Antibiotic Resistance Genes of Class 1 Integrons in Chicken Microbiomes Modulated by Prebiotics

BY

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Abstract

Class 1 integrons are genetic elements of bacteria that contribute to the abundance of antibiotic resistance. Poultry products are thought to be one of the most important reservoirs for transmission of antimicrobial resistance bacteria (AMR) via foodborne zoonotic pathogens reducing the effectiveness of antimicrobial treatments. Prebiotics have attracted attention as an aid to reduce pathogen loads and support the intestinal health of poultry. However, whether prebiotics directly reduce the expansion of bacterial populations carrying antibiotic resistance genes (ARG) or reduce their transmission from livestock, is not known. This thesis investigates the potential role of prebiotic galactooligosaccharides (GOS) in the mitigation of ARG prevalence in the microbiomes of broiler chickens reared under biosecure or commercial conditions.

This study identified and estimated the prevalence of three conserved genes present in class 1 integrons (*intI1*, *sul1* and *qacEΔ1*) from broiler chicken caecal contents reared in biosecure and commercial condition. The prevalence of gene cassettes (GCs) featuring ARGs were examined from birds fed either standard control or an isocaloric diet supplemented with GOS. Six GC types were identified by sequencing long-range PCR products: GCs-A (*aadA2*, *linF*), GCs-B-1 (*dfrA1*, *ORF1*, *aadA24*), GCs-B-2 *(dfrA1*, *aadA1*), GCs-C-1 (*aadA1*), GCs-C-2 (*aadA1*), and GCs-F (*aadA9*). The predominant GC in biosecure birds was GC-B2, whilst GC-A was more prevalent in commercial birds. Phenotypic antimicrobial resistance of the caecal microbiota was estimated by evaluation of trimethoprim coliform resistant populations and parallel determination of integrase gene copy number for birds fed either control or GOS diets. Alpha and beta diversities of the caecal bacterial communities were also determined using a 16S rRNA sequencing approach. Differences in the caecal communities were calculated using AMOVA and

differentially abundant Operational Taxonomic Units (OTUs) identified by LEfSe (Linear discriminant analysis effect size).

Feeding prebiotic GOS to 22 days of age mitigated the expansion of antibiotics resistance populations observed in the control microbiome composition for broiler chickens reared in a biosecure environment. Colonization by zoonotic *Salmonella* Enteritidis demonstrated that both *Salmonella* and GOS feed influence the structure of the gut microbiome. GOS treatment altered the proportions of specific OTUs in infected bird compared to non-infected. These important changes resulted in a faster clearance of *Salmonella* infection in GOS-fed birds compared to control fed birds, which was associated with a significant increase on Negativicutes at the expense of Clostridiales. It is hypothesized that this change restricts the abundance of Proteobacteria carrying antimicrobial resistance due to the depletion of oxygen. Thus, GOS feed modulates the broiler microbiome, which can have a positive impact on the safety of poultry products by reducing the incidence of foodborne pathogens, mitigating the antibiotic resistance load, and improving overall public and animal health.

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AGs	Aminoglycosides
AMOVA	Analysis of Molecular Variance
AMR	Antimicrobial Resistance
AR	Antibiotic Resistance
ARGs	Antibiotic Resistance Genes
Bio	Biosecure Birds
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
ºC	Degrees Celsius
CFU	Colony Forming Unit
Com	Commercial Birds
Ct	The Cycle of Threshold
Ctl	Control Fed Birds
da	Day
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthetase
DMSO	Dimethyl Sulfoxide
dfrA1	Dihydrofolate Reductase
ESBLs	Extended-Spectrum Beta-Lactamases
EU	European Union
ExPEC	The Multidrug Resistant Extra Intestinal Pathogenic *E. coli*
FUTIs	Foodborne Urinary Tract Infections
g	Gram
GCs	Gene Cassettes
GI	Gastrointestinal
GOS	Galactooligosaccharides
GP	Growth Promotors

HGT	Horizontal Gene Transfer
IRQ	Interquartile Range
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
log	Logarithm
mg	Milligram
MIs	Mobile Integrons
ml	Mililiters
MRD	Maximum Recovery Diluent
MGEs	Mobile Genetic Elements
Nonc	Non-Colonised
µg	Microgram
µl	Microliter
OD600	Optical Density at a Wavelength of 600 Nanometers
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
QACs	Quaternary Ammonium Compounds
RO water	Reverse Osmosis
rRNA	Ribosomal Ribonucleic Acid
Sal	*Salmonella* Colonised Birds
SCFA	Short-chain Fatty Acid
*S*E	*Salmonella* Enteritidis
SIs	Super Integrons
TEs	Transposable Elements
WGS	Whole Genome Sequencing
% v/v	Percentage Volume per Volume
% w/v	Percentage Weight per Volume

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The high plasticity of bacterial genomes that enables bacteria to adapt to most ecosystems, has meant that bacterial evolution has been widely investigated. This feature that allows bacteria to re-arrange and exchange genomic sequences to acquire new characteristics, has been widely demonstrated with antibiotic resistant bacteria (Stalder et al., 2012). Human impact extends to the change of microbial distribution and abundance of species and dynamics of microbial genome evolution. One of the most important groups that have flourished in the modern world, are antimicrobial resistant bacteria. Thus, to control this process, understanding the dissemination and evolutionary selection is important in order to identify the reasons for the success this phenomenon. Currently, the growing rate of antibiotic resistant bacteria is a major public health issue (Davies and Davies, 2010). In addition, many studies have highlighted the source of resistance genes of clinical interest, as an environmental resistome (D'Costa et al., 2006;Aminov and Mackie, 2007;Martínez, 2008;Wright, 2010). While the occurrence of mutations involved in bacterial adaptation is partially responsible, horizontal gene transfer seems to be the more frequent cause of the rapid dissemination of antibiotic resistance genes (ARGs) across a wide range of bacteria. Horizontal gene transfer is prevalent in natural ecosystems (Aminov, 2011). One mechanism of horizontal gene transfer is through mobile genetic elements (MGEs) which are diverse and prevalent in bacteria (Wozniak and Waldor, 2010;Bertels and Rainey, 2011). During horizontal gene transfer, they act as functional platforms which can be acquired or lost, contributing a significant part to the processes of rapid bacterial adaptation and resistance development (Stalder et al., 2012).

There are various genetic elements that are involved in the adaptation of bacteria. The most significant contributor in dissemination of antibiotic resistance is the class 1 integron. The way in which they have spread shows similarities to the way in which virulent species invade new environments. Many have been isolated from clinically important human pathogens. More than 70 clinically relevant bacterial species have been invaded by class 1 integrons including common human gut resident bacteria and those from domesticated animals. They possess an invasive nature that have facilitated their dispersal to every continent of the world and every environment. Therefore, they are considered now as significant environmental pollutants (Gillings, 2017).

Poultry production for both meat and eggs has been increasing speedily worldwide (Scanes, 2007). It is thought to be one of the most important reservoirs for transmission of antimicrobial resistance bacteria (AMR) via foodborne zoonotic pathogens reducing effectiveness of antimicrobial treatments (Kaakoush et al., 2014;Card et al., 2017). Dietary interventions have been described as promising approach for modulating chicken microbiome (Ranjitkar et al., 2016). Galactooligosaccharide (GOS) prebiotic has attracted attention in poultry production for its role in reducing pathogen loads and supporting body health (Pourabedin and Zhao, 2015). However, whether they directly reduce expansion of bacterial population carried antibiotic resistance genes (ARGs) or reduce their transmission from livestock foodborne pathogens into the food supply is not known. This thesis describes the investigation of the potential role of GOS in the mitigation of the prevalence of ARGs in the broiler chicken microbiome, via a 16S rRNA sequencing approach in order to track changes in abundance of *Enterobacteriaceae*. In addition to reducing AMR, understanding the role of GOS on the microbiome may have a positive impact on the safety of poultry products by reducing the incidence of foodborne pathogens thus improving overall public and animal health.

1.2 literature review

1.2.1 Antibiotics

Antibiotics remain the most effective treatment to deal with infectious diseases caused by bacteria and are considered the cornerstones of modern medicine (Surette and Wright, 2017). They are biologically active molecules with diversife structures and different origins. Antibiotics are natural products, semi-synthetic derivatives, or chemically synthetic compounds possessing different modes of action (Molinari, 2014). They are used to treat both humans and animals for several purposes such as disease treatment, disease prevention, and (animals) as growth promotion in livestock animals (Rolain, 2013). Today, most of the antibiotics used are generated from the phylum Actinobacteria with nearly 80% of actinobacterial antibiotics derivates produced by soil resident bacteria of the genus *Streptomyces* (Barka et al., 2016).

Antibiotics are classified, based on their structure and degree of affinity to target sites, into Penicillin's, Cephalosporins, Tetracyclines, Aminoglycosides, Macrolides, Sulfonamides, Quinolones, Diaminopyrimidines, Polymyxin and Carbapenems (Sengupta et al., 2013;Bi et al., 2015;Liu et al., 2016). The majority of antibiotics have a specific effect toward different bacterial species that impact essential microbial functions either by: (i) inhibiting cell wall synthesis (β-lactams), (ii) by affecting protein synthesis machinery by interacting with ribosomal subunits (Tetracycline, Chloromphenicol, Aminoglycosides), (iii) targeting nucleic acid machinery (Rifampcin, Fluoroquinolones), (iv) interfering with metabolic pathways (Folic acid analogues, sulfonamides), and (v) by damaging bacterial membrane structure such as Polymyxins (Sultan et al., 2018).

The widespread distribution of multi-drug resistant pathogens to the major classes of antibiotics have become increasingly prevalent worldwide (Stalder et al., 2019). Drug resistance flourishes because of the injudicious use of

antibacterials in human medicine, extensive veterinary usage of antibacterials in the livestock food industry as growth promoters, and the presence of resistant microbes in the environment and food (Molinari, 2014). Selection pressure due to the overuse of antibiotics in clinical and agricultural settings is thought to have promoted the evolution and prevalence of antibiotics resistance genes (ARGs) that confer resistance, regardless of their origins (Allen et al., 2010).

The emergence of bacterial resistance mechanisms such as; mutations in topoisomerase including all fluoroquinolones (Jacoby, 2005), metallo- and beta-lactamases (comprising all β-lactams) (Bush, 2010), 16S rRNA methylases (including nearly all aminoglycosides) (Zhou et al., 2010) and up-regulation of resistance nodulation and division (RND) efflux pumps compromising multiple drug classes (Nikaido and Takatsuka, 2009), has left few to no antibiotics active against multi-resistant bacteria. A serious concern is the recent worldwide rise of resistant bacterial pathogens like *Klebsiella pneumoniae* expressing carbapenemase-2(KPC-2), New Delhi metallo-β-lactamase-1 (NDM-1), and plasmid-mediated colistin resistance MCR-1 (Xiong et al., 2018b). This rise in antimicrobial resistance is much faster than the development of and approval of new antibiotics, creating an urgent need for new antibiotics (Molinari, 2014).

1.2.2 Antibiotic resistance

The increasing levels of AMR is threatening the health progress achieved by antibiotics and is recognized as a global crisis (Ventola, 2015). During the period of 2011–2014 in Europe, a significant increase has been observed in the percentage of both *Klebsiella pneumoniae* and *Escherichia coli* infections that were found to be resistant to fluoroquinolones, third-generation cephalosporins or aminoglycosides, as well as multiple resistance to all three antibiotic groups (ECDC, 2015). Currently, AMR is estimated to be responsible for 50 000 deaths annually across the US and Europe (O'Neill, 2014), and by 2050, the estimated

annual deaths number of AMR bacteria could reach 10 million, surpassing the deaths associated with cancer (Tagliabue and Rappuoli, 2018).

Antibiotic resistance (AR) is a natural phenomenon developed by bacteria that are protecting themselves from antibiotic-producing bacteria in order to increase their survival chances in highly competitive environments (D'Costa et al., 2011). The organisms that produce antibiotics have self-resistance mechanisms against their own antibiotics. Additionally, co-existence of antibiotic producing and non-producing bacteria is also thought to have led to co-evolution of resistance mechanisms in non-producer environmental bacteria (Kaur and Peterson, 2018) and both groups are thought to have led to the emergence of resistance in pathogenic clinical isolates (Surette and Wright, 2017;Kaur and Peterson, 2018). An enormous diversity of resistance mechanisms have been identified in both antibiotic-and non-antibiotic producing bacteria to practically all antibiotic families (D'Costa et al., 2006;Bhullar et al., 2012).

Bacteria that have encoded antibiotic resistance determinants obviously have a selective advantage over those antibiotic-sensitive bacteria in presence of antibiotics (Sengupta et al., 2013). However, some of the multidrug resistance genes confer resistance to a number of structurally unrelated compounds such as, quaternary ammonium compounds, ethidium bromide, the DNA-intercalating mutagen acridine, the anionic detergent sodium dodecyl sulfate and uncouplers such as carbonyl cyanide m-chlorophenyl hydrazone. Hence, it seems that ARGs have a greater role in bacterial physiology than just antibiotic resistance. Furthermore, they may also confer resistance to some chemical substances generated by the host, like bile acids or may being a part of some unknown physiological roles (Martínez and Rojo, 2011;Sengupta et al., 2013).

Resistance to antibiotics can be categorised into three main groups: intrinsic, adaptive, and acquired resistance (Fernández et al., 2011;Blair et al., 2015). The intrinsic resistance is ancient in origin and has complex mechanisms that are have been adapted through evolution (Rolain, 2013). Additionally, these antibiotics

naturally produced by organisms can act as signalling molecules in nature and homeostasis of bacterial communities (Martínez and Rojo, 2011;Baquero et al., 2013). Thereby, production of antibiotics by naturally resistant organisms maintains an ecological balance in the environment (Cordero et al., 2012). For instance, the natural low permeability of the bacterial cell wall of Gram-negative bacteria is an example of the intrinsic antibiotic resistance, which limits the uptake of many antimicrobials including aminoglycosides (Garneau-Tsodikova and Labby, 2016).

Adaptive antibiotic resistance takes place due to an environmental trigger (for example, changes in nutrient concentration or sub-inhibitory levels of antibiotics) that leads to temporary genetic changes and/or changes in protein expression levels involving in the antibiotic tolerance (Garneau-Tsodikova and Labby, 2016). Bacterial growth on surfaces as biofilms creates an adaptive resistance (often referred to as tolerance) to antibiotics (de la Fuente-Núñez and Hancock, 2015). Finally, acquired antibiotic resistance may occur by the integration of exogenous genetic element such as a plasmid, conferring multiple resistance genes, or by mutation of existing genes. Both intrinsic and acquired resistances are carried by genetic elements that are passed vertically during bacterial reproduction, while adaptive resistance is transient, which typically means reverting upon when the environmental trigger has been removed. Additionally, resistance genes carried on plasmids may be transmitted horizontally from one bacterium to another which is the main mechanism of the dissemination of antibiotic resistance genes among various bacterial species (Garneau-Tsodikova and Labby, 2016).

Recently, genomic and metagenomic researche in humans, animals, food and in the environment have revealed that there is a huge reservoir of ARGs named the "intrinsic resistome" which represents a large subset of non-acquired ARGs that have multiple and complex functions in nature (Rolain et al., 2012;Baquero et al., 2013).

1.2.3 The mobile resistome

The continuous appearance of resistance genes present in the environmental, antibiotic producing, and pathogenic bacteria, led to the concept of the 'resistome' which is defined by Wright (2007) as "the collection of all the antibiotic resistance genes, including those usually associated with pathogenic bacteria isolated in the clinics, non-pathogenic antibiotic producing bacteria and all other resistance genes". Mobilisation of resistance genes can be driven between distantly related bacteria by their association with mobile genetic elements (MGEs). These MGEs that seem vary in their appearance or absence within a bacterial population, hence they are not encoding essential genes for cell function. Figure 1.1 shows how resistance genes can be acquired from any source (Wellington et al., 2013).

Mobilisation of MGEs are associating with the stress response, which means their mobility can be activated by exposure to antibiotics and environmental pollutants (Wellington et al., 2013). When bacteria are exposed to sublethal doses of antibiotics, whether in a clinical setting due to treatment of an infection being not completed (patient non-compliance), or when there is limitation in the drug's accessibility to certain tissues such as bone or cerebrospinal fluid (Bryskier, 2005a). Outside the clinical setting, bacteria may be exposed to sublethal doses of antibiotics when manure of livestock fed diet supplemented with antibiotics are then released into soil and aqueous environment. All these mechanisms of exposure, have highly influenced the dissemination of multidrug resistance (Sengupta et al., 2013). Additionally, low antibiotic doses are likely to be associated with enhancing horizontal gene transfer (HGT) by MGEs that mediate the dissemination of antibiotic resistance genes such as sub-minimum inhibitory concentrations of tetracycline (Celli and Trieu-Cuot, 1998;Sengupta et al., 2013).

Finally, transmission of genetic material between bacterial populations is also potentially triggered by stressors like metals and biocides (Seier-Petersen et al., 2014;Zhang et al., 2017;Bengtsson-Palme et al., 2018).

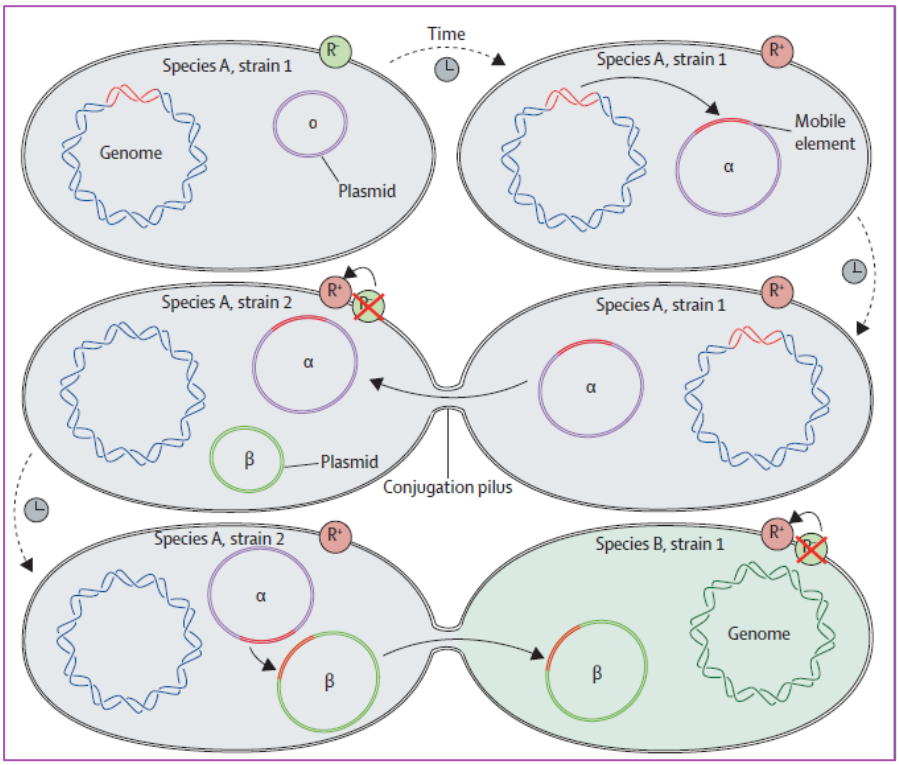


Figure 1.1 The mobilome of ARGs between closely and distantly related bacteria of mobile genetic elements shared resistance gene pool. The ARGs (red) evolving on the chromosome and moving by transposition to the plasmid. Narrow host range plasmids (α) permitting spread between strains whereas broad host range plasmids (β) allowing transfer to distantly related bacteria. R−=sensitive phenotype. R+=resistant phenotype (Wellington et al., 2013).

Many antibiotic resistance genes have been shown, by having a perfect nucleotide sequence homology, to have been transferred between environmental bacteria and clinical pathogens including those that confer resistance to β-lactams, aminoglycosides, tetracyclines, sulfonamides, and phenicoles (Forsberg et al., 2012). These genes orthologous to ARGs have been recognised on MGEs in resistant pathogenic bacteria which make up the environmental resistome; a potential reservoir for transferring ARGs to pathogens (Wright, 2010;Sengupta et al., 2013).

However, some phylogenetic studies have suggested that current clinical resistance genes cannot be determined in antibiotic producers, and their emergence in clinical pathogens are not explained by recent horizontal gene transfer from these organisms (Baquero et al., 2013). It is suggested that they may have undergone gene duplications and frequent horizontal gene transfer that predate the industrial release of antibiotics (Baquero et al., 2013). Anthropogenic action (the amount of released antibiotics into microbial environments by human action), has certainly increased the amount of antimicrobials that enter the environment and interact with bacteria (Baquero et al., 2013), but its involvement in the dissemination of antibiotic resistance remains controversial (Bhullar et al., 2012). Evidence of the occurrence of antibiotic resistance in environments that are apparently antibiotic-free suggests the situation is more complex (Sengupta et al., 2013). For example, bacteria from a region of the Lechuguilla Cave, New Mexico, that has been isolated for over 4 million years, were resistant to 14 different commercially available antibiotics (Bhullar et al., 2012).

Phylogenetically related gut microbiota are known to frequently transfer resistance genes between commensals and pathogens. Surprisingly, some studies indicated that fairly large numbers of resistance genes harboured in human microbiome have not (yet) been transmitted to human pathogens (Sommer et al., 2009;Sommer et al., 2010;Bengtsson-Palme et al., 2018). The main causes for this are unknown, however, it seems there are strong barriers preventing transmission. One of these barriers could be the oxygen requirements. For instance, many uncultured, anaerobic microorganisms in the human gut microbiota have a higher proportion of undiscovered genes that give resistance to aminoglycosides, amphenicols, beta-lactams and tetracyclines, that have not been transmitted to human pathogen due to difference in oxygen needs (Sommer et al., 2009). Resistance genes found in the cultivable human gut bacteria, have homologues to resistance genes in aerobic bacterial human pathogens (Bengtsson-Palme et al., 2018).

1.2.4 The use of antibiotics in poultry

Poultry production is one of the most important worldwide food industries, with over 90 million tons of chicken meat produced per year (FAO, 2017;Nhung et al., 2017). Several different antimicrobials are still used to raise poultry in most countries (Agunos et al., 2012;Landoni and Albarellos, 2015), generally by the oral administration, to prevent and treat disease, as well as to enhance growth and productivity (Page and Gautier, 2012;Nhung et al., 2017). The use of antibiotics in growth promotion (GP) has been banned by the European Union (EU) in 1999 (Casewell et al., 2003). Numerous studies have linked the ban of antibiotic usage in food animal production with a decrease of AR prevalence (Marshall and Levy, 2011). However, the use of antibiotics as growth promoters and therapeutics in animals is not monitored in some countries in the world such as China and United States, despite a high occurrence of AR bacteria (Zhu et al., 2013). The large number of antibiotics used in food animal production have been used also in the treating of human bacterial infections. Thereby, the usage of one specific antibiotic in animal farms can accelerate the development of AMR in both pathogens and commensal organisms (Nhung et al., 2017). Furthermore, it also causes cross-resistance with antibiotics used in medicine and possibly that could select for multiple ARs to functionally unrelated antibiotics because ARGs could be associated with transferable plasmids and transposons (Marshall and Levy, 2011). Additionally, residues of antibiotics used in poultry production are also of concern to human health (Nhung et al., 2017).

1.2.5 Food animals as source of antibiotic resistance in human pathogen

The interplay between food animals, the environment, pathogenic bacteria and humans have been involved in shaping the evolution of infectious diseases. Livestock play a crucial role in the dissemination and development of antibiotic

resistance in pathogenic bacteria (Surette and Wright, 2017). The relationships between antibiotic usage in animals and the emerging of AR bacteria in animal and human infections was studied during the previous decades (Marshall and Levy, 2011). Administration of antimicrobial agents in food-animals as prophylaxis (growth promoters) or as treatment has been recognised to act as selector for AR bacteria that might be transmitted to clinical human pathogens since the 1970s. Utilisation of oxytetracyclines as growth promoters in chickens showed an increased selection of tetracycline-resistant *Escherichia coli* colonisation in the poultry and in the gut of the farm family (Levy et al., 1976a;Levy et al., 1976b). Another example of bacterial cross-resistance through antibiotic usage in animal feed production and those used in humans is vancomycin-resistant enterococci (VRE) which has emerged as a result of using avoparcin with dramatic increases in this resistance amongst human pathogens (Hammerum et al., 2010). The use of avoparcin as a growth promoter has been banned in the EU since 1997, which subsequently caused a decrease in the carriage of glycopeptide-resistance in *Enterococcus faecium* (Kazimierczak and Scott, 2007). However, although resistance has generally decreased, persistence was observed in some cases: for example, in Denmark in pigs administered tylosin (a macrolide antibiotic). As a consequence, the *ermB* and *vanA* genes were found to be encoded on the same mobile genetic elements (Aarestrup, 2000). Thus AR bacteria are selected in chickens, pigs, and cattle or food animals and are likely to be transmitted to the human intestinal microbiota via the food chain (Salyers et al., 2004). Moreover, along with the detection of numerous AR genes, Aziz et al. (2010) found most of these genes were carried on transposons with some of them acquiring by mobility of ARGs, posing an increased risk of LGT of ARGs from livestock animals to human pathogens (Zhu et al., 2013).

