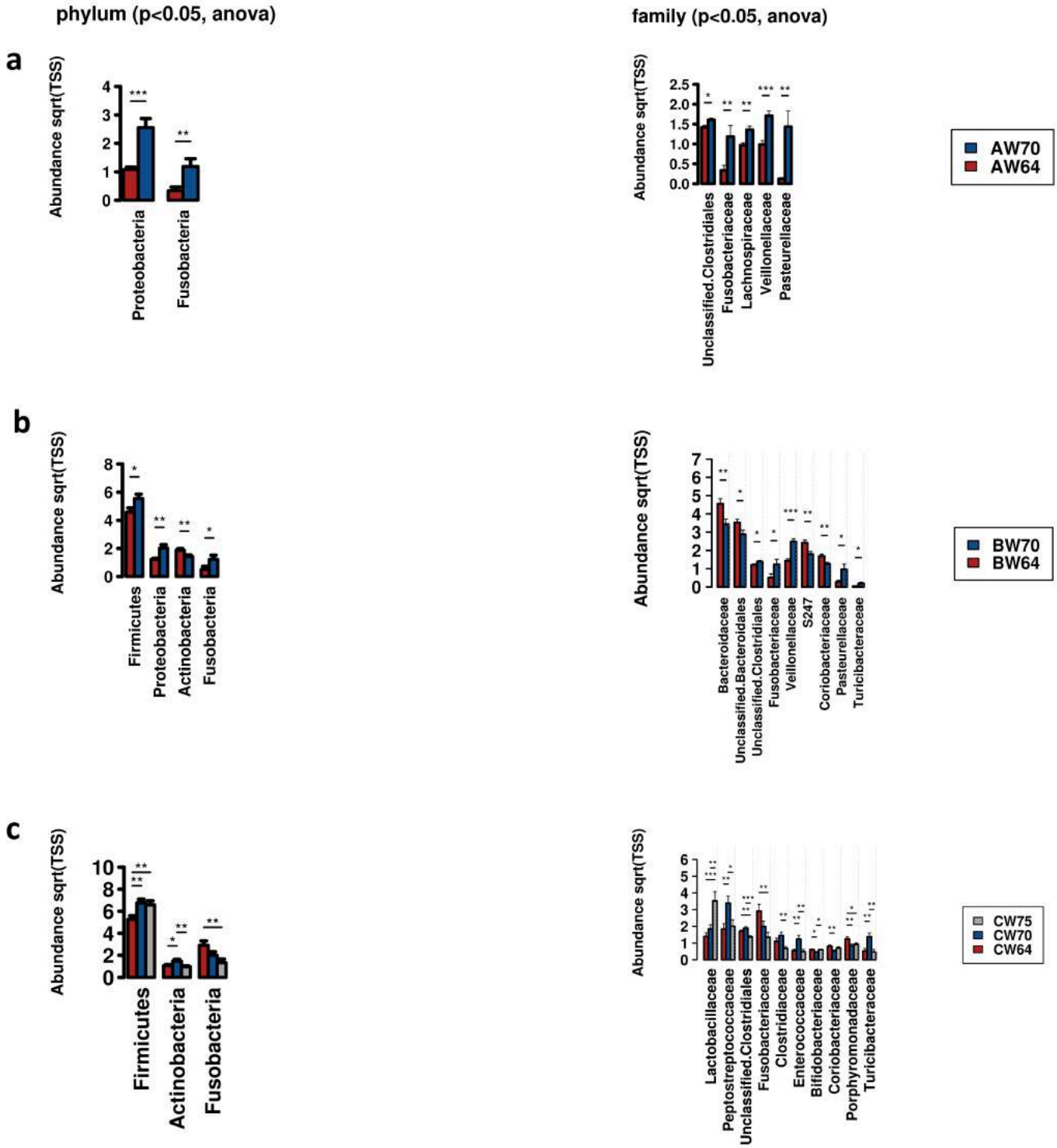
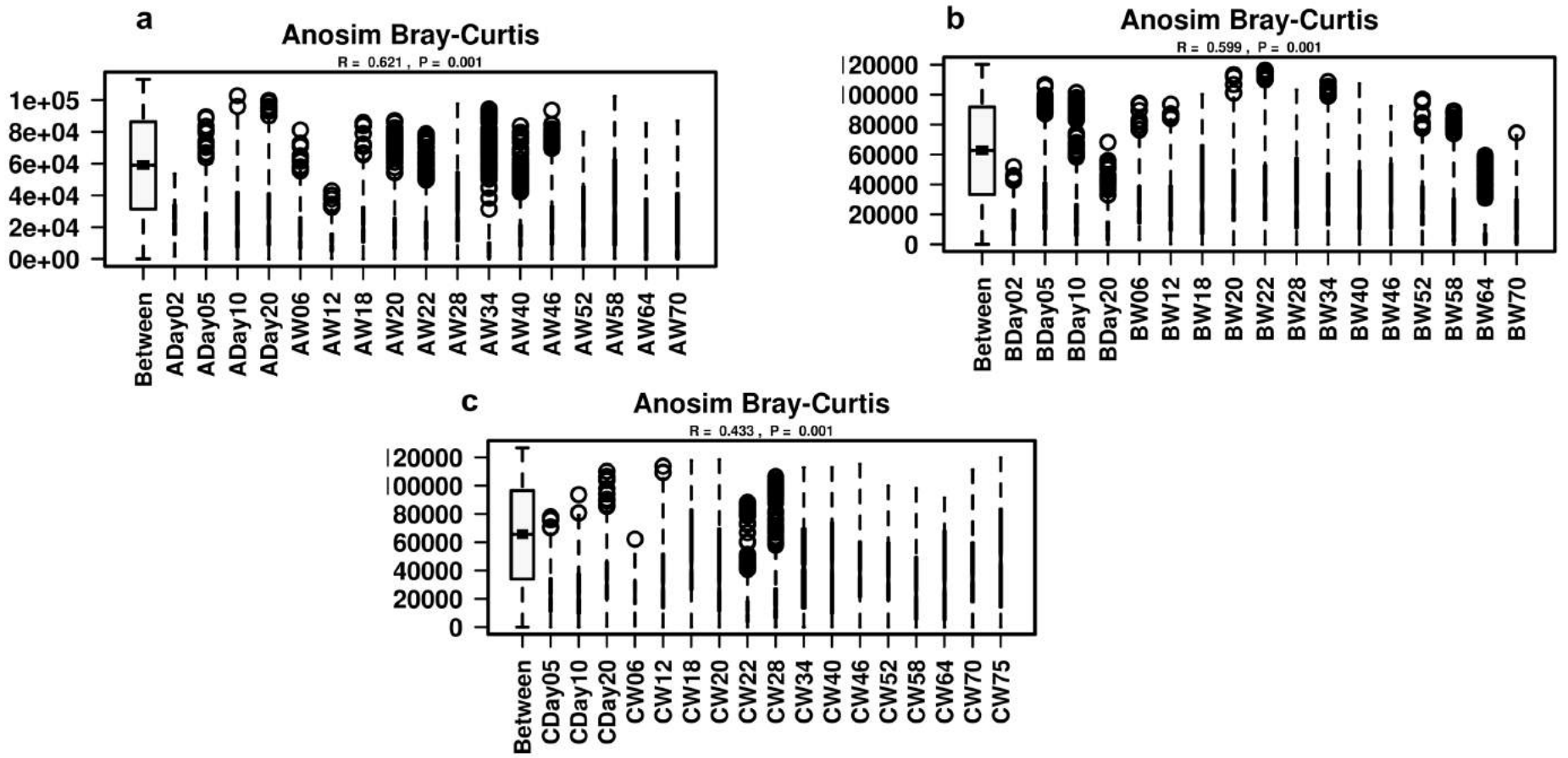
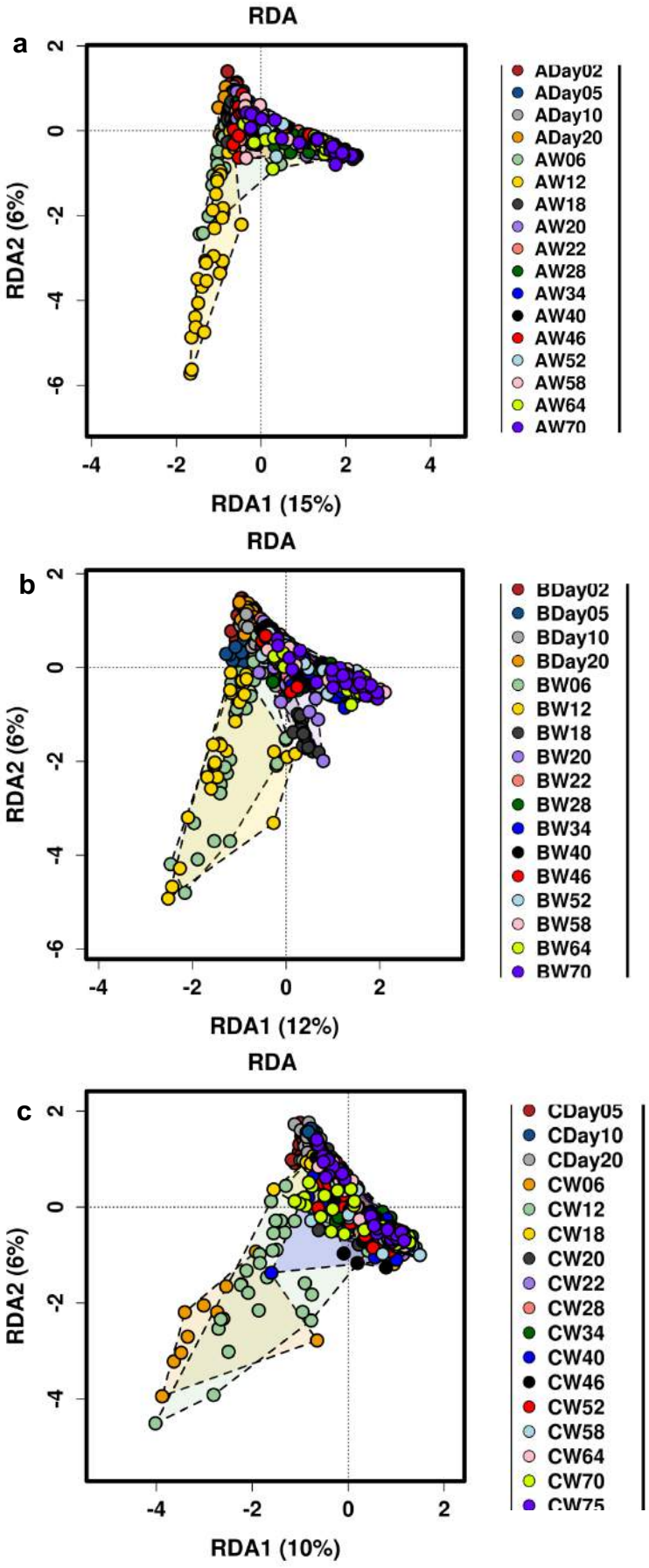


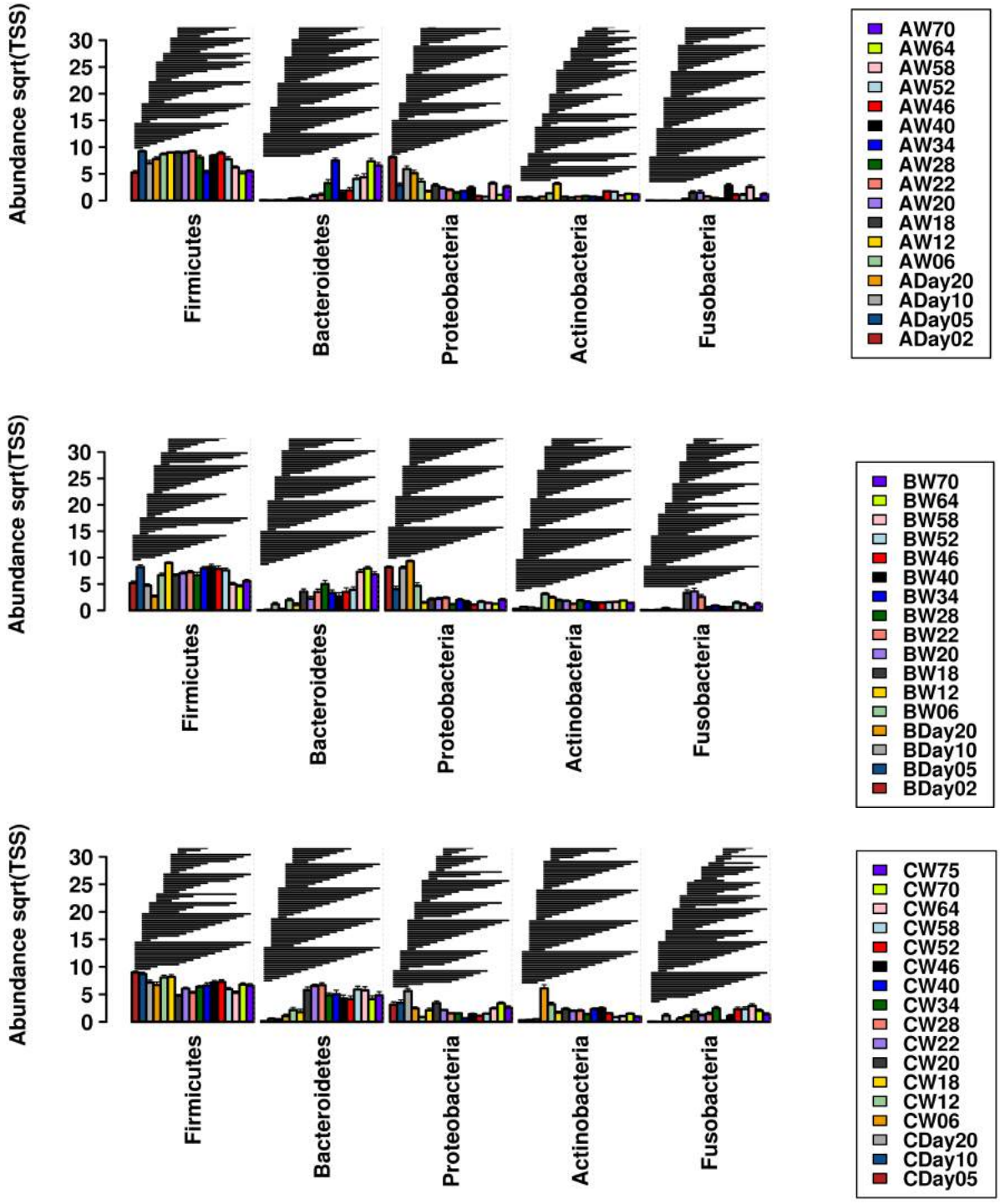
Figure 3.8: Comparative analysis of significant differences (*P* <0.05) in the abundance of gut microbiota at the phylum level (3.8A) and the family level (3.8B) in flocks A, B, and C in the late lay phase (week 60 to week 75). The bar charts represent the abundance square root of a specific family and its comparative analysis at each time point. The different colors of the bar chart denote samples collected at various time points. The asterisk sign denotes significant differences between the abundance of families at specific time points.



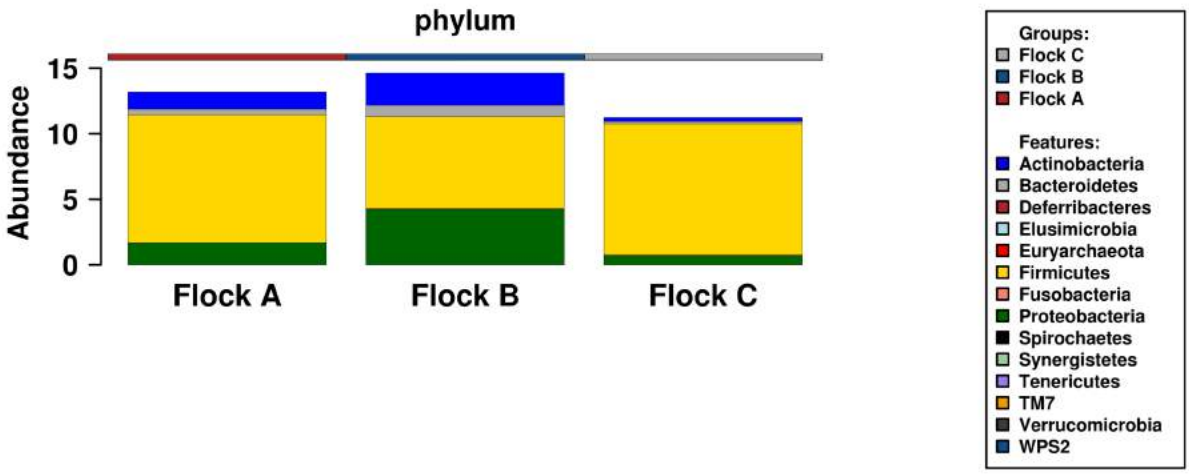
Supplementary Figure 3.1 Beta diversity analysis using ANOSIM showing significant effect of age on gut microbiota composition in flock A (a), B (b), C (c). The box plot shows the dissimilarity of gut microbiota between different time points. Each time shows the dissimilarity within the samples at specific time points.



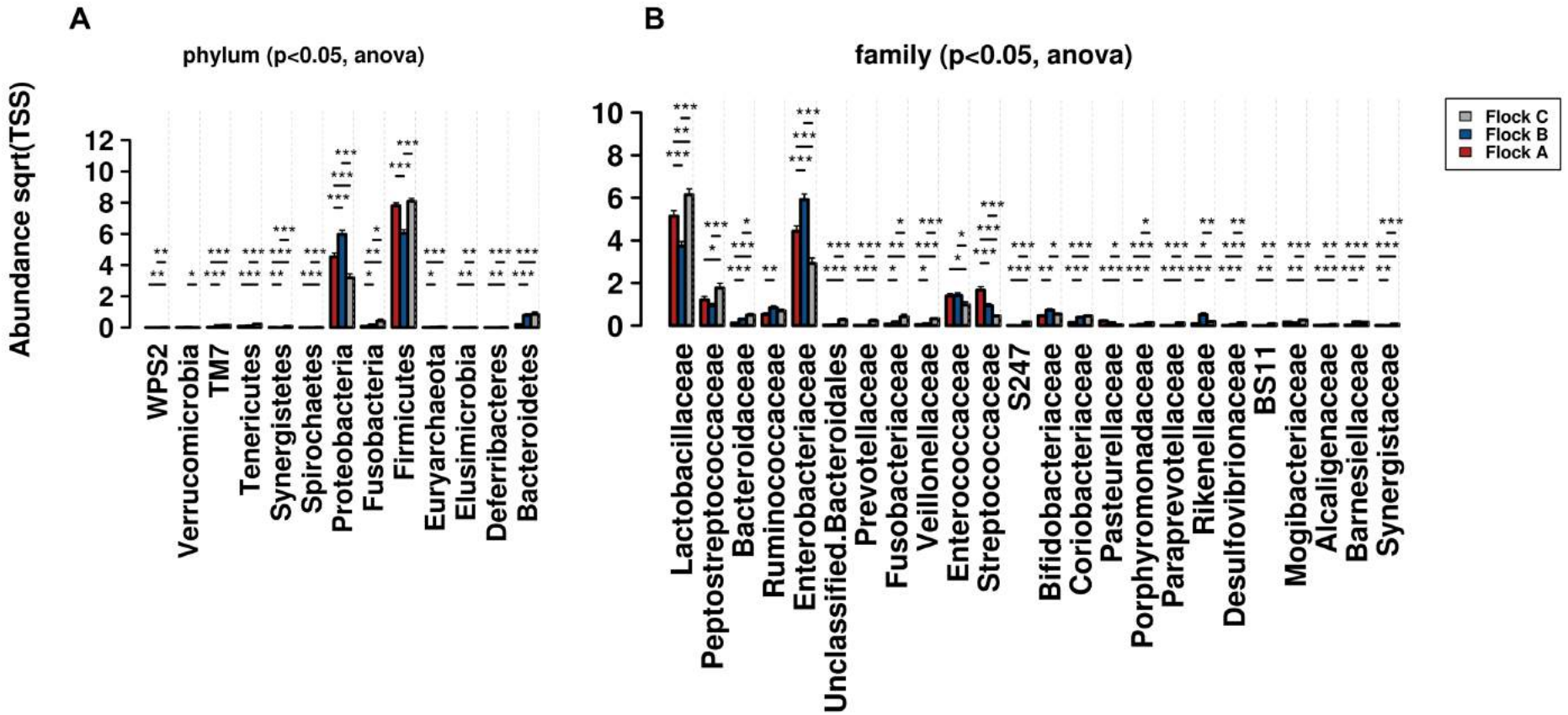
Supplementary Figure 3.2 RDA+ analysis showing significant effect of age on gut microbiota in flock A (a), B (b) and C (c). Each dot represents the individual sample and different colours indicate samples collected at different timepoints.