Recently, foodborne urinary tract infections (FUTIs) has been considered as a new source for antimicrobial-resistant foodborne illness (Nordstrom et al., 2013). For instance, the multidrug resistant extra-intestinal pathogenic *E. coli*

(ExPEC) causes community-acquired urinary tract infections (Vincent et al., 2010). These include a trimethoprim–sulfamethoxazole-resistant *E. coli* UTI outbreak in women from the United States (Manges et al., 2001), as well as the community-outbreak of clonally related extended-spectrum beta-lactamases (ESBLs) type CTX-M infections worldwide (Pitout et al., 2005;Pitout and Laupland, 2008). These outbreaks have been linked to contaminated meat and other foods as a reservoir of the strains causing UTI; suggesting that the use of antimicrobials in food animal production can select for AR strains of ExPEC. Furthermore, a widespread contamination by AMR ExPEC in retail foods has been detected, specifically in turkey products obtained from grocery stores in retail markets from the United States (Johnson et al., 2005a).

There are several examples of foods that act as source of AR bacteria and could be transfered to humans via the food chain. Johnson et al. (2005b) have reported that retail foods may be a proxy for community transmission of AR ExPEC, which are recently considered as clinically significant foodborne pathogens. This has also been found in Canada in retail chicken, meat, and pork (Manges et al., 2007;Vincent et al., 2010). Antibiotic resistance in *E. coli* isolated from chicken have also been founded in Spain, Barcelona, Minnesota, Wisconsin and the United States, suggesting that foodborne AR ExPEC transmission is a very predominant phenomenon acting as a reservoir of ARGs transmissible to the human microbiome via the food chain (Johnson et al., 2006;Johnson et al., 2007).

1.2.6 Resistance mechanisms

The mechanisms of action and resistance of the major antimicrobial agents' categories are described below.

1.2.6.1 Aminoglycosides (AGs)

Since streptomycin was first isolated from *Streptomyces griseus* and introduced for clinical use in 1944, this class of antibiotics has become a

cornerstone of antibacterial chemotherapy. Aminoglycosides are broad-spectrum agents targeting protein synthesis. They exhibit activity against various Gram-positive and Gram-negative organisms (Krause et al., 2016). They are particularly potent against members of the *Enterobacteriaceae* family which include *E. coli*, *K. pneumoniae* and *K. oxytoca*, *Enterobacter cloacae* and *E. aerogenes*, *Providencia* spp., *Proteus* spp., *Morganella* spp., and *Serratia* spp. (Ristuccia and Cunha, 1985;Aggen et al., 2010;Landman et al., 2010).

The mechanism of action of AGs involves inhibition of protein synthesis by high affinity binding to the A-site on the 16S ribosomal RNA of the 30s ribosome (Kotra et al., 2000). Different classes of AGs have different specificities for different regions on the A-site, most of them altering its conformation. As a consequence of this interaction, the antibiotic induces mistranslation by promoting codon misreading on delivery of the aminoacyl transfer RNA. As a result of this error prone protein synthesis allowing the wrong amino acids to assemble into a polypeptide, the cell membrane becomes damaged (Davis et al., 1986;Mingeot-Leclercq et al., 1999;Ramirez and Tolmasky, 2010;Wilson, 2014). Some AGs can also disrupt protein synthesis by blocking elongation or by direct inhibition of initiation (Davis, 1987;Kotra et al., 2000;Wilson, 2014).

Mechanisms of aminoglycoside resistance include enzymatic modification, target site modification via an enzyme or chromosomal mutation, and efflux pumps. Each mechanism has different effects on the various AG classes and often several mechanisms are involved in the resistant phenotype (Krause et al., 2016). The most common mechanism of AG resistance is inactivation by a family of enzymes named aminoglycoside modifying enzymes (AMEs). These enzymes are classified into three types according to the kind of modification involved. They are the acetyltransferases (AAC), adenyl transferases or the nucleotidyl transferases (ANT), and the phosphotransferases (APH;(Kotra et al., 2000;Ramirez and Tolmasky, 2010)). The ANT group of enzymes, products of the *aadA* gene,

encoded by integrons and are commonly found in streptomycin resistant Gram-negative bacteria (Sultan et al., 2018). Resistance to AGs may also occur by decreasing antibiotic uptake through outer membrane proteins (intrinsic barrier). This is achieved by a decrease in membrane permeability by acquiring lipid modifications which causes repulsion of AGs. Furthermore, even if some AG molecules enter the bacterial cell, their intercellular concentrations may still be low because of the active ejection of AGs out of the cell by efflux pumps (Fernández and Hancock, 2012;Garneau-Tsodikova and Labby, 2016).

Resistance to AGs can be gained by mutations of the ribosomal target of aminoglycoside antibiotics. This may occur by modification of the ribosome by a family of ribosomal methyltransferase enzymes (Wilson, 2014). Most pathogenic bacteria develop resistance to AMEs via horizontal gene transfer (HGT). This is due to the high mobility of AMEs because their genes are transferred on mobile genetic elements like plasmids, integrons, transposons, and other integrative genetic elements. Genes involved in resistance to AGs are often associated with other resistance genes such as β-lactamases, "*bla*" genes (Garneau-Tsodikova and Labby, 2016).

1.2.6.2 Lincosamides

Lincomycin was the first lincosamide isolated from soil in 1962 in Lincoln, Nebraska. It was produced by *Streptomyces lincolnensis* ssp. *Lincolnensis* (MacLeod et al., 1964;Bryskier, 2005b;Schwarz et al., 2016). Lincosamides have a wide antimicrobial spectrum, against Gram-positive bacteria, most anaerobes, but not Gram-negative aerobes, and some mycoplasmas and protozoa (Greenwood, 2010). Lincosamides may act as bacteriostatic agents, which slow or inhibit the growth of bacteria but do not kill them, or as bactericidal antibiotics, which actively kill bacteria, based on drug concentration, bacterial species, and pathogen concentration (Das and Patra, 2017). The mechanism of lincosamides action is by protein synthesis inhibition in sensitive bacteria. This is achieved by

blocking the activation of amino acid monomers by aminoacyl-tRNA synthesis, elongation, and chain termination of the grown polypeptides on the ribosome. Therefore, this class of antibiotics cause interruption to the timing and specificity of these steps, leading to either deceleration of growth or death of the microorganism (Spížek and Řezanka, 2017). Clindamycin is the main prescribed lincosamide antibiotic in clinical practice (Spížek and Řezanka, 2017).

The main route of resistance to lincosamides is achieved by modifying the 23S rRNA in the 50S ribosomal subunit, which is the same resistance mechanism to macrolides and streptogramin B. Resistance to lincosamides can occur through three different mechansims: (1) mutation in the antibiotic ribosomal target that prevents its binding or by modification of the target-site by methylation, (2) efflux of the antibiotic, and (3) by inactivation of the drug. These mechanisms have been observed in lincosamide producer microorganism to protect themselves against the antimicrobial products that they produce. However, in pathogenic microorganisms, the effectiveness of these three mechanisms is not equal in terms of incidence and clinical implications. The broad-spectrum of lincosamides resistance is caused by modification of the ribosomal target whereas efflux and inactivation affect only some of the molecules in this class (Leclercq, 2002). Bacterial outer membrane permeability is an important factor for the intrinsic resistance of *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. (Kwon, 2017). Hence, Gram-negative microorganisms are generally resistant to lincomycin and clindamycin (Spížek and Řezanka, 2017) so the use in human medicine is limited. In veterinary use, lincomycin is approved for therapeutic use of various infections in dogs, cats, and swine, in combination with spectinomycin, but it is not approved for poultry, ruminants, and pigs (Schwarz et al., 2016).

1.2.6.3 Quinolones

Resistance to quinolones has emerged since nalidixic acid was used in clinical medicine (Jacoby, 2005). The mode of quinolone action is by targeting two essential bacterial enzymes. These are DNA gyrase and DNA topoisomerase IV, inhibition of which lead to impairment of DNA synthesis (Jacoby, 2005). Mechanisms of resistance occurred by mutation and acquisition of resistance-conferring genes. Resistance through mutations in one or both enzymes commonly taken place in a specific domain of the GyrA and ParE subunits leading to reduced drug binding to the enzyme-DNA complex (Hooper and Jacoby, 2015). The other mechanism of resistance is mediated by mutations in proteins that encode the regulatory genes controlling the transcription or expression of native efflux pumps or porin genes located in the bacterial membrane (Hooper and Jacoby, 2015). These efflux pumps have a broad substrate range including quinolones, other antimicrobials, disinfectants, and dyes. Mutations of both types can increase with the presence of selection pressure and produce highly resistant strains. Resistance genes that are acquired on plasmids are likely to confer low-level resistance that induces the selection of mutational high-level resistance (Hooper and Jacoby, 2015). The chromosome-encoded resistance causes a decline in outer-membrane permeability which is linked with loss of porins. While over expression of the naturally present efflux pumps causes antimicrobials to be pumped out of cells. Additionally, mutations in the molecular targets of quinolones: DNA gyrase and topoisomerase IV confer resistance to them (Hooper, 2000;Jacoby, 2005;Sultan et al., 2018).

1.2.6.4 Sulfonamides and Trimethoprim

Sulfonamides were first effectively used as antibacterial agents, in the United States, during the 1930s, whereas trimethoprim was introduced to clinical medicine at the end of the 1960s (Sköld, 2010). The spectrum of activity of each agent is bacteriostatic alone however the combination (synergistic effect) between

both of them (sulfamethoxazole) is bactericidal (Sykes and Papich, 2013). Both antibiotics affect bacterial folic acid synthesis. Sulfonamides target the inhibition of dihydropteroate synthetase (DHPS), which act as a catalyst for forming of dihydrofolate from para-aminobenzoic acid in metabolic pathway of folic acid biosynthesis. In the next step of this pathway, trimethoprim acts as an inhibitor for dihydrofolate reductase (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate. These steps follow each other and result in a sequential blockade. Resistance to both these drugs can be transferable, thus, they are often linked to each other, an example of which are the transposons of the Tn*21* family (Eliopoulos and Huovinen, 2001).

Several mechanisms mediate resistance to sulfonamides and trimethoprim as follows: (1) efflux pumps and or their impermeability to the drugs, (2) intrinsic lack of the enzymes target, (3) spontaneous chromosomal mutations leading to overproduction of the host DHFR caused by promoter mutation, a consequence of this is that a greater concentration of trimethoprim is required for the inhibition (found in *Enterobacteriaceae*), (4) mutations in structural gene of the DHFR (*streptococci, staphylococci*). The last two mechanisms often occur in *Enterobacteriaceae* and *Haemophilus influenzae* causing high-level resistance. The acquisition of exogenous *dfr* genes horizontally, that encode resistant DHFRs, are also involved in resistance (Eliopoulos and Huovinen, 2001;Bergmann et al., 2014;Rossolini et al., 2017).

Enterobacteria that possess a high-level of resistance to trimethoprim is often due to acquisition of a genetic element encoding trimethoprim resistant DHFR with an alteration of the active site. To date, many different trimethoprim resistant DHFRs have been described in Gram-negative organisms that belong to at least two groups encoding the *dfrA* and *dfrB* genes. These genes are commonly carried on mobile gene cassettes embedded in integrons described in *Enterobacteriaceae* (Brolund et al., 2010;Rossolini et al., 2017).

Campylobacter jejuni and *Helicobacter pylori* are naturally resistant to trimethoprim because they lack the chromosomal gene for dihydrofolate reductase and therefore, the target for trimethoprim is absent (Myllykallio et al., 2003). Many pathogenic bacteria such as *Escherichia coli* have been detected that carry chromosomal resistance to trimethoprim that is due to 100-fold overproduction of chromosomal dihydrofolate reductase. This overproduction is caused by several types of mutations, which contribute to an increase in the expression of the enzyme gene via increased promoter efficiency, optimization of ribosome binding and more efficient codon usage (Sköld, 2010).

Resistance to sulfonamides is acquired by a single amino acid chromosomal mutation in the *dhps* gene. This mutation is naturally prevalent among many clinical pathogens (Eliopoulos and Huovinen, 2001). Resistance may lead to an increase of *para*-aminobenzoic acid production as well as to alteration of DHPS which reduces the enzyme affinity for sulphonamides (Rossolini et al., 2017). Resistance is also commonly associated with harbouring plasmids that encode a drug-resistant DHPS (Chen, 2004). There are three types of DHPS resistance, encoded by the *sulI, sulII* and *sulIII* genes. These genes have been identified in Gram-negative enteric bacteria (Rossolini et al., 2017). The *sulI* gene is frequently associated with other resistance genes and is locating in conserved segments of integrons in Tn21-like elements carried by large conjugative plasmids (Eliopoulos and Huovinen, 2001). The *sulII* gene is genetically linked to a streptomycin resistance gene that moves around on broad host-range plasmids and on small non-conjugative plasmids (Chen, 2004). These genes encoding DHPS confer high-levels of resistance (Rossolini et al., 2017).

1.2.7 Resistance mediated by mobile genetic elements (MGEs)

The acquisition of antibiotic resistance in bacteria occurs through two principal routes: chromosomal mutation and the acquisition of MGEs by horizontal

gene transfer (Alekshun and Levy, 2007;San Millan, 2018). However, the acquisition of ARGs frequently takes place via mobility of DNA which can be loosely defined as any segment of DNA that is able to translocate from one part of a genome to another or between genomes (Van Hoek et al., 2011). This includes a wide range of mobile genetic elements that are described below.

1.2.7.1 Resistance mediated by plasmids

A plasmid is a circular DNA molecule replicating independently of the chromosome and can be transmited horizontally between bacteria via conjugation. This DNA segment (plasmid) plays a vital role in both bacterial evolution and distribution of antibiotic resistance genes among the most serious clinical pathogens (Alekshun and Levy, 2007;Carattoli, 2013). Conjugative plasmids are the most important drivers of ARGs dissemination among bacterial population such as *Enterobacteriaceae* and *Enterococcaceae* including some of the most significant nosocomial pathogens (Vincent, 2003;Boucher et al., 2009;Rozwandowicz et al., 2018).

Plasmids which have been detected in almost all bacteria, include an origin of replication as well as genes that encode replication functions as their simplest elements (Chambers et al., 1988). Some other plasmids commonly harbour an origin of transfer and genes encoding functions that allow them to transmit to new hosts via conjugation (Smillie et al., 2010). Thus, plasmids that contain conjugation genes are called conjugative while plasmids that are only composed of an origin of transfer (*oriT*) without conjugation genes are called mobilizable plasmids (Van Hoek et al., 2011). Conjugative plasmids are either broad host range, being not restricted to host within their division, or narrow host range being limited to a small number of related bacterial groups (Sultan et al., 2018). Moreover, some plasmids have the ability to transfer to a specific host, but they are unable to replicate in the new host or cannot replicate well. Consequently, this type of plasmid is likely to be lost, but if this plasmid contains resistance genes on

a transposon, this genetic element can contribute to translocation of this resistance to the bacterial chromosome and be preserved in the absence of the plasmid. Consequently, plasmids do not necessarily need to be maintained in a specific host to be involved in the spread of resistance (Van Hoek et al., 2011).

Additionally, functions in replication and transfer plasmids commonly encode antibiotic resistance. Therefore, resistance genes carried by a conjugative or mobilizable plasmid have the potential to transfer to new hosts (Van Hoek et al., 2011). The association between plasmids and bacterial clones is ubiquitous, with certain AR plasmids that strongly linked to specific bacterial lineages (San Millan, 2018).

1.2.7.2 Resistance mediated by transposons

Transposable elements (TEs) or conjugative transposons, are also called integrative conjugative elements (Roberts et al., 2008). They are similar to conjugative plasmids, in that transposons have an origin of transfer and the required genes for the conjugation apparatus but they do not carry an origin of replication. They need to be incorporated into a replicon that can be either a plasmid or chromosome, in order to be maintained. This gives transposons an advantage over plasmids because they do not need to have replication machinery, therefore, they tend to have a larger host range than plasmids (Van Hoek et al., 2011).

TEs are divided into two classes: composite transposons and complex transposons. Composite transposons have a range of resistance genes possessing identical structural and functional characteristics, but little DNA homology to each other. Complex transposons comprise three dissimilar but interrelated families; Tn*3*, Tn*21* and Tn*2501* (Schmitt, 1986;Wiedemann et al., 1986;Lafond et al., 1989;Sultan et al., 2018). Composite transposons, Tn*5*, Tn*9*, Tn*10*, Tn*903*, Tn*1525*, and Tn*2350* are found in Gram-negative bacteria while Tn*1*, Tn*3*, Tn*21*, Tn*501*, Tn*1721*, and Tn*3926*, found among both Gram-negative and Gram-

positive bacteria, are classed as complex resistance transposons. These components are capable of "jumping" within a DNA molecule or from one DNA molecule to another (Bennett, 2008). The most studied example is Tn*21* which carries OXA (a carbapenems, possessing oxacillinase activity) and PSE (β-lactam gene with *Pseudomonas* specific enzyme) determinants that confer resistant to aminoglycoside antibiotics (Sultan et al., 2018). Tn*21* also encodes resistance to mercury compounds (Brown et al., 1986) and trimethoprim, imparted by *dhfr* II and V (Sundström et al., 1988).

1.2.7.3 Resistance mediated by integrons

1.2.7.3.1 Properties of integrons

Integrons are genetic elements that in conjunction with transmissible plasmids and transposons, can integrate and express various genes including resistance genes increasing antibiotic resistance in bacteria. Screening for their presence may therefore be used as an indicator for the presence of antibiotic resistance genes. They are non-mobile themselves, but they can be carried within mobile genetic elements like transposons and plasmids, facilitating their horizontal transfer into a broad range of pathogens. They are ancient elements that can be recovered from all parts of the environment. More than 15% of bacteria that have been sequenced have integrons in their genomes (Gillings, 2017). They are classified to five different types based on the integrase gene sequence (class I-V). The most studied and prevalent integrons among commensal and pathogenic bacteria are the class I integrons. They possess a high conserved sequence of the *intI1* gene which is classed among mobile integrons in clinical isolates however; it has variability in environmental mobile integrons (Gillings et al., 2008b). They are found widely in clinical isolates that are resistant to commonly used antibiotics (Gillings et al., 2008b;Gillings, 2017). Hence, integrons enable bacteria to face the challenge of antibiotic treatment by aiding rapid adaptation. They can be categorised into two main categories. The first are the resistance integrons or

mobile integrons (MIs) which are located either on the chromosome or on plasmids that carry gene cassettes that encode resistance to antibiotics. The second category are the super-integrons (SIs), which are located on the chromosome and carry gene cassettes with a variety of functions (Fluit and Schmitz, 2004). Figure 1.2 summarised different mechanisms of antibiotics resistance

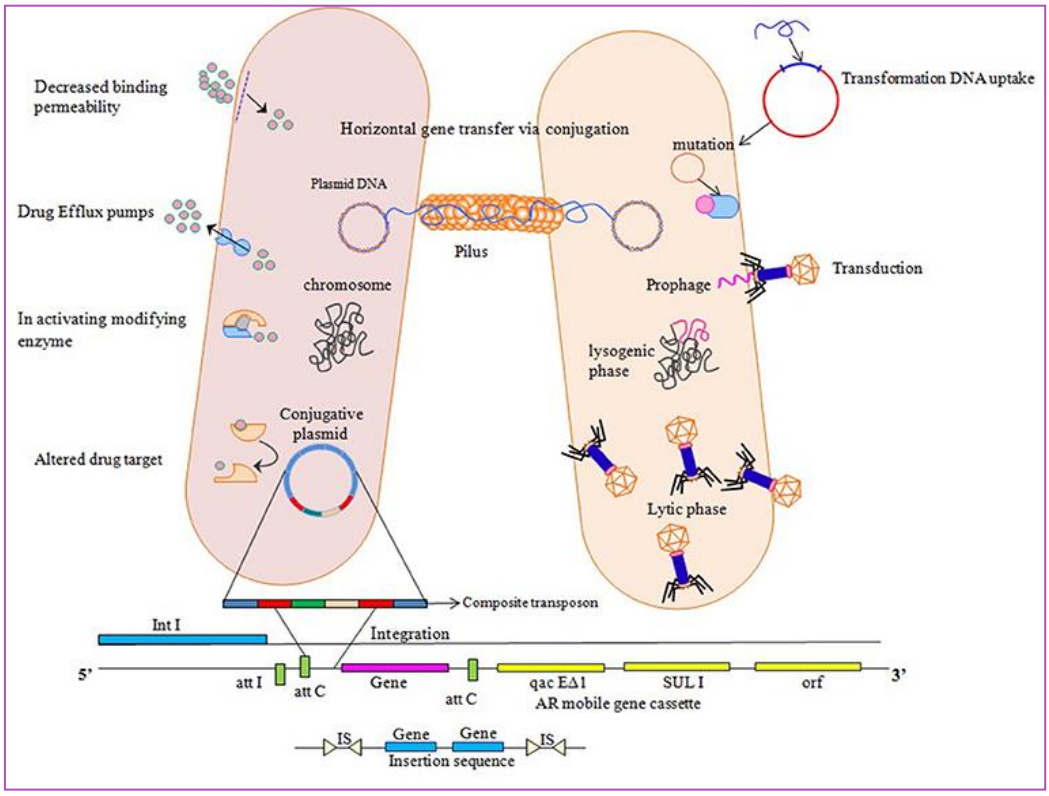


Figure 1.2 Different routes of resistance mechanisms to face effect of antibiotics. Lateral gene transfer is involved in transferring and exchanging of genetic elements among bacterial cells. Transformation participates in direct uptake of DNA segments from the surrounding environment by competent recipient carrying chromosomal set of proteins. Transduction allows DNA insertion into chromosome as a prophage which then replicates, packages host DNA alone or in combination with the host cell chromosome. Conjugative plasmids use a conjugative pilus to form a connection with the recipient cell to transfer these plasmids into the recipient cell that is eventually transferred and copied with the entire bacterial chromosome, multicopy plasmid or a small DNA piece to a recipient cell. These genetic elements are embedded into the chromosome or independently replicated if compatible with the inhabitant plasmids. Integrons possess site specific recombination mechanism which it encodes a promoter for gene cassettes for genetic exchange and

dissemination. Transposons and insertion sequences integrate into new sites on the chromosome or plasmids by non-homologous recombination and increase the copy number of transferred genes giving rise to chromosomal mutations, deletions and rearrangements (Sultan et al., 2018).

1.2.7.3.2 Class 1 integrons

The class 1 integron is the most ubiquitous platform and remains the main focus of numerous studies (Deng et al., 2015). Class 1 integrons located are on MGEs are called mobile integrons (MI) which are widely distributed in 22% to 59% of Gram-negative bacterial pathogens in clinical settings (Labbate et al., 2009;Cambray et al., 2010). They are often involved in carrying and disseminating antibiotic resistance genes (Naas et al., 2001;Gillings, 2014;Li et al., 2017;Kaur and Peterson, 2018). They originated from Tn*402* and their stable structure consists of two conserved sequence regions, called 5'-CS region and 3'-CS region, with a variable gene cassette (GCs) region between them (Cambray et al., 2010). The 5'-CS region consists of three key sequences; the integrase gene (*intI*) controlled by P_{int} promoter, the specific site for recombination of gene cassettes (*attI*) and a cassette promoter region that is located within *int1*. This common promoter (Pc) of integrase, controls the expression of all cassettes that are incorporated within the integrons (Gillings, 2017). Each GCs carrying DNA sequences is associated with a recombination site (*attC*), and these cassettes are promoterless (Nivina et al., 2016). The *Int1* genes encode the integrase protein which is a member of tyrosine recombinase family and is closely related to Xer proteins (Cury et al., 2016). The 3'-CS region carries resistance to sulphonamides (*sul1*), the quaternary ammonium compound resistance gene (*qacEΔ1*) which provides detergent resistance, and two open reading frames, *orf5* and *orf6*, encoding unknown functions proteins (Karah, 2008) Figure 1.3 shows the structure of a class 1 Integron and its activity.

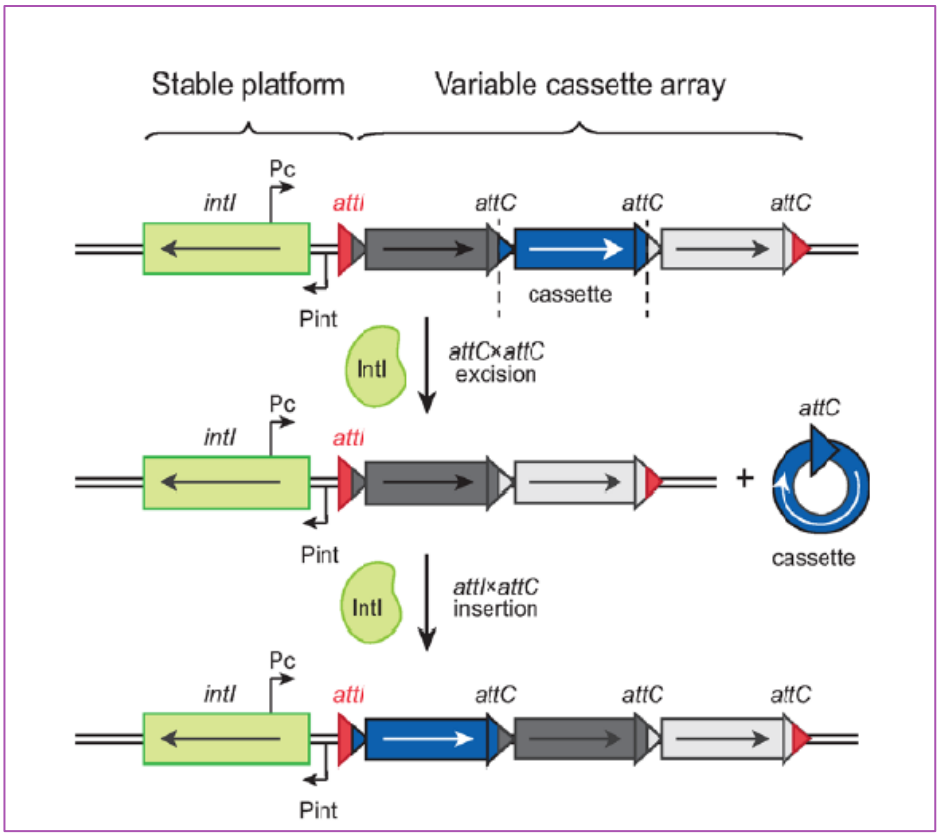


Figure 1. 3 The structure and activity of the class 1 integron (Nivina et al., 2016). Schematic representation of the class 1 integron. The stable platform consists of a gene coding for the integron-integrase gene (*intI*) and its promoter Pint, a cassette promoter (*Pc*), and an integron recombination site (*attI*). Circular, mobile gene cassettes encoding DNA sequences can be excised through an intramolecular *attC*×*attC* reaction mediated by the integrase to regenerate circularized gene cassette, which may insert through an *attI*×*attC* reaction mediated by the integrase to form an array of GCs of variable size.

1.2.7.3.3 Gene cassette structures

Gene cassettes (GCs) exist as circular, non-replicating DNA molecules when moving from one genetic site to another (Bennett, 1999). They are only considered to be part of the integron after the integration event. They usually consist of a single gene and a short sequence of 59 bases, located downstream of the gene termed *attC.* The function of *attC* is a specific recombination site capable of binding

with the integron at the *attI site.* The attachment site of cassettes has imperfect inverted repeats.

Each integrated cassette is flanking by two boundaries with the sequence GTTRRRY, that are recognised by integrase (*intI*), for the recombination process to proceed. Two sites with different structures are used by integrase to exchange DNA between the non-palindromic *attI* and palindromic *attC*. The process of integration is carried out by integrase between the *attC* site in a closed circular cassette and the *attI* site of the integron. The excision process of the captured cassettes occurs between two *attC* sites; one of them associated with the previous cassette located upstream and the other one downstream of the excised cassette, which regenerate to form a circular structure of the cassette. The consequence of integration is a chimeric product between the *attI/attC* sites upstream the GC gene on one side and chimeric *attC/attC* sites downstream of the gene on the other side of the cassette. This results in a clusters of resistance genes with chimerical *attC* sites, with similar palindromic structures. The size of the cassettes is small, around 500–1000 bp, and the genes carried on the gene cassettes are usually promoter-less and are expressed from a common promoter on the integron (Karah, 2008;Larouche and Roy, 2011). Distant cassettes from the Pc promoter may not be expressed (Collis and Hall, 1995;Jové et al., 2010), however they can be excised and reintegrated at the *attI* site in which their expression is increased (Barraud and Ploy, 2015). Hence, several cassettes can be stockpiled in an array to constitute a low-cost memory reservoir of functions for the host cell (Escudero et al., 2018). Thus, this activity that enables integrons to create genomic and phenotypic diversity (Ghaly et al., 2020).

Specifically, the cassettes are composed of the inverse core site which carries two domains 1L (RYYYAAC) and 2L (GTTRRRY) sequences and the core site which contains 2R (RYYYAAC) and 1R sequence (G↓TTRRRY), where R is G or A (a purine), Y is C or T (a pyrimidine). GCs are generally defined by the presence of sequence between the RYYYAAC inverse core site 1L and the GTTRRRY core site

R1 (Stokes et al., 1997;Larouche and Roy, 2011). While the *attI* sites of class 1 integrons carry non palindrome sequences at R and L regions as well as two further integrase binding sites which consist of direct repeats, termed DR1 and DR2 presented in Figure 1.4 A (Gillings, 2014). The incorporation point of incoming GCs occurs either between the G and TT in the right core site or between the AA and C on the complement strand of DNA (Hansson et al., 1997) the vertical arrows show the site of recombination point (Figure 1.4 A and B).

These two pairs (1L-2L and 2R-1R) of the inverted repeat binding sites are in opposite directions and form simple putative integrase binding sites termed LH and RH. They are recognised depending on the type of tyrosine recombinase, recombination sites and are described as conserved sequences in several resistance cassette *attC* sites (Stokes et al., 1997;Biskri et al., 2005). These sites are separated by a central sequence region of variable length. This internal homology enables *attC* to form secondary structures or enclosed hairpin DNA which play a vital role in recognition and recombination by *Int1* as shown in Figure 1.4C (Gillings, 2014).

The size of the *attC* recombination sites ranging from 57 to 141 bp and it is currently the main feature for a*ttCs* classification (Recchia and Hall, 1995;Recchia and Hall, 1997). The insertion of several cassettes in tandem in the same integron always in the same orientation creates an array of cassettes (Partridge et al., 2009). Furthermore, the marked base in 2L (the fourth base; asterisk in Figure. 1.4 B) act to direct the orientation of the inserted strand to ensure that cassettes are inserted in the right orientation (Cambray et al., 2010;Bouvier et al., 2005). Integration between *att1* and *attC* takes place in the bottom strand only of the captured *attC*, and the single stranded recombination structure is then resolved by replication (Bouvier et al., 2009;Loot et al., 2012). The activity of *Int1* is dependent on the *Int1* protein structure, not on the sequence, which is why *Int1* proteins are able to mobilize diverse gene cassettes with very different *attC* sequences (MacDonald et al., 2006).

GCs are frequently associated with resistance to a variety of antibiotics including the *aad*A gene that encodes streptomycin-spectinomycin resistance and trimethoprim resistance (Deng et al., 2015). Moreover, Lu et al. (2003b) highlighted that class 1 integrons commonly carried the streptomycin resistance gene cassette (*aad*A1) in avian *E. coli*. Interestingly, the therapeutic use of this drug has stopped in both human and veterinary medicine. Therefore, streptomycin resistance could be maintained even in the absence of a selection pressure (Ochman et al., 2000). Additionally, Ebner et al. (2004) suggested that the high-level prevalence of streptomycin resistance could act as an interesting model in studying how predominance of antibiotic resistance did not always correlate with withdrawal of the antibiotic from the bacterial environment.

Resistance to trimethoprim determinants is also found frequently (Fluit and Schmitz, 2004;Mazel, 2006;Cambray et al., 2010). These resistance genes determinants show prevalence of 22–59%, and reported among various groups of Gram-negative bacteria including *Escherichia, Klebsiella, Aeromonas, Enterobacter, Providencia, Mycobacterium, Burkholderia, Alcaligenes, Campylobacter, Citrobacter, Stenotrophomonas, Acinetobacter, Pseudomonas, Salmonella, Serratia, Vibrio,* and *Shigella* (Ramírez et al., 2005;Crowley et al., 2008;Partridge et al., 2009;Xu et al., 2009;Xu et al., 2011;Sultan et al., 2018). The study by Heir et al. (2004) was conducted on 192 clinically relevant *Enterobacteriaceae* of blood culture isolates, indicating that the most common gene cassette types determined were trimethoprim resistance genes *dfr*A followed by aminoglycoside resistance *aad*A genes.

Despite much research the regulatory control and dynamics of cassette recombination remain unclear. Several studies reported that the expression of class 1 mobile integron was controlled by the SOS response (Guerin et al., 2009). This response controlled by a repressor protein termed (LexA) and induced by the presence of damaged ssDNA fragments that can arise from various environmental factors. These DNA segments non-specifically bind to universal recombination

protein (RecA) that induce LexA inactivation by autocatalytic cleavage (Sassanfar and Roberts, 1990;Little, 1991;Cambray et al., 2011).

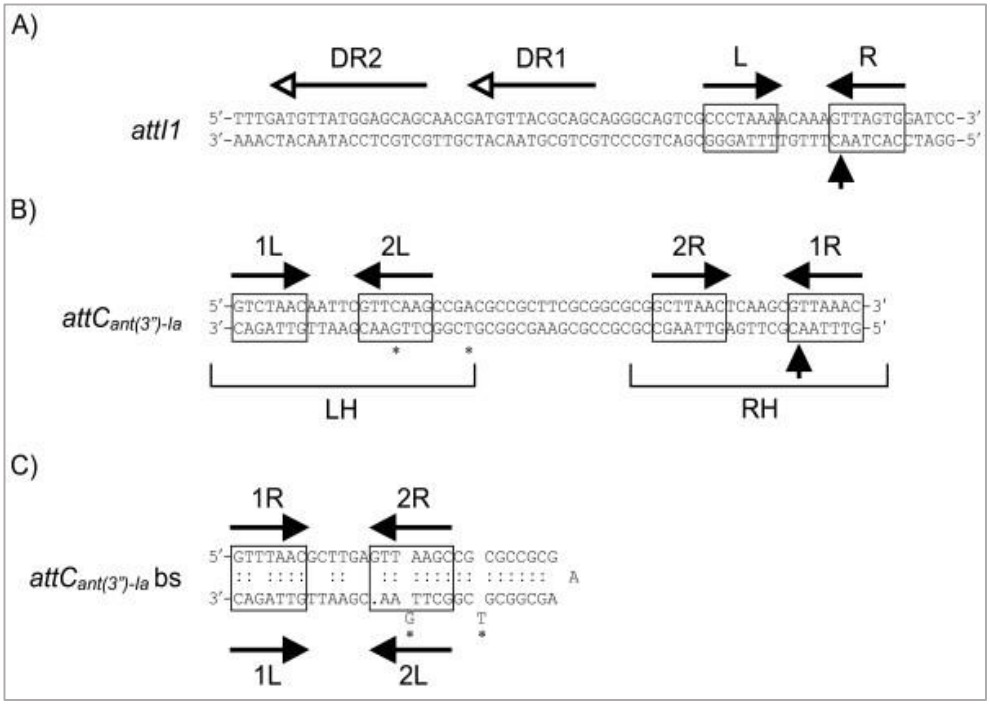


Figure 1.4 Structure of recombination sites of *attI1* and *attC* of class 1 Integron. **(A)** Represents the sequence of the double strand (ds) *attI1* site. **(B)** represents sequence of the ds $attC_{ant(3'')-Ia}$ site. **(C)** Secondary structure of the folded bottom strand of the $attC_{ant(3'')-Ia}$ site, according to MFOLD (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi). The inverted repeats regions (L, 1L, and 2L, R, 1R and 2R) are marked by horizontal black arrows whereas the *attI1* direct repeats are shown by horizontal lines with an empty arrowhead. The recombination positions are indicated by vertical arrows and the extrahelical bases are identified by asterisks (Larouche and Roy, 2011).

1.2.8 Role of horizontal gene transfer (HGT) in transmission of antibiotic resistance genes ARGs

The genetic exchange mechanisms that are involved in transmission of ARGs between bacterial populations are: transformation of free DNA, transduction by bacteriophages, and conjugation via plasmids (Wright, 2007;Hu et al., 2017), collectively named as the mechanisms of HGT (Kaur and Peterson, 2018). These

HGT mechanisms occur widely in nature, although certain bacterial species are likely to use one mechanism more exclusively than others (Barlow, 2009). For instance, transformation is an effectively utilized mechanism in streptococci because they can become naturally competent whereas enterobacteria commonly use conjugative plasmids for exchanging of genetic information. Johnston et al. (2014) indicated that the most efficient method in Gram-positive *Streptococcus pneumoniae* and *Bacillus subtilis* is transformation although many Gram-negative bacteria also become competent. The bacterial nutrition status (Claverys et al., 2006) and environmental stressors, such as antibiotics or DNA damaging agents (Prudhomme et al., 2006) are considered the main factors that control competence. However, genetic transfer mechanisms mediated by conjugative plasmids are likely to be more prevalent in the spreading of resistance genes in nature than either transformation or transduction. This is because of plasmids are able to autonomously replicate, and they carry ARGs against all major classes of antibiotics (Kaur and Peterson, 2018). Plasmids can encode a collection of ARGs as part of transposons, thus simultaneously confer resistance to various classes of antibiotics and metal ions (Nikaido, 2009). Therefore, they can disperse ARGs over long genetic distances to different species, genera, and even kingdoms relying on plasmid host range (Kaur and Peterson, 2018).

A study by Volkova et al. (2014) showed that conjugation seems to be 1000-fold more common than transduction as a mechanism for the transfer of resistance genes. Carattoli (2013) demonstrated that the transmission of ARG determinants by conjugation is responsible for their contribution to worldwide prevalence in both community and hospital environments. The process of genetic exchange can be encouraged in "hot-spot" environments, such as wastewater treatment plants and sewage, agricultural and slaughterhouse waste and hospital effluents, due to the high density of bacteria, phages, and plasmids in these settings (Kenzaka et al., 2010;von Wintersdorff et al., 2016).

The three mechanisms of HGT each have limitations including: the host range of the incoming plasmid or the phage, the host restriction modification systems, formation of cell-to-cell contacts, fitness cost of acquired new gene, as well as the ability of incoming DNA recombination with DNA of the host (Thomas and Nielsen, 2005;Domingues et al., 2012). Furthermore, the capability of MGEs to incorporate into a population is also dependent on its ability to autonomously replicate and achieve vertical transmission. For instance, the incompatibility group IncP, are the most successful conjugative plasmids that have a broad host range (Davies and Davies, 2010) facilitating their dissemination and maintenance in distantly related phyla (Klümper et al., 2015). Also, HGT exhibits success in the persistence of MGEs or DNA to in the environment (Kaur and Peterson, 2018).

Recently, a novel mechanism termed 'carry-back' was proposed for inter-phylum genetic exchange (Jiang et al., 2017). This mechanism involves conjugation mediated by a broad-host range conjugative plasmid (Klümper et al., 2015) that may transfer a DNA fragment from commonly spread class 1 integrons of Proteobacteria to Actinobacteria, followed by recombination, consequently creating actinobacterial DNA flanked by proteobacterial DNA. Dead cells of Actinobacteria can release these actinobacterial DNA molecules flanked by proteobacterial DNA into the environment, and Proteobacteria can incorporate this DNA into their genome by transformation and homologous recombination (Kaur and Peterson, 2018).

1.2.9 Chicken gut microbiota

1.2.9.1 Chicken gut microbiota as sources of AR

The system of animal production has been linked to the prevalence and evolution of AMR of organisms such as *E. coli* (Tadesse et al., 2012;Simoneit et al., 2015;Luna-Galaz et al., 2016), *Enterococcus* spp., *Staphylococcus aureus* (Bortolaia et al., 2016) and other foodborne zoonotic pathogens, like non-typhoidal *Salmonella* (Luna-Galaz et al., 2016;Vickers, 2017;Nhung et al., 2017)

and *Campylobacter* spp. (Richter et al., 2015). Comparatively, little is known regarding the dissemination and mechanisms of AMR in pathogenic bacteria in food animal production including poultry. As reviewed previously (1.2.4) there is an increasing global consumption of poultry meat and eggs (Mishra and Jha, 2019), thereby poultry production is one of the fastest growing animal industries involved in nutrition and global food security.

Some important human pathogens such as *Campylobacter* and *Salmonella* are commonly detected within the chicken microbiota where they are largely non-pathogenic to chickens (Newell et al., 2011). However, they can be a source for disseminating pathogens to humans as well as a pool for transferring antibiotic resistance (Nhung et al., 2017). Since the commensal bacteria share the same habitat as pathogens, commensals may act as reservoir of multidrug resistance genes that are acquired by conjugative transfer from pathogenic bacteria.

Poultry products are one of the most significant reservoirs for transmission of foodborne disease such as *Campylobacter* (Kaakoush et al., 2014). The abundance of this organism on poultry farms and in the surrounding environment is not surprising because the majority of warm-blooded domestic animals, wild animals and birds shed viable *Campylobacter* species in their faeces. Colonisation of broiler flocks by *Campylobacter* spp takes place at between 2–3 weeks of age and positive birds often remain colonised until slaughter (Kaakoush et al., 2014).

1.2.9.2 Gastrointestinal tract of chicken

The gastrointestinal (GI) tract of chickens is densely populated by a diverse microbiota that plays a vital role in digesting and absorbing nutrients, developing the immune system, and reducing the opportunity of pathogenic bacteria colonisation via attachment to the epithelial walls of the enterocytes acting as a protective barrier (Yeoman et al., 2012;Pan and Yu, 2014;Wang et al., 2016). The bacterial microbiota produces vitamins (e.g., vitamin K and vitamin B groups), short chain fatty acids (acetic acid, butyric acid and propionic acid), organic acids

(e.g., lactic acid) and antimicrobial agents (such as bacteriocins), lower triglyceride, and induction non-pathogenic immune responses, which all provide both nutrition and protection for the host (Jeurissen et al., 2002;Apajalahti, 2005;Dibner and Richards, 2005;Yegani and Korver, 2008;Shang et al., 2018b).

The GI tract of the chicken consists of the crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, large intestine, and cloaca (Yeoman et al., 2012). Each part of GI tract has different metabolic functions that are shaped by the resident microbial communities and thereby it is important to determine sampling location and study design (Shang et al., 2018b). Generally, the most representative phylum in GI tract of the chicken microbiota is Firmicutes followed by a small abundance of Proteobacteria and Bacteroidetes phyla, and a low abundance of Actinobacteria and Tenericutes members (Waite and Taylor, 2014).

Microbial colonisation in the GI tract of chickens is highest in the caecum with a much greater bacterial diversity than found in the upper GI tract (Oakley et al., 2014). The caecum is considered the key site for bacterial fermentation of non-digestible carbohydrates as well the organ most targeted by pathogens for colonisation. In chickens this organ consists of two paired caeca, both harbouring similar bacterial communities (Stanley et al., 2015). The caecum microbiota is a stable, rich and diverse microbial community including many anaerobes (Salanitro et al., 1974;Videnska et al., 2013).

The predominant residents of the caecal microbiota are members of the *Clostridia* genus followed by the genera *Lactobacillus* and *Ruminococcus* (Gong et al., 2007). Most *Clostridia* found in the caecum belong to three main families which are *Clostridiaceae, Lachnospiraceae* and *Ruminococcaceae* (Danzeisen et al., 2011). *Enterococcaceae*, *Enterobacteriaceae* and *Bacteroidaceae* are also abundant families in the caecal microbiota (Yin et al., 2010), which is also dominated by unknown and unclassified bacterial members (Stanley et al., 2013).

Factors which affect the chicken gut microbiota include: diet (Torok et al., 2008), gender (Lumpkins et al., 2008), genetic background (Zhao et al., 2013), housing condition (Nordentoft et al., 2011), litter managements (Torok et al., 2009;Cressman et al., 2010), feed restriction (Callaway et al., 2009) and density of rearing birds (Guardia et al., 2011).

Research in recent years has focussed on the way in which the caecal microbiota develops with time from hatching to adult birds. A study by Oakley et al. (2014) documented significant shifts in caecal microbial communities from first day of hatching until 6 weeks of age in commercial broilers (Oakley et al., 2014;Oakley and Kogut, 2016). Typically, the cecum richness and diversity increase through these 6 weeks, and the taxonomic composition of these microbial communities quickly changes from Proteobacteria, Bacteroides, and Firmicutes, to almost entirely Firmicutes by 3 weeks of age (Oakley et al., 2014;Oakley and Kogut, 2016). On the other hand, Kumar et al. (2018) found that Firmicutes were the most abundant phylum in both caeca and ileum at all the ages (day 0 to day 42) except d 42 in the caeca where Bacteroidetes were abundant. The variation in reported bacterial composition can be explained because of differences in extraction of the nucleic acids, protocol used, primers, sequencing approach, environmental factors, dietary types/ composition, breed, and geographical conditions between different studies (Shang et al., 2018b).

Some bacterial population may appear or disappear over time in the GI microbiota of older chickens whereas others remain stable during the life. Young chickens are dominated by Firmicutes species while adult birds (older than 7 months) commonly colonised by Bacteroidetes (Callaway et al., 2009;Videnska et al., 2014).

1.2.9.3 16S RNA Genes Technology

Culture based microbial studies have provided a limited understanding of microbial communities, because less than 1% microbial organisms can be easily cultured (Kellenberger, 2001). The sequences of the small unit of ribosomal RNA gene in prokaryotes has become the most widely used technique for deducing the phylogenetic evolution among microbial species (Lane et al., 1985;Sharpton et al., 2011). Ribosomal RNA genes are an ideal tool for studying microbial phylogeny because they contain both highly conserved regions across all bacteria and 9 hypervariable regions which vary in terms of length, position and taxonomic discrimination (Van de Peer et al., 1996). The amplification of the hypervariable regions is carried out using the polymerase chain reaction with universal primers designed to bind to the conserved regions (Klindworth et al., 2013). These variable regions have different power levels of discrimination dependent on the microbial group and the short target regions (<300 bp). The most hypervariable informative part is the V4 region (Soergel et al., 2012).

The nucleotide sequences of these amplified products can distinguish among bacteria to the genus or species level (Weisburg et al., 1991;Flint et al., 2006). The relative abundance of each sequence reflects the abundance of the bacterium in the original sample. Thus, the 16S rRNA genes sequencing gives a true census of a bacterial community by identifying the bacterial types present in a sample with its relative abundances. DNA sequencing technology has developed in the last few years, to the point that can enable complete census of the richness and diversity of complex communities (Shang et al., 2018b). This revolution of molecular sequencing biotechnology has shaped the view of microbial diversity and composition of various environments including the human gut, soil, and salt lakes as well as prediction function and interaction in various sections of the GI tract (Chen et al., 2013;Shang et al., 2018b). The Illumina Miseq is one of the high-throughput sequencing technologies that has allowed gene amplicon sequencing in microbial ecology studies where millions of paired-end reads can be

sequenced directly from the relevant environments (Chen et al., 2013;Wen et al., 2017). This next generation sequencing (NGS) platform has offered an accurate, convenient, rapid, and inexpensive method for genomic research (Pettersson et al., 2009;Park et al., 2013). Likewise, it is the most frequently utilised system used in recent chicken gut microbiome and metagenomic research (Shang et al., 2018b). However, this platform suffers from some limitations such as short read assembly and high cost (Kumar and Pitta, 2015).