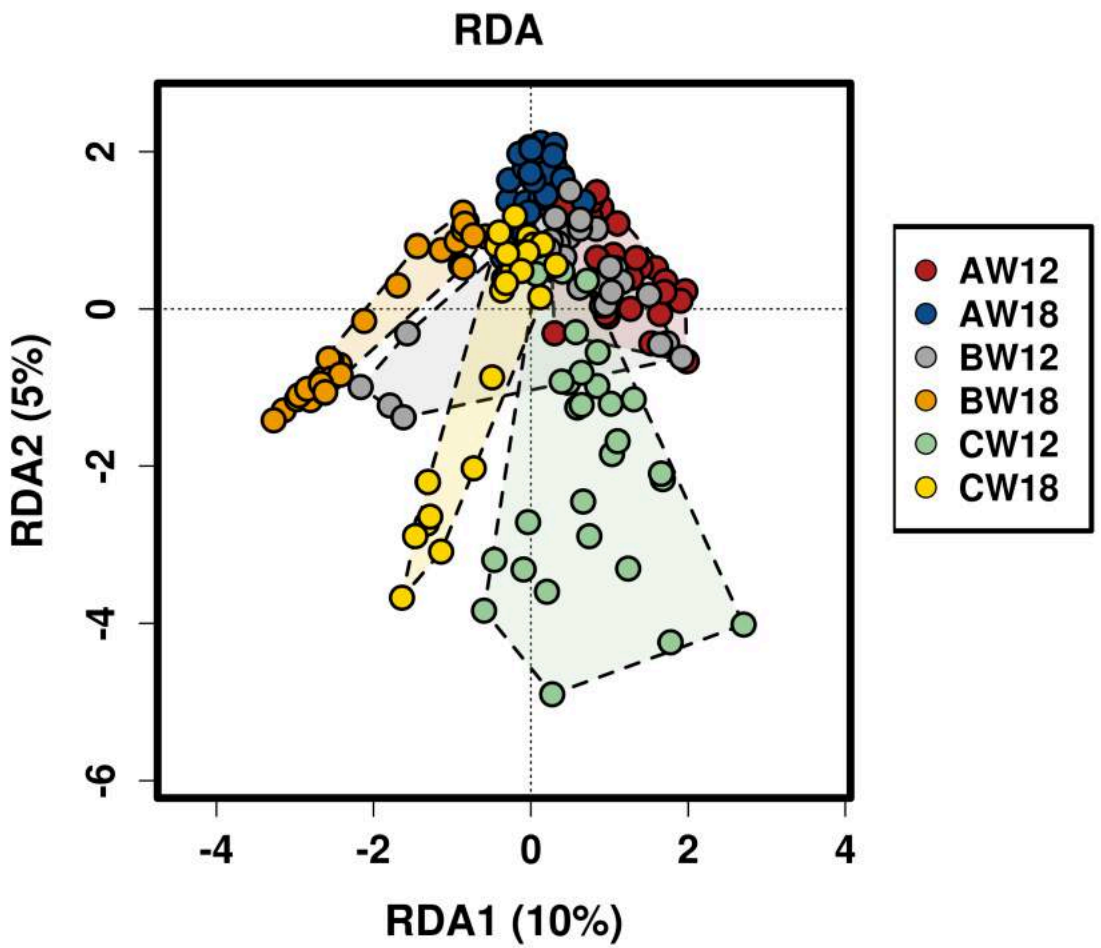
Supplementary Figure 3.3 Bar charts showing significant differences from day 2 to week 75 ($P < 0.05$) in gut microbiota at phylum in flock A (a), B (b) and C (c). The bar chart shows the square root abundance of different phyla at different time points denoted by different colors. The asterisk sign shows significant differences in the abundance of phyla at different time points.



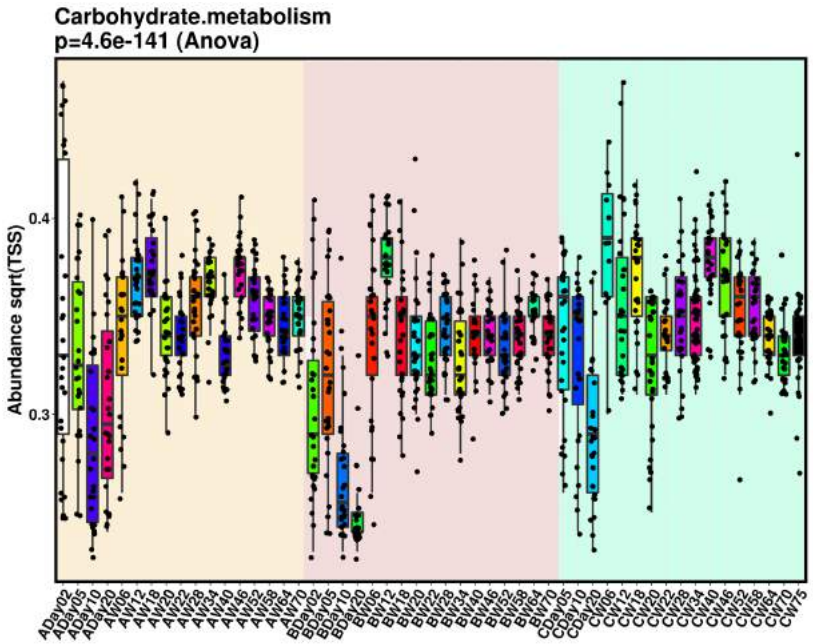
Supplementary Figure 3.4 Stacked bar charts showing abundance of different phyla in meconium samples in flock A, B and C.

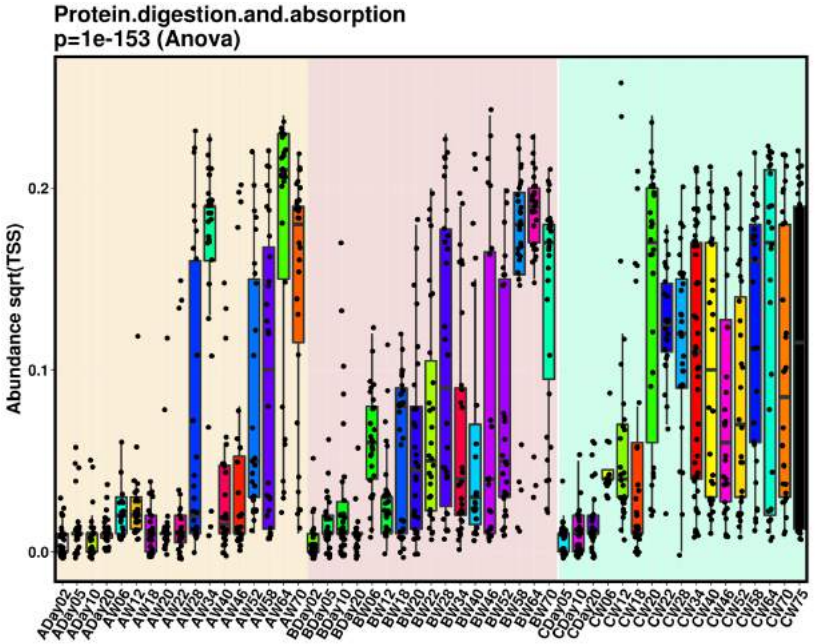


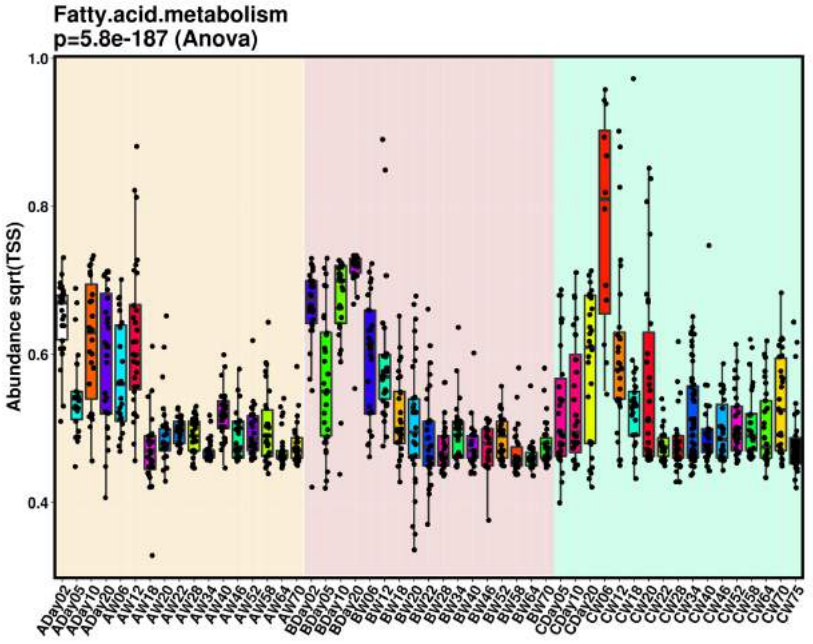
Supplementary Figure 3.5 Comparative analysis of significant differences (*P* <0.05) in the abundance of gut microbiota at the phylum level (A) and family level (B) in flocks A, B, and C in the rearing phase (up to week 12). The bar chart shows the square root abundance of different phyla and families in different flocks denoted by different colours. The asterisk sign shows significant differences in the abundance of phyla and family at different time points.

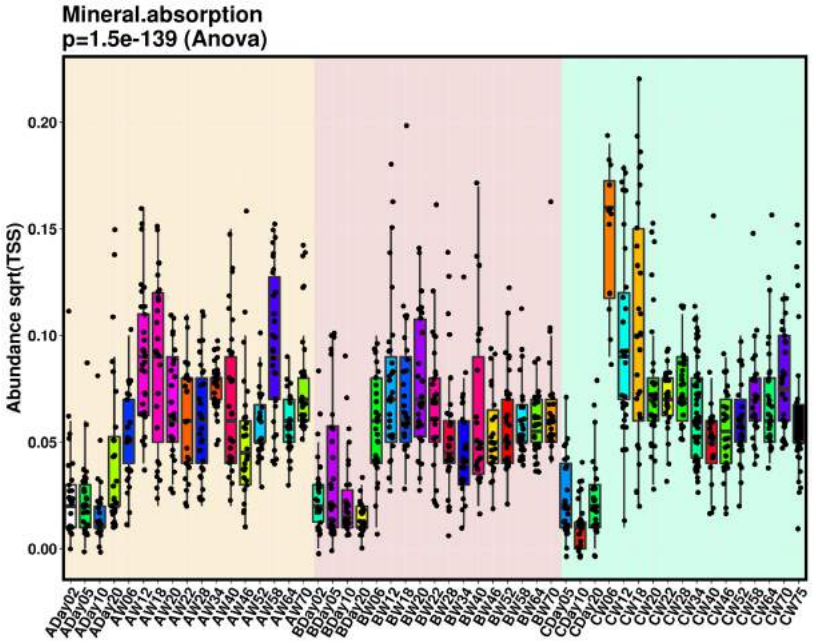


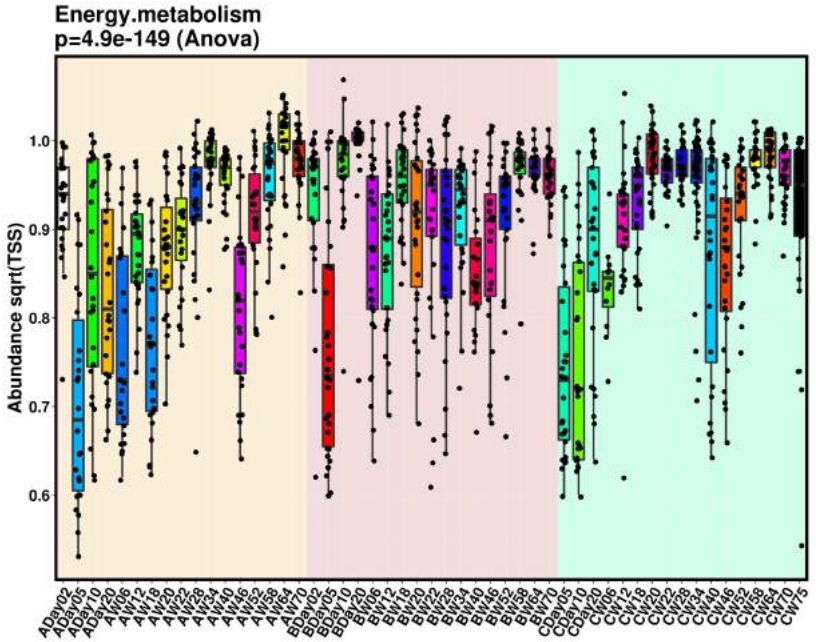
Supplementary Figure 3.6 RDA+ analysis showing significant time and flock dependant variation in gut microbiota composition between week 12 and week 18 in flock A, B and C. Each dot shows the individual sample collected at week 12 and 18. The different colours shows that samples are collected from different flocks at two different time points.

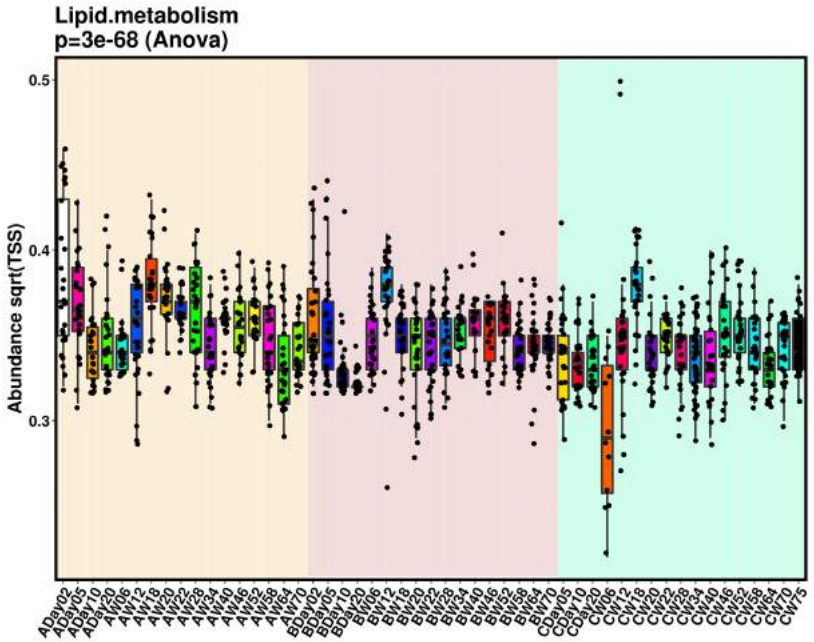

Carbohydrate.metabolism
p=4.6e-141 (Anova)
Abundance sqrt(TSS)
0.4
0.3

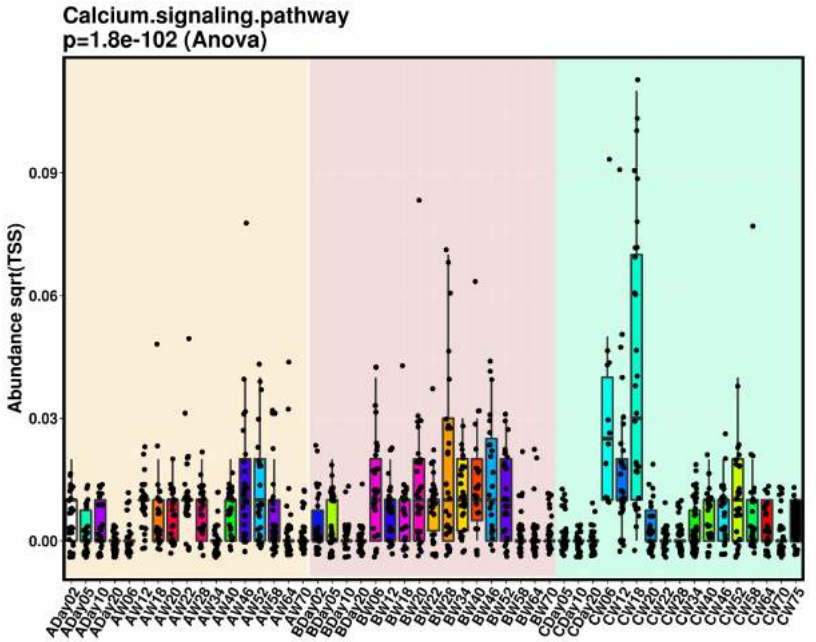

Protein.digestion.and.absorption
p=1e-153 (Anova)
Abundance sqrt(TSS)
0.2
0.1
0.0