1.2.10 Modulation chicken gut microbiota by prebiotics

To cope with the need for reducing the usage of antimicrobial growth promoters (AGPs) after their use was banned in poultry in the EU since 2006, several strategies involving different feed additives have been evaluated (Teng and Kim, 2018). Ideally these should promote growth but without encouraging antimicrobial resistance (AMR) and the transfer of ARGs from animal to human microbiota (Castanon, 2007;Yadav and Jha, 2019;Richards et al., 2020). Addition of prebiotics into broiler diets are one of these approaches that have been studied (Teng and Kim, 2018).

Prebiotics have been defined as non-digestible food ingredients that when metabolised by gut microorganisms, modulates the composition or activity of the gut microbiota, thereby conferring a beneficial physiological effect on the host (Bindels et al., 2015;Pourabedin and Zhao, 2015). The main characteristics of prebiotics described by Patterson and Burkholder (2003). This includes the definition that prebiotics should be considered nondigestible food ingredients, not capable of being hydrolysed by host enzymes or even directly absorbing by host cells in GIs. Prebiotics should selectively stimulate one or limited numbers of health promoting bacteria, have the ability to change the intestinal microbiota and their activities, and improve the host immune system’s response against pathogenic bacteria.

The potential advantages of dietary supplemention with prebiotic oligosaccharides in the gut microbiota are summarised in Figure 1.5, which includes: reduction of pathogens by competitive exclusion (Callaway et al., 2008); improving gut morphological structure (Pourabedin et al., 2014;Chee et al., 2010b) and increasing the production of SCFA which modulates host immune response and metabolism (Saulnier et al., 2009;Roberfroid et al., 2010). Additionally, the formation of SCFA in the intestines involves: lowering pH, bioavailability of calcium and magnesium, and inhibiting of potentially harmful bacteria (Teitelbaum and Walker, 2002;Wong et al., 2006).

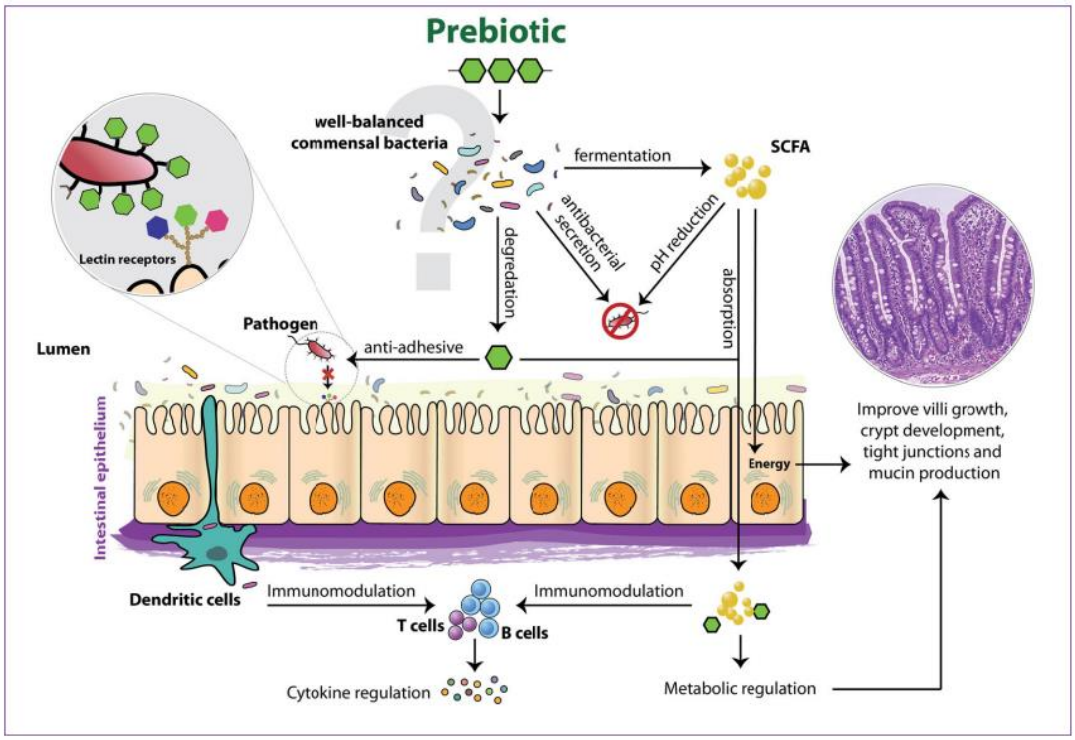


Figure 1.5 Potential mechanisms of prebiotics action.

Metabolisation of prebiotics by the gut commensal microbiota. Prebiotics can be fermented into short-chain fatty acids (SFCA) by the action of gut microbiota, mainly acetate, propionate, and butyrate. SCFA lowering the luminal pH, providing energy sources for epithelial cells, and have deep effects on inflammation modulators and metabolic regulations. Intestinal mucosal structure can also be improved by a well-balanced bacterial community. Some bacterial strains produce antimicrobial factors or induce the immune system by signalling dendritic cells. Both monosaccharides and oligosaccharides can decrease pathogen colonisation by blocking the receptor sites utilised by pathogens for

attachment to the epithelial cell surface. This figure is taken from (Pourabedin and Zhao, 2015).

Generally fermented prebiotics in the intestine produce SCFAs which include butyrate that are considered as significant fuel for colonocytes, enhancing the growth and absorptive capacity of the epithelium in the colon and to suppress the growth of colonic carcinoma cells (Van Craeyveld et al., 2008;Pourabedin and Zhao, 2015;Teng and Kim, 2018). Additionally, fermentation of prebiotics produce lactic acid and some antibacterial substances, such as bacteriocin against pathogenic bacteria (Bogusławska-Tryk et al., 2012).

In the gut microbiome, bacterial population that are thought to be specifically induced by prebiotic supplemented diet are lactobacilli and bifidobacteria. As in the intestine of young broilers, the administration of prebiotics in diets shows enhancement of the abundance of lactobacilli and bifidobacteria as well as reducing the titers of coliforms (Yang et al., 2008;Chee et al., 2010a). The enhancement of the populations of these beneficial bacteria leads to suppression of levels of pathogenic bacteria, such as *Clostridium pefringens* and *E. coli*, in the intestinal microbiome of broiler chickens (Xu et al., 2003;Kim et al., 2011;Ricke, 2015). The increased availability of sequencing information regarding the microbial community means there may be other unidentified bacteria that are also selectively stimulated by certain prebiotics (Kaplan and Hutkins, 2000;Hutkins et al., 2016). Furthermore, inhibition of pathogen colonisation by prebiotics can decrease harmful molecules produced by pathogens, which have been recognised as exogenous signals (Tizard, 2013). These signal molecules are known as pathogen-associated molecular patterns (PAMPs) which can be identified by pattern recognition receptors (PRR), such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), that are expressed on the surface of sentinel cells (Kogut, 2013). When the PRRs recognise PAMPs, sentinel cells including epithelial cells, macrophages, mast cells, and dendritic cells, are activated, thereby cytokines produced for regulation of further innate immune responses. Likewise, prebiotics

themselves can act as non-pathogenic antigens which means they can be detected by receptors of immune cells leading to beneficial modulation of host immunity (Teng and Kim, 2018).

Galacto-Oligosaccharide (GOS) is a valuable product that can be produced from the cheese whey industry. Whey is considered the most significant by-product waste from the dairy industry because it can cause significant environmental pollution problems (Smithers, 2008). This by-product corresponds to 85–95% of the milk volume and comprises of 55% of milk nutrients with a 4.5-5% w/v abundance of lactose (Chandrasekaran, 2012;Geiger et al., 2016). The process of bioconversion of lactose hydrolysates by using the catalytic activity of β-galactosidases to form valuable products is known as transgalactosylation. This reaction leads to the formation of oligosaccharides of different chain lengths and glycosidic linkages that act as prebiotics and possess structural similarity to oligosaccharides in human breast milk (Fischer and Kleinschmidt, 2018). Therefore, they are widely used in infant formulas because bovine milk contains only very small amounts of oligosaccharides (Sangwan et al., 2011). GOS are non-digestible carbohydrates (lactose-derived compounds) with known prebiotic activity. GOS generally consists of one or more galactose units that are usually linked to a terminal glucose. It shows variable degrees of polymerisation ranging from 2 to 8 monomeric units (Geiger et al., 2016). Thus, GOS can act as fermentable substrates for some members of the gut microbiota that have been determined to modulate the colonic flora by promoting of beneficial bacteria, such as bifidobacteria and lactobacilli, and inhibition of less desirable bacteria (Holzapfel and Schillinger, 2002;Rastall et al., 2005;Macfarlane et al., 2008;Geiger et al., 2016).

Studies have reported that the injection of GOS *in ovo* broilers 34 days after hatching is likely to increase body weight (Pruszynska-Oszmalek et al., 2015). In addition, GOS administration also modulated the intestinal microbiota as Park et

al. (2017a) found that GOS treatment showed higher abundance of *Alistipes* genus, *Lactobacillus intestinalis* and *Faecalibacterium prausnitzii* in broilers caeca compared with the control group. It has been reported that adding GOS to broiler diets could increase counts of *Bifidobacteria* members in faeces (Jung et al., 2008;Teng and Kim, 2018).

1.2.11 *Salmonella*

1.2.11.1 Salmonella Background

Salmonella is a genus of Gram-negative bacteria, usually motile, facultative anaerobic and non-spore forming bacilli, which belong to the *Enterobacteriaceae* family (Coburn et al., 2007;Dunkley et al., 2009;Agbaje et al., 2011). It grows at temperatures between 5–45°C in a pH range of 4–9 and with water activity above 0.94 (Guthrie, 1992). These bacteria are sensitive to heat and are killed at temperatures of 70°C or above. Salmonellae are resistant to drying and may survive for years in dust and dirt. This genus includes two species, *Salmonella bongori* and *Salmonella enterica*. *S. enterica* has six subspecies: *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica,* and *enterica* (Wisner, 2011;Hooton et al., 2014). These subspecies can be further classified based on their lipopolysaccharide (LPS) O antigen component and flagellar (H) antigen into more than 2500 serovars (Sabbagh et al., 2010;Wisner, 2011). *Salmonella* are capable of colonizing the GI tracts of many mammals, birds and reptiles and can persist in the environment as well (Callaway et al., 2008). Most *Salmonella* serovars are host-restricted, but some are not. Host-restricted serovars tend to cause systemic disease that threatens the life of the host, while non-host-restricted strains often produce gastroenteritis in many different host species (Wisner, 2011).

Salmonella enterica is a leading cause of foodborne human diseases with poultry considered as the main source of human illnesses (Havelaar et al., 2015;Hughes et al., 2017). Annually, Salmonellosis is responsible for 78 million incidences and 59 thousand foodborne-related deaths (Havelaar et al., 2015).

Yearly, in the United States, non-typhoidal Salmonellosis is responsible for 1 million cases, 19 thousand hospitalizations, and 378 deaths (Scallan et al., 2011). Most susceptible to *Salmonella* infection are children (≤5 years) and the elderly whom frequently require medical treatment (Bythwood et al., 2019).

The severity and length of illness can be treated by antibiotic therapeutics (Hu et al., 2014). However, the emergence of antimicrobial-resistant *Salmonella* has reduced the efficacy of commonly prescribed antimicrobials that treat salmonellosis (Wasyl et al., 2015;Iwamoto et al., 2017;Tyson et al., 2017;Duong et al., 2018). The documented cases of treatment failure (nontyphoidal *Salmonella*) have become a significant public health concern (Collard et al., 2007;Tribble, 2017;Duong et al., 2018).

Contaminated meat and eggs are frequently associated with salmonellosis. The acquisition of *Salmonella* by chicks takes place via vertical transmission from parents as well as horizontal transfer from the rearing environment and feed (Rothrock Jr et al., 2015). The majority of the initial infection occurs at an early stage of life (post-hatch); however, *Salmonella* infection can happen during any stage of the production cycle (Byrd et al., 1999;Shanmugasundaram et al., 2019). *Salmonella* prevention within poultry flocks is difficult since cleaning and disinfection fail to eradicate *Salmonella* in poultry (Davies and Wray, 1996;Shanmugasundaram et al., 2019). Therefore, it is important to control *Salmonella* infection within poultry products to increase the safety of human consumed food.

S. Enteritidis is considered the most predominant *Salmonella* serovar in human cases associated with the contamination of poultry products in US (Morningstar-Shaw et al., 2016). *S.* Enteritidis has historically been linked to poultry and it has been proposed to succeed due to niche displacement of the closely related host-specific poultry *Salmonella* serovars Gallinarum and Pullorum, which had previously been removed from commercial flocks (Louis et al., 1988;Bäumler et al., 2000;Control and Prevention, 2000;Porwollik et al.,

2005;Martelli and Davies, 2012;Ricke, 2017). Several controlling strategies on-farm have been evaluated/investigated for preventing *Salmonella* shedding in poultry, include vaccination (Varmuzova et al., 2016). However, these strategies have not succeeded in controlling *Salmonella* contamination in chicken (Koutsoumanis et al., 2019), and thereby, it is important to identify alternative on-farm approaches to manage *Salmonella* infection in broilers (Shanmugasundaram et al., 2019).

1.2.11.2 Salmonella Enteritidis pathogenicity in the GI tract of chicken

Although the infection of zoonotic *Salmonella* in poultry is largely asymptomatic (Kogut and Arsenault, 2017), *S. enterica* serovars, specially *S.* Enteritidis and *S.* Typhimurium have been shown to colonise the lower part of GI tract in the first days of life, and stimulate a low-level systemic infection in chickens (Beal et al., 2005). The persistence of *Salmonella* colonisation may extend for several weeks without giving any clinical signs of disease in the GI tract of chickens. While the process of pathogenesis by *S. enterica* serovars includes adhesion, colonisation, invasion, and intracellular replication, which can lead to the systemic spread of the organism to internal organs such as the liver and spleen. The first important step in colonisation and persistence is efficient adhesion to the epithelial layer of the GI tract. The cecum is predominantly colonised by *S. enterica* serovars, however, their translocation to internal organs is often limited, mainly in adult birds (Van Immerseel et al., 2003;Beal et al., 2005). Generally, the primary source of *S*E infection and transmission in chickens is through the faecal-oral route (Shah et al., 2017). SE frequently colonises the crop after ingestion, (Hargis et al., 1995;Turnbull and Snoeyenbos, 1974), but less frequently in the proventriculus and duodenum. However, the most preferential and persistent sites are in the lower ileum, cecum, and cloaca (Turnbull and Snoeyenbos, 1974). Subsequently, *S*E is able to invade the intestinal epithelium and localise in the submucosa within 4 hours of infection (Berndt et al., 2007).

The process of invasion happens via M-cells that sample the intestinal lumen and nonphagocytic cells. The invasion of intestinal prompts inflammation that is characterised by the infiltration of heterophils, macrophages, red blood cells, and other immune cells into the lamina propia and caecal luminal exudate (Porter Jr and Holt, 1993;Macri et al., 1997;Van Immerseel et al., 2002). When inflammation proceeds, *S*E produces virulence factors that support *Salmonella* survival within macrophages, which are thought to be responsible for the systemic spread to colonise internal organs including the liver and spleen, and in laying hens this also results in colonisation of the reproductive tract organs (Berchieri Jr et al., 2001;Higgins et al., 2007;He et al., 2010).

The kinetics of *S*E infection varies depending on the age or type of chickens (Shah et al., 2017). The infection of *Salmonella* in chickens seems to be dependent on age. Early infected birds by *Salmonella* in day 1 of life have long extended periods of carrying *Salmonella* with high numbers compared to those infected at day 8 of life (GAST and Beard, 1989). As *Salmonella* clearance is slower in early challenged birds than those challenged later in life 3–6 weeks (Beal et al., 2004). Moreover, detectable changes in the cecum microbiome have been recognised in birds that are exposed to *Salmonella* in the first 4 days post hatching (Juricova et al., 2013).

1.2.11.3 Prevalence of antibiotic resistance Salmonella in poultry

The presence of *Salmonella* on poultry meat products is associating with their prevalence in the poultry farms (Choi et al., 2014). Although several methods have been applied to eradicate *Salmonella* colonisation on breeding farms, including vaccination, use replacement systems of all-in/all-out on broiler farms, and antimicrobial free approaches, high rates of *Salmonella* expansion and antimicrobial-resistance are still common place on broiler farms (Ishihara et al., 2009;Rayamajhi et al., 2010). Poultry litter, has been documented as indicator for the presence of *Salmonella* in poultry farms (Shang et al., 2018a).

Despite representing a minor population of chicken microbiota and litter, poultry sources of zoonotic *Salmonella* not only represent a source of infection but are a repository for antimicrobial resistance in food. Bythwood et al. (2019) documented abundant AMR *Enterobacteriaceae* in poultry and poultry litter in the absence of antibiotic treatments. Several studies have reported the similar findings (Fairchild et al., 2005;Smith et al., 2007;Agga et al., 2016;Liljebjelke et al., 2017).

As *Salmonella* that colonise chickens easily acquire antimicrobial resistance genes from the resident bacteria present in chickens, even if antimicrobial use is absent (Bythwood et al., 2019). Poultry litters are enriched in bacteria with a high abundance of class 1 integrons at 1 copy per 1-100 bacterial genomes (Nandi et al., 2004). The most prevalent integrons carrying antimicrobial resistance genes in poultry litter are remarkably similar to those present in clinical and food isolates of *Salmonella* (Nandi et al., 2004;Liljebjelke et al., 2017).

The appearance of *Salmonella* that possess extended-spectrum β-lactam/cephalosporin resistance has been assigned to acquisition of conjugative plasmids (Winokur et al., 2000). These plasmids often carry other resistances, such as chloramphenicol, florfenicol, streptomycin, and tetracycline (Winokur et al., 2000;Doublet et al., 2004;van Loon et al., 2015). Antimicrobial resistant (AMR) *Salmonella* with high abundances of streptomycin resistance, either alone or in combination with other antibiotics (36.3%), has been recovered from commercial broiler chicken farms. Streptomycin and sulfadimethoxine resistant *Salmonella* tend to carry the determinants on the transposon Tn*21* (Liljebjelke et al., 2017). This transposon encodes the *merA* gene for mercury resistance; *aadA1* resistance gene for streptomycin; and *sul1* resistance gene for sulfadimethoxine resistance (Liebert et al., 1999). Tn*21* is frequently involved in the dissemination of mercury and antimicrobial resistance in nature (Liebert et al., 1999), and is widespread in poultry *Salmonella* and *E. coli* (Bass et al., 1999). The association

between the *merA* and *aadA* resistance genes and streptomycin/ sulfadimethoxine resistance was observed in 17.72% of streptomycin resistant *Salmonella* (Liljebjelke et al., 2017).

1.2.11.4 Prebiotics for Salmonella control in poultry

Controlling *Salmonella* proliferation in poultry is a key element to reduce the contamination of poultry products consumed by humans (eggs and meat). Efficient alternative control strategies are required to decrease the hospitality of the cecum to *Salmonella* colonisation (Hughes et al., 2017). Feed additive prebiotic, represents one of these strategies that has attracted the attention of the poultry industry. Besides its role in promoting bird health, prebiotics can improve the safety of poultry products by making intestinal environment unfavourable for foodborne pathogens including *Salmonella* (Micciche et al., 2018). The most frequently studied prebiotics used in poultry production are fructo-oligosaccharides (FOS), mannan-oligosaccharides (MOS) and galacto-oligosaccharides (GOS) (Mundt et al., 2015;Pourabedin and Zhao, 2015;Ricke, 2015;Gibson et al., 2017). Despite this, GOS has not received the same level of attention for poultry farm use as compared with FOS and MOS (Pourabedin and Zhao, 2015).

Numerous studies have documented the role of increases in short chain fatty acids (SCFAs) concentrations with the reduction of *Salmonella* proliferation (Durant et al., 2000b;Durant et al., 2000a), which can be achieved by prebiotic administration (Cummings and Macfarlane, 2002;Ricke, 2015). Prebiotic protection against *Salmonella* colonisation is proposed to be accomplished by competition for binding sites (Durant et al., 2000b) and increasing the levels of the SCFAs concentrations in the intestine (Ricke, 2015).

The most extensively studied oligosaccharides against *Salmonella* are MOS (Pourabedin, 2015). For instance, a study by Fernandez et al. (2002) on chickens

challenged with *S.* Enteritidis showed a reduction in caecal *Salmonella* concentration. While Lourenço et al. (2015) elucidated that feed supplemented with MOS boosted lymphocyte counts (CD4+ and CD8+) in the ileum and cecum, and reduced *Salmonella* shed in chicken faeces when challenged with *S.* Enteritidis. Stanley et al. (2016) also demonstrated a 1-3 log reduction of caecal *Salmonella* counts in 21-day old chicks supplemented 0.05% MOS and MgSO4. Fructooligosaccharides supplements have been demonstrated to enhance *Lactobacillus* and *Bifidobacterium* populations, resulting in an increasing in SCFAs and lactate that are presumed to improve the immune system and the reduce *Salmonella* colonisation (Cummings and Macfarlane, 2002;Bogusławska-Tryk et al., 2012;Emami et al., 2012;Ricke, 2015). The elucidated mechanism of FOS action is achieved by fermentation of FOS by *Lactobacillus* and *Bifidobacterium*, which leads to increase both SCFAs and lactate in the cecum resulting in a reduced *Salmonella* population (Cummings and Macfarlane, 2002;Ricke, 2015).

The influence of the GOS on the caecal microbiome and the carriage of *Salmonella* was reported by Hughes et al. (2017) indicated that *Salmonella* counts were reduced in the GOS treated birds challenged with a cocktail of *Salmonella* Typhimurium and *Salmonella* Enteritidis. They found that the prebiotic GOS could modify caecal tonsil immune gene expression and the caecal microbiome, proposing that this treatment could be a useful tool for lowering the carriage of *Salmonella* in poultry. Azcarate-Peril et al. (2018) also identified the role of GOS in acceleration of *Salmonella* clearance via modifying the gut microbiome. Moreover, a study by Searle et al. (2010) indicated that the existence of GOS within the GI tract has been shown to decrease the adherence and invasion of *Salmonella* in human enterocytes. Thus, modulation of the microbiome through the using prebiotics has been shown to have an effective impact in the gut microbiome and the development pathogen resistance.

1.3 Aims and Objectives

This research aimed to test the hypothesis that GOS supplemented feed can alleviate the expansion of antibiotic resistance gene carriage in the bacterial populations of the broiler chicken microbiome in addition to the established role of maintaining gut health.

Particularly, the aims of the current study are to investigate the overall prevalence of ARGs in the caecal contents of broiler chickens reared in controlled biosecure conditions and to compare these with birds reared for commercial production by using class1 integrons as a bioindicator for antibiotic resistance. In this context, the gene cassette (GCs) contents of class 1 integrons will be reported from birds reared under the different regimes. Profiles of the 16S rRNA genes of the caecal microbiota of these birds will be investigated and the patterns of resistance evaluated with respect to the relative abundance of the intestinal bacterial populations. Phenotypic antimicrobial resistance will be estimated by examining trimethoprim coliform resistant populations. The effect of prebiotic galactooligosaccharide feed on microbiome composition will be carried out in order to investigate the prebiotic's role in modifying the gut microbiota and whether there was any reduction in resistance gene carriage by comparing control and GOS diets. In addition, the involvement of GOS in the overall reduction of intestinal colonization of zoonotic *Salmonella* Enteritidis (PT4) 125109 will be assessed with regard to the prevalence of ARGs within the broiler caecal microbiota.

CHAPTER 2

MATERIAL AND METHODS

2.1 General bacterial growth and storage media preparation

All details for the preparation of media and antibiotics described in this thesis are presented in Appendix 1. The suppliers for all media and chemical substances are listed in Table 2.1.