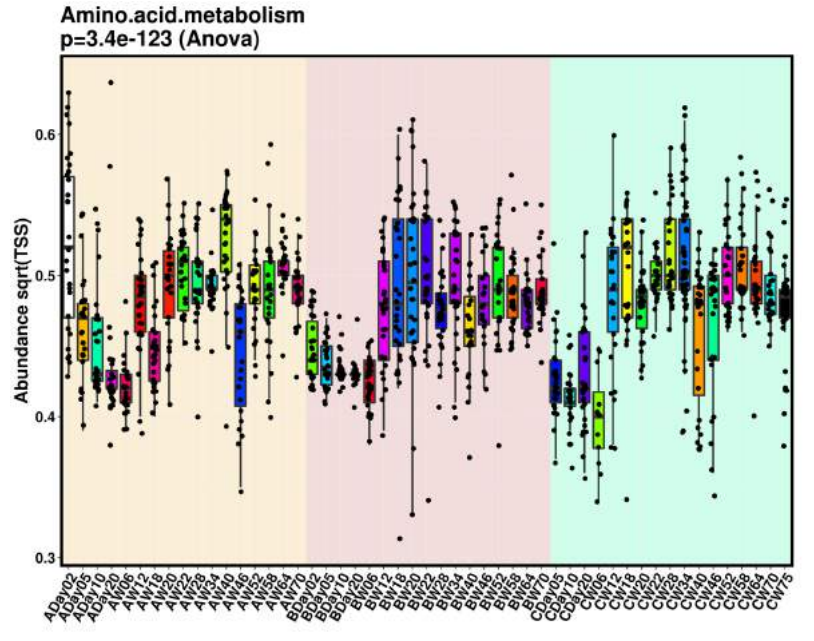

Fatty.acid.metabolism
p=5.8e-187 (Anova)
Abundance sqrt(TSS)
1.0
0.8
0.6
0.4

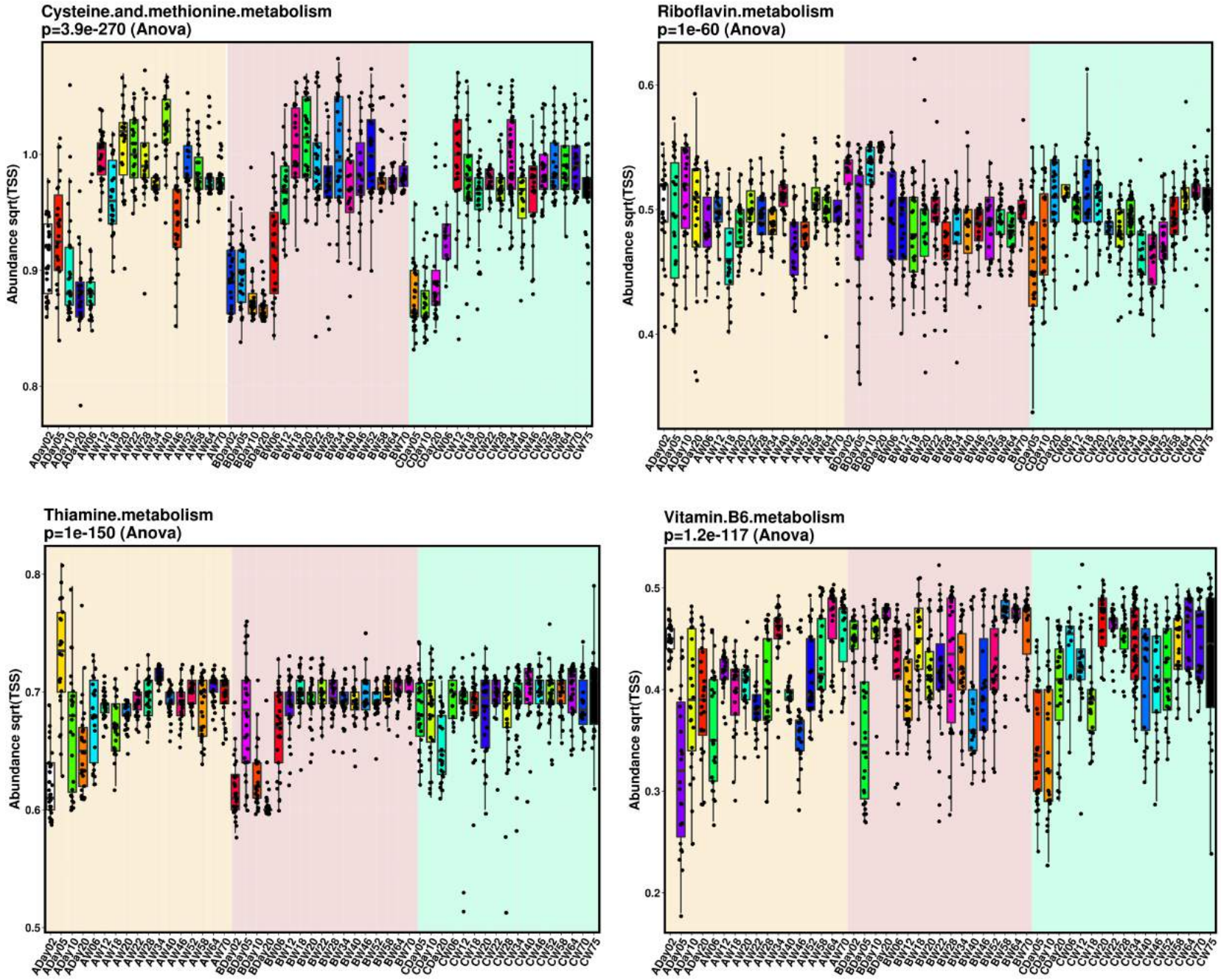

Mineral.absorption
p=1.5e-139 (Anova)
Abundance sqrt(TSS)
0.20
0.15
0.10
0.05
0.00


Energy.metabolism
p=4.9e-149 (Anova)
Abundance sqrt(TSS)


Lipid.metabolism
p=3e-68 (Anova)
Abundance sqrt(TSS)


Calcium.signaling.pathway
p=1.8e-102 (Anova)
Abundance sqrt(TSS)


Amino.acid.metabolism
p=3.4e-123 (Anova)
Abundance sqrt(TSS)



Supplementary Figure 3.7: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States showing significant differences metabolic pathways in flock A, B and C at different time points.

Chapter 4 Understanding the effects of intramuscular injection and feed withdrawal on *Salmonella* Typhimurium shedding and gut microbiota in pullets.

Statement of Authorship

Title of Paper	Understanding the effects of *Salmonella* Typhimurium and stress on gut microbiota in commercial pullets
Publication Status	√ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Joat, N. N., Khan, S., & Chousalkar, K. K. (2021) Understanding the effects of *Salmonella* Typhimurium and stress on gut microbiota in commercial pullets. Journal of Animal Science and Biotechnology.

Principal Author

Name of Principal Author (Candidate)	Nitish Narendra Joat		
Contribution to the Paper	Contributed to study design. Performed animal trial, sample processing, data analysis and drafting the manuscript		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/02/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Samiullah Khan

Contribution to the Paper	Contributed to study design, animal trials, data analysis and critical revision and edition of manuscript.		
Signature		Date	22/02/2021

Title of Paper	Understanding the effects of *Salmonella* Typhimurium and stress on gut microbiota in commercial pullets	
Publication Status	√ Published	☐ Accepted for Publication
	Submitted for Publication	Unpublished and Unsubmitted work written in manuscript style
Publication Details	Joat, N. N., Khan, S., & Chousalkar, K. K. (2021) Understanding the effects of *Salmonella* Typhimurium and stress on gut microbiota in commercial pullets. Journal of Animal Science and Biotechnology.	

Name of Co-Author	Kapil K. Chousalkar		
Contribution to the Paper	Contributed to study design, animal trial, sample processing, data analysis and critical revision and editing manuscript.		
Signature		Date	22/2/2021

4.1 Abstract

Background

Gut microbiota plays a key role in health, immunity, digestion, and production in layers. Factors such as environment, diet, diseases, stress, and flock management significantly affect gut microbiota; however, it is not known how potential stressors such as intramuscular injections or feed withdrawal alter the composition of gut microbiota that result in increased shedding level of foodborne pathogens. In the current study, the effects of intramuscular corticosterone injection and feed withdrawal were evaluated to understand their role in *Salmonella* Typhimurium shedding and changes in the composition of gut microbiota in layers.

Results

Salmonella shedding was observed for 8 weeks post-infection. There was a significant increase in *Salmonella* Typhimurium count after intramuscular injection and feed withdrawal. The *Salmonella* infected, and the negative control groups showed significant differences in the abundance of different genera at week 1 and up to week 7 post-infection. The infected group showed a significant reduction in alpha diversity of gut microbiota. Firmicutes reduced significantly ($P < 0.05$) after intramuscular injection, while the feed withdrawal groups did not cause any significant changes in the Proteobacteria-Firmicutes ration. Furthermore, intramuscular injection resulted in a significant change in the alpha diversity of gut microbiota.

Conclusions

Salmonella Typhimurium infection disrupted the gut microbiota composition immediately after infection. The potential stress of intramuscular injection and feed withdrawal significantly increased the *Salmonella* Typhimurium count in faeces. The

intramuscular injection also resulted in significant alteration of the Proteobacteria-Firmicutes ratio which could increase the risk of dysbiosis.

Keywords: Corticosterone injection, Feed withdrawal stress, Gut microbiota composition, Layer chicken, *Salmonella* Typhimurium infection.

4.2 Introduction

The gut microbiota is a subject of investigation owing to its key role in homeostasis, nutrient digestion, immune modulation and conferring protection against pathogen colonisation in the host [1]. In the last two decades, significant advances have been made in gut microbiota research due to tools such as 16S rRNA and whole-genome sequencing [2]. Because of the diverse functions of gut microbiota, especially its role in the immune system modulation, nutrient digestion, and colonisation resistance to pathogens, it is essential to understand its role in food-producing animals such as layer chickens.

Layer farming is one of the leading food-producing sectors in the world because of the acceptability and high demand for egg and egg products. Foodborne pathogens such as *Salmonella* and *Campylobacter* are global concerns for the egg industry. *Salmonella enterica* is commonly related to contaminated eggs and egg products that act as a source of infection in humans. *Salmonella* Enteritidis is the dominant serotype globally [3]; however, in Australia, *Salmonella* Typhimurium is the dominant serotype, which has been responsible for multiple egg and egg product related outbreaks [4]. *Salmonella* Typhimurium infection in adult hens does not induce clinical signs; therefore, the hens act as a carrier by continuously shedding it to the environment [5]. It has also been reported that *Salmonella* shedding in hens is intermittent, and the bacterium can remain undetected for several weeks [5]. In porcine alveolar

macrophages and mice, repeated subcutaneous dexamethasone injections have been shown to increase the shedding of *Salmonella* Typhimurium [6].

The impact of *Salmonella* Enteritidis and *Salmonella* Typhimurium colonisation on the diversity of gut microbiota in chicks has been reported earlier [7, 8]. However, the *Salmonella* Enteritidis challenge study was conducted over a short period, and the *Salmonella* Typhimurium study was conducted in adult hens. The life cycle of commercial layer birds is up to 80 weeks that involves rearing for up to 16 weeks. Thus, it is essential to understand how the colonisation of *Salmonella* Typhimurium during rear affects the gut microbiota composition. Furthermore, pullets go through multiple potential unavoidable stressors during rearing that includes vaccinations (intramuscular or sub-cut injections), transport to production shed and on-set of lay. These stressors activate the hypothalamic-pituitary-adrenal axis (HPA axis) in the host [9]. This triggers the neuroendocrine pathway that leads to the secretion of stress hormones (glucocorticoids and catecholamines) [9-11]. Out of these hormones, corticosterone (glucocorticoid) is the main stress hormone in birds [12]. The corticosterone affects gut microbiota and results in the under-representation of beneficial (Firmicutes) and pathogenic (Mycoplasma, Campylobacter, Pseudomonas) microbial taxa in wild birds [12]. An earlier study in broilers also reported that acute stressors could affect the normal intestinal microbiota and intestinal structure, which could lead to increased *Salmonella* Enteritidis colonisation [13].

Based on relevant studies described earlier, it is evident that stressors in adult hens can lead to the replication of *Salmonella* Typhimurium and can negatively impact the gut microbiota. The effects of stress on gut microbiota and *Salmonella* Typhimurium shedding have been documented in the literature [12, 14]. Furthermore, the effects of *Salmonella* Typhimurium colonisation on gut microbiota in adult hens is also reported

[8]. However, there is a scarcity of data on the interaction between gut microbiota and *Salmonella* Typhimurium during stressful events. Therefore, the objective of this study was to investigate the effects of *Salmonella* Typhimurium challenge on the gut microbiota of pullets. This study also investigated the effects of feed withdrawal and intramuscular injection on gut microbiota composition and *Salmonella* Typhimurium shedding pattern in pullets.

4.3 Methods

4.3.1 Animal Ethics Committee approval

The animal trial was approved by the Animal Ethics Committee at The University of Adelaide under approval number: S-2019-004.

4.3.2 Hatching and rearing of *Salmonella* free laying chicks

Fertile eggs of an Isa-Brown parent flock were obtained from a commercial hatchery. The eggs were fumigated and incubated for 21 days at 37.5°C. Relative humidity in the incubator was 55% until day 18 and was increased to 60% until day 21. A total of 77 chicks were raised in different pens during the experiment. The minimum number of birds in each group was 11. Earlier studies included 3 [15-18] and 7 [8] birds per group to understand the effect of *Salmonella* Typhimurium infection on gut microbiota composition in chickens. The chicks were housed in separate rooms at the Roseworthy Campus, The University of Adelaide. The housing conditions (size of pens, stocking density, lighting program) were according to Australian animal welfare standards and guidelines [19]. The birds were reared on the mesh floor and had some access to faeces. Swabs were collected from the incubator after hatch and processed for *Salmonella* isolation. Before the placement of chicks, pens and facility were

cleaned and decontaminated using SaniGuard (Chemetall, Australia) followed by formaldehyde fumigation. The chicks were provided with *ad libitum* feed and water throughout the experiment [20]. Feed was fumigated. The chicks were divided into six different treatment groups, as outlined in Table 4.1.

4.3.3 Bacterial strains and challenge

Bacterial strain KC 30 (*Salmonella* Typhimurium phage type 9) was cultured on nutrient agar overnight at 37°C. This strain was isolated from an egg farm during a previous epidemiological investigation [5]. Twenty-four hours before infecting the chicks, a single colony was incubated in 10 mL Luria Bertani (LB) broth for 6 h with shaking (180 rpm). From this culture, 10 µL was subcultured in 30 mL LB broth at 37°C overnight. On the day of the chicks challenge, the broth was centrifuged, and the bacterial pellet was washed with phosphate-buffered saline (PBS). The pellet was resuspended in 25 mL PBS, and the optical density of the suspension was measured at 600 nm.

In the challenged groups, individual chicks received 10^3 colony forming units (CFUs) of *Salmonella* Typhimurium orally at day 7 of chicks' age (treatment groups- INF_CORT, INF-PBS, PC, INF_FW), while the control groups were sham inoculated with sterile PBS (treatment groups – NC_CORT, NC_FW). The dose rate was kept low to understand the effect of *Salmonella* Typhimurium colonisation on gut microbiota [15, 16]. The chicks were monitored for clinical signs of salmonellosis for 8 weeks. The leftover inoculum was maintained on ice, serially diluted and plated onto Xylose Lysin Deoxycholate agar (XLD) to confirm the exact dose received by the individual chicks. Faecal samples were collected every week from the individual chicks to monitor the

Salmonella Typhimurium shedding. A subset of faecal samples was stored at -20°C for DNA extraction and 16S rRNA sequencing.

4.3.4 Intramuscular injections and feed-withdrawal at week 7 of chicken age

An injectable solution was prepared by dissolving the corticosterone-HBC complex (Merck, Australia) in water to make the final dose as 1.5 mg/kg body weight. At week 7 post-infection, chickens in treatment groups negative control corticosterone (NC_CORT) and *Salmonella* Typhimurium corticosterone (INF_CORT) received an intramuscular injection of corticosterone, while group *Salmonella* Typhimurium PBS (INF_PBS) was injected PBS. The week 7 post-infection time point was selected to mimic field conditions to understand the effects of potential stress caused by intramuscular injection. The common intramuscular vaccines administered in layer chickens in the field are Fowl Cholera, Egg Drop Syndrome, and Newcastle Disease [21]. In this study, birds did not receive any vaccine. Group negative control feed withdrawal (NC_FW) and group *Salmonella* Typhimurium feed withdrawal (INF_FW) were subjected to feed withdrawal for 4 h immediately after night photoperiod. It has been noted that commercial layers are likely to go through feed withdrawal stress at some point in 80 weeks of lifespan [22]. The feed withdrawal groups were included to mimic the field conditions. The group positive control (PC) was not subjected to any physical and hormonal treatment. From all the treatment groups, faecal samples were collected 24 h after the injection or feed withdrawal, bacterial enumeration, and the chickens were humanely sacrificed 72 h following the injection or feed withdrawal. At the time of sacrifice, cloacal swabs and tissue samples from the liver, spleen, ileum, caecum and colon were collected from all the chickens for quantification of *Salmonella* Typhimurium through the bacterial culture method.

4.3.5 *Salmonella* detection in faeces and swabs

All the groups (positive and negative) were tested weekly for *Salmonella* Typhimurium according to a previously published protocol [5]. Briefly, faecal samples from pens or swabs collected from the hatching incubator were incubated in buffered peptone water (BPW) (Oxoid, Australia) at 37ºC overnight and 100 µL of it was transferred to Rappaport- Vassiliadis soya peptone (RVS) broth (Oxoid, Australia). The RVS samples were incubated at 42ºC for 24 h and streaked on xylose lysine deoxycholate agar (XLD) (Oxoid, Australia) plates and incubated overnight at 37°C. The *Salmonella* suspected colonies from XLD were subcultured onto Brilliance *Salmonella* agar (BSA) plates (Oxoid, Australia).

4.3.6 *Salmonella* Typhimurium enumeration in tissues

Ileum, caecum, colon, liver and spleen were collected at 8-week post-infection for the enumeration of *Salmonella* Typhimurium. Briefly, 0.1 to 0.2 g of tissue was collected in sterile 1.5 mL microcentrifuge tubes containing stainless steel beads (0.5-2 mm) and PBS. The tissue samples were homogenised using bullet blender (Next Advance, USA) on full speed for 5-10 min. Serial ten-fold dilutions were prepared in PBS, and 100 µL of the homogenates was spread plated on XLD media and incubated overnight at 37ºC. *Salmonella* load in tissue was expressed as mean $\log_{10}$ CFU/g of tissue. The negative samples from the *Salmonella* Typhimurium challenged groups after direct plating were enriched in BPW and RVS as described [5]. The samples that showed positive for *Salmonella* after enrichment were noted as “1”, and the negative samples were noted as “0” for calculating the proportion of positive samples.

4.3.7 DNA extraction from faeces

DNA was extracted and purified from faecal samples (*n* = 584) using the QIAamp Fast DNA Stool Mini kit (Qiagen, Australia) with a modified protocol [23] that was further optimised for this study. Briefly, 200 mg faecal samples were vortexed after adding 1 mL of preheated (70°C) InhibitEx buffer. The samples were homogenised after the addition of glass beads (acid washed, 450-600 μm &106 μm, equal quantity), using a bullet blender for 5 min. The samples were heated at 95°C for 7 min and then incubated on ice for 30 seconds. The samples were centrifuged for 2 min, and the supernatant was collected and further processed for DNA extraction according to the modified QIAamp Fast DNA Stool Mini kit protocol [23]. DNA was eluted in 100 μL of ATE buffer and stored at -20°C until used for qPCR and 16S rRNA sequencing.

4.3.8 Quantitative PCR and optimization of standard curve for *Salmonella* Typhimurium

The extracted DNA samples were subjected to quantitative PCR for *Salmonella* Typhimurium quantification from week 1 post-infection until sacrifice. The qPCR was performed using SensiFAST SYBER HI-ROX Kit (Bioline, Australia) following the manufacture's protocol. The qPCR reaction was performed in 72 well rotor-gene disc (Qiagen, Australia). The master mix was prepared and added to the disc through the Corbett CAS1200 robot (Corbett Life Science, Australia). The 10 μL final reaction volume contained 5 μL SensiFAST SYBER Hi-Rox mix, 1 μL each of the forward (5′-TTTACCTCAATGGCGGAACC) and reverse (5′-CCCAAAAGCTGGGTTAGCAA) TSR3 primers, 1 μL RNase-free water and 2 μL DNA template. The cycling conditions were initial denaturation at 95ºC for 3 min, then 40 cycles of denaturation at 95ºC for 5 s, annealing and extension at 59ºC and 72ºC for 20 and 30 s, respectively.

A standard curve was constructed by spiking non-infected control faeces with a known amount of *Salmonella* Typhimurium. Briefly, serial ten-fold dilutions of *Salmonella* Typhimurium grown overnight in LB broth were used to spike the faecal samples. The DNA was extracted from the spiked faecal samples to construct a standard curve, and a limit of detection was established.

4.3.9 16S rRNA library preparation and illumina sequencing

Out of 584 extracted DNA samples, 360 DNA samples were submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for 16S rRNA sequencing and generation of operational taxonomic units (OTUs) table using barcoding PCR. The total reaction volume was 25 µL containing 12.5 µL KAPA HiFi HotStart Readymix (Kapa Biosystems), 1 µL of each of the forward and reverse primers, 1 µL DNA sample and 10.5 µL PCR grade water. This PCR was used to generate 2 × 300 bp pair-end reads in Illumina. To generate the reads, V3-V4 region specific primer pair (341F 5'-CCTACGGGNGGCWGCAG-3'; 805R 5'-GACTACHVGGGTATCTAATCC-3') was used. The thermal cycling conditions for the PCR were as follows: initial denaturation 95ºC for 3 min, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s and elongation at 72ºC for 30 s, ending with final elongation at 72ºC for 5 min. PCR products were normalised and pooled using SequalPrepTM Normalization Plate Kit (ThermoFisher) according to the manufacturer's instructions. The library was purified using Axygen AxyPrep Mag PCR Clean-UpKit (Fisher Biotech) as per the manufacturer's instructions. The concentration and quality of the pooled library were checked with Qubit, and the library size was assessed in an Agilent 2200 TapeStation instrument. The Agencourt AMPure XP Bead Clean-up kit was used on the pool to reduce/remove the presence of primer

dimers. The library pool was sequenced on the MiSeq using a MiSeq Reagent Kit v3 with a 2 × 300 bp run format, using default run parameters including adaptor trimming. For these runs, custom primers were added to the reagent cartridge for Read1, Index, & Read2.