Table 2.1 Media and chemical substances that were used in this study

Compound	Supplier
Agar Bacteriological (Agar No.1) LPOOII Maximum Recovery Diluent (MRD) (CM0733) Xylose-Lysine-Desoxycholate (XLD) agar (CM0469) Modified Semi-solid Rappaport Vassiliadis (MSRV) agar CM1112 MacConkey agar No 3 (Mac_03) CM115 De man, Rogosa and Sharpe (MRS) agar CM0361 Nutrient Agar (NA) Nutrient broth No. 2 CM0067 Buffered peptone water (BPW; CM509)	Oxoid, UK Ltd
Tryptone Yeast extract	BiTek™, USA
LB broth	Thermo Fisher, UK Ltd
Novobiocin Nalidixic acid Lincomycin Proteinase kinase Trimethoprim Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Sodium chloride	Fisher Scientific, UK Ltd
Ethanol absolute	Acros Organics, UK Ltd

2.2 Salmonella

2.2.1 *Salmonella* growth conditions

Salmonella enterica 125109 was selected to be the model strain used to colonise broiler chickens, as it is nalidixic acid resistant and this could be used to easily select and enumerate it. *Salmonella* were cultured on m-XLD plates (Oxoid, UK) or nutrient agar in aerobic incubator at 37°C for 24 h or 50 in YT broth (Appendix 1.1.10) incubated at shaker incubator at 200 rpm for 24h. *Salmonella enterica* U288 was used as positive control in PCR usually cultured on m-XLD and then on nutrient agar plates for DNA extraction.

2.2.2 *Salmonella* serotype test

Three types of *Salmonella* antisera (Poly O, O9 and O4 antisera; Pro-Lab Diagnostics, USA) were used to confirm *Salmonella* serotype. The slide agglutination test was performed by adding two separate loopfuls (10 μl each) of PBS (Appendix 1.2.3) on a clean glass slide. Then, one or two bacteria colonies were emulsified in one of the drops, while the positive control *Salmonella* was emulsified in the other drop. A 10 μl aliquot of PBS was added to the first test subject (a negative control). Then, 10 μl of antisera was added to the remaining test subject and 10 μl of Poly O antisera was added to the positive control *Salmonella* drop. The glass slide was gently rocked for a minute and agglutination was checked. Negative results showed no agglutination while positive results showed agglutination with Poly O. *Salmonella enterica* 125109 is serotype O9 so agglutinated with the O9 serum, the Poly O serum but not the O4 serum.

2.2.3 Preparation of *Salmonella* inoculum

To prepare the inoculum*, Salmonella* Enteritidis 125109 was grown on m-XLD and LB agar plates (Appendix 1.1.1 and 1.1.6) and incubated for 24 h at 37 ºC. A colony was inoculated into 50 ml of LB broth in a 250 ml conical flask and

incubated at 37°C with shaking at 200 rpm for 16 h. The cells were washed twice with 50 ml of MRD.

The suspension was diluted 1:10, then the OD_{600} was adjusted to 0.34 (approximately 10^8 CFU /ml). The actual dose is determined by decimal dilution of the suspension 10^{-6} with 0.1 ml spread on XLD in triplicate using the 10^{-4} to 10^{-6} dilutions.

2.3 Experimental birds

Two trials involving experimental chickens are described in this thesis. The first trial was conducted under biosecure conditions in the Bio-Support Unit (BSU) at the University of Nottingham, Sutton Bonington Campus. The trial started from hatching until 35 days of age (da), with four sampling days (22, 24, 28, 35 da). The second trial was conducted using birds reared under commercial conditions with two sampling days at 30 and 37 da. Both trials were designed and carried out according to the ARRIVE (Animal Research: Reporting of *in Vivo* Experiments) guidelines (Kilkenny et al., 2010) which is a system to improve the design, analysis and reporting of research using animals.

2.3.1 Ethics statement

The use of birds in experiments were subjected to an approval process under national guidelines by the United Kingdom Home Office. This project was approved under United Kingdom Government Home Office Project Licensing ASPA 86. The University Ethics Committee internally reviewed all project licenses prior to submission to the Home Office. This includes the handling of animals, scrutiny of welfare and ethics.

2.3.2 Selection of broilers and *Salmonella* free test

The male Ross 308 birds were selected because they are a modern broiler, and their growth rate is in accordance with Home Office regulation in terms of size restrictions of pens for experimental birds. All male birds were supplied at one day old from P.D. Hook Ltd.

In order to ensure that the birds were *Salmonella* free, a test was performed by collecting all the papers on which the birds had been transported. The papers were shredded and added to BPW for *Salmonella* enrichment as described below in 2.4.1.3 to check that the birds were *Salmonella* free.

2.3.3 Pre-trial test for selecting antibiotic resistance genes and its concentration

One sample of caecal contents from a chicken, reared under commercial conditions, was randomly selected for the pilot study. Approximately 0.2 g of caecal contents was weighed and MRD was added to give a 10% suspension. The suspension was mixed, and then serial dilutions were prepared to 10^{-4}. Three types of media were used: MRS, Mac-03 and m-XLD. The agar plates were supplemented with trimethoprim or lincomycin used in three different concentrations. For trimethoprim these were 5, 10 and 20 µg/ml. For lincomycin these were 30, 50, 70 µg/ml. Each serial dilution was inoculated on to each of the three different media, with three different concentrations, of the two different antibiotics. The workflow illustrated in Figure 2.1.

2.3.4 Trial 1 design (Biosecure or controlled housing birds, CH)

On arrival at the BSU, the 112 birds were randomly penned at two rooms. Control birds (non-infected) in Room 1 in two groups, G1 (standard feed or non-GOS feed) and G2 (standard feed supplemented by GOS). Birds to be colonised

with *Salmonella* were penned in Room 2 also in two groups, G3 (standard feed) and G4 (GOS feed). Feed and water were supplied *ad libitum* with alternating light and dark regimes of 12 hours.

The birds were fed starter standard diet (G1 and G3) or standard diet with 3% w/w GOS (G2 and G4), until day 10 da. Groups G1 and G3 were then changed to standard grower diet while groups G2 and G4 were fed GOS grower diet, where the amount of GOS in the diet was reduced to 1.5% w/w, for economic reasons. At 22 da all birds in G1, G2, G3 and G4 were fed a standard finisher diet until 35 da. Birds in groups G3 and G4 were given 4.6 $x10^8$ CFU/ml *Salmonella* Enteritidis 125109 (nalidixic acid resistant), at 20 da, by oral gavage, while birds in G1 and G2 were given an equivalent mock dose of MRD. Each group contained at least seven birds per sample point to be able to measure a significant 1 $\log_{10}$ reduction in intestinal *Salmonella* colonisation at 95% confidence. During the experiment, temperature and humidity were controlled in both rooms. Euthanasia of birds were carried out by exposure to rising CO_2 gas according to Schedule 1 of the UK Animals (Scientific Procedures) Act. All birds remained healthy throughout the trial.

Table 2.2 Experimental design of Trial 1

Groups	Diet	Sampling date and Bird number
Group 1	Uninfected / standard feed	(n = 7 at 22, 24, 28 and 35 da, total n = 35 birds)
Group 2	Uninfected/GOS feed	(n = 7 at 22, 24, 28 and 35 da, total n = 35 birds)
Group 3	*Salmonella* challenge/ standard feed	(n = 7 at 22, 24, 28 and 35 da, total n = 35 birds)
Group 4	*Salmonella* challenge/ GOS feed	(n = 7 at 22, 24, 28 and 35 da, total n = 35 birds)

2.3.5 Trial 2 design using commercial birds

A total of 40 Ross 308 broiler chickens were obtained from a commercial source (Moy Park, UK), 20 birds fed standard control diet (ctl), and 20 fed galacto-oligosaccharides diet (GOS). Birds were placed in the barn as day olds and brooded at a temperature of 32°C and relative humidity of 60% to 70%. During rearing, starter GOS diet (3% w/w) or starter control diet was given until 11 da then the diet was changed to grower control or a GOS diet with less GOS (1.5% w/w). At 22 da all birds were fed control diet until 37 da. The chickens were raised in barns in commercial conditions and GOS fed birds kept separately from control birds. The thinning process (reducing the number of birds to allow for more space or a proportion of birds are transported or taken from the main flock to lower stocking density before birds are finally removed for slaughter) was carried out at 30 da and 20 birds collected at random. The birds were transferred to The University of Nottingham where the chickens were humanely euthanized by carbon dioxide asphyxiation. Caecal content and tissue samples were collected for further analysis. The samples were stored at -80°C until processed. Both experiments were performed according to the guidance of The Institutional Animal Care and Use Committee at the UK, and it was approved by the Local Ethics Committee in the University of Nottingham and achieved under Home Office license.

Table 2.3 Trial 2 commercial chicken experiment design

Groups	Diet	Sampling date and Bird number
G1	standard feed	(n = 10 birds, at 30 da)
G2	GOS control feed	(n = 10 birds, at 30 da)
G3	standard feed	(n = 10 birds, at 37 da)
G4	GOS control feed	(n = 10 birds, at 37 da)

2.3.6 Feed composition and quality control

The starter diet comprised of wheat (59.9% [wt/wt]), soya meal (32.5% [wt/wt]), soyabean oil (3.65% [wt/wt]), limestone (0.6% [wt/wt]), calcium phosphate (1.59% [wt/wt]), sodium bicarbonate (0.27% [wt/wt]), the enzymes phytase and xylanase (dosed according to the manufacturer's instructions; DSM Nutritional Products Ltd., Basel, Switzerland), and a vitamin mix containing salt, lysine hydrochloride, DL-methionine, and threonine. The wheat content has increased in both the grower and finisher diets at the expense of soya meal by 2 and 5% (wt/wt), respectively. The prebiotic GOS was supplied as Nutrabiotic (GOS, 74% [wt/wt] dry matter, Dairy Crest Ltd., Davidstow, Cornwall, UK). The preparations of GOS contained a mixture of monosaccharides (glucose and galactose) and oligosaccharides (DP2 to DP8). The disaccharide lactose, a reactant in the manufacture of galacto-oligosaccharides, is not a galacto-oligosaccharide otherwise all other disaccharides and longer oligosaccharides (DP3+) are galacto-oligosaccharides and nondigestible. The starter feed was supplemented with 3.37% (wt/wt) GOS and isocaloric adjustments made in the wheat (54% [wt/wt]) and soybean oil (4.88% [wt/wt]) contents. The grower and finisher feeds contained 1.685% GOS with respective adjusted wheat contents of 57.7% (wt/wt)

and 63.3% (wt/wt) and soybean oil contents of 6.14% (wt/wt) and 6.22% (wt/wt). The final feeds were isocaloric (metabolizable energy including enzyme contribution) and contained the same crude protein levels and Degussa poultry digestible amino acid values which are lysine, methionine, methionine plus cysteine, threonine, tryptophan, isoleucine, valine, histidine, and arginine (Richards et al., 2020).

2.4 Sample collection and treatment

The procedure of treating all samples is summarised in Figure 2.2.

2.4.1 Caecal contents

Approximately one gram of caecal content was immediately transferred into cryovial tubes, frozen in liquid nitrogen and then stored at -80°C for genomic DNA extraction. Another one gram of fresh material was collected from caeca combined into pre-weighed universals. Each collected sample was diluted in MRD to give a 10 % w/v suspension. The sample was then serially 10-fold diluted (20 µl to 180 µl) in the same medium to 10^{-5} in 96 well plates. Aliquots of 100 µl of appropriate dilutions (detailed below) were spread on the appropriate different agar plates (detailed below) in triplicate and incubated under the appropriate conditions (detailed below). The average values of colony-forming units (CFU) were used for the statistical analysis. Colonies was counted after overnight incubation by coulter counter (Digital colony counter, Stuart SC6 Colony counter

- Stuart Equipment, protected by BioCote, UK). The CFU/ml was calculated using following formula,

CFU ml^{-1} = (number of colonies x dilution factor) / sample volume

2.4.1.1 Enumeration of coliforms and trimethoprim resistant coliforms in caecal contents

For coliform enumeration (this include counts of lactose fermenters and non-lactose fermenting bacteria), 100 µl of serial dilutions 10^{-4}-10^{-6} were spread on Mac-03. For trimethoprim resistant coliforms, 100 µl of dilutions 10^{-1}-10^{-4} were spread on Mac-03 with trimethoprim (20 µg/ml). Both types of plate were incubated under aerobic conditions at 37°C for 24 h before colonies were counted. Mac-03 media contain crystal violet and bile salts as selective agents for preventing the growth of Gram-positive bacteria.

2.4.1.2 Salmonella enumeration in caecal contents modified from Atterbury et al. (2007)

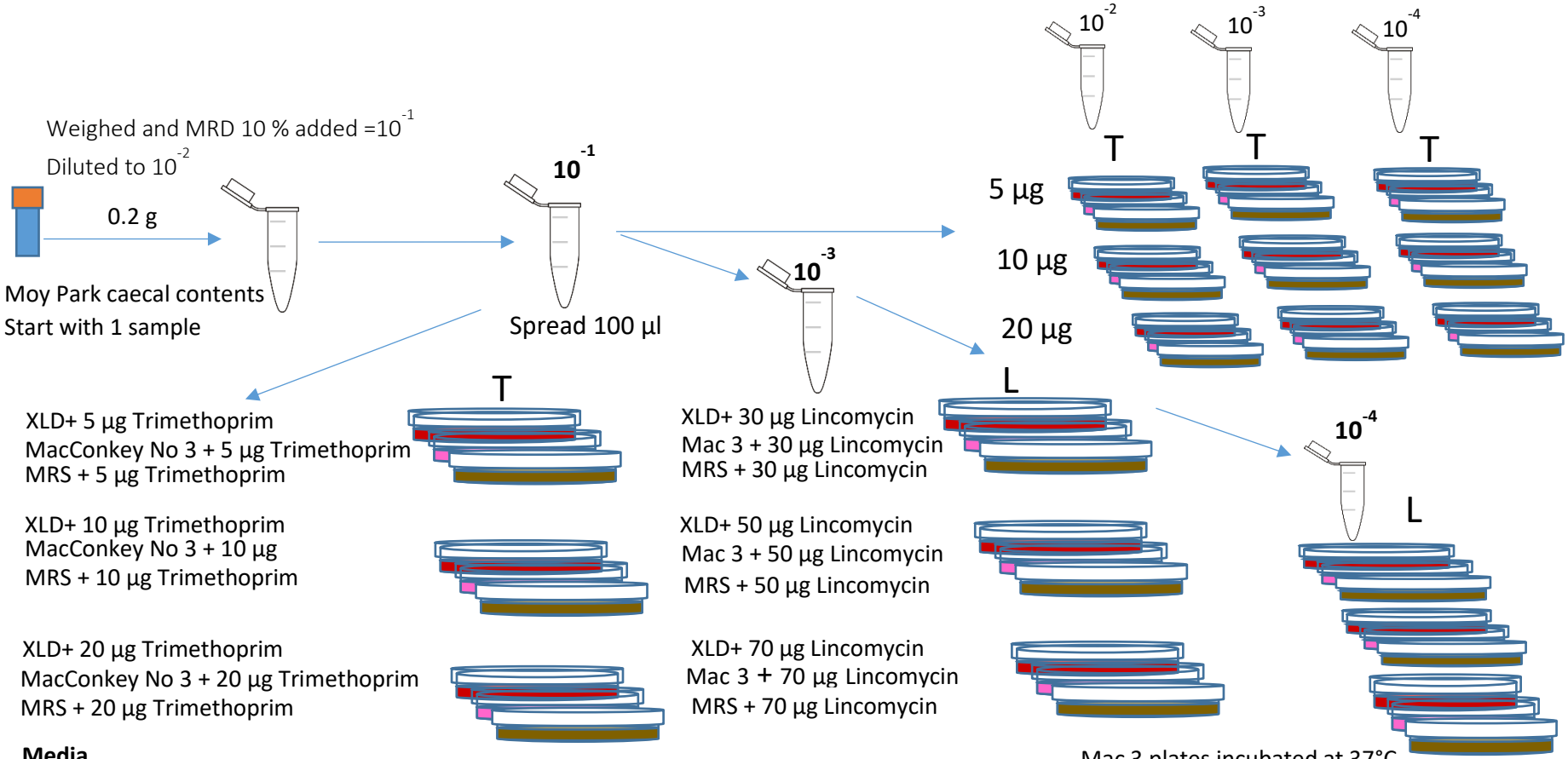
For *Salmonella* enumeration, 100 µl of serial dilutions 10^{-2} to 10^{-5} were spread in triplicate onto m-XLD plates (XLD with 1 µg/ml of Novobiocin and 12.5 µg/ml Nalidixic acid added) and incubated at 37°C for 24 h. Before counting, a confirmation test was done on representative *Salmonella* colonies (typically black) by slide agglutination tests with Poly O, and O9 serotype-specific antisera (Pro-Lab Diagnostics, Cheshire, United Kingdom).

2.4.1.3 Salmonella enrichment from caecal contents

To ensure *Salmonella* were not present in the non-infected control groups, pooled swabs of approximately 0.1 g of caecal contents were transferred to 50 ml BPW (Appendix 1.2.1) for *Salmonella* enrichment for 16-20 h at 37 °C. Three 100 µl aliquots of the incubated BPW suspension were dispensed on to MSRV plates and incubated without inversion at 42°C for 24 h. Any growth was sub-cultured to XLD plates (with no nalidixic acid or novobiocin added) and incubated at 37°C. If any suspected colonies were identified they were checked with Poly O antiserum as described in Section 2.2.2.

2.4.2 Liver and spleen

Approximately 1-5 g of liver and spleen tissues were added to weighed stomacher bags, reweighed and BPW to 10 % w/v was added. The bag was sealed, and the contents stomached for 1 min on medium speed, in a Seward Stomacher® 80 (Seward Biomaster; UK). All bags were incubated at 37°C for 16-20 h. For enumeration, 100 µl of the 10 % tissue suspension was spread on m-XLD incubated at 37°C for 24 h. For enrichment three 100 µl aliquots of the incubated tissue BPW suspension were dispensed onto MSRV plates and incubated without inversion, as mentioned in Appendix 1.1.3



Media

500 ml of each media was made. 25 ml of molten agar was transferred to universals kept war in water bath, and antibiotics at different were added and quickly media pour into Petri dish.

Figure 2.1 Pre-trial experiment for detecting the suitable antibiotics concentration and dilution factor. The Figure was obtained from Philippa Connerton.

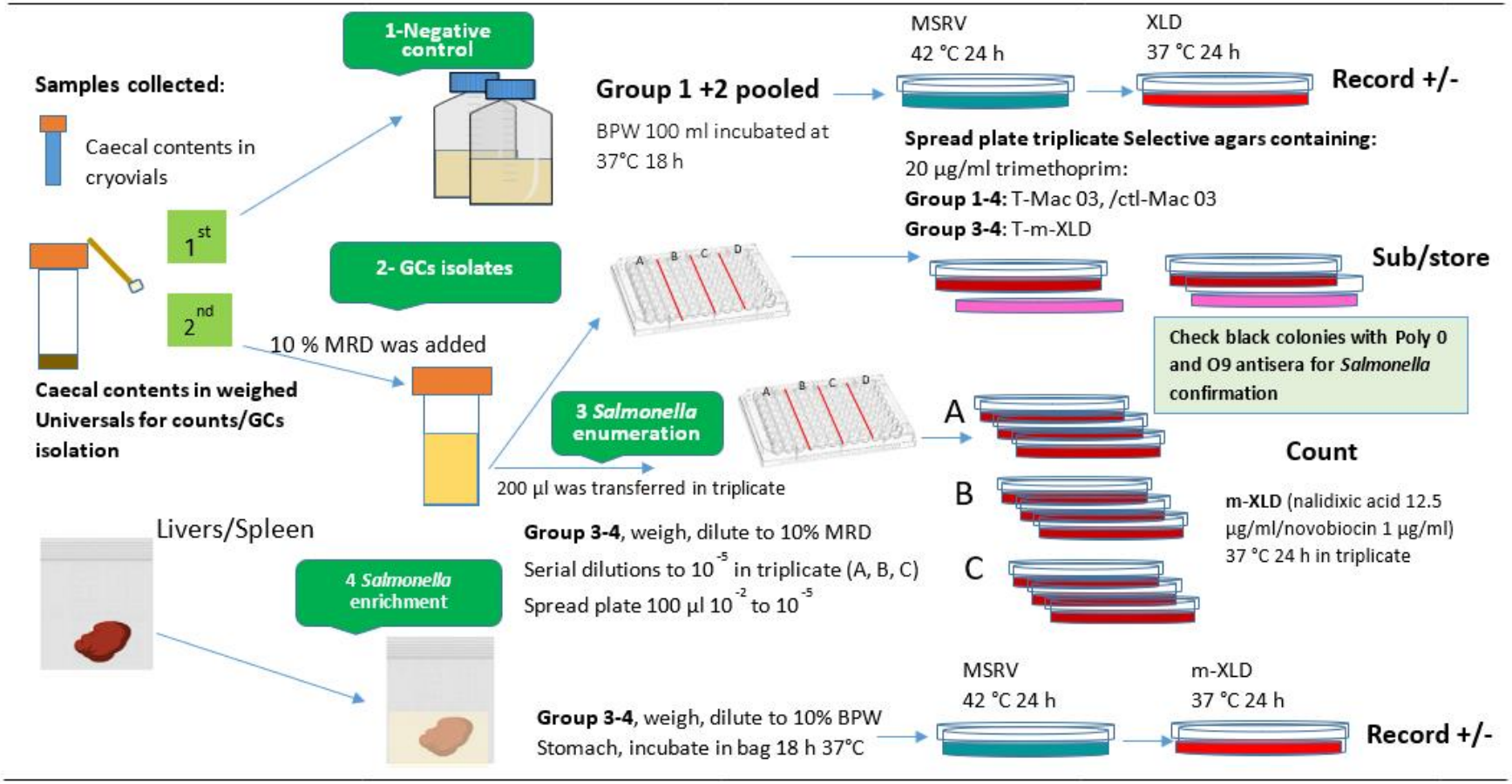


Figure 2.2 Summary of workflow in Trial 1 (controlled housed birds) for studying the effect of GOS diet on *Salmonella* colonisation, persistence, and antibiotic resistance genes. The Figure was obtained from Philippa Connerton.

2.5 Nucleic acid extraction and analysis

2.5.1 Gel electrophoresis

2.5.1.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for routine analysing of DNA fragments produced from PCR in this thesis. To prepare an agarose gel, 0.8% or 1% w/v agarose (Sigma-Aldrich) was firstly added to 100ml of 1x TAE buffer (40 mM tris-acetate adjusted with glacial acetic acid to pH 8, 1 mM disodium ethylene-diamine-tetra-acetic acid) in a 250ml sterile conical flask. This mixture was completely melted by microwave and kept until it cooled to approximately 50°C with gentle mixing every 5 min. Ethidium bromide (Fisher) was added at a final concentration of 0.4 µg/ml and gently mixed by swirling the flask. This mixture was poured into a pre-assembled casting tray provided with desired comb. The gel was allowed to solidify at room temperature for approximately 20-30 min, and the rubber dams and comb were carefully removed from the casting tray. The tray with gel was then placed in a gel electrophoresis tank containing TAE buffer, and DNA samples were loaded into the wells. In order to estimate the size of DNA samples either a 100 bp or 1 kb DNA ladder (New England Biolabs, UK) were also loaded to wells. These samples were run at 75V-80V for approximately 45-60 min until bromophenol blue dye reached ¾ length of the gel. Finally, samples were visualised under UV light using gel documentation system (Gel Doc XR+ System, Bio-Rad, Hertfordshire, UK).

2.5.2 Genomic DNA isolation

2.5.2.1 GenElute Bacterial Genomic DNA kit

Genomic DNA of gram-negative bacteria (*S. enterica* U288, *S. enterica,* P125109 and *E. coli* J53 pMG101 and trimethoprim resistance isolates) were extracted from cells using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) according to manufacturer's instructions. The overnight growth of bacterial cells was harvested from LB agar plates or nutrient ager (Appendix 1.1.6 and 1.1.8) and suspended in 1.5 ml sterile RO water using a sterile cotton swab. The suspension was centrifuged in a bench top centrifuge (HETTICH MIKRO 185; Germany) at 12,000 x *g* for two min. The supernatant was removed, and the pellet was thoroughly re-suspended in 180 µl of Lysis Solution T followed by addition of 20 µl of 20 mg/ml DNase-free RNase. Then, 20 µl of 20 mg/ml proteinase K (Sigma-Aldrich / Appendix 1.1.13) was added and this suspension was incubated in heat block (Grant QBT2; UK) for 30 min at 55°C. After incubation, 200 µl of Lysis Solution C was added to the mixture and thoroughly vortexed for 15 s followed by re-incubating at 55°C for 10 min. During incubation, 500 µl of column preparation solution was added to the column to maximize DNA binding to the membrane and centrifuged at 12,000 x *g* and the flow-through was discarded. After incubation of lysed suspension, 200 µl of 100% ethanol was added and thoroughly vortexed for 10 s. Then, the homogenous suspension was transferred into the binding column by using a wide-bore pipette to avoid shearing DNA and the column was then centrifuged at 6,500 x *g* for 1 min. The flow-through was discarded and 500 µl of First Wash Solution was transferred into the column. The column was then centrifuged at 6,500 x *g* for 1 min and the flow-through was discarded. Then, 500µl of Second Wash Solution was added into the column and it was centrifuged at 12,000 x *g* for3 min. The collection tube was discarded and was replaced with a new tube. An aliquot of 100 µl of Elution Solution was loaded into the centre of column and left for 1-5 min to elute the DNA. The column was

then centrifuged at 12,000 x *g* for 1 min and the flow-through was collected. Extracted DNA concentration was measured using a Nano-drop ND1000 spectrophotometer (ThermoScientific, Wilmington, USA). Finally, isolated genomic DNA was stored in a-20°C freezer for long term storage.