4.3.10 Bioinformatics analysis

The reads were analysed according to MiSeq [24] protocol using mothur (v1.39.5) [25]. Briefly, the reads were quality filtered and assigned to their respective samples. After trimming, samples with the length between 405 and 495 bp were retained. Samples with homopolymers longer than 8 bp were removed. Chimera.vsearch script in mothur [26] was used to remove chimeric sequences and SILVA reference alignment (v132) [26] was used to align and classify the sequences. The lineages not targeted by the primer pair (i. e. archaea, chloroplast, eukaryote, mitochondria and unknown sequencing errors were removed. The sequences were grouped into OTUs based on 97% similarity using OptiClust algorithm [27] and subsampled based on the sample with the lowest number of sequences. ZymoBIOMICS Microbial Community Standards was used as a control in each sequencing run to assess sequencing error. Krona [28] was used to create interactive OTU plots from the subsampled data, and mothur_krona_XML.py script [29] was used to generate OTU richness plot. For the generation of diversity plots, OTUsamples2krona.sh script [30] was used by providing a reformatted mothur biom file.

4.3.11 Statistical analysis

The *Salmonella* Typhimurium load in organs was analysed in SPSS software (version 26), considering treatment as the main effect. For the quantification of *Salmonella* Typhimurium from faeces, treatment and time points were considered as main effects. The level of significance was determined by Fisher's protected least significant difference (PLSD) at $P < 0.05$. The gut microbiota data were analysed in Calypso software [31] using ANOVA and diversity analysis (Shannon index). The total sum normalised method was used to transform the data. The visualisation of data (q-PCR and comparative analysis of gut microbiota) was performed in GraphPad Prism (version9.0.2).

4.4 Results

4.4.1 Mortality and clinical signs of salmonellosis

There were no visible clinical signs in the infected chickens. There was no mucoid faeces or diarrhoea observed in any of the treatment groups. The swabs collected from the incubators were *Salmonella* negative. All the chicks were tested for *Salmonella* negative before the challenge. Mortality (n =3) was observed in the PC group in week 1 after infection. However, no clinical signs or post-mortem lesions were observed in the birds.

4.4.2 *Salmonella* Typhimurium detection in faecal samples

A total of 584 faecal samples were collected over the experimental period and monitored for *Salmonella* Typhimurium. All the chickens from the *Salmonella* negative control groups (NC_CORT and NC_FW) were negative, and all the chickens from the

Salmonella infected groups (INF_CORT, INF_PBS, PC and INF_FW) were positive from the time of post-infection until the end of the trial.

4.4.3 *Salmonella* Typhimurium quantification from tissue samples

All the tissue samples collected from the negative control groups were negative for *Salmonella* Typhimurium. Liver and spleen collected from the infected groups did not show any significant difference in $\log_{10}$ CFU count of *Salmonella* Typhimurium (INF_CORT, INF_PBS, PC and INF_FW). There was no significant difference in $\log_{10}$ CFU count of *Salmonella* Typhimurium in caecum and colon in all infected groups (Supplementary table 1). In the ileum, the significant difference in the bacterial count was observed only between-group INF_PBS and PC ($P = 0.0487$). Negative samples after direct plating were enriched in buffer peptone water and RVS. After enrichment, intestinal organs (liver, spleen and ileum) showed significant differences in *Salmonella* detection level (Supplementary Table 1). Post enrichment *Salmonella* positive rate in the liver in group INF_PBS was significantly lower ($P = 0.0346$) than the PC group. The spleen samples from INF_CORT treatment group showed significantly low *Salmonella* positive samples as compared to INF_PBS ($P = 0.0268$) and INF_FW ($P = 0.0451$) groups. Furthermore, *Salmonella* positive spleen samples post enrichment in the INF_PBS group was significantly lower ($P = 0.0491$) compared to the INF_FW group.

4.4.4 Enumeration of *Salmonella* Typhimurium in faecal samples by quantitative PCR

Salmonella Typhimurium load (Log_{10} CFU/gram) was detected using q-PCR from faecal samples collected at different time points. The cut-off Ct value was 31.

Samples were collected from all 6 groups at weeks 1, 2, 3, 4, 5, 6, 7, 8, 8.3 post-infection. The samples collected from negative control groups (NC_CORT, NC_FW) were negative for *Salmonella* Typhimurium throughout the experiments. These results were confirmed by testing negative samples by gel electrophoresis. Significant effect of time (P <0.0001) and treatment groups (P <0.0001) was observed on *Salmonella* Typhimurium load in faecal samples (Figure 4.1).

After the injection and feed withdrawal (at week 8post infection), no significant differences in Log_{10} CFU of *Salmonella* Typhimurium were observed between the groups except in between INF_PBS vs PC (P = 0.0292). A significant increase in *Salmonella* Typhimurium shedding was observed within the groups at week 8 compared to week 7 post-infection in INF_CORT (P<0.0001), INF_PBS (P<0.0001), PC (P=0.0005), INF_FW (P<0.0001) treatment groups.

4.4.5 *Salmonella* Typhimurium affects the abundance and diversity of gut microbiota at week 1 post infection.

In total, 360 DNA samples were sequenced to understand the effects of *Salmonella* Typhimurium infection on gut microbiota composition. In this trial, we did not observe significant bird variation in gut microbiota composition. Therefore, the data from infected groups (INF_CORT, INF_PBS, PC and INF_FW) and negative control groups (NC_CORT and NC_FW) for week 1 post-infection and up to week 7 post-infection were combined for analysis and presentation.

The comparative analysis of gut microbiota between the infected (INF) and the negative control (NC) groups showed significant differences in the abundance of various genera week 1 post-infection compared to day 7 (Figure 4.2 a, b). At week 1 post-infection, the infected group showed a significantly (P<0.05) higher abundance

of genera including *Trabulsiella*, *Melissococcus*, *Lactobacillales*_ unclassified and *Enterobacteriaceae*_ unclassified compared to the negative control group. The *Salmonella* Typhimurium infected group showed a significantly lower abundance of genera, including *Ruminococcus*_torques_group, *Ruminococcaceae*_unclassified, *Ruminococcaceae*_UCG014, *Lachnospiraceae*_unclassified, *Enterococcus*, *Blautia,* compared to the negative control group. The infected groups showed a significant reduction in genera *Ruminococcus*_torques_group and *Ruminococcaceae*_UCG013 at week 1 post-infection compared to day 7 (Figure 4.2a, b).

On day 7, the alpha diversity of microbial communities within individual samples of the NC and INF was not significantly different (Figure 4.3a) as compared to week 1 post-infection (Figure 4.3b), where the alpha diversity in infected groups reduced significantly (P = 0.0014). Alpha diversity analysis within the groups between day 7 and week 1 post-infection showed no significant differences (P= 0.081) in the negative control group (Figure 4.3c); however, the infected group showed a significant reduction (P= 1.5e-09) in alpha diversity (Figure 4.3d).

4.4.6 Abundance and diversity of gut microbiota up to week 7 post infection in negative control and *Salmonella* Typhimurium infected groups.

The gut microbiota from the negative control group and *Salmonella* Typhimurium infected group was compared from week 1 to week 7 post-infection to analyse the abundance and diversity at the genus level (Figure 4.4a). In *Salmonella* Typhimurium infected group, the abundance of microbial communities including *Streptococcus*, *Romboutsia*, *Rikenellaceae*_RC9_gut_group, *Peptostreptococcaceae*_unclassified and *Butyricicoccus* was significantly higher compared to the negative control group (Figure 4.4a). In negative control groups, abundance of the genus including

Enterobacter, CHKCI001, C*aproiciproducens*, and *Blautia* were significantly higher as compared to *Salmonella* Typhimurium infected group (Figure 4.4a). Alpha diversity measured by Shannon index did not show any significant difference in the overall gut microbiota up to week 7 post-infection between the infected and negative control groups (Figure 4.4b).

4.4.7 Effect of the injection and feed withdrawal on gut microbiota diversity and Proteobacteria-Firmicutes ratio.

To understand the effects of an intramuscular injection and feed withdrawal on the composition of gut microbiota, data obtained at week 7 and week 8 were compared within the respective treatment groups. Compared to week 7 (Figure 4.5a), intramuscular injections significantly affected the alpha diversity of gut microbiota among the negative control, and *Salmonella* Typhimurium challenged groups at week 8 of chickens age (Figure 4.5b). Compared to week 7 (Figure 4.5c), feed withdrawal did not impact the alpha diversity of gut microbiota within and between the groups (Figure 4.5d).

After intramuscular injections, significant differences were observed in the abundance of phyla Proteobacteria and Firmicutes in groups NC_CORT, INF_CORT and INF_PBS (Figure 4.6 a-f). The abundance of Proteobacteria increased significantly at week 8 post-infection in NC_CORT (mean = 7.76), INF_CORT (mean = 6.33), INF_PBS (mean = 6.53) as compared to week 7 post-infection NC_CORT (mean = 4.88), INF_CORT (mean = 3), PC_PBS (mean = 4.66). The abundance of Firmicutes significantly decreased at week 8 in NC_CORT (mean = 4.47), INF_CORT (mean = 6.21), INF_PBS (mean = 5.8) as compared to week 7 in NC_CORT (mean = 6.61), INF_CORT (mean= 8.22), INF_PBS (mean = 7.36) groups. The significant differences

in group NC_CORT, INF_CORT and INF_PBS in gut microbiota at the genus level were also observed (Figure 4.7a, b). A significantly higher abundance of microbial communities, including *Klebsiella* and *Gammaproteobacteria*_unclassified, was observed in the non-infected stress-induced group (NC_CORT) at week 8 post-infection (Figure 4.7b). In the *Salmonella* Typhimurium infected and corticosterone injected group (INF_CORT), the abundance of genera including *Ruminococcaceae*_UCG014, *Ruminococcaceae*_UCG013, Melissococcus, *Lachanoclostridium*, and *Acinetobacter* was significantly higher at week 8 post-infection. In the feed withdrawal groups (NC_FW, PC and INF_FW), no significant differences were observed in the gut microbiota after feed withdrawal at phylum level; however, the gut microbiota in the feed withdrawal group showed significant differences in abundance at the genera level after induction of stress (Figure 4.7c, d) The abundance levels of genera *Lactobacillus* and *Faecalibacterium* were higher in non-stressed *Salmonella i*nfected group (PC).

4.5 Discussion

The present study was conducted to understand the effects of intramuscular injection and feed withdrawal on *Salmonella* Typhimurium shedding and gut microbiota composition in pullets. In an earlier infection trial, chicks inoculated with *Salmonella* Typhimurium showed persistence in caeca until the age of week 7 post-infection. However, the chicks were inoculated with 10^6 to 10^7 CFU of *Salmonella* Typhimurium [32]. In this study, we observed that all the *Salmonella* Typhimurium inoculated birds were positive until the end of the trial, i.e., up to week 8 post-infection despite a low challenge dose of *Salmonella* Typhimurium (10^3 CFU). This could suggest that *Salmonella* Typhimurium can colonise and shed persistently in faeces at least up to

week 8, even at low doses of infections. Some studies reported the detection of *Salmonella* Typhimurium from infected hens up to week 15 [33] and week 16 [34] post-infection; however, the birds in those studies were inoculated with 10^9 CFU of *Salmonella* Typhimurium and the studies were conducted in older birds. In an earlier study, 10^3 CFU was used to infect chicks on day 7, which reported colonisation of *Salmonella* Typhimurium in caeca, liver and spleen. However, this study was conducted for 1 week and did not focus on shedding patterns in faecal samples [15]. During this study, birds did not show any clinical signs after *Salmonella* Typhimurium infection. This finding is in agreement with a previous study [15, 35].

In this study, we did not find any significant differences in bacterial load in the spleen and liver from the *Salmonella* Typhimurium positive groups after direct plating. These findings were in contrast to the earlier studies where a significant *Salmonella* load was detected in the liver and spleen [8, 15, 34]. The caeca and colon did not show significant differences in *Salmonella* load between the *Salmonella* infected groups. The negative ileum samples for *Salmonella* Typhimurium showed significant differences in *Salmonella* load on enrichment in RVS except for between-group INF_PBS and PC. This can be attributed to the potential stress induced by intramuscular PBS injections in the INF_PBS group. An earlier study has reported that stress results in increased *Salmonella* shedding in animals [36]. Earlier *Salmonella* colonisation studies were focused on the caecum, liver and spleen [8, 15, 34]. However, our study suggests that ileum and colon could also be the potential sites for colonisation of *Salmonella* Typhimurium in chickens.

The significant increase in *Salmonella* count in faeces at week 8 post-infection in the groups INF_CORT, INF_PBS, PC and INF_FW groups can be co-related to the potential stress caused by intramuscular injection and feed withdrawal induced 24

hours prior. Interestingly, the *Salmonella* count in faeces after induction of stress did not show any significant differences in INF_CORT and INF_PBS groups, which was not expected. However, the increased shedding in the INF_CORT group (7.805±0.151) was 2.323 log CFU higher at week 8 as compared to increased shedding in INF_PBS (7.439±0.149), where it was higher by 1.744 log CFU at week 8. It is unclear why the INF_PBS group showed an increase in shedding. However, it does support the notion that stress induced by handling or intramuscular injection, in this case, increased *Salmonella* Typhimurium shedding in faeces. If a *Salmonella* infected flock is to be vaccinated for other diseases, the increase in bacterial shedding in faeces is to be expected, and hence, the personal hygiene of the bird handling crew and farm managers is important to reduce the risk of zoonosis. The *Salmonella* count in faecal samples did not show a significant difference between groups INF_FW which was subjected to feed withdrawal and PC. Although there was no significant difference in both the groups, the increase in *Salmonella* Typhimurium shedding in the INF_FW group (7.845± 0.207) was 1.284 log CFU higher at week 8 as compared to the PC group (8.025±0.228), where it was higher by 1.164 log CFU at week 8. All four groups showed a significant increase in *Salmonella* Typhimurium shedding at week 8 post-infection i. e, after induction of stress, as compared to week 7 post-infection. These results could not be compared as there are no such studies conducted on chickens. A study monitoring *Salmonella* Typhimurium load in pigs after feed withdrawal and transport stress showed an increased number of *Salmonella* Typhimurium load in the gut; however, in that study, feed withdrawal was for 24 h and instead of corticosterone, dexamethasone was used because of its longer half-life as compared to corticosterone to induce stress [36]. The current study demonstrated that the intramuscular injections and feed withdrawal resulted in increased *Salmonella* count

in faecal samples. However, this study did not investigate the effect of intramuscular injections and feed withdrawal on corticosterone levels in chickens. Thus, further studies are needed to understand the effects of intramuscular injections and feed withdrawal on corticosterone levels in chickens. However, in an earlier study in mice, handling and intraperitoneal saline injection have been correlated with increased plasma corticosterone levels [37]. The increased faecal *Salmonella* count could be an indicator of recrudescence of *Salmonella* Typhimurium; however, multiple studies are required to understand the actual mechanism of recrudescence in chickens.

In this study, the gut microbiota showed significant changes in diversity due to *Salmonella* Typhimurium infection. The alpha diversity of gut microbiota was estimated by using the Shannon index that measures the richness and evenness in the distribution of species in the given gut microbiota community [31]. Furthermore, the *Salmonella* Typhimurium infected group showed a significant reduction in the alpha diversity at week 1 post-infection. In growing chicks, normally, the diversity of gut microbiota increases with age [38]. In the current study, *Salmonella* Typhimurium infection was negatively co-related with *Ruminococcus_torques, Ruminococcaceae_unclassified, Runimoclostridium_9, Oscillibacter, Lactobacillus, Lachnospiraceae_unclassified, Flavonifractor, Erysipelatoclostridium, Eisenbergiella and, Caproiciproducens* and *Blautia*. The reduction in these genera in infected groups after 1 week of infection could suggest that not all the bacterial genera can compete with *Salmonella* Typhimurium infection and leads to the reduction in their abundance. These findings are partially supported by an earlier study that analysed the effect of *Salmonella* Typhimurium on gut microbiota modulation in pullets [8]. However, in the current study, the abundance of many different genera was also affected. This could be attributed to bird-to-bird differences as well as age which is an important influencing

factor for gut microbiota [39]. Further, in this study, chicks were challenged at day 7 compared to 18-week-old pullets, which had resilient and developed microbiota [38]. Thus, it could be assumed that *Salmonella* Typhimurium infection at an early age could have lingering effects on gut microbiota composition. However, further studies are required to understand this hypothesis. *Ruminococcaceae* (*Ruminococcus_torues*, *Ruminococcaceae_unclassified*, *Ruminoclostridium_9*) is a natural inhabitant of the chicken gut and plays a key role in a breakdown in complex carbohydrates [8]. *Ruminococcaceae* and *Lachnospiraceae* (*Lachnospiraceae_unclassified, Eisenbergella*) are major producers of butyric acid, which is a preferred substrate of intestinal epithelial cells [38]. *Blautia* is the most abundant member of gut microbiota responsible for the production of butyric and acetic acid. The lower abundance of *Blautia* has been co-related to obesity, diabetes, liver cirrhosis, rectal cancers in humans [40]. Reduction of these beneficial genera at week 1 post-infection in *Salmonella* infected group could have adverse effects on health and also affect the performance of the birds.

The *Salmonella* infection also reduced the abundance of *Falvinofractor* and *Erysipelitoclostridium* in infected groups. *Flavinofractor* is a known opportunistic pathogen known to cause infections in immunocompromised patients [41]. *Erysipelitoclostridium* has been co-related to Crohn's disease and *Clostridium difficile* infections in humans [42]. Thus, from the available data, it could be concluded that *Salmonella* Typhimurium infection at an early age has a complex interaction with normal gut microbiota in chickens, and further studies are required to analyse it in detail.

In this study, the abundance of *Melissococcus* and *Enterabacteriaceae_unclassified* increased in *Salmonella* Typhimurium infected groups. Both these genera belong to

the family *Enterobacteriaceae*, which is contains known pathogens. *Melissococcus pluton* is also known to cause European foulbrood in honeybees [43].

The analysis of gut microbiota composition between week 1 post-infection to week 7 post-infection showed increased abundance of *Streptococcus*, *Peptostreptococcaceae_unclassified*, *Romboutsia*, *Megamonas, Anaerostipes* in gut microbiota. *Streptococcus, Romboutsia* and *Peptostreptococcaceae_unclassified* are known pathogenic genera. An increase in abundance of *Rombutsia* was in contrast to the earlier reports, which suggested that *Salmonella* Typhimurium load was negatively co-related to its abundance [8]. Genus *Romboutsia* plays a role in the synthesis of vitamins and cofactors and the fermentation of single amino acids [44]. Earlier studies suggested that genus *Anaerostipes* could have a protective effect against colon cancer in humans [45]. However, more studies are required to understand its role in chickens. Further, the alpha diversity analysis using the Shannon index between the infected and negative control group did not show any significant differences. This data suggests that over the period (up to week 7 post-infection), gut microbiota could have recovered from disruption caused by *Salmonella* Typhimurium infection.