2.5.2.2 DNA extraction from poultry caecal contents (Trial 2) by MP Biomedicals Fast DNA SPIN Kit

Total genomic DNA was extracted from approximately 250 mg of total caecal contents samples using the Fast DNA Spin extraction kit (MP Biomedicals; USA) according to manufacturer's instructions protocol. Approximately 250 mg of caecal contents was weighted into a 2 ml Lysing Matrix E tube, 825 µl of Sodium Phosphate Buffer was added followed by 275 µl of PLS solution. The mixture was then vortexed for 10-15 s. Sample tubes were centrifuged at 14,000 x *g* for 5 min and the supernatant aseptically decanted. An aliquot of 978 µl of sodium phosphate buffer was added followed by 122 µl of MT Buffer. The mixture was vigorously mixed by vortexing, and samples homogenized in the FastPrep® 24 5G instrument set at 6.0 m/s for 40 s. Samples were centrifuged at 14,000 x *g* for 5 min and supernatant transferred to a clean 2.0 ml centrifuge tube. Then, 250 µl of PPS solution was added, vigorously mixed (not vortexed), and incubated at 4°C for 10 min. After incubation, samples were centrifuged at 14,000 x *g* for 15 min. During centrifuging, 1 ml of binding matrix solution was mixed and added to a clean 2 mL conical tube (not supplied). The supernatants from the samples were then transfered to the binding matrix solution in the 2 mL conical tube, then tubes gently mixed by hand for 3-5 min. All tubes were centrifuged at 14,000 x *g* for 2 min and the supernatant decanted. The binding mixture pellet was washed by re-suspending with 1 ml Wash Buffer 1. The following step required two centrifugation steps. First, approximately 600 µL of the binding mixture was transferred to a SPIN Filter tube and centrifuge at 14,000 *g* for 1 min. The Catch tube was emptied, and the remaining binding mixture was added to the SPIN Filter tube and

centrifuged as before. The Catch tube was emptied again and 500 µl of prepared Wash Buffer 2 was added to the SPIN Filter tube and gently re-suspend using the force of the liquid from the pipette tip to re-suspend the pellet without vortexing tubes. Samples were centrifuged at 14,000 x *g* for 2 min and flow-through discarded. Residual ethanol was extracted by centrifuging the sample again for 2 min to dry the sample from the binding matrix. The SPIN Filter was transferred to a clean 1.9 ml Catch tube. The pellet was suspended in 60-100 µl of TES. Samples were centrifuged at 14,000 x *g* for 2 min to elute the purified DNA into the clean Catch tube. The SPIN filter was discarded, and DNA stored at -20°C (for extended periods) or 4°C until use. The purity and concentration of the extracted DNA was measured on the Nanodrop spectrophotometer.

2.5.2.3 DNA extraction from poultry caecal contents (Trial 1) by QIAcube HT

The majority of DNA samples in Trial 1 were extracted by QIAcube® HT (Qiagen; UK) using the QIAamp Power Faecal protocol. In brief, 100 mg of caecal sample was weighed into a Pathogen Lysis Tube L (cat. no.19092). Then, 650 µl pre-warmed Buffer PW1 was added to each sample. Each sample was thoroughly homogenized twice by FastPrep® 24 5G instrument (MP, USA) set at 6.5m/s for 45 s separated by 5 min. Then samples were centrifuged at 14,000 x *g* for 1 min. Approximately 400-µl supernatant from previous step was pipetted into a new tube, avoiding transferring any solid material, re-centrifuged the sample again. Then, 150 µl of Buffer C3 was added to the supernatants and mixed thoroughly by carefully vortexing or pipetting and incubated for 5 min at 4 °C. All samples were centrifuged at 14,000 x *g* for 1 min. For each sample, 20 µl Proteinase K was added to a new S-Block well. Then, 300 µl of each supernatant from previous step was transferred to these wells, mixed and incubated for 10 min at room temperature (15–25°C). Finally, the S-Block was placed in the QIAcube HT and the QIAamp DNA Protocol followed.

2.5.2.4 Manual DNA isolation for poultry caecal contents (Trial 1) by QIAamp® PowerFecal® DNA Kit.

Some samples fail to yield DNA by the QIAcube HT method, therefore DNA from these samples was manually isolated. Approximately 0.10 g of caecal contents were weighted into the Bead Tube. Then, 750 µl of Power Bead Solution was added. followed by addition of 60 µl of Solution C1 and vortexed briefly. Then, the tubes were heated at 65°C for 10 min. Secured tubes were horizontally Vortexed using an adapter (cat. no. 13000–V1–24) at maximum speed, for 10 min. The tubes then were centrifuged at 13,000 x *g* for 1 min. The supernatant (between 400 to 500 µl) was transferred to a clean 2 ml Collection Tube. Then, 250 µl of Solution C2 was added and mixing by brief vortexing. The tubes were incubated at 2–8°C for 5 min. The tubes then were centrifuged at 13,000 x *g* for 1 min. Supernatant (600 µl) was transferred to a clean 2 ml Collection Tube avoiding disturbing the pellet. Then, 200 µl of Solution C3 was added and vortexed briefly. The tubes were incubated at 2–8°C for 5 min. The tubes were centrifuged at 13,000 x *g* for 1 min. The supernatant was transferred (not more than 750 µl) to a clean 2 ml collection tube, avoiding the pellet. An aliquot of 1200 µl of Solution C4 was added to the supernatant and vortexed for 5s. Then, 650 µl of each supernatant was loaded onto an MB Spin column and centrifuged at 13,000 x *g* for 1 min. The flow-through was discarded and this was repeated until all the supernatants had been finished. After that, 500 µl of Solution C5 was added and centrifuged for 1 min at 13,000 x *g*. The flow-through was discarded and centrifuged again for 1 min at 13,000 x *g*. The MB Spin Column were placed in a clean 2 ml collection tube. An aliquot of 100 µl of Solution C6 was added to the centre of the white filter membrane and centrifuged at 13,000 x *g* for 1 min and the MB Spin Column was discard. The DNA samples were stored in a –20 °C freezer.

2.5.2.5 Wizard genomic DNA purification Kit

PCR amplification products of gene cassettes were isolated using Wizard Genomic DNA purification kit (Promega, UK) according to manufacturer's instructions. After running the PCR products on 0.8% agarose gel, the required size bands (Approximately 2000 bp) were excised in a minimal amount of agarose on a UV wavelength transilluminator, and then placed into weighed 1.5 ml microcentrifuge tube. All tubes were re-weighed, and the weight of the excised DNA gel slices calculated. Then an equal volume of membrane binding solution was added (approximately 10 µl of membrane Binding Solution was added per 10 mg of agarose gel fragment) then vortexed and incubated at 50–65°C in heat block (Grant QBT2; UK) for 10 min or until the gel slice was thoroughly dissolved. During incubation, all tubes were briefly vortexed to maximize the rate of agarose gel melting. After incubation, all samples were briefly centrifuged to ensure that the DNA particles were at the bottom of the tube. Dissolved DNA was transferred into SV Minicolumns in a single Collection Tube for each dissolved gel slice and incubated for 1 min at room temperature.

The SV Minicolumn was centrifuged at 16,000 × *g* for 1 min and flow-through collection tubes discarded. The column was washed by adding 700 µl of previously diluted membrane wash solution (with 95% ethanol) to the SV Minicolumn. The SV Minicolumn was centrifuged for 1 min at 16,000 × *g* and the collection tube was emptied. A second wash was performed by adding 500 µl of Membrane Wash Solution to the SV Minicolumn and centrifuging for 5 min at 16,000 × *g*. The SV Minicolumn and the Collection Tube was emptied and re-centrifuged for 1 min with opened lid to allow evaporation of any residual ethanol. The SV Minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of Nuclease-Free Water was added directly into the centre of the column without touching the membrane with the pipette tip. The column was incubated at room temperature for 1 min and then centrifuged for 1 min at 16,000 × *g*. The SV Minicolumn was

discarded, and the elution containing DNA in the microcentrifuge tube was stored at 4°C or –20°C. All genomic DNAs were stored at -20°C for long term storage.

2.5.3 Polymerase Chain Reaction (PCR)

2.5.3.1 PCR Primers

The PCR oligonucleotides designed in this thesis are listed in Tables 2.5 and 2.6. Generally, the length of all designed oligonucleotides was in ideal range of 18–22 base pairs, melting temperature in the range from 48 to 66°C, the content of GC between 40 and 70%, and usually termination of primers was either a guanine or cytosine residue at the 3' end unless they were obtained from another resource. After designing the primers, all primers were checked by oligo analyser (Integrated DNA Technologies) for possible formation of stable primer secondary structures, self-dimer and hairpins. For calculating the melting temperature for each primer, the equation below is used,

Tm = 4°C (G+C) + 2°C (A+T)

Primers applied in this thesis were synthesised by Eurofins MWG Operon (Ebersberg, Germany). All oligonucleotides for GCs listed in this thesis were designed based homogeneity of the majority of sequence data reads obtained from first primer reads and then divided into groups based on that.

2.5.3.2 PCR running conditions

The DreamTaq Green 2x PCR Master Mix (ThermoScientific) was used in all PCR reactions carried out in a BIOER XP thermal cycler. The DreamTaq DNA Master Mix containing of 2 U of polymerase, 2x DreamTaq Green buffer, 0.4 mM of dNTPs each and 4 mM of magnesium chloride. Each PCR reaction (25 µl) contained 12.5 µl of 2× DreamTaq Green PCR Master Mix (ThermoScientific) mixed with approximately 1 µl of ±50 ng DNA template; 0.5 µM forward and reverse primers; and 9 µl water. In general, the PCR setting conditions for each reaction were

carried out as follows: initial denaturation at 95°C for 5 min; 35 cycles of PCR amplification including denaturation at 95°C for 30 s, annealing routinely set at 64°C unless otherwise indicated for 30 s, extension at 72°C for 1 or 3 min; and the reaction ended with a final extension at 72°C for 5 min. The extension time through PCR amplification cycles was adjusted depending on the size of amplified product, with 1 min per kilo base pair of PCR product. Agarose gel electrophoresis (Section, 2.5.1.1) for running amplified PCR products was carried out to verify product size either using a DNA standard ladder and taking a digital image or to cut out desired bands for purification. PCR products were stored in -20°C.

2.5.3.3 Real time quantitative PCR (RT-qPCR)

The Quantitative PCR was preformed using PowerUp SYBR Green Master Mix (ThermoFisher) according to manufacturer's instructions. In brief, a mixture of (0.5 µl, 1x) 10pmol forward (ACGAGCGCAAGGTTTCGGT) and reverse oligonucleotides (CGTTCCATACAGAAGCTGG) target specific location in integrase gene added to (5 µl, 1x) PowerUp SYBR Green Master Mix (ThermoFisher) and (3 µl, 1x) PCR water (Invitrogen) in a total volume of 10 µl, then 9 µl of this mixture was dispensed into 384-Well white qPCR Plate. One µl of extracted DNA samples (described in 2.5.2.3) transferred into required well into the plate. The plate then was sealed and mixed briefly three times for few second by mini spinner centrifuge (Axygen; ThermoFisher). The q-PCR reaction was carried out using a Light Cycler 480 instrument (Roche). The qPCR conditions are setting as follow: denaturation programme at 95°C for 5 min; followed by amplification programme including 45 repeated cycles of denaturation at 95°C for 15 s, annealing at 66°C for 1 min; and then one cycle of melting curve including incubation at 95°C for 5 s, 65°C for one minute and 97°C for 30 s with a continuous fluorescence measurement; and finally the run was finished by cooling programme to 40°C for 10 s. All results were analysed using Light Cycler 480 software for detecting the cycle threshold value

(Ct value) as well as absolute quantification of integrase expression value which was then calculated based on control results.

2.5.4 Class 1 integron gene detection

The presence of class 1 integrons were determined using PCR primers that detect three conserved sequences of class 1 integrons which are: *intI1*, *qacEΔ1* and *sul1*. The oligonucleotides pairs were chosen using data from published papers which are outlined in Table 2.4 (Holmes et al., 2003;Ebner et al., 2004;Gillings et al., 2015;Ravi et al., 2015) and purchased from Eurofins (Ebersberg; Germany). Each PCR reaction (total volume 25 µl) contained 2× DreamTaq Green PCR Master Mix (ThermoScientific, Wilmington, USA); 0.5 µM forward and reverse primers; 1 µl of sample DNA and the remainder PCR grade water (Roche; 03315959001; Germany). The PCR reaction was run on XP Thermocycler, (Bio-Rad, UK). The control template DNA was prepared by extracting DNA from *S*. typhimurium U288 that carries class 1 integrons, using the GenEluteTM Bacterial Genomic DNA Kit for gram-negative bacteria (Sigma–Aldrich, UK) in accordance with the manufacturer's instructions (see 2.5.2.1). The extracted DNA was diluted to 50 ng/ul and were used immediately for PCR. The PCR conditions were: 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 5 min. The PCR products separated by agarose gel electrophoresis using 1 % agarose (Pharmacia Fine Chemicals, UK) in TAE buffer and stained with ethidium bromide for visualisation by UV Transilluminator (Bio-Rad, UK).

Table 2.4 Primers used for detecting three conserved sequences of class 1 integrons

Primer	Sequence (5'-3')	Gene Target	product size (bp)	Reference
F-*intI1* Ravi R-*inti1* Ravi	ACGAGCGCAAGGTTTCGGT GAAAGGTCTGGTCATACATG	*intI*1 gene	546	(Ravi et al., 2015)
F-*intI1* EB R-*intI1* EB	CCTTCGAATGCTGTAACCGC ACGCCCTTGAGCGGAAGTATC	*intI*1 gene	254	(Ebner et al., 2004)
F-HS464 R-HS463a	ACATGCGTGTAAATCATCGTCG CTGGATTTCGATCACGGCACG	*intI*1 gene	471	(Holmes et al., 2003)
Intl1 F165 *Intl1* R476	CGAACGAGTGGCGGAGGGTG TACCCGAGAGCTTGGCACCCA	Clinical Integron	311	(Gillings et al., 2015)
F-*sul1* *R-sul1*	ATCAGACGTCGTGGATGTCG CGAAGAACCGCACAATCTCG	Sulfonamide Resistance	346	(Ebner et al., 2004)
F-*qacE*Δ1 R-*qacE*Δ1	GAGGGCTTTACTAAGCTTGC ATACCTACAAAGCCCCACGC	Quaternary Ammonium Compound Resistance	200	(Ebner et al., 2004)

2.5.5 Amplification and mapping of gene cassettes (GCs)

The amplification of inserted gene cassettes was performed by long range PCR (XP Thermocycler, BioRad; UK). Firstly, four primer pairs were tested to start the amplification of GCs with gradient PCR to detect optimum conditions summarised in Table 2.5. Secondly, specific walk primers, that flanking the integrated gene cassettes from the 5' and 3' conserved ends of class 1 integrons, were designed and are shown in Table 2.6 and 2.7. Each PCR reaction (25 µl) contained 2× DreamTaq Green PCR Master Mix (ThermoScientific); 0.5 µM forward and reverse primers; 1 µl of sample DNA and PCR grade water (Roche). The *S. enterica* Typhimurium U288 genomic DNA was used as a positive control and water as a negative control. PCR conditions were 95 °C for 5 min and 35 cycles of 95°C for 30 s, 64 °C for 30s, 72 °C for 3 min and final extension 72 °C for 5 min. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System kit

(2.5.2.5) and then the purified DNA sequenced by the Eurofins MWG Value Read service. The resulting sequences reads were organised and grouped by using BioEdit (Sequence Alignment Editor version 7.0.5.3), then the reads were assembled into contigs using CLC Work Bench by Prof Ian Connerton, under the default settings and then submitted to the NCBI database using the BlastN search program, for identifying each gene.

Table 2.5 Primers tested for identifying integrated gene cassette sequences of class 1 integrons

Gene	Sequence	Product size	Reference
F-Ravi-long	GGCATCCAAGCAGCAAG	na	(Ravi et al., 2015)
R-Ravi-long	AAGCAGACTTGACCTGA		
F-MRG284	GTTACGCCGTGGGTCGATG	n/a	(Gillings et al., 2009)
R-MRG285	CCAGAGCAGCCGTAGAGC		
F-ntf2	ACACCGTGGAAACGGATGAAG	n/a	(Ebner et al., 2004)
R-qcr2	ACCGATTATGACAACGGCGG		
F-Ravi	ACGAGCGCAAGGTTTCGGT	n/a	(Ravi et al., 2015)
R-qacEΔ1	ATACCTACAAAGCCCCACGC		(Ebner et al., 2004)

Table 2.6 Primers used for walking in integrated gene cassette sequences of class 1 integrons obtained from biosecure broiler chicken caecal samples (Trial 1)

Primer	Sequence	Product size	Reference
F-ntf2	ACACCGTGGAAACGGATGAAG	n/a	Ebner *et al.,* 2004
F2-GCs-B	CTGTTGGTTGGACGCAAGAC	n/a	This study
F2-GCs-F	ACCATGCGAAAGCTCAATACTC	n/a	This study
F2-GCs-C	ATTCTTGCAGGTATCTTCGAGC	n/a	This study
F3-GCs-B-1	AGGTTTGCGATCCGCTGTG	n/a	This study
F3-GCs-B-2	GAGCTTTGATCAACGACC	n/a	This study
F3-GCs-C	GCTGGCTTTTTCTTGTTATC	n/a	This study
R2-GCs-B-1	CAAATTGCAGTTCTCGCT	n/a	This study
R-qcr2	ACCGATTATGACAACGGCGG	n/a	Ebner *et al.,* 2004

Table 2.7 Primers used for walking in integrated gene cassette sequences of class 1 integrons in commercial broiler chicken caecal samples (Trial 2)

Primer	Sequence	Product size	Reference
F-ntf2	ACACCGTGGAAACGGATGAAG	n/a	Ebner *et al.,* 2004
F2-GCs-A	CAGCCATGATCGACATTGATC	n/a	This study
F2-GCs-B	GTTAACCTCTGAGGAAGAATTG	n/a	This study
F2-GCs-C	CAACGATGTTACGCAGCAGG	n/a	This study
F3-GCs-A1	GTTCTCTGATATCGAATTCGC	n/a	This study
F3-GCs-A2	AGAAGATCACTTGGCCTCAC	n/a	This study
F3-GCs-B	CATCATGAGGGAAGCGGTG	n/a	This study
F3-GCs-C	CCACGATCGACATTGATCTG	n/a	This study
F4-GCs-B	ATCGCTTGGCCTCGCGCGC	n/a	This study
R-qcr2	ACCGATTATGACAACGGCGG	n/a	Ebner *et al.,* 2004

2.5.6 16S rRNA Gene sequences and analysis

The amplification of 16S rRNA was carried out by using specific primers: 515f (50 GTGCCAGCMGCCGCGGTAA 30) and 806r (50 GGACTACHVGGGTWTCTAAT 30) that are designed to flank the hypervariable V4 region of the bacterial 16S rRNA genes (Caporaso et al., 2011). Amplicons were sequenced on the Illumina MiSeq platform using 2 × 250 bp cycles according to the manufacturer's instructions by Source Bioscience, Nottingham UK. All sequences data of the 16S rRNA gene were quality filtered and then clustered into operational taxonomic units (OTUs) by Mothur (V1.39.5, Schloss et al., 2009) through the Schloss lab. MiSeq SOP2 (Kozich et al., 2013).

The functions required to perform analysis pipeline are available on the Mothur website (http://www.mothur.org/wiki/MiSeq_SOP) 22 and it was carried out following the approach of (Kopylova et al., 2016) and (Schloss, 2016). In brief, sequences paired reads was assembled into contigs and ambiguous bases of any contigs was removed or picked out. Then, sequences were aligned against the SILVA reference database, and any incorrect alignment (non-bacterial origin) of sequences were picked out. The ends of the sequences were trimmed in order to start and end at the same alignment position of all sequences (Schloss, 2013). Then, identification of the unique sequences and their frequency per each amplicon was carried out. De-noise sequences of each amplicon were performed by using pre-clustering algorithm (Schloss et al., 2011). To decrease sequences errors, the sequences were checked for chimeras using VSEARCH. Classification of each sequence was implemented against the Ribosomal Database Project (RDP) 16S rRNA gene training set by a naive Bayesian classifier used to classify (version 14) which required 80% confidence score (Schloss et al., 2011). This classifier was customised to cover rRNA gene sequences of mitochondria and *Eukaryota* in order to exclude any undesirable sequences that classified as *Archaea*, *Eukaryota*, chloroplasts, or mitochondria. Finally, sequences were categorised into groups according to their taxonomic level of phylum and assigned to operational

taxonomic units (OTUs) at a 3% dissimilarity level. Coverage was calculated in mothur by Good's coverage at 98%. Bacterial sequences were normalised to equal sequence counts, and these normalised OTU tables were used in all further analysis (Westcott and Schloss, 2017).

The α- and β-diversity were analysed using Mothur (V1.39.5). The alpha diversity was assessed by Chao index for OTU richness, while the overall diversity (evenness) was measured with the inverse Simpson's index. Differences in α -diversity was tested using Wilcoxon rank sum test. For the Beta-diversity, assessed by metric calculated by Mothur and significance was tested by using analysis of molecular variance (AMOVA) implemented also by Mothur. Distribution of gene cassettes was presented by phylogenetic tree of Gram-negative bacteria (Proteobacteria) and rarefaction curve was constructed by Mothur. Linear discriminant analysis effect size (LefSe) was also performed by Mothur.

2.5.7 Bioinformatics software utilised

To assemble and analyse all sequencing data generated from 16S RNA as well as whole metagenome sequence, several bioinformatics techniques were used. Bioinformatics software utilised throughout the study are listed Table 2.8.

2.5.8 Statistical analysis

All data that related to bacterial count first converted to log $_{10}$ (cfu/ml) for bacterial counts and treated by parametric statistics using T. test. All the results related to profiling microbiota were tested for normality using the Shapiro-Wilk test and expressed as bar charts by using median or percentage or box plots. Analysis of significance was carried out based on normality test results by non-parametric tests (Mann Whitney U test for unpaired data or Wilcoxon test). Each data was compared to another to show if there are any differences between the mean or ranks. Statistical analysis was carried out using GraphPad Prism V8

(GraphPad Software, Inc, CA, USA) and Microsoft® Excel 2016/XLSTAT©-Pro (2013.4.03, Addinsoft, Inc., Brooklyn, NY, USA) statistical package at 95 % confidence level. The values were considered as statistically significant when p values were less than 0.05.

Table 2.8 Showing the different softwares that were used to generate the assembled annotated genomes and the downloaded site.

Software	Use	Citation	Link
BLAST	Basic Local Alignment Search Tool that was used to search genes or proteins against the NCBI database or against specified sequences.	(Altschul et al., 1990)	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Clustal Omega	General multipurpose alignment program for nucleotides and proteins; finds the best alignment over the entire length of each sequence submitted.	(McWilliam et al., 2013)	http://www.ebi.ac.uk/Tools/msa/clustalo/
BioEdite 7.2.6	Sequence alignment editor	(Hall, 1999)	http://www.mybiosoftware.com/bioedit-7-0-9-biological-sequence-alignment-editor.html
Mothur V1.39.5	Powerful software package for analysing sequencing data.		https://github.com/mothur/mothur/releases/tag/v1.39.5
RStudio 1.1419	Data organization and visualization	(RStudio, 2018)	http://www.rstudio.org/
Snap gene viewer	Data organization and visualization	(SnapGene®)	http://www.snapgene.com

2.5.8.1 Statistical analysis for quantification of integrase copy number

The cycle of threshold (Ct) of reaction is value at which the fluorescence can be emitted appropriately above the background fluorescence and then this value can be detected. The baseline signal and threshold signal of fluorescence were determined automatically by the PCR machine. The Ct values for each reaction were detected and the calculation was carried out in Microsoft Excel 2016. According to Higuchi et al. (1993) method, the absolute quantification of integrase copy number was calculated based on standard curve of positive control *S*. Typhimurium U288. Fivefold serial dilution was done (10^{0}-10^{-7}) and then $\log_{10}$ gene numbers were plotted versus Ct values and straight line was generated (the standard curve). In brief, the number of copies for template was calculated by using this website (https://cels.uri.edu/gsc/cndna.html) for determining the number of copies of a template and then $\log_{10}$ was used to plot values against Ct value. Then, quantitation of the amount of integrase copy in the 'unknown' samples of interest is conducted by measuring Ct and using the standard curve to determine starting copy number (Arya et al., 2005). T-test was performed in GraphPad Prism 8.0 and Microsoft Excel 2016 to compare the difference of copy number between G1 and G2, G3 and G4 for each sampling points.

2.5.8.2 16S RNA PCR

The 16S RNA sequence was used to select coliform isolates for whole genome sequencing. The PCR conditions and reactions were carried out as described in 2.5.3.2. According to Hugerth et al. (2014) the optimum oligonucleotides for amplifying 16S RNA are: forward primers was (341F:CCTACGGGNGGCWGCAG) and reverse primer (R805: GGACTACHVGGGTWTCT) which target amplification of the V3-V4 region (Hugerth et al., 2014). Prior whole genome sequence, 16S RNA sequence of approximately 35 bacterial isolates that collected from CH birds

(Trial1) were used to select different isolates of coliforms based on sequence data for whole genome sequencing. PCR conditions of 30 cycles of amplification include: denaturation 95°C for 5 min, 95°C 20s, annealing 51°C 20s, extension 72°C 20s, final extension 72°C 1 min. In addition, this step was followed by PCR for both screening integrase and gene cassettes presence. 6 sample then were selected for whole genome sequence.

2.5.9 DNA sequencing

2.5.9.1 PCR product sequencing

In order to get sequence reads for GCs obtained from broiler caecal contents of biosecure housed and commercial birds, the concentrations of purified PCR products (2.5.2.5) were measured by Nano drop and diluted if needed to 10 ng/µl in total volume 15µl of required PCR product. In addition, another 15µl of required primer at 10pmol µl-1 was transferred to 1.5 ml microcentrifuge tube with extra 5µl volume of required primer for each additional sequencing reaction. PCR samples and primers were sent to Eurofins genomics for sequencing.

2.5.9.2 Whole genome sequencing of gDNA samples

Bacterial DNA was first isolated as described in 2.5.2.1. After extraction, approximately 10 µl of bacterial DNA sample was transferred into 1.5 ml microcentrifuge tubes. Selected samples were sequenced by illumina Sequencing (Food science, Sutton Bonington, Nottingham, UK) for whole genome sequencing via the MiSeq platform.

Briefly, genomic DNA fragments are sheared into 200-300 bp fragments. A sequencing library is generated by mixing the adapters with its complements and allowing DNA to attach on the surface of flow cell oligonucleotides. Each DNA

fragment molecule was iso-thermally amplified to simulate generation of millions of clusters in a flow cell, and this process is known as bridge amplification. The flow cell is a glass slide containing lanes which are coated with a lawn consisted of two types of oligonucleotides to allow template fragment binding. The process of sequencing is initiated by binding of the first primer to generate the sequence reading. Then, the fluorescent tagged nucleotide that is integrated into the synthesised complementary chain of the template fragment in each sequencing cycle. The fluorescent labels also act as the terminator for polymerisation. After insertion of one nucleotide, the fluorescent dye is triggered by a laser and the fluorescent signal is emitted. These signals produce images which are then analysed by the MiSeq image control software. To enable the incorporation of the next nucleotide the fluorescent label is enzymatically cleaved, and the fragment length is detected by the number of the sequencing cycles, and the base calling is done based on the emission wavelength together with the signal intensity. The previous steps are summarised in Figure 2.3. All data or paired end reads were *de novo* assembled via the CLC genomic workbench by Prof. Ian Connerton.

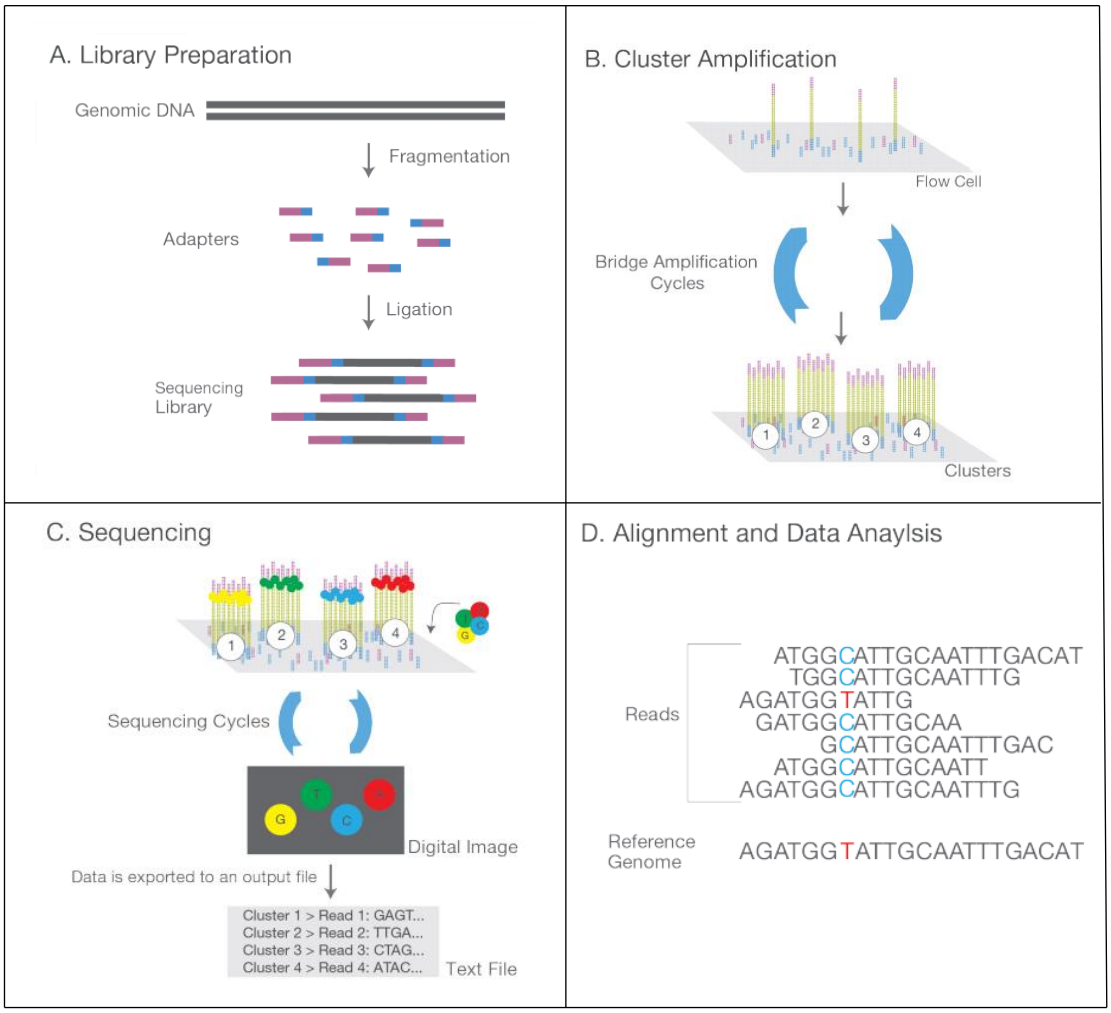


Figure 2.3 Next generation sequencing basic steps of Illumina MiSeq.

A. Fragmentation of gDNA sample and ligation with specialised adaptors to both fragment ends. B. loading library into flow cell to hybridise the fragments to the flow cell surface. Amplification of each bound fragment into a clonal cluster through bridge amplification. C. sequencing biosynthesis through sequencing reagent (fluorescently labelled nucleotides) are added and the first base is incorporated, and the flow cell is imaged. The emission from each cluster is recorded. The emitted wavelength and intensity are used to identify the base. The repeated cycle ‘’n’’ times create a read length of ‘’n’’ bases. D. reads are aligned to reference sequence with bioinformatics software. Differences between reference genome and the newly sequenced reads can be identified after alignment (Illumina, 2015).

CHAPTER 3

INVESTIGATION OF THE PREVALENCE OF CLASS 1 INTEGRONS IN BROILER CHICKEN CAECAL CONTENTS REARED UNDER BIOSECURE AND COMMERCIAL CONDITIONS

3.1 Introduction

Mobile genetic elements are one of the most significant factors that influence the spread of antibiotic resistance genes (ARGs) by the acquisition, expression, and dissemination of these ARGs in clinical settings and environmental habitats. Class 1 integrons are bacterial genetic elements that widely contribute to the emergence the multidrug resistance in human medicine, which themselves are frequently located on mobile genetics elements including plasmids and transposons (Ruiz et al., 2003;Nardelli et al., 2012;Jechalke et al., 2014). This has facilitated integron mobility and dispersal among similar or different species within bacterial communities (Escudero et al., 2015). Indeed, mobile integrons (MIs) are evolvable elements for the rapid adaptation of bacteria to environmental changes shaped by humans. In the 1960s, MIs played a significant role in the early rise of multidrug resistance among clinically important bacteria, leading to the discovery of integrons in the late 1980s (Escudero et al., 2015). Integron classes 1 and 3 (Arakawa et al., 1995) are found to be correlated with Tn*402* (Collis et al., 2002;Xu et al., 2007), whereas class 2 integrons are almost exclusively associated with Tn*7* derivatives (Ramírez et al., 2010).

The embedded gene cassettes (structure of GCs, Chapter 1, Section 1.2.7.3.3) are frequently associated with a variety of resistances for almost all antibiotic families, including trimethoprim, aminoglycosides, lincosamides, beta-lactams, chloramphenicol, fosfomycin, macrolides, rifampicin, and quinolones (Stalder et al., 2012). Besides antibiotics that have played vital roles in selection and dissemination of class1 integrons, the selective pressure of resistance to quaternary ammonium compounds (QACs) used in cleaning and disinfection products, has also been reported to be a contributory factor in their dissemination. The *qac* gene encoding resistance determinants to QACs is commonly observed on class 1 integrons (Jechalke et al., 2014;Stalder et al., 2012). QACs are commonly used in food processing because of their biocidal effect in addition to their non-

toxic, non-tainting, and non-corrosive properties (Holah et al., 2002). Morente et al. (2013) stated that there is a potential concern with regard to the cross-resistance observed between antibiotics and QACs in environmental and food-associated bacteria.

Poultry meat production faces a range of challenges to satisfy growing demand. To meet the demand the world poultry production would need to grow by 2.5% per year up to 2030, and as much as 3.4% in developing countries (Ricke, 2016). Consumption in the UK is reported as 83 million birds per year (GOV.UK, 2020). Thus, the poultry industry is important part in human food chain that requires careful monitoring with respect to AMR in order to achieve desirable outcomes for food safety and quality.

However, livestock are considered a significant reservoir for AMR expansion due to the possible horizontal spread between commensal bacteria and zoonotic pathogens, which is real concern to human health (Munk et al., 2017). Several studies have demonstrated that the use of antimicrobials in domestic animals, often as growth promoters, leads to an increase in the occurrence of AMR and reductions in their use positively decreases the observed resistance (Munk et al., 2017). In commercial broiler chicken production birds are typically reared in barns of 20,000 birds or more, which allow the dispersion of microorganisms throughout the flock representing an important source of microbiota to other flocks on the farm (Connerton et al., 2018). Therefore, class I integrons encoding ARGs can easily disseminate between flocks, which enter the human food chain for acquisition by human commensal and pathogenic bacterial populations. Human interaction with the environment can recycle the ARGs to create a continuous process of acquiring new gene cassettes with sequential rearrangements of host mobile elements. These events make the natural environment a prolific reservoir for the acquisition of new resistance genes to the advantage of opportunistic pathogens (Waldron and Gillings, 2015). Moreover, differences in agricultural

practice have the potential to effect class 1 integron carriage and gene cassette arrangements (GCs). Monitoring how human activities (anthropogenic pollutants) participate in recycling resistance to food chain animals and how these activities contribute to worldwide crisis in the management of bacterial infections are critical to our understanding (Gillings et al., 2008a).

Due to limited knowledge of the gene flow in these environments or the genetic platforms that support the mobility of class 1 integrons, making predictions of their impact on food-associated bacterial communities is difficult. The broiler chicken caecal microbiome is an important reservoir of class 1 integrons, where three conserved genes are frequently observed: class 1 integrase (*intI1),* heavy metal or quaternary ammonium compounds (*qacEΔ1)* and sulphonamides resistance (*sul1*). This chapter therefore aims to use the class 1 integron as a biomarker for the acquisition and spread of antibiotic resistance in commercially hatched chicks reared in either low density biosecure conditions or commercial production.

3.2 Results

3.2.1 Detection of class 1 integrons in biosecure chicken flocks

In order to determine the prevalence of class 1 integrons, genomic DNAs were extracted from a total of 111 caecal samples from broiler chickens reared in controlled housing from one-day old chicks. Three conserved genes of the integron-integrase system (*intI1*, *qacEΔ1* and *sul1*) were PCR amplified and the products analysed using standard agarose gel electrophoresis. The *intl1* Ravi primer (Ravi et al., 2015) was selected as main indictor for presence of class 1 integrons from four different primers used for detecting the prevalence of *intl1* noted in Table 2.4 (Chapter 2). The locations of the integrase primers within *intl1* are indicated in Table 3.1 and 3.2. Validation of the primers for these three genes was performed using *Salmonella enterica* Typhimurium strain U288 as a positive control for the *intI1* gene and *E. coli* J53 (pMG101, obtained from Dr Jon Hobman's group, University of Nottingham) as a positive control for the *qacEΔ1* and *sul1* genes. *S.*T.U288 was PCR negative for the *sul1* gene and also it gives undeniable band with *qacEΔ1* results shown in Figure 3.1. Gradient PCR was used to optimise annealing temperature Figure 3.2 (A, B and C).

The PCR conditions for each reaction were carried out as follows: initial denaturation at 95°C for 5 min; 35 cycles of PCR amplification including denaturation at 95°C for 30 s, annealing routinely set at 64°C (one degree reduced from above optimisation) for 30 s, extension at 72°C for 1 and the reaction ended with a final extension at 72°C for 5 min. Generally, the results show that 99 % (110/111) of biosecure chicken caecal samples were positive for integrase gene based on the *intl1* Ravi and *intl1* HS464 primers, whereas *qacEΔ1* and *sul1* genes were detected in 93.7% (104/111) and 94.5% (105/111) of chicken caecal genomic DNA samples respectively. Only one sample appeared to be negative for the class 1 integron. The full results are shown in Appendix 2.1 Table 2.1.1 and Figures in Appendices 2.2-2.6.

Table. 3.1 Sequence of class 1 integron gene clarify positions of used oligonucleotides coloured according to their positions in the *IntI1* sequence.

Sequence of class 1 integrons obtained from positive control *Salmonella* Typhmurium U288
>STU288_RS24150 STU288_RS24150 class 1 integron integrase *IntI1* gene sequence 109469:110482 forward ATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTG CGTGAGCGCATACGCTACTTGCATTACAGCTTACCAACCGAACAGGCTTATGTCCACTGG GTTCGTGCCTTCATCCGTTTCCACGGTGTGCGTCACCCGGCAACCTTGGGCAGCAGCGAA GTCGAGGCATTTCTGTCCTGGCTGGCGAACGAGCGCAAGGTTTCGGTCTCCACGCATCGT CAGGCATTGGCGGCCTTGCTGTTCTTCTACGGCAAGGTGCTGTGCACGGATCTGCCCTGG CTTCAGGAGATCGGAAGACCTCGGCCGTCGCGGCGCTTGCCGGTGGTGCTGACCCCGGAT GAAGTGGTTCGCATCCTCGGTTTTCTGGAAGGCGAGCATCGTTTGTTCGCCCAGCTTCTG TATGGAACGGGCATGCGGATCAGTGAGGGTTTGCAACTGCGGGTCAAGGATCTGGATTTC GATCACGGCACGATCATCGTGCGGGAGGGCAAGGGCTCCAAGGATCGGGCCTTGATGTTA CCCGAGAGCTTGGCACCCAGCCTGCGCGAGCAGCTGTCGCGTGCACGGGCATGGTGGCTG AAGGACCAGGCCGAGGGCCGCAGCGGCGTTGCGCTTCCCGACGCCCTTGAGCGGAAGTAT CCGCGCGCCGGGCATTCCTGGCCGTGGTTCTGGGTTTTTGCGCAGCACACGCATTCGACC GATCCACGGAGCGGTGTCGTGCGTCGCCATCACATGTATGACCAGACCTTTCAGCGCGCC TTCAAACGTGCCGTAGAACAAGCAGGCATCACGAAGCCCGCCACACCGCACACCCTCCGC CACTCGTTCGCGACGGCCTTGCTCCGCAGCGGTTACGACATTCGAACCGTGCAGGATCTG CTCGGCCATTCCGACGTCTCTACGACGATGATTTACACGCATGTGCTGAAAGTTGGCGGT GCCGGAGTGCGCTCACCGCTTGATGCGCTGCCGCCCCTCACTAGTGAGAGGTAG

Table. 3.2 Oligonucleotides that were used for detecting the class 1 integron

Gene	Colour	Sequence	Gene Target	Product size	Ref.
HS464 HS463a	Dark pink	ACATGCGTGTAAATCATCGTCG CTGGATTTCGATCACGGCACG	*intI1* gene (H)	471	(Holmes et al., 2003)
IntI1 F165 *IntI1* R476	Red	CGAACGAGTGGCGGAGGGTG TACCCGAGAGCTTGGCACCCA	Clinical *intI1* (C)	311	(Gillings et al., 2015)
F-*intI1* Ravi R-*inti1* Ravi	Gray	ACGAGCGCAAGGTTTCGGT GAAAGGTCTGGTCATACATG	*intI1* gene (Ravi)	564	(Ravi et al., 2015)
F-*intI*1 EB R-*intI*1 EB	Blue	CCTTCGAATGCTGTAACCGC ACGCCCTTGAGCGGAAGTATC	*intI1* gene (EB)	254	(Ebner et al., 2004)

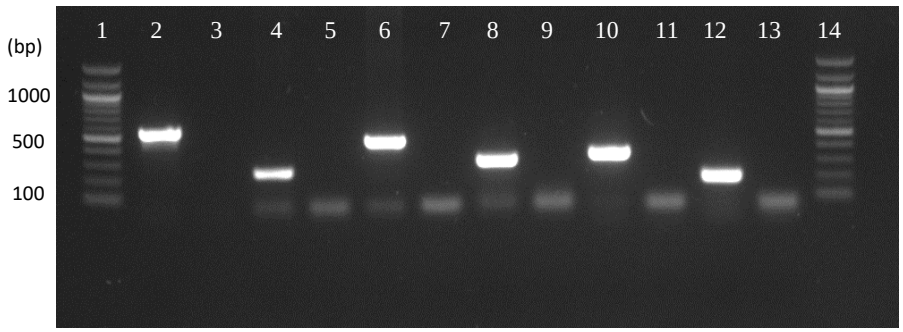


Figure 3.1 Positive control verification of PCR primers.

Agarose gels (Chapter 2, Section 2.5.1.1) showing target genes amplification products of PCRs from positive controls bacteria (*S*.U288 for *IntI1* genes and *E.coli* J53 for *sul1* and *qacEΔ1* genes) electrophoresed on a 1 % TAE gel. The annealing temperature was 64 **°**C. All odd numbered lanes respective negative controls.

Lane 1 100 bp ladder, Lane 2 the 546 bp PCR product of Ravi *intl1,* Lane 4 the 254 bp gene amplicon of EB *intl1,* Lane 6 the 471 bp of *intl1* HS464, Lane 8 the 311 bp clinical *intI1* (C), Lane 10 346 bp the *sul1* gene (J53), Lane 12 the 200 bp *qacEΔ1 gene* (J53). The PCRs were carried out using the primers listed in Table 2.4, Chapter 2. The DNA molecular weight markers (100-1,517 bp) used were 100 bp DNA Ladder (New England Biolabs, Ipswich, UK).

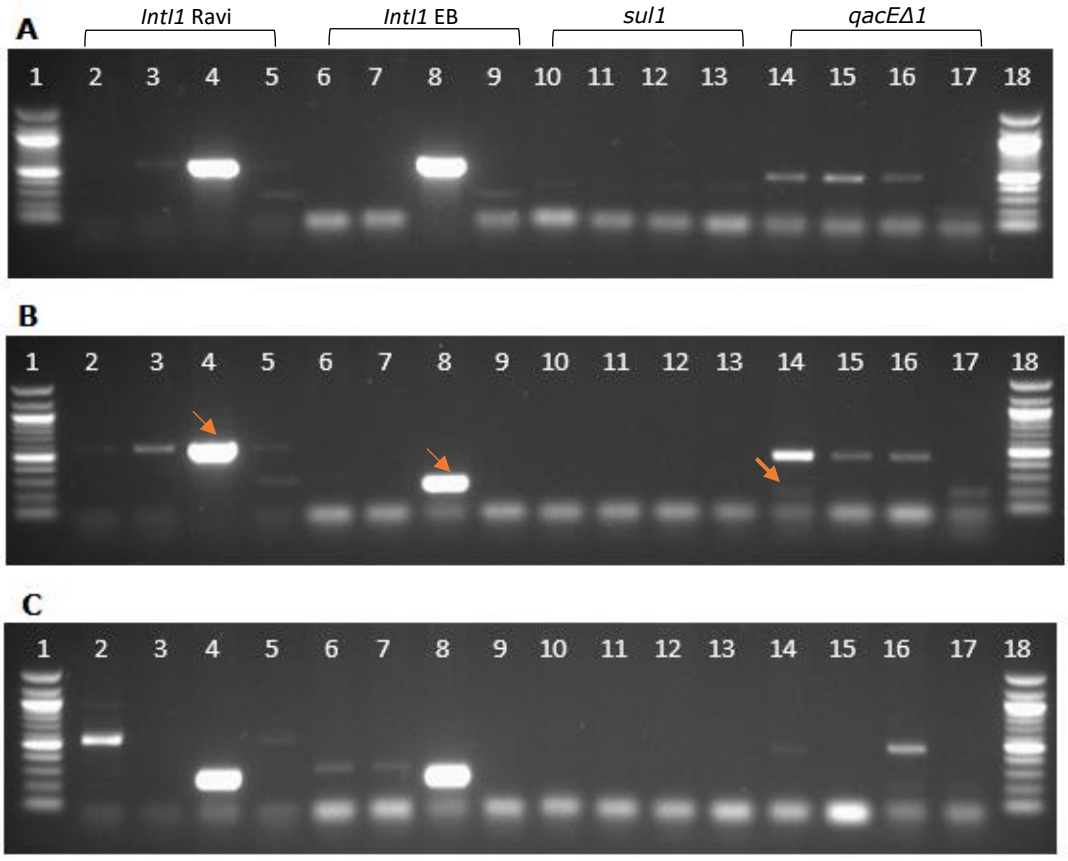


Figure 3.2 Gradient PCR products amplified under three different annealing temperatures A 65 °C, B-61.4 °C, C 55 °C. Agarose gels (Chapter 2, Section 2.5.1.1) demonstrate target genes amplification products of PCRs electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder, lane2 *intl1* Ravi *S*.E.125109, lane 3 *Intl1* Ravi sample1, lane 4 *Intl1* Ravi *S*.T.U288, lane 5 *int1* Ravi –ve control, lane 6 *int1* EB *S*.E.125109, lane 7 *int1* EB sample1, lane 8 *intl1* EB *S.*T.U288, lane 9 *int1* EB –ve control, lane 10 *sul1 S*.E.125109, lane 11 *sul1 S*.E. sample1, lane 12 *sul1 S*.T.U288, lane 13 *sul1–ve* control, lane 14 *qac*EΔ1 *S*.E.125109, Lane 15 *qac*EΔ1 samples1, lane 16 *qacE*Δ1 *S*.T.U288 Lane 17 *qac*EΔ1–ve control, lane 18 100 bp ladder DNA. The primers used listed in Table 2.4, Chapter 2 and DNA obtained from different *salmonella* isolates used in this gradient PCR. The DNA molecular weight markers used were 100 bp DNA Ladder (New England Biolabs, Ipswich, UK).