The feed withdrawal did not show any significant difference in diversity of the gut microbiota; however, the intramuscular injection (of both PBS and corticosterone) reduced the diversity of gut microbiota, which has been documented earlier [46]. Furthermore, stress-induced in groups (NC_CORT, INF_CORT, and INF_PBS) by intramuscular injection showed a significant decrease in phylum Firmicutes and a significant increase in phylum Proteobacteria at week 8 post-infection. The feed withdrawal did not result in any significant variation in both the phyla. There are limited studies in chickens that assess the effect of different stressors on the host gut microbiota. The data available in chickens is focused on the effect of heat stress on

gut microbiota in chickens [47]. A study in layers showed that exposure to a high temperature significantly reduced the abundance of phylum Firmicutes [48]. However, these results are contradicted by studies in broilers and in Shaoxing ducks in which exposure to high temperature showed an increased abundance of phylum Firmicutes [49, 50]. A study in humans suggests that the effect of stressors on gut microbiota vary across stressors [51]. Layers are exposed to multiple potential stressors throughout their life span such as vaccination, transport, beak trimming, change in feed, extreme weather. Thus, studies are required to focus on the effect of different stressors on gut microbiota in chickens. Although the immune functions were not the focus of this study, it is known that stress adversely impacts the immune functions by suppression of immunity, altering the intestinal morphology that intern can affect the gut microbiota in the host[49, 52-54]. The results in groups INF_CORT and INF_PBS also co-relate with a significant increase in faecal *Salmonella* Typhimurium count at week 8 post-infection. Earlier studies reported that stress (induced by corticosterone) leads to replication of *Salmonella* Typhimurium in pigs by modulating the immune system [6]. This replication is driven by the *sscA* gene, which under the influence of cortisol, induces macrophage cytoskeletal rearrangement [6]. This facilitates the intracellular replication of *Salmonella* Typhimurium in macrophages [6]. The mechanism of *Salmonella* replication in chickens under stress is unknown. The stress associated with feed withdrawal and transport has been shown to increase the caecal carriage of *Salmonella* in broilers [55, 56]. Further, the undetected infection in pigs leads to the continued shedding of *Salmonella* Typhimurium due to stress caused by transportation and feed withdrawal [57-59]. However, it can be assumed that potential stress due to intramuscular injections could have increased *Salmonella* shedding in this study. The increase in the count of *Salmonella* could have contributed to an

increased abundance of phylum Proteobacteria. This could be because “like will to like” concept proposed by Stretcher et. al which may help to understand the increase in closely related bacterial species in the gut [60]. Phylum Proteobacteria includes the *Enterobacteriaceae* family, which contain know enteric pathogens like *Shigella*, *Escherichia coli* [7]. Thus, an increased abundance of phylum Proteobacteria could increase susceptibility to the infection [7]. The significant increase in phylum Proteobacteria can affect the Proteobacteria-Firmicutes ratio, which could lead to dysbiosis in the gut [60]. A significant decrease in phylum Firmicutes after intramuscular injection in *Salmonella* infected group was not only the result of the infection but can also be the predisposing factor for the growth of *Salmonella*. Phylum Firmicutes contains families like *Ruminococcaceae* and *Lachnospiraceae*, which are reported to be inversely proportional to the abundance of family *Enterobacteriaceae* [7]. These families produce butyric acid, a short-chain fatty acid that down-regulates *Salmonella* pathogenicity island 1 expression. This could lead to a reduced invasion capability of bacteria in a host [61].

4.6 Conclusions

In conclusion, our findings suggest that exposure to a relatively lower dose of *Salmonella* can lead to persistent shedding in pullets. Furthermore, our data suggest that colon and ileum could be potential anatomical sites for colonisation and replication of *Salmonella* Typhimurium in chickens; however, detailed studies are required to test this hypothesis. The intramuscular injections mimicking field practises (such as vaccinations), handling (shifting) and feed withdrawal could increase *Salmonella* shedding in infected or carrier pullets. The study also concludes that *Salmonella* Typhimurium infection at an early age significantly reduces the diversity of gut

microbiota and disrupts the microbiota composition. Furthermore, intramuscular injections altered the gut microbiota composition by significantly changing the Proteobacteria- Firmicutes ratio, which could increase the risk of dysbiosis.

4.7 Declarations

Ethics approval

The animal trial for this study was approved by the Animal Ethics Committee at The University of Adelaide under approval number: S-2019-004.

Consent for publications

Not applicable

Availability of data and materials

The 16S rRNA sequence data are available from the NCBI SRA under the BioProject accession number PRJNA647923.

Competing interests

The authors declare that they have no competing no financial competing interests.

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Authors' contributions

NNJ, KKC and SK contributed to the study design, animal trials, samples processing and critical revision of the manuscript. NNJ, SK and KKC performed the animal trials, NNJ processed the samples, analysed the data and drafted the manuscript. SK and

KKC critically edited the manuscript. All the authors approved the manuscript for publication.

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Table 4.1 Details of various treatment groups used in the study

Sr. No.	Treatment Code	Treatment group	No. of chicken
1	NC_CORT	Negative control + CORT	11
2	INF_CORT	*Salmonella* Typhimurium+ CORT	14
3	INF_PBS	*Salmonella* Typhimurium + PBS	13
4	NC_FW	Negative control + Feed Withdrawal	12
5	PC	*Salmonella* Typhimurium	14
6	INF_FW	*Salmonella* Typhimurium + Feed withdrawal	12

CORT is corticosterone; PBS is phosphate buffered saline

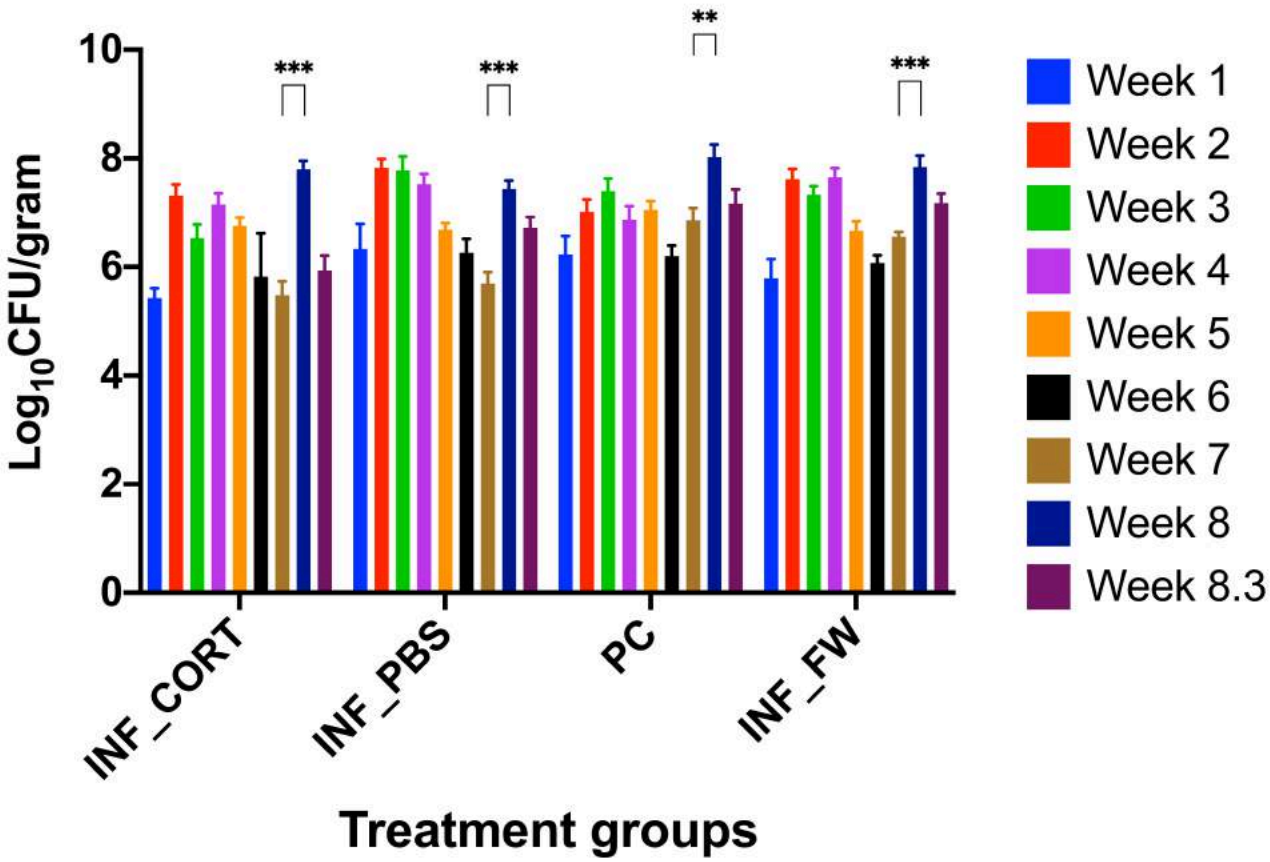

Figure 4.1 Bacterial load was quantified using qPCR from faecal samples collected from chickens infected with *Salmonella* Typhimurium from week 1 to week 8 post-infection. Groups INF_ CORT and INF_PBS received an intramuscular injection of corticosterone and PBS, at week 7 post-infection while group INF_FW was subjected to feed withdrawal which showed a significant ($P < 0.0001$) increase in *Salmonella* Typhimurium count at week 8 post-infection. Group PC also showed a significant increase ($P = 0.0005$) in Salmonella Typhimurium count at week 8 post-infection. *Salmonella* Typhimurium was detected using a TSR3 gene-specific primer pair and the data are present as Log CFU ± S.E.M. The data was visualised in GraphPad Prism v.8.0.0. Asterisks (*, ** and ***) show P values at 0.01, 0.001 and 0.0001 respectively.

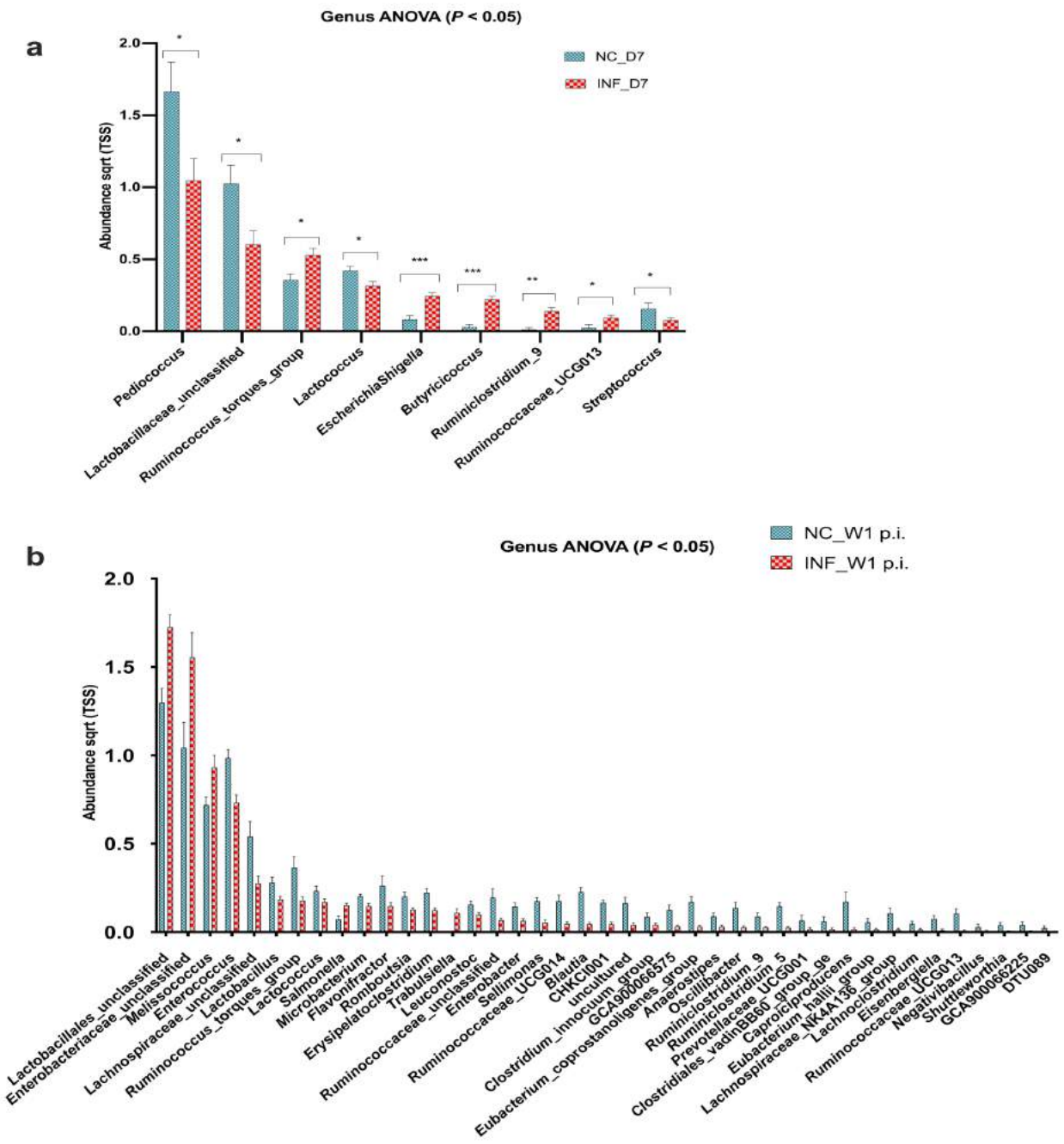

Figure 4.2 The relative abundance of different genera in the negative control (NC) and *Salmonella* Typhimurium infected (INF) groups at day 7 post-hatch and week 1 post-infection. (a) The relative abundance of significant genera at day 7 post-hatch. (b) The abundance of significant genera at week 1 post-Salmonella Typhimurium infection. The relative abundance levels (in log2 cumulative sum scaling) were calculated in Calypso software and the data were visualised in GraphPad Prism v.8.0.0. Asterisks (*, ** and ***) show P values at 0.01, 0.001 and 0.0001 respectively.

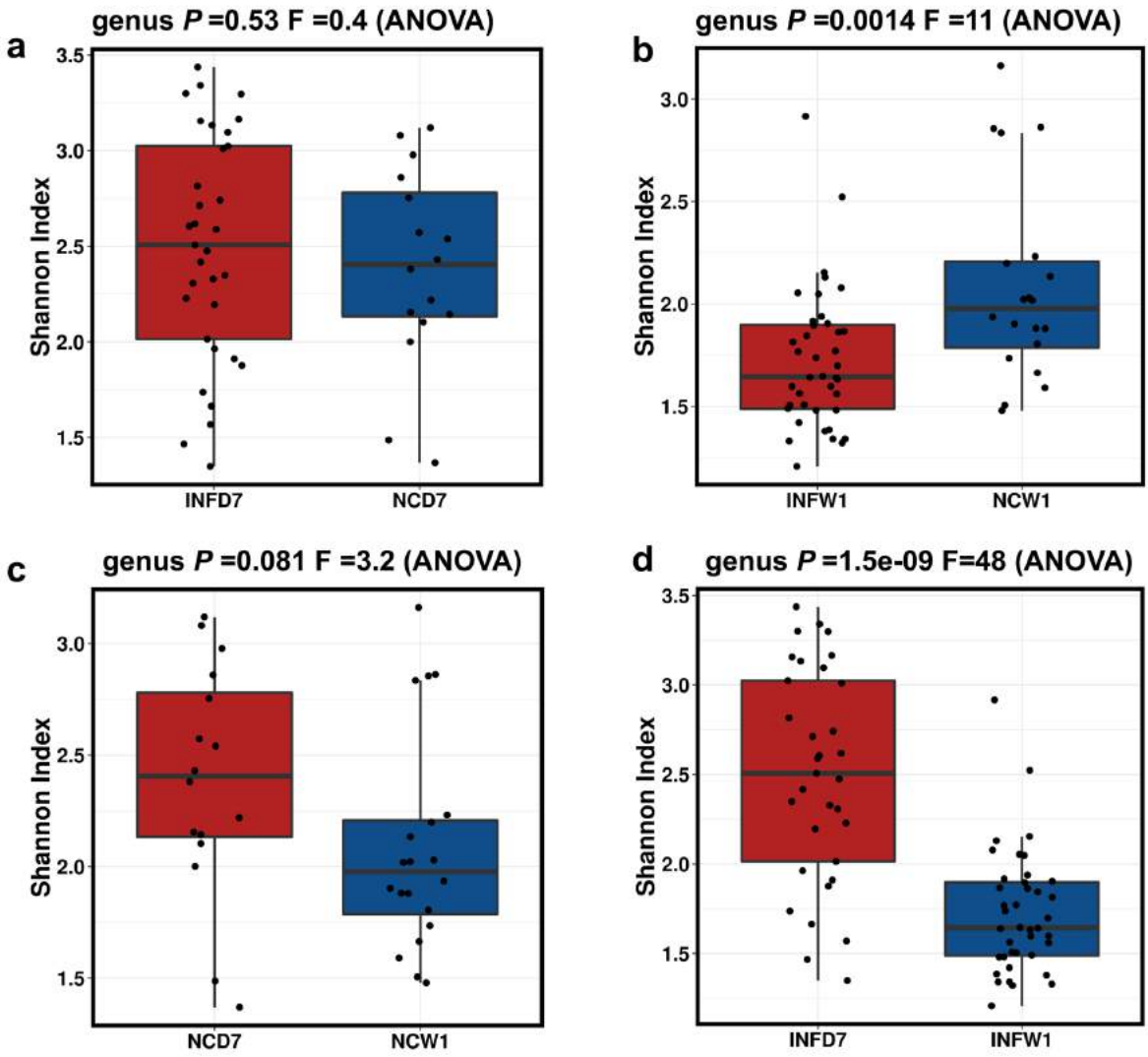

Figure 4.3 Alpha diversity of gut microbiota at genus level affected by *Salmonella* Typhimurium infection. (a) Alpha diversity between negative control (NC) and *Salmonella* Typhimurium infected (INF) groups at day 7 post-hatch. (b) Alpha diversity between NC and INF groups at week 1 post-infection. (c) Alpha diversity of NC at day 7 and week 1 post-infection. (d) Alpha diversity of INF at day 7 post hatch and week 1 post-infection. Alpha diversity was measured by the Shannon index in Calypso software.

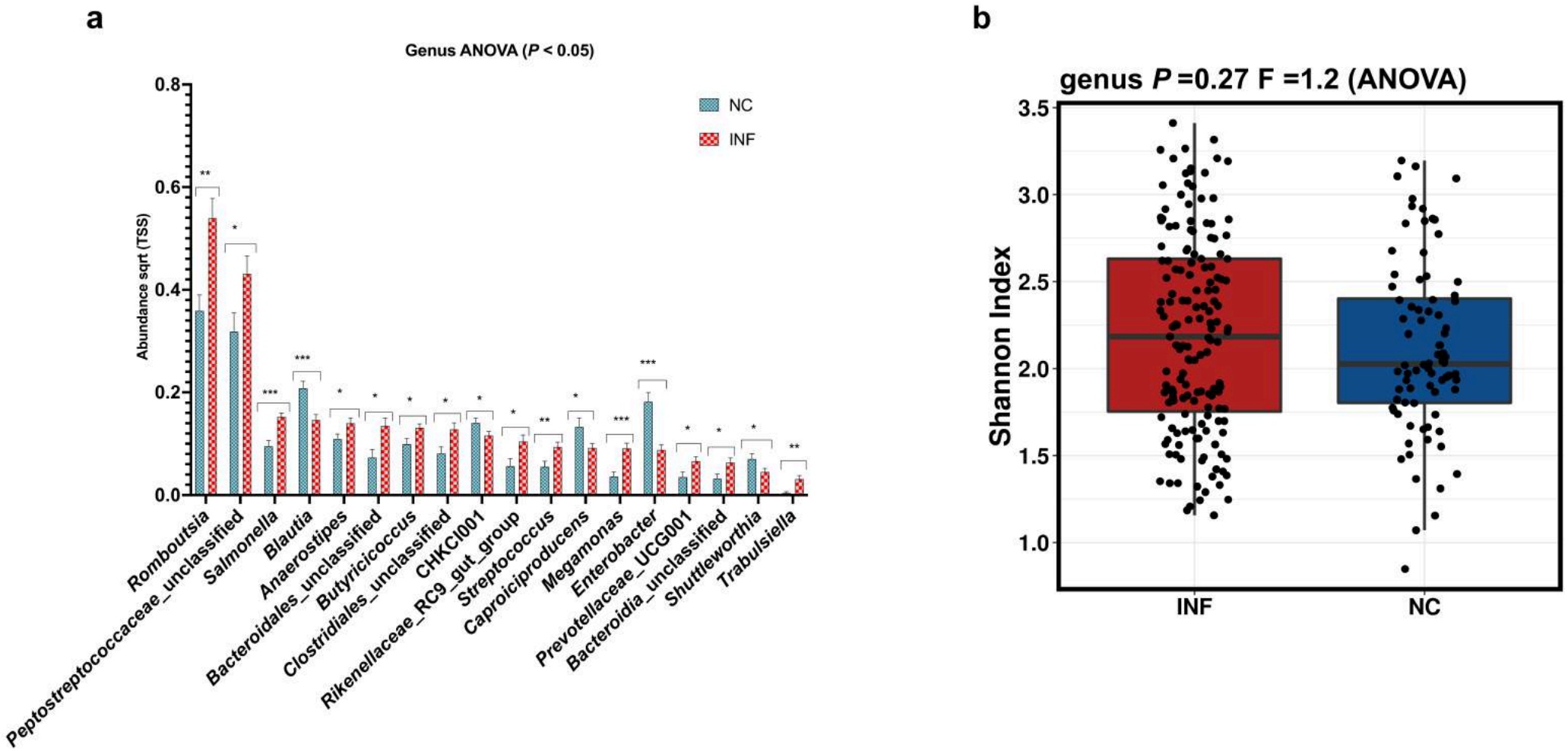

Figure 4.4 Relative abundance and diversity of gut microbiota. (a) The abundance of different genera in the negative control (NC) and *Salmonella* Typhimurium infected (INF) treatment groups from week 1 to week 7 post infection. (b) Comparative analysis of alpha diversity between NC and INF groups using Shannon index. The relative abundance levels (in $\log_2$ cumulative sum scaling) were calculated in Calypso software and the data were visualised in GraphPad Prism v.8.0.0. Asterisks (*, ** and ***) show P values at0.01, 0.001 and 0.0001 respectively.

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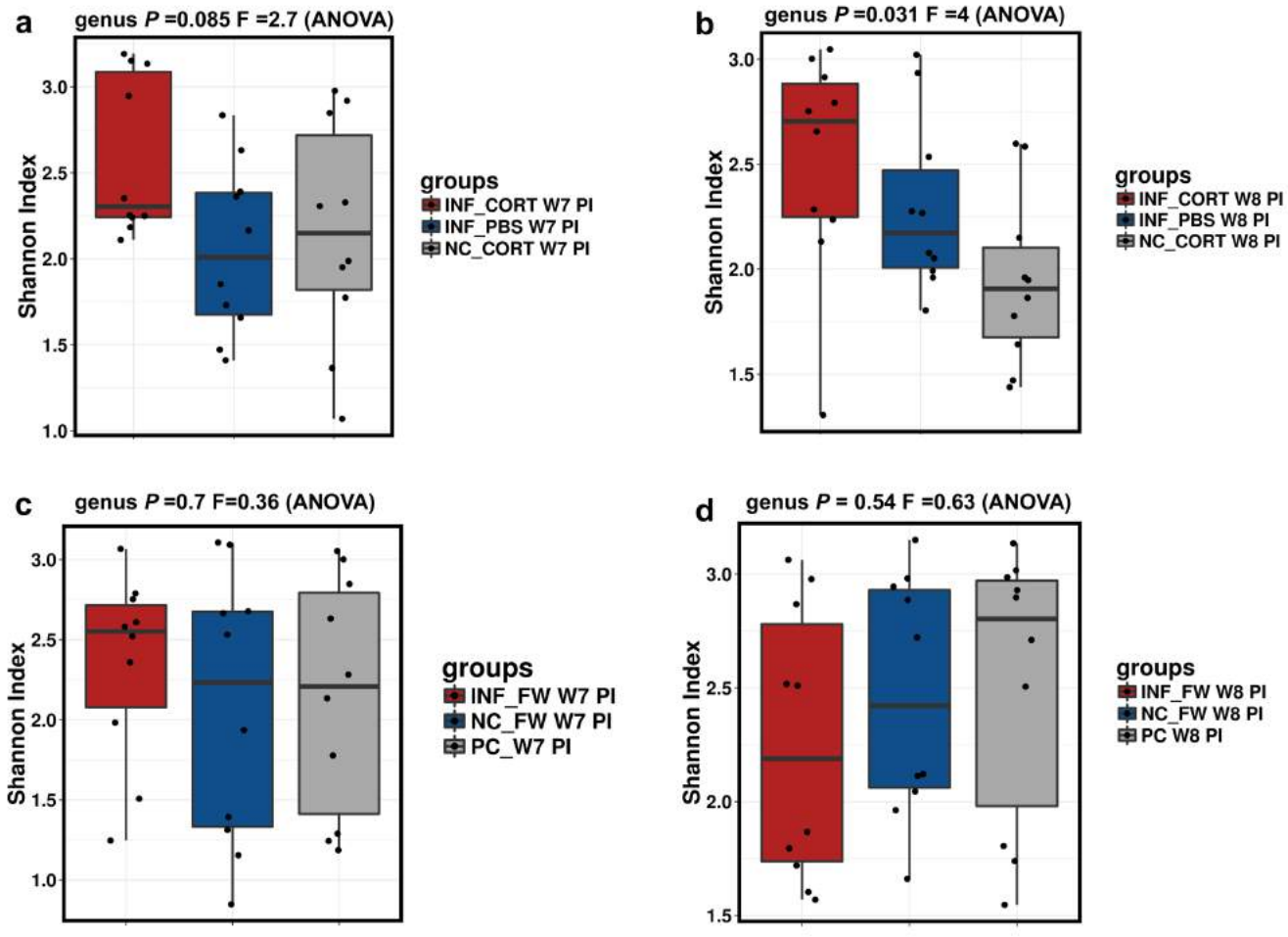

Figure 4.5 Alpha diversity of gut microbiota at genus level affected by *Salmonella* Typhimurium infection, intramuscular injection and feed withdrawal. (a) Alpha diversity of gut microbiota before the intramuscular injection (week 7 *Salmonella* Typhimurium post-infection). (b) Alpha diversity of gut microbiota after intramuscular injection (week 8 *Salmonella* Typhimurium post-infection). (c) Alpha diversity of gut microbiota before the feed withdrawal (week 7 *Salmonella* Typhimurium post-infection). (d) Alpha diversity of gut microbiota after the feed withdrawal (week 8 *Salmonella* Typhimurium post-infection). Alpha diversity was measured by the Shannon index in Calypso software. For treatment group details, refer to Table 1.

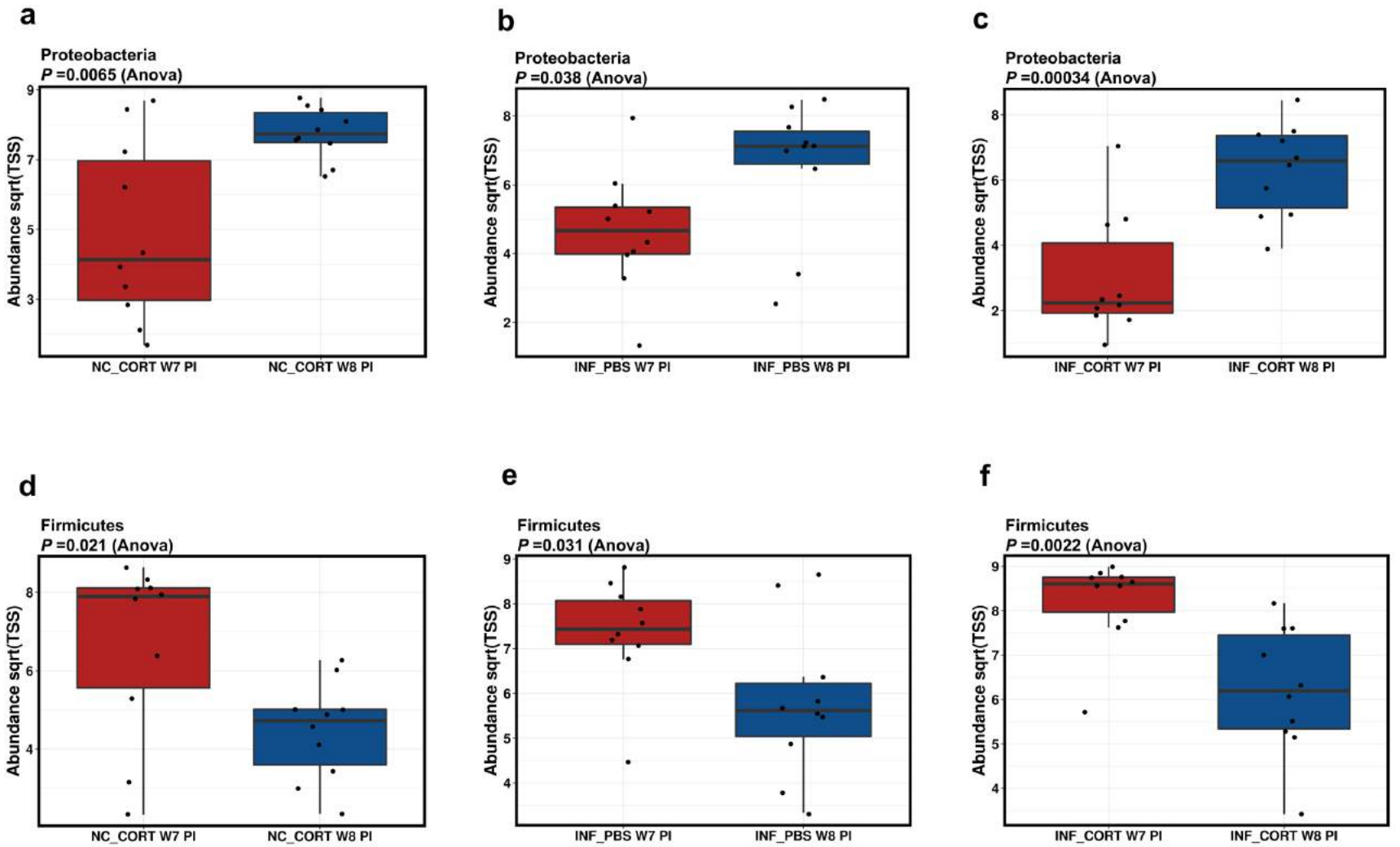

Figure 4.6 Alpha diversity of Proteobacteria and Firmicutes affected by intramuscular injection. (a) Proteobacteria in negative control and corticosterone injected group (NC_CORT) at week 7 and week 8 post *Salmonella* infection. (b) Proteobacteria in *Salmonella* Typhimurium infected treatment (INF_PBS) group at week 7 and 8 post-infection. (c) Proteobacteria in *Salmonella* Typhimurium infected and corticosterone injected treatment (INF_CORT) group at week 7 and 8 post-infection. (d) Firmicutes in negative control and corticosterone injected treatment group (NC_CORT) at week 7 and 8 post -infection. (e) Firmicute in *Salmonella* Typhimurium infected (INF_PBS) treatment group at week 7 and 8 post-infection. (f) Firmicutes in *Salmonella* Typhimurium infected and corticosterone injected treatment group (INF_CORT) at week 7 and 8 post-infection. NC_CORT and INF_CORT received an intramuscular injection of corticosterone, while group INF_PBS received an intramuscular injection of PBS.

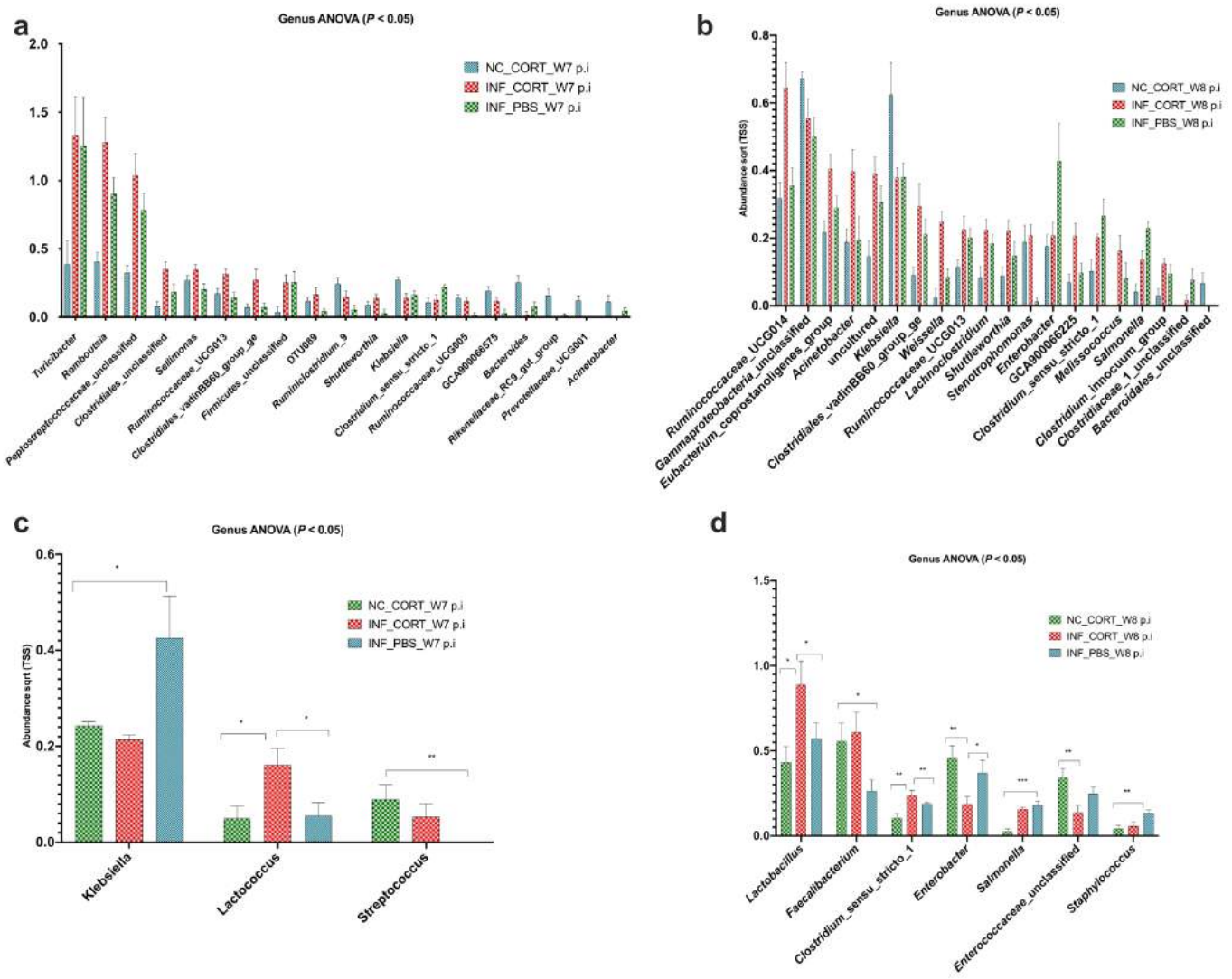

Figure 4.7 The relative abundance of microbial communities at the genus level is affected by intramuscular injection and feed withdrawal. (a) The relative abundance of gut microbiota before the induction of intramuscular injection (week 7 *Salmonella* Typhimurium post-infection). (b) The abundance of gut microbiota after the intramuscular injection (week 8 *Salmonella* Typhimurium post-infection). (c) The abundance of gut microbiota before feed withdrawal (week 7 *Salmonella* Typhimurium post-infection). (d) The abundance of gut microbiota after feed withdrawal (week 8 *Salmonella* Typhimurium post-infection). For treatment group codes, refer to Table 4.1.

Supplementary Table 1 Comparative analysis of *Salmonella* Typhimurium positive tissue samples between direct plating and post enrichment *Salmonella* Typhimurium load (Log10CFU/g) in organ at the time of cull.

Sr. No	Organs	**INF_CORT**		**INF_PBS**		**PC**		**INF_FW**	
		Direct plating	**Post-enrich**	**Direct plating**	**Post-enrich**	**Direct plating**	**Post-enrich**	**Direct plating**	**Post-enrich**
1	**Liver**	0/13	2/13	1/13 (1.83±0.00)	1/12	0/11	1/11	3/12 (1.98±0.08)	1/9
2	**Spleen**	0/13	1/13	1/13 (2.649± 0.0)	1/12	0/11	3/11	2/12 (1.54±0.00)	1/10
3	**Ileum**	5/13 (3.22±0.26)	3/8	4/13 (4.077±0.25)	6/9	6/11 (2.54±0.25)	0/5	3/12 (4.44±0.00)	1/9
4	**Caeca**	13/13 (6.21±0.16)	0/0	13/13 (6.83±0.12)	0/0	11/11 (6.33±0.27)	0/0	12/12 (6.08±0.57)	0/0
5	**Colon**	8/13 (4.52±0.39)	5/5	13/13 (4.51±0.29)	0/0	8/11 (4.07±0.44)	2/3	12/12 (4.02±0.41)	0/0

Log_{10} CFU data are expressed as mean ± S.E.M.

Chapter 5 General Discussion

5.1 Introduction

The gut microbiota plays important role in modulation of immunity, physiology, and nutrient digestion in the host (Khan et al., 2020). In last two decades, research in gut microbiota increased exponentially mainly due to the availability of new techniques such as 16S rRNA and whole genome sequencing (Shang et al., 2018). The influence of gut microbiota on overall health and pathogenic diseases especially in food producing animals has also been reported earlier (Stanley et al., 2012b, Diaz Carrasco, Casanova and Fernández Miyakawa, 2019). Thus, it is also important to understand the development and composition of gut microbiota in food producing animals such as chickens. Chicken meat and eggs are highly consumed animal food products in the world because of their acceptability across different cultures and religions. However, most of the gut microbiota research was conducted in commercial broilers owing to their shorter life span and direct economic importance (Stanley et al., 2012b, Stanley et al., 2012a, Mookiah et al., 2014). In comparison, the availability of data on the development and composition of gut microbiota in commercial layer flock is limited because of the longer life span (80 weeks).

5.2 Temporal development, richness pattern and age dependent gut microbiota in cage hens.

In chapter 2, the study was focused on the development of gut microbiota in commercial layers raised in cages from day 1 to week 75 of the flock's age. Many supermarkets in Australia impose the standards which require the cage egg producing

flock to be raised in cages from day old. In Australia, 40% of total eggs are produced in cage production systems (AECL, 2019).

In this study, the richness of gut microbiota increased with age up to the early lay phase after which it started stabilizing. A significant increase in gut microbiota richness was observed at week 12 of the flocks age as compared to the earlier time points. The richness of gut microbiota has been co-related with improved gut health in earlier studies (Stanley et al., 2013a, Stanley, Hughes and Moore, 2014). There was a significant reduction in the richness of gut microbiota after transporting flock from rearing to production farm which could be attributed to stress due to transport, the onset of lay, change in feed etc. The reduced richness has been co-related with diminished gut health (Valdes et al., 2018). It has been reported that after transport, at week 18, pathogens tend to colonise in the gut in layer birds (Gole et al., 2014). This study reported an increase in abundance of phylum *Fusobacteria* and significant increases in abundance of family *Peptostreptococcaceae* from phylum *Firmicutes* at week 18. In phylum *Firmicutes,* the family *Peptostreptococcaceae* are known as pathogenic taxa. In this study, gut microbiota was dominated by phyla *Firmicutes* and *Proteobacteria* in the rearing phase. Further in our study, in phylum *Firmicutes*, the dominant family was *Lactobacillaceae* as compared to *Ruminococcaceae* and *Lachnospiraceae* as reported in the earlier study (Videnska et al., 2014). From the early lay phase, phylum *Bacteroidetes* started replacing phylum *Proteobacteria*. In late lay phase *Bacteroidetes* became the dominant phylum replacing *Firmicutes*. These results were in partial agreement with the pattern of gut microbiota development reported earlier in layers (Videnska et al., 2014, Ngunjiri et al., 2019). However, the abundance and composition of each phylum were significantly different. The High abundance of phylum *Firmicutes* in the rearing and early lay phase could be attributed

to its functions. Phylum *Firmicutes* include families responsible for the production of butyrate which is a preferred substrate for intestinal epithelial cells (Videnska et al., 2014). There is a need for high energy for rapid growth and production which can be co-related with a high abundance of phylum *Firmicutes* during rear. However, from the mid lay phase, the abundance of phylum *Bacteroidetes* increased replacing phylum *Firmicutes*. The primary products of phylum *Bacteroidetes* are acetate and propionates which are not the preferred substrates of colonocytes (Wrzosek et al., 2013, Yang et al., 2013). Thus, it could be assumed that energy requirement in mid and late lay is less as it is required for production and maintenance of egg quality compared to the rear and early lay phase in which energy is required for overall growth and egg production. So, high abundance of phylum *Bacteroidetes* can be correlated to the energy demand of the host in mid and late lay. Further, it could be assumed that reduction in the abundance of *Firmicutes* and or *Bacteroidetes* could affect the physiological functions. For example, at an early age decline in the abundance of phylum *Firmicutes* could negatively affect the growth which in the future could influence the production and overall health. The composition and abundance of microbiota can be influenced by multiple factors such as feed, housing, environment, and the host itself (Kers et al., 2018). Factors such as feed, housing and environment have been discussed in earlier chapters. The role of the host itself could be critical in gut microbiota composition and shift. The host-microbiota communication and age dependent variation have been described in previous studies (Ngunjiri et al., 2019, White et al., 2020) and discussed in detail later in this chapter.

5.3 Comparative analysis of gut microbiota in hens from different housing systems shows similarities and significant differences.

In Australia, the egg production in the Free range system is 47% and in the Barn is 11% (AECL, 2019). These two systems together constitute most of the egg production in Australia. The use of these production system has been increasing in the world. European Union has already banned cage production system and a few other countries (Canada, New Zealand) are planning to phase out cage production system in coming years (Appleby, 2003, Hartcher and Jones, 2017). Further, in Australia, multi-age cage system is also practiced in commercial layer industry. Many commercial farms transfer pullets at point of lay from different batches to the same production shed. Thus, birds are exposed to other birds of different ages which could affect the gut microbiota and increases the chances of pathogen infection.

Thus, it is essential to understand the development of gut microbiota in layer birds raised in free range and barn systems. Chapter 3 focused on the development of gut microbiota in three commercial layer flocks raised in the different housing systems. The flocks were raised in Free range (flock A), Barn (flock B) and Cage (flock C) production systems. The development pattern and composition of gut microbiota in all three flocks were significantly different. The richness of gut microbiota was significantly different in all three flocks. In flock A and B richness of gut microbiota increased up to mid lay phase after which it stabilised while in flock C gut microbiota increased significantly at week 20 after which it stabilised till week 75. These differences could be attributed to the management system. Flock A and B were raised in an all-in all-out system while flock C was housed in multi-age cage system. This could have exposed flock C to gut microbiota from older hens and could have affected the richness. As described earlier the gut microbiota higher richness is co-related with

improved gut health in the earlier studies (Stanley, Hughes and Moore, 2014). This study observed that in flock C, the richness of gut microbiota decreased at week 75. This decrease could be caused due to new flock (pullets 18 weeks of age) introduced to the multi-age shed. This study also noted the significant effect of age on the gut microbiota. Age is the most important influencing factor in the development of gut microbiota which has been reported by an earlier study (Ngunjiri et al., 2019). The development of gut microbiota in production phases (Early, Mid and Late lay phase) was also significantly different in all three flocks. Further, the abundance and composition of gut microbiota were also significantly different. The production phases (Early, Mid, and Late-lay phase) were dominated by *Firmicutes-Bacteroidetes* phyla. In flock A and B, *Firmicutes* was the dominant phylum in the early lay phase while in flock C, *Bacteroidetes* was dominant at some timepoints. Similar significant variations were noted in the mid lay and late phase in all three flocks. The significant finding in the production phase was the dominance of phyla *Firmicutes* in flock C, at week 70 and & 75. All the earlier studies have noted the significant abundance of phylum *Bacteroidetes* in late lay phases in hens (Callaway et al., 2009, Videnska et al., 2014). This shift to phylum *Firmicutes* could have caused due to a new pullet flock in the shed. In pullets, *Firmicutes* is the dominant phylum in gut microbiota. Because of this the abundance of *Firmicutes* in the environment would have increased and it has been reported earlier that environmental conditions affect gut microbiota significantly (Kers et al., 2018).

In this study, *Campylobacter hepaticus (C. hepaticus)* infection which causes Spotty Liver Disease was recorded in flock A at week 34. The flock was treated for the next two weeks with chlortetracycline and egg production was declined by 20% during this period. The gut microbiota in flock A at week 34 showed a shift to phylum

Bacteroidetes. *C. hepaticus* is known to colonise the small intestine and caeca of infected birds. However, the effect of *C. hepaticus* infection on gut microbiota has not been studied in depth and further studies are necessary to understand the impact of the Spotty Liver infection on gut microbiota of hens. The gut microbiota richness in flock A dropped significantly at week 40. This could have been caused due to *C. hepaticus* infection. Althoughthe impact of *C. hepaticus* infection is unknown, *C. jejuni* infection is known to cause the reduction in gut microbiota in chickens (Han et al., 2017). It has been also reported that the host immune response to *C. jejuni* infection in layers is dependent on feed composition (Han et al., 2017). Further, the flock was treated for two weeks with chlortetracycline. It has been reported that chlortetracycline reduces gut microbiota diversity (Ma et al., 2021). The antibiotic treatment for two weeks could have reduced the richness of gut microbiota at week 40. Thus, it is important to understand the effect of commonly used antibiotics on gut microbiota in chickens. Furthermore, it is also possible that reductions in gut microbiota richness might have been due to the compounding effect of *C. hepaticus* infection and chlortetracycline treatment. Therefore, to understand *C. hepaticus*, chlortetracycline, and gut microbiota interaction further studies are required.

5.4 Intramuscular injection and *Salmonella* Typhimurium infection affects the gut microbiota composition and diversity.

Multiple factors influence the gut microbiota which leads to significant differences in the composition, however, some factors are known to affect gut microbiota adversely such as enteric pathogens (Khan and Chousalkar, 2020a, Khan and Chousalkar, 2020b) and stress. Earlier studies have reported the far-reaching effects of stress on gut microbiota, immunity, and growth performance in layers (Song et al., 2018, Xu et

al., 2018, Shi et al., 2019). The layer birds experience multiple potential stressors throughout the life span such as feed withdrawal, intramuscular vaccination, transportation, etc. some of which are unavoidable such as intramuscular vaccinations and feed withdrawal. In this study we used intramuscular injections to mimic intramuscular vaccination and feed withdrawal to mimic the field events to induce the stress. Further, the role of enteric pathogens like *Salmonella* in the reduction in diversity and richness of microbiota in chickens has been reported (Mon et al., 2015, Khan and Chousalkar, 2020b). In Australia, *Salmonella* Typhimurium is an important enteric pathogen in layers due to its colonisation and zoonotic potential. In chapter 4, the effects of intramuscular injection and feed withdrawal and *Salmonella* Typhimurium on gut microbiota in pullet were studied. All the *Salmonella* Typhimurium infected chickens showed persistent shedding of bacteria up to week 8 post infection. This study showed that the diversity of gut microbiota was reduced significantly at an early age after inoculation of *Salmonella* Typhimurium. It also showed the significant reduction in the abundance of beneficial genera in gut microbiota which are essential for the growth and overall health of the birds. The early weeks in a chick's life are critical for the establishment and development of gut microbiota, thus disruption of gut microbiota at an early age could cause lingering effects. Further, study also concluded that handling and intramuscular injection alter the *Firmicutes-Proteobacteria* ratio which could lead to dysbiosis in chickens. Potential stress due to feed withdrawal and intramuscular injections significantly increased the *Salmonella* Typhimurium shedding in carrier birds. The potential stress related shedding can spread infection in the flock and to humans via eggs. In earlier studies in broilers and pigs feed withdrawal and transportation stress has been co-related with increased *Salmonella* shedding (Rigby and Pettit, 1980, Bolder and Mulder, 1983, Isaacson et al., 1999, Boyen et al., 2008,

Martín-Peláez et al., 2009). *Salmonella* Typhimurium enumeration from the tissue also suggested that colon and ileum could be potential anatomical sites for colonisation and replication of *Salmonella* Typhimurium, however, details studies focusing on the stress induces replication and shedding of *Salmonella* Typhimurium in chickens are needed to test the hypothesis.

In chapter 2 and 3, a total 2190 samples were collected and analysed from hatch to the end of the production cycle in four commercial layer flocks raised in different housing systems. All the four flocks showed significant variation in the composition and development of gut microbiota. Further, the gut microbiota composition varied significantly over the period in all four flocks. The richness of gut microbiota and its development pattern was also significantly different in all four flocks. These differences could have been due to different feed, housing systems, or management protocols.

These studies were focused on commercial flocks which give the advantage to understanding the effects of multiple variables on the gut microbiota mimicking the field conditions. The data obtained from these studies can be directly used to modify the feed composition, use specific feed supplements (Prebiotics/Probiotics), and in the future use gut microbiota as a therapeutic application. In contrast, the data obtained from controlled pen trials will provide limited information because the number of variables are limited and controlled experimentally. Further, the controlled trials havea shorter period and a limited sample size. It is always not possible to have similar feed composition, management, and housing systems in all the commercial/field setups. Thus, the data generated from field-based trials is valuable because it gives a real time understanding of the effects of multiple variables and their interaction on gut microbiota. Therefore, to understand the establishment and development of gut microbiota, field-based trials are more applicable in food-producing animals compared

to the control studies. The controlled studies are more beneficial to understand the specific objective such as the effect of specific prebiotic or feed on gut microbiota composition.

Earlier studies have reported the effect of multiple host and environmental factors on the development of gut microbiota in chickens (Kers et al., 2018). The richness of gut microbiota increased with age which was significantly low in the 1st week of the flocks. From this, it could be concluded that newly hatched chickens do not have a high abundance of the microbiota. Naturally, the newly hatch chicks will acquire gut microbiota from the nest environment and the hens. However, in modern practices, the chicks are hatched in hatcheries which are cleaner and have a low microbiota load. Further, the newly hatched chicks are not exposed to adult birds which reduced the initial microbiota acquisition. Thus, the primary source for birds to receive microbiota is the farm environment or transportation environment. There is a possibility to inoculate chicks with beneficial bacterial taxa at hatch which could lead to consistent and healthy gut microbiota. This hypothesis is supported by a study conducted in broilers which showed improved weight gain and reduction of pathogenic taxa in at hatch probiotic administered chicks (Baldwin et al., 2018). The development pattern of gut microbiota composition and richness was distinct in all four flocks. However, flock C, which was multi-age cage system, the gut microbiota richness stabilised from week 20 onwards. This flock also showed dominance of phylum *Firmicutes* in late lay phase with reduction in richness at week 75. This could be attributed to the types of rearing systems followed in the flocks. Three flocks i. e flock from chapter 2 and, flock A and B from chapter 3 were reared in an all-in-all-out system where all the birds are housed at same time and culled at the same time. However, in flock C was raised in a multi-age cage system which is significantly different and would have caused

significant differences in gut microbiota. The multi-age cage systems expose pullets to older birds which have different gut microbiota and immune status. Thus, exposure to older birds increases the risk of pathogen colonisation and stress. It also limits the implementation of cleaning protocols which are important for bird's health. As there will always be birds in the shed due to multi-age rearing system, through cleaning of a shed will have its limitation. This could increase the risk of pathogen colonisation to chickens. In this study, the richness of gut microbiota in three flocks was stabilised in the late lay phase and production was optimal at week 70 and 75 of flocks' age. Generally, layers are culled at week 80 because of the possibility of lower production. However, the results of these studies put forward the possibility of extending the life cycle of layer birds. It can lead to huge economic profits. Although, more studies focused on this aspect are needed.

In all four flocks, the richness of gut microbiota reduced significantly at week 18 i. e after transportation to the production farm as compared to week 12. This could have been caused due to transportation stress, onset of lay, change in feed, or change in management. The reduced richness could lead to pathogen colonisation. It has been reported earlier that at week 18, after transportation, pathogenic bacteria such as *Salmonella* tend to colonise the gut in layer birds (Gole et al., 2014). The source for these pathogens could be the shed environment. Thus, the cleaner shed and implementation of biosecurity protocol could reduce the chances of pathogen colonisation post transportation. This data is also useful to plan intervention strategies to minimise the impact of stress at week 18 in birds. These strategies could be the supplementation of probiotics/prebiotics/ multivitamins to enhance gut health and reduce the stress in the birds. Further, transportation distance, climatic conditions and supplementation of water and feed during transportation could also impact gut

microbiota composition. Selecting the route with minimum distance between rearing to production farms, considering climatic conditions such as temperature, humidity and time of the day, supplementation of feed and water during transport could help to strategically minimise effects of stress on gut microbiota composition in layers.

The data from chapters 2 and 3 concludes that the changes in gut microbiota composition were not limited to a single genus or phylum but took place throughout the gut microbiota. Thus, there is a possibility that the use of consortia of bacteria tailored for particular stages of bird growth as probiotic as compared to use a single isolate to enhance the gut health and production. Although, the development and composition of gut microbiota in all four flocks were significantly different, some common patterns in the development of gut microbiota were identified. For example, gut microbiota was dominated by phylum *Proteobacteria* and *Firmicutes* at an early age. In mid and early lay, *Firmicutes* and *Bacteroidetes* phylum dominate the gut microbiota which shows the shift to *Bacteroidetes* in the late lay phase. These changes could be related to the age of the birds. Age has been considered as an influencing factor on gut microbiota composition in layer birds (Ngunjiri et al., 2019). This information could be used to design new intervention strategies (such as using *Firmicutes* probiotics at an early age up to mid lay, shift to *Bacteroidetes* probiotics from mid lay phase) to enhanced gut health and production performance of the flock. This strategy will also help to populate gut microbiota with similar bacterial taxa following the “like will to like” principle by Stecher et al. (2010). Stecher et al. (2010) in their study concluded that “the presence of closely related species can increase the chance of invasion of newly incoming species in to gut ecosystem”. This mechanism could be used to populate gut microbiota with beneficial bacterial communities by supplementation of closely related beneficial species. The age-based changes in gut

microbiota composition also point towards the possible host microbiota communication mechanism. Study in humans and animals suggests that host controls the gut microbial population by producing and releasing various molecules (White et al., 2020). These host molecules include miRNA (miR-199a-5p, miR-1226), hormones (Insulin, Estradiol, Noe-epinephrine), Cytokines (IL-1ß, TNF-α, IFN-γ), host molecules (NLRP6), metabolic signalling pathways and metabolites (FXR signalling agonist (GW4064) and antagonist (Gly-MCA), Fucose) (White et al., 2020). However, the exact communication mechanism in the host responsible for the shift in gut microbiota is not yet understood.

Although all 4 flocks had significantly different microbiota, the production performance (% egg production, body weight gain, mortality) were optimal. This indicates that there is no one ideal gut microbiota. Birds with significantly different gut microbiota can show optimal production performance. The difference in the gut microbiota might have been due to different housing system, feed composition or managemental protocols. Studies conducted earlier also showed significant differences in gut microbiota in layers (Videnska et al., 2014, Ngunjiri et al., 2019, Adhikari et al., 2020). Some of these studies also compared gut microbiota composition in birds reared in different housing systems showed significant variation in gut microbiota (Adhikari et al., 2020, Hubert et al., 2019, Cui et al., 2017). A study in broilers with the same feed, housing and management showed variable gut microbiota between the flocks (Stanley et al., 2013b). However, these studies were conducted with controlled variables which are not possible in the commercial industry. Thus, results from controlled gut microbiota studies have limitations. In the commercial industry, housing, feed composition, genetic origin, managemental protocols and geoclimatic conditions are significantly different and it is not practically possible to mimic similar standards throughout the

industry. This study addressed this specific problem how gut microbiota develops in layers in field conditions and generated data which will be helpful for commercial layer industry to develop new strategies to develop and modulate diet for optimal gut health. The role of stress and enteric pathogens like *Salmonella* Typhimurium in gut microbiota was not studied in detail in layers. Although earlier studies have reported effects of *Salmonella* Enteritidis infection and heat stress on the gut microbiota of layer chickens, the scope of these studies was limited (Mon et al., 2015, Zhu et al., 2019). This study assessed the effect of *Salmonella* Typhimurium, a prevalent *Salmonella* serotype in Australia (Moffatt et al., 2016) and stressors such as feed withdrawal and corticosterone induced stress to mimic vaccination on gut microbiota. The effect of *Salmonella* Typhimurium showed reduced diversity of gut microbiota and intramuscular injections induced stress showed significant changes in the *Proteobacteria-Firmicutes* ration which could potentially lead to dysbiosis in layers (Stecher et al., 2010). This information could be used to design strategies during critical time points in a life span of layers to reduce the adverse effects of stress on gut microbiota composition. The stress due to intramuscular injections and feed withdrawal also increased the shedding of *Salmonella* Typhimurium in chicken faeces. Thus, the testing strategies for *Salmonella* Typhimurium in layer flocks could be modified to include potential stress points (vaccinations, transport, a sudden change in climatic conditions) to avoid potential human transmissions.

5.5 Conclusions

This study produced an important data set and contributed to the knowledge of development, establishment, and maturation of gut microbiota in field settings. This will help to formulate long term strategies such as supplementation of different pro and

prebiotics (single or consortium of bacteria) according to the age, critical time points such as intramuscular vaccinations, transport, onset of lay to modulate and establish consistent healthy gut microbiota in chickens. The gut microbiota composition changed significantly at week 18 which is a critical time point in a flock's life as birds experience multiple stressors for example shifting from rear to production, onset of lay, change in feed. The richness of gut microbiota in the birds decreased after shifting to the production shed which led to the colonisation of pathogenic genera. The gut microbiota from all the four flocks showed similarities such as the dominance of phylum *Firmicutes* at an early age which aligns with high energy requirement for weight gain and phylum *Bacteroidetes* at later ages supporting ongoing production and sustenance. The taxonomic composition of gut microbiota varied considerably between all 4 flocks. It shows that there is no single ideal microbiota for high productivity flocks: different communities can produce similar productivity outcomes for the host. The richness of gut microbiota was significantly low in the first week of age which could suggest that the number of microbial communities after hatch are significantly low, and chicks acquire gut microbiota from the exposed environment. Thus, the administration of beneficial genera at hatch could help to establish healthier and more consistent microbiota in chickens. The stabilised richness of gut microbiota can be co-related to feed efficiency in layers however, further studies are required to assess its effect on production performance and the possibility of extending the production cycle. The changes in gut microbiota composition are not focused on a single genus or phylum but take place throughout the gut microbiota. Thus, in the future possibility of using consortia of bacteria tailored for stages of bird growth, rather than single probiotic isolates, to enhance production and gut health could be explored. Further, effect of *C. hepaticus* infection, commonly used antibiotics (chlortetracycline)

and gut microbiota interaction must be evaluated. The *Salmonella* Typhimurium infection at an early age significantly reduces the diversity and disrupts the gut microbiota composition in chicks. It also reduces the abundance of beneficial genera which could affect the growth and overall health of the birds. In infected birds, stressors such as intramuscular injections and feed withdrawal significantly increased the shedding of *Salmonella* Typhimurium in faeces which could increase the chances of infection in the flock and to the human working in a farm environment. The stress due to intramuscular injections mimicking vaccinations disrupts the gut microbiota by increasing the abundance of *Proteobacteria* significantly. This could lead to dysbiosis of the gut in chickens. The effect of different stressors on gut microbiota composition (heat stress, cold stress, feed withdrawal, transportations, vaccination, feed change) could be significantly different and requires further studies. These stressors highlight the critical points of vulnerability for flock health and indicate the potential value of formulating strategies to modify diet (providing prebiotics, probiotics, vitamins) at critical stress points in the life cycle of laying hens to reduce the risk of gut pathogens.

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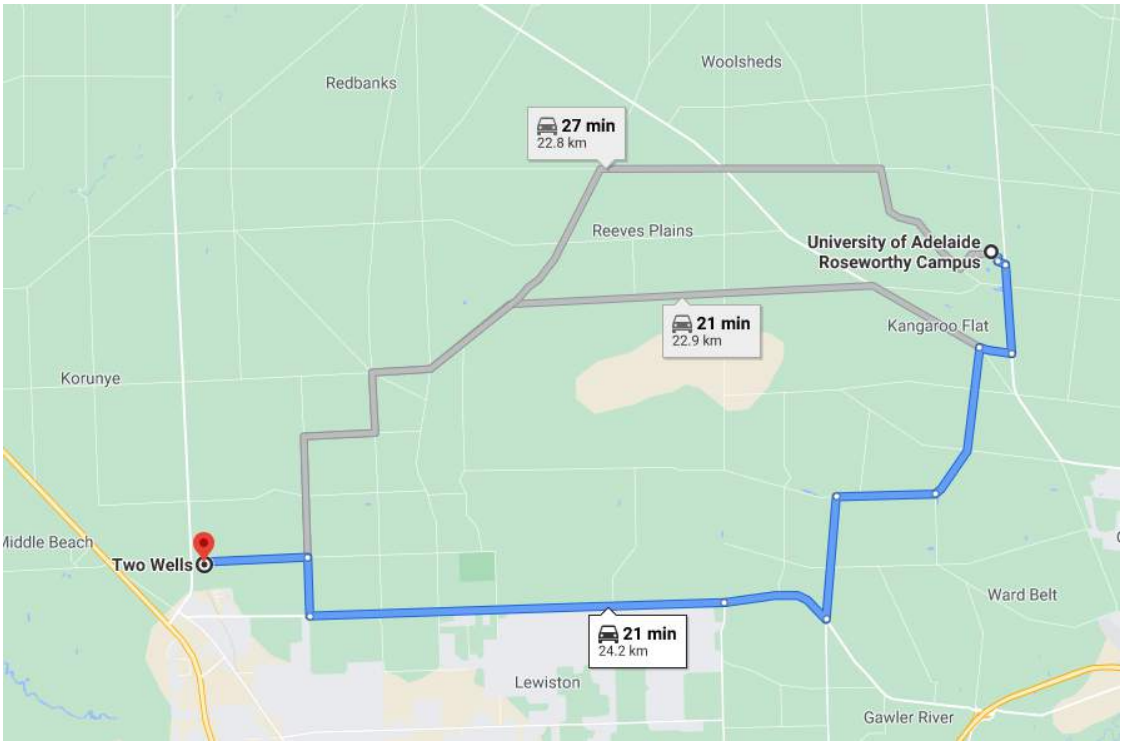
Appendix 1

A



B

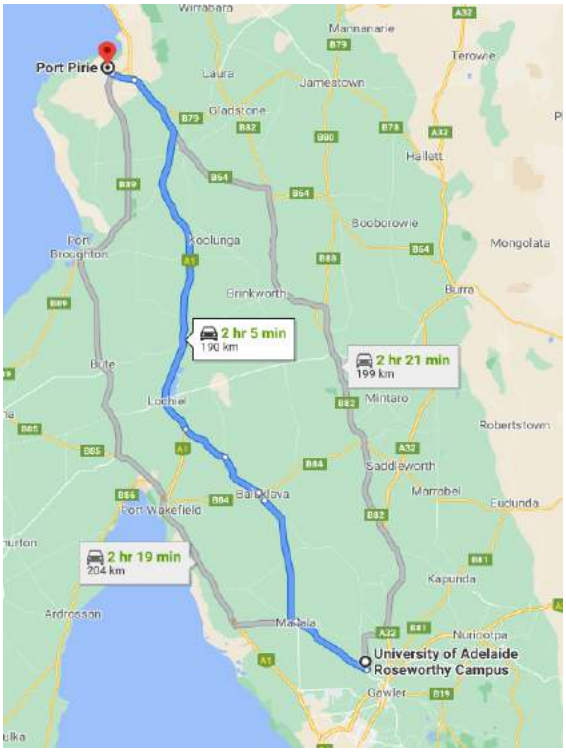
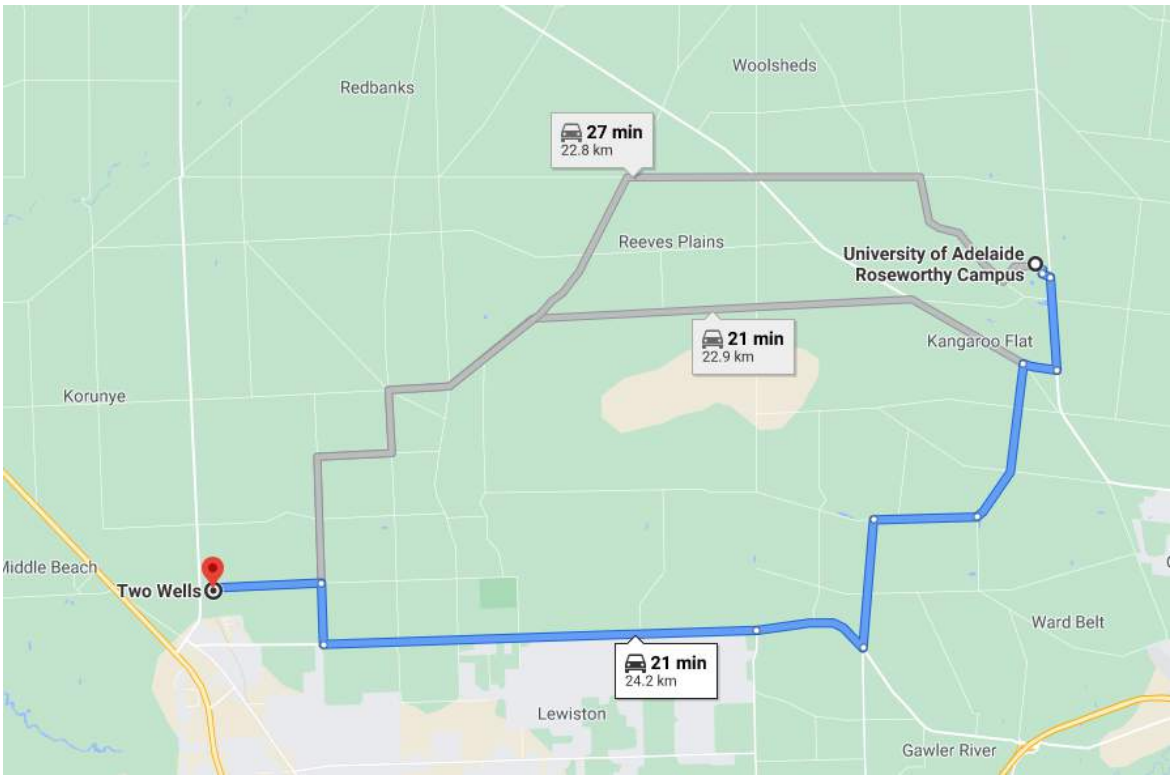


Figure 1 Google Maps showing locations of rearing (A) and production (B) farm of cage flock.

A



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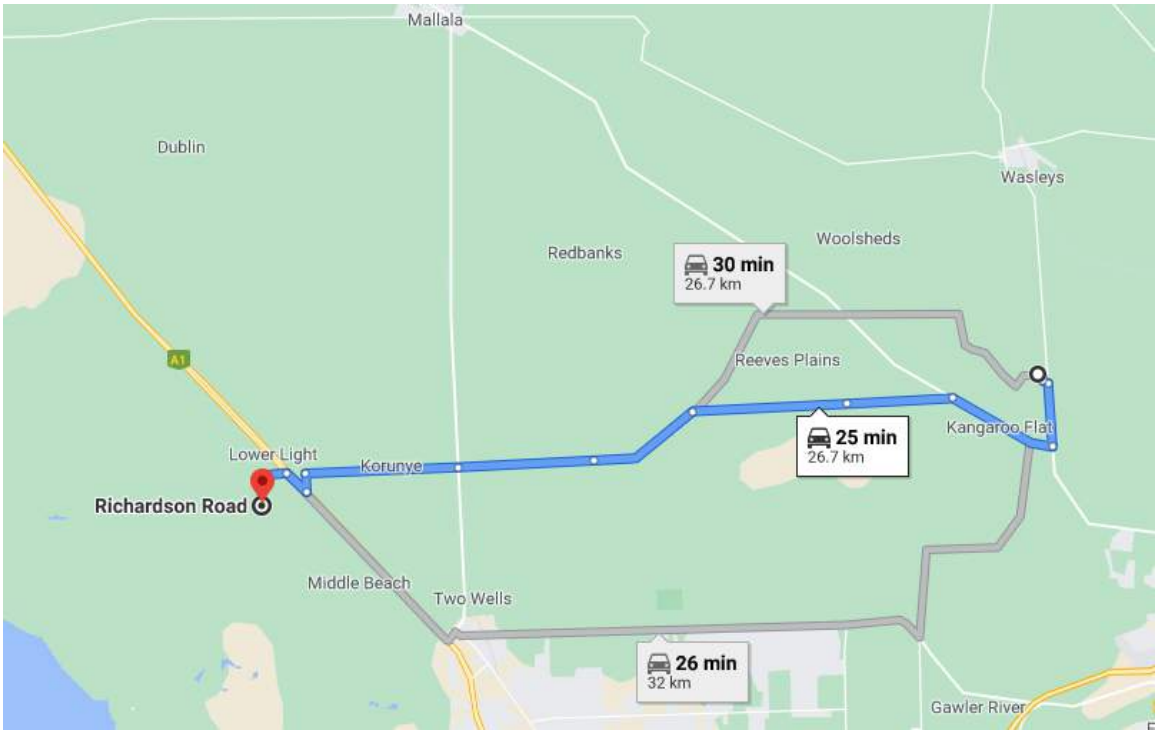
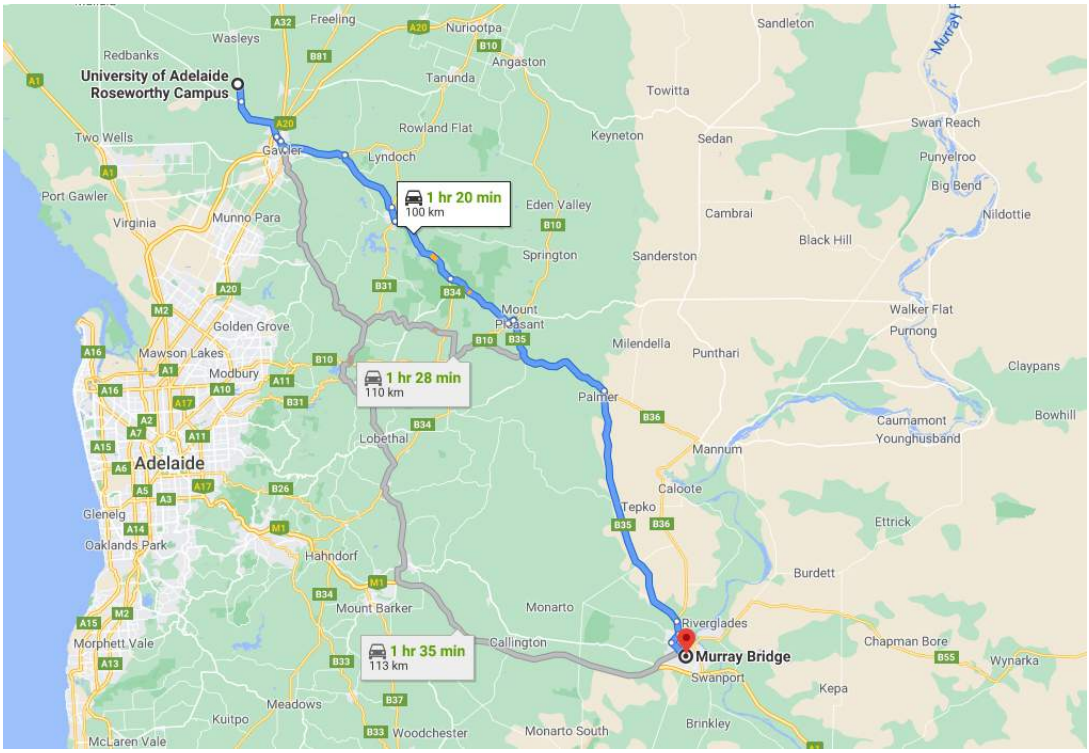


Figure 2 Google Maps showing locations of rearing (A) and production (B) farms of flock A.

A



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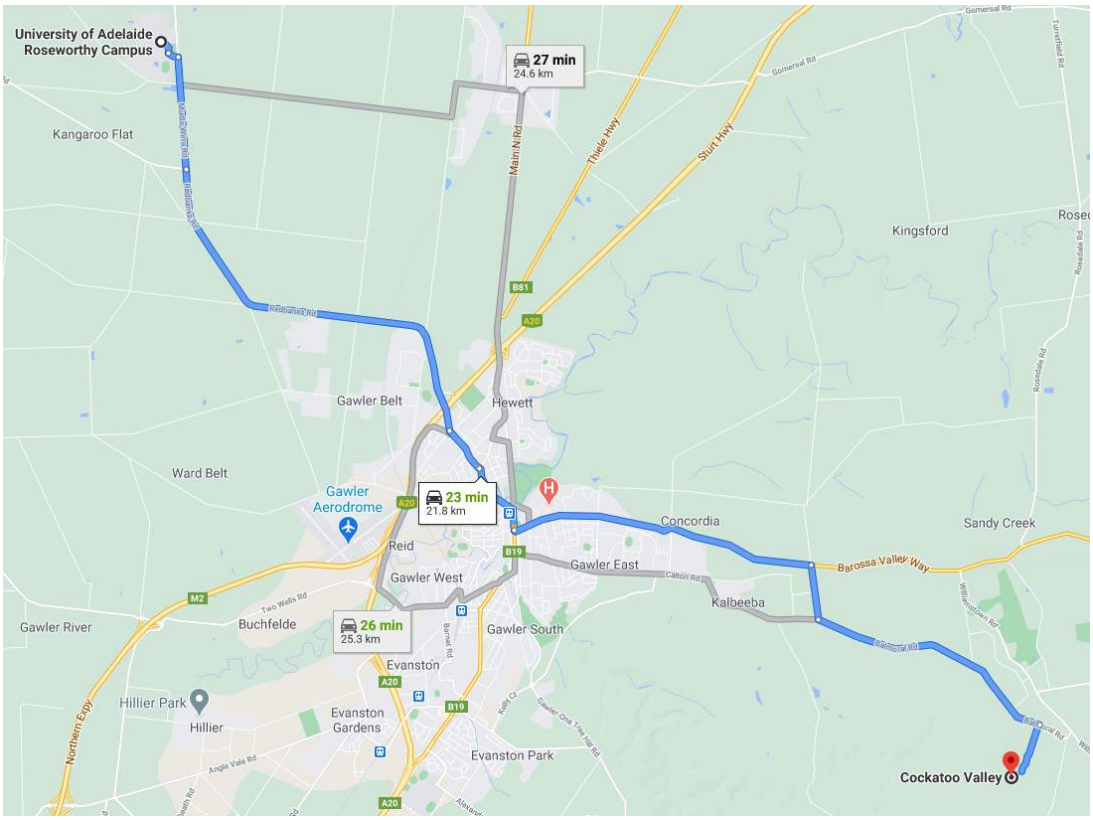

Figure 3 Google Maps showing locations of rearing (A) and production (B) farms of flock B.

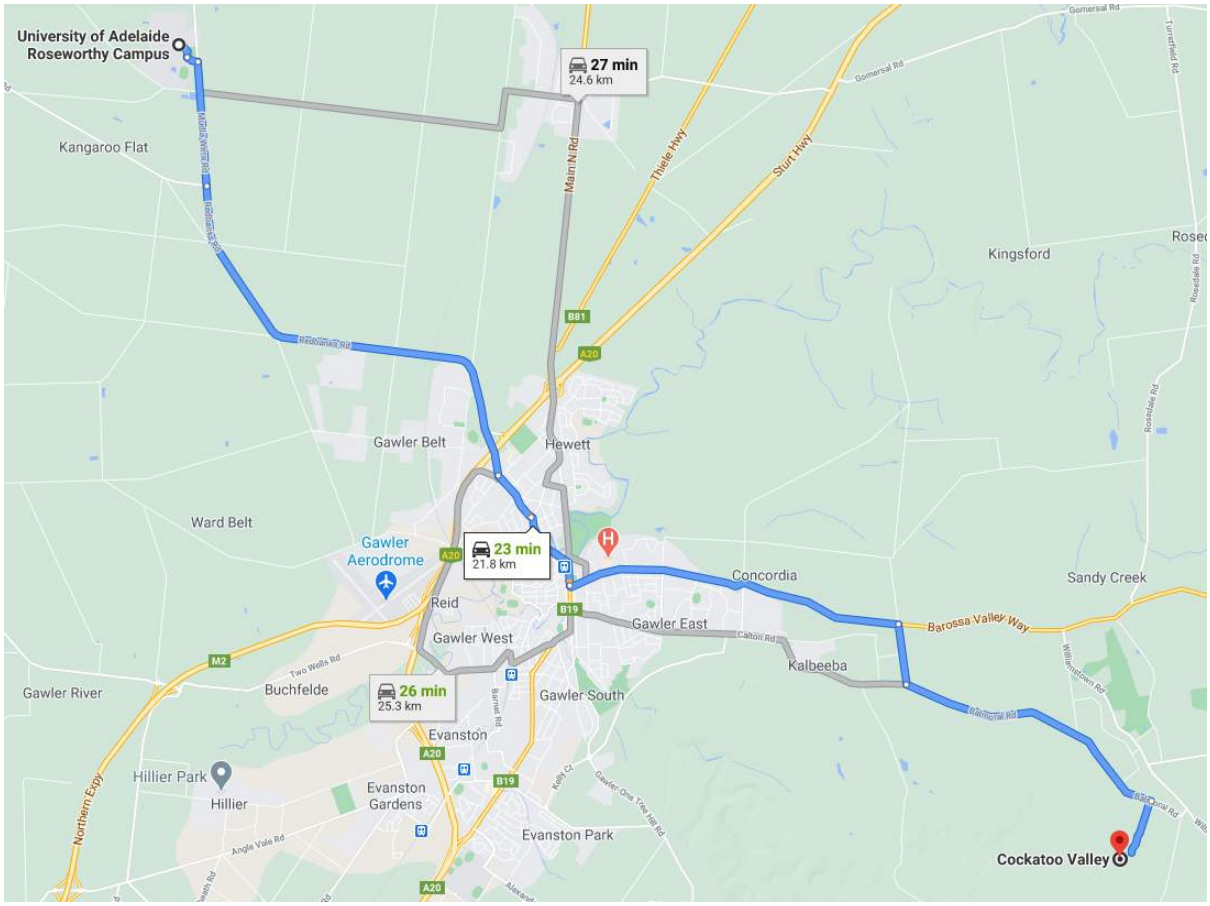

Figure 4 Google Maps showing locations of rearing and production farm of flock C.