3.2.2 Detection of class 1 integrons in commercial chicken flocks

A similar approach was performed with animals reared under normal commercial conditions to investigate how prevalent these genes are in commercial setting. A total of 40 broiler chicken caecal samples, fed either control or GOS diets were harvested at two different sampling times from the same barns at thinning (30 da) and depopulation (37 da) as frequently practiced in broiler production. The birds were transported to the University of Nottingham before euthanasia and sampling of the caecal contents for genomic DNA isolation and investigation of the three conserved genes (*intI1*, *qacEΔ1* and *sul1*).

The prevalence of *intI1* was determined by using four different primers as noted in Table 2.4 (Chapter 2). The *IntI1* Ravi primer was selected as the main primer for detecting the presence of class 1 integrons. In this trial *S*.T.U288 was used as a positive control for detecting integrons genes and as negative control for sulphonamide resistance (*sul1*). The amplicon products of the PCR results are shown in Appendix 2.7 (Table 2.7.1) and the corresponding Figures presented in Appendices 2.8-2.11, which illustrate that 92.5% (37/40) of the commercial chicken faecal samples were positive for the integrase gene whereas the *qacEΔ1* and *sul1* genes were detected in 100% (40/40) and 97.5% (39/40) of the broiler chicken caecal samples respectively. It appears that only three samples were negative for class 1 integrons.

3.2.3 Characterisation of antibiotic resistance gene cassettes of class 1 integrons of caecal content microbiota of biosecure birds

Further investigation was performed to determine the distribution of antibiotic resistance gene cassettes (GCs) in the class 1 integrons. A total of 111 luminal caecal samples, including samples negative for the class 1 integrase gene, were investigated for GCs using long range PCR. Four different primers (long range Ravi, MRG 284/285, ntf2/qcr2 and F-Ravi/R-qacEΔ1) for the gene cassettes arrays are noted in Table 2.5 (Chapter 2), and were tested at different extension times(1 min and 3 min) to select the optimum primers for amplifying the gene cassettes and determine the optimal PCR conditions (Appendix 2.12 Figure 2.12 1A, 2A, and 2.12 1B, 2B). The primers F-ntf2/R-qcr2 were chosen for amplifying GCs of both trials with annealing temperature of 64 ^{o}C because this primer pairs give one clear band compared to the others. The extension time was selected at 3 min because expected maximum size of GCs proximality 1.5-2kb.

The contents of all the PCR positive amplicons for the GCs were characterised at nucleotide sequence level by primer walking through the amplified fragment using the Eurofins dye-terminator sequence service. In general, the results of DNA sequencing data from primer walking of the GCs (in most cases 3 forward reads and one reverse read with the exception of gene cassettes type B-1 for which two reverse primers were used) were first, classified based on similarity of sequence data via Bioedit, second, by assembling all reads from which consensus contigs were created and then verified to each GC, and finally the integron embedded GCs were annotated and verified using Basic Local Alignment Search Tool (BlastN). The results revealed that five different types of gene cassettes arrays were present in the biosecure reared broilers that could be categorised into four groups or types: GCs-B-1, GCs-B-2, GCs-C-1, GCs-C-2 and GCs-F. The results are summarised in Table 3.3.

In respect of totals, the most predominant cassette was type GCs-B-2 at 50.45 % (56/111) that contained *dfrA1*, and confers resistance to dihydrofolate reductase (trimethoprim-resistant dihydrofolate reductase type I) and aminoglycoside 3'' adenylyltransferase (*aadA1*). The next most frequent type was group C-1, which contained the *aadA1* gene alone (6.3 %; 7/111), whilst GCs placed in group C-2 were detected in 3 samples with 2.7% (3/111). The GC group F was found in only 4.5% (5/111), which contained the *aadA9* gene alone. The GCs group B-1 were present at 3.6% (4/111). The B-1 GCs were found in only four samples (3.22.5-1, 3.28.5-1, 4.22.7-1 and 4.24.7-1), and this gene cassette shared sequence identity to GCs-B-2 but with an insertion of approximately 500 bp between the *dfrA1* and *aadA1* genes. The relative abundance of each gene is presented in Tables 3.4 A and B.

The PCR amplicons for the GCs were analysed by gel electrophoresis and are shown in Figures 3.3-3.6. What was notable in this trial was that 12 samples had 2 integrase cassette bands, and from these samples 4 had GCs-B-1 & GCs-B-2, 3 had GCs-B-2 & GCs-C1, 2 GCs-B-2 & GCs-C-2 and 3 had GCs-B-2 & GCs-F. Moreover, there was a band of 700 bp that was analysed and found not to be related to class 1 integrons as indicated in Figure 3.4 by the yellow arrow.

The mapping of the whole genes cassettes is illustrated in Figure 3.7.

Agarose gel electrophoresis of PCR amplified GC products.

Each group is represented by one image, the first row contains samples of 22 and 24 da old birds, whereas the second row contains samples of 28 and 35 da old birds.

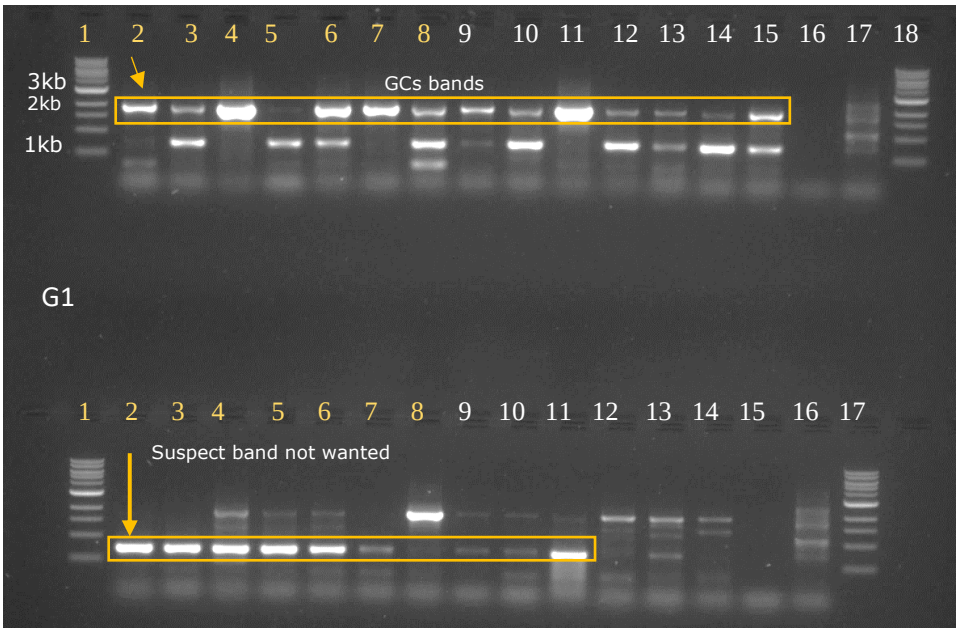


Figure 3.3 The GC amplicon products of caecal DNA samples of birds fed the standard control diet (Group1). The agarose gel electrophoresis performed at 3 min extension time, 64 C° annealing temperature and primer used was F-ntf2/R-qcr2 electrophoresed on 0.8% TAE agarose gel.

Row 1, Lane 1 Kb bp ladder (BioLab), Lane 2 GCs sample 1.22.1, Lane 3 GCs sample 1.22.2, Lane 4 GCs sample 1.22.3, Lane 5 GCs sample 1.22.4, Lane 6 GCs sample 1.22.5, Lane 7 GCs sample 1.22.6, Lane 8 GCs sample 1.22.7, Lane 9 GCs sample 1.24.1, Lane 10 GCs sample 1.24.2, Lane 11 GCs sample 1.24.3, Lane 12 GCs sample 1.24.4, Lane 13 GCs sample 1.24.5, Lane 14 GCs sample 1.24.6, Lane 15 GCs sample 1.24.7, Lane 16 GCs -ve control, Lane 17 GCs +ve control, Lane 18 100 bp ladder (BioLab).

Row 2, Lane 1 100 bp ladder (BioLab), Lane 2 GCs sample 1.28.1, Lane 3 GCs sample 1.28.2, Lane 4 GCs sample 1.28.3, Lane 5 GCs sample 1.28.4, Lane 6 GCs sample 1.28.5, Lane 7 GCs sample 1.28.6, Lane 8 GCs sample 1.28.7, Lane 9 GCs sample 1.35.1, Lane 10 GCs sample 1.35.2, Lane 11 GCs sample 1.35.4, Lane 12 GCs sample 1.35.5, Lane 13 GCs sample 1.35.6, Lane 14 GCs sample 1.35.7, Lane 15 GCs -ve control, Lane 16 GCs +ve control, Lane 17 100 bp ladder (BioLab).

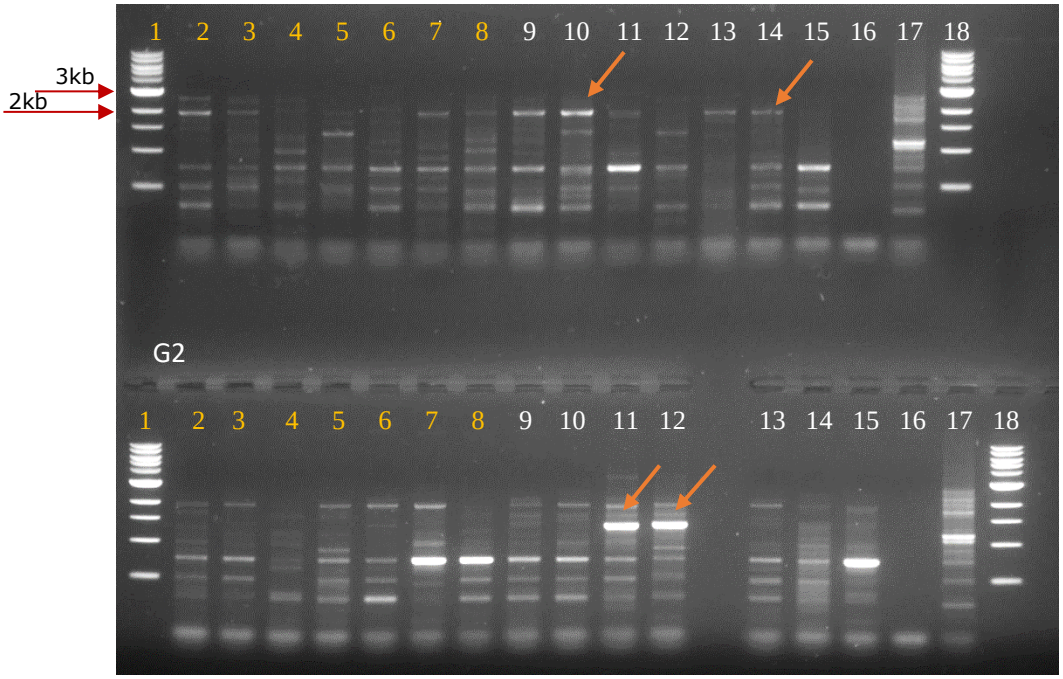


Figure 3.4 The GC amplicon products of caecal DNA samples of birds fed the GOS supplemented diet (Group2). The agarose gel electrophoresis performed at 3 min extension time, 64 C° annealing temperature and primer used was F-ntf2/R-qcr2 electrophoresed on 0.8% TAE agarose gel. The pointed orange bands were only the identified GCs in this group.

Row1, Lane 1 Kb bp ladder (BioLab), Lane 2 GCs sample 2.22.1, Lane 3 GCs sample 2.22.2, Lane 4 GCs sample 2.22.3, Lane 5 GCs sample 2.22.4, Lane 6 GCs sample 2.22.5, Lane 7 GCs sample 2.22.6, Lane 8 GCs sample 2.22.7, Lane 9 GCs sample 2.24.1, Lane 10 GCs sample 2.24.2, Lane 11 GCs sample 2.24.3, Lane 12 GCs sample 2.24.4, Lane 13 GCs sample 2.24.5, Lane 14 GCs sample 2.24.6, Lane 15 GCs sample 2.24.7, Lane 16 GCs -ve control, Lane 17 GCs +ve control, Lane 18 100 bp ladder (BioLab).

Row 2, Lane 1 100 bp ladder (BioLab), Lane 2 GCs sample 2.28.1, Lane 3 GCs sample 2.28.2 Lane 4 GCs sample 2.28.3, Lane 5 GCs sample 2.28.4, Lane 6 GCs sample 2.28.5 Lane 7 GCs sample 2.28.6, Lane 8 GCs sample 2.28.7 , Lane 9 GCs sample 2.35.1, Lane 10 GCs sample 2.35.2, Lane 11 GCs sample 2.35.4, Lane 12 GCs sample 2.35.5, Lane 13 GCs sample 2.35.6, Lane 14 GCs sample 2.35.7, Lane 15 GCs -ve control, Lane 16 GCs +ve control, Lane 17 100 bp ladder (BioLab).

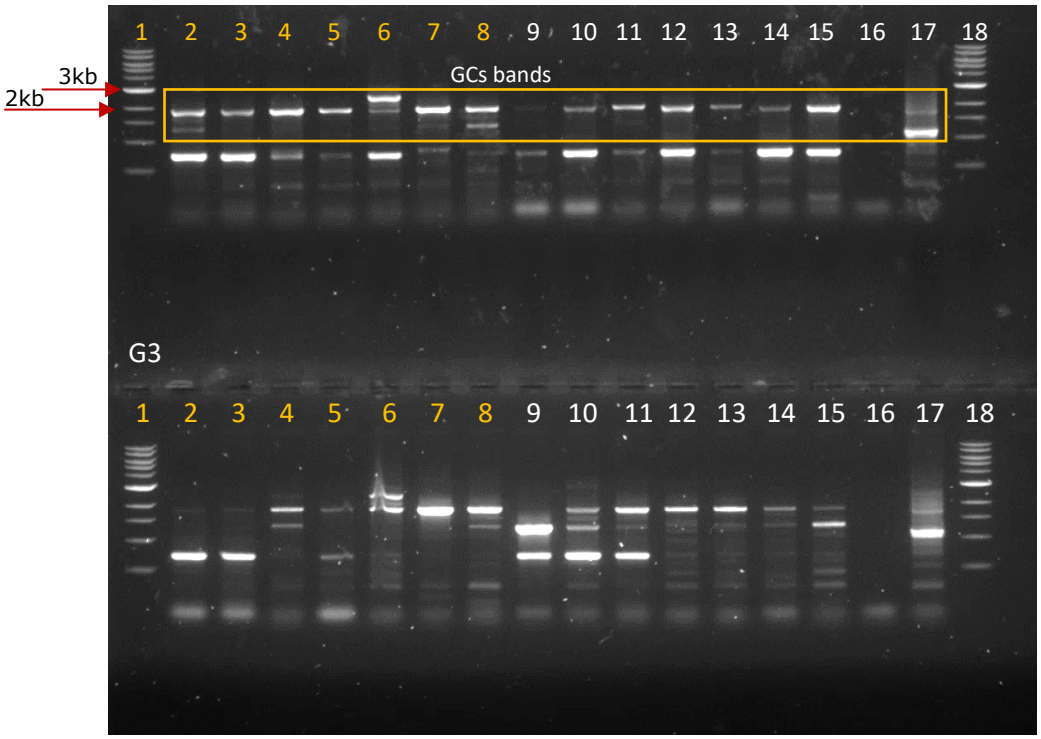


Figure 3.5 The GC amplicon products of caecal DNA samples of birds fed the standard control diet challenged by *Salmonella* (Group3). The agarose gel electrophoresis performed at 3 min extension time, 64 C° annealing temperature and primer used was F-ntf2/R-qcr2 electrophoresed on 0.8% TAE agarose gel.

Lane 1 Kb bp ladder (BioLab), Lane 2 GCs sample 3.22.1, Lane 3 GCs sample 3.22.2, Lane 4 GCs sample 3.22.3, Lane 5 GCs sample 3.22.4, Lane 6 GCs sample 3.22.5, Lane 7 GCs sample 3.22.6, Lane 8 GCs sample 3.22.7, Lane 9 GCs sample 3.24.1, Lane 10 GCs sample 3.24.2, Lane 11 GCs sample 3.24.3, Lane 12 GCs sample 3.24.4, Lane 13 GCs sample 3.24.5, Lane 14 GCs sample 3.24.6, Lane 15 GCs sample 3.24.7, Lane 16 GCs -ve control, Lane 17 GCs +ve control, Lane 18 100 bp ladder (BioLab).

Row 1 Lane 1 100 bp ladder (BioLab), Lane 2 GCs sample 3.28.1, Lane 3 GCs sample 3.28.2, Lane 4 GCs sample 3.28.3, Lane 5 GCs sample 3.28.4, Lane 6 GCs sample 3.28.5, Lane 7 GCs sample 3.28.6, Lane 8 GCs sample 3.28.7, Lane 9 GCs sample 3.35.1, Lane 10 GCs sample 3.35.2, Lane 11 GCs sample 3.35.4, Lane 12 GCs sample 3.35.5, Lane 13 GCs sample 3.35.6, Lane 14 GCs sample 3.35.7, Lane 15 GCs -ve control, Lane 16 GCs +ve control, Lane 17 100 bp ladder (BioLab).

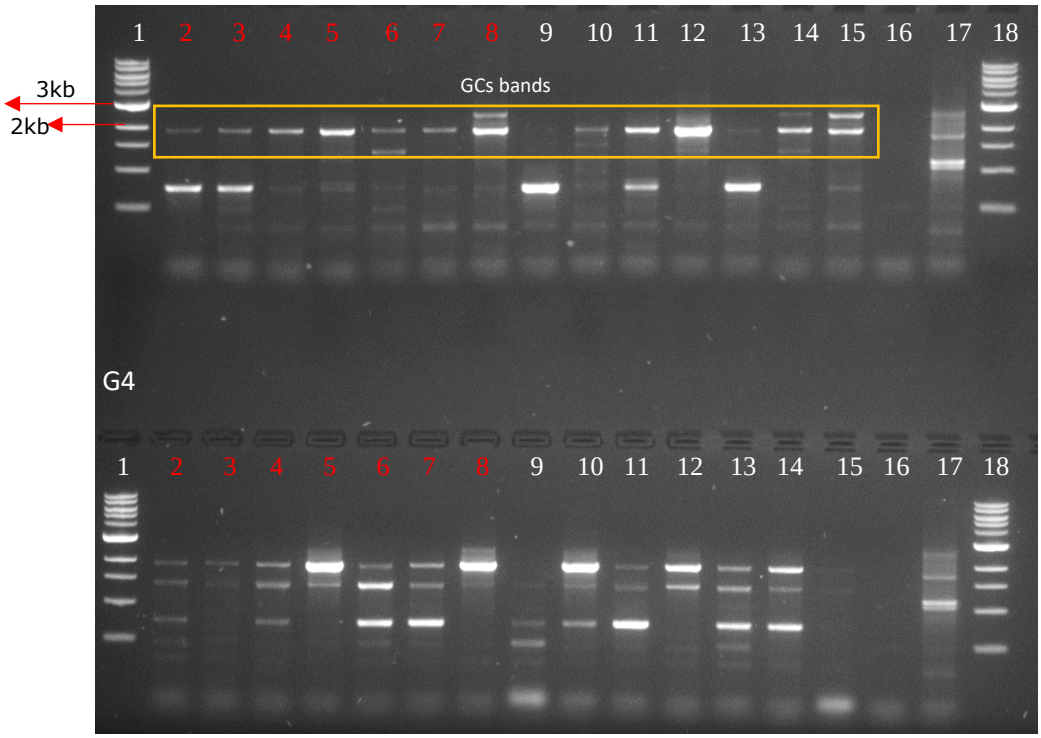


Figure 3.6 The GC amplicon products of caecal DNA samples of birds fed the GOS supplemented diet challenged by *Salmonella* (Group 4). The agarose gel electrophoresis performed at 3 min extension time, 64 C° annealing temperature and primer used was F-ntf2/R-qcr2 electrophoresed on 0.8% TAE agarose gel

Row 1, Lane 1 Kb ladder (BioLab), Lane 2 GCs sample 4.22.1, Lane 3 GCs sample 4.22.2, Lane 4 GCs sample 4.22.3, Lane 5 GCs sample 4.22.4, Lane 6 GCs sample 4.22.5, Lane 7 GCs sample 4.22.6, Lane 8 GCs sample 4.22.7, Lane 9 GCs sample 4.24.1, Lane 10 GCs sample 4.24.2, Lane 11 GCs sample 4.24.3, Lane 12 GCs sample 4.24.4, Lane 13 GCs sample 4.24.5, Lane 14 GCs sample 4.24.6, Lane 15 GCs sample 4.24.7, Lane 16 GCs -ve control, Lane 17 GCs +ve control, Lane 18 1 Kb ladder (BioLab).

Row 2, Lane 1 kb ladder (BioLab), Lane 2 GCs sample 4.28.1, Lane 3 GCs sample 4.28.2, Lane 4 GCs sample 4.28.3, Lane 5 GCs sample 4.28.4, Lane 6 GCs sample 4.28.5, Lane 7 GCs sample 4.28.6, Lane 8 GCs sample 4.28.7, Lane 9 GCs sample 4.35.1, Lane 10 GCs sample 4.35.2, Lane 11 GCs sample 4.35.3, Lane 12 GCs sample 4.35.4, Lane 13 GCs sample 4.35.6, Lane 14 GCs sample 4.35.7, Lane 15 GCs sample 4.35.5, Lane 16 GCs -ve control, Lane 17 GCs +ve control, Lane 18 kb ladder (BioLab).

Table 3.3 Genetic content of GCs arrays identified by long range PCR of DNA caecal samples of birds reared in biosecure condition

Sample	feed type	age	Gene cassette
1.22.1	ctl-nonc	22	GroupB-2
1.22.2	ctl-nonc	22	GroupB-2
1.22.3	ctl-nonc	22	GroupB-2
1.22.4	ctl-nonc	22	ND
1.22.5	ctl-nonc	22	GroupB-2
1.22.6	ctl-nonc	22	GroupB-2
1.22.7	ctl-nonc	22	ND
1.24.1	ctl-nonc	24	GroupB-2
1.24.2	ctl-nonc	24	ND
1.24.3	ctl-nonc	24	GroupB-2
1.24.4	ctl-nonc	24	ND
1.24.5	ctl-nonc	24	ND
1.24.6	ctl-nonc	24	ND
1.24.7	ctl-nonc	24	GroupB-2
1.28.1	ctl-nonc	28	ND
1.28.2	ctl-nonc	28	ND
1.28.3	ctl-nonc	28	ND
1.28.4	ctl-nonc	28	ND
1.28.5	ctl-nonc	28	ND
1.28.6	ctl-nonc	28	ND
1.28.7	ctl-nonc	28	GroupB-2
1.35.1	ctl-nonc	35	ND
1.35.2	ctl-nonc	35	ND
1.35.4	ctl-nonc	35	ND
1.35.5	ctl-nonc	35	GroupB-2
1.35.6	ctl-nonc	35	ND
1.35.7	ctl-nonc	35	ND
2.22.1	GOS-nonc	22	ND
2.22.2	GOS-nonc	22	ND
2.22.3	GOS-nonc	22	ND
2.22.4	GOS-nonc	22	ND
2.22.5	GOS-nonc	22	ND
2.22.6	GOS-nonc	22	ND
2.22.7	GOS-nonc	22	ND
2.24.1	GOS-nonc	24	ND
2.24.2	GOS-nonc	24	GroupB-2
2.24.3	GOS-nonc	24	ND
2.24.4	GOS-nonc	24	ND
2.24.5	GOS-nonc	24	ND
2.24.6	GOS-nonc	24	GroupB-2
2.24.7	GOS-nonc	24	ND

Continued Table 3.3 Genetic content of GCs arrays identified by long range PCR of DNA caecal samples of birds reared in biosecure condition

Sample	feed type	age	Gene cassette
2.28.1	GOS-nonc	28	ND
2.28.2	GOS-nonc	28	ND
2.28.3	GOS-nonc	28	ND
2.28.4	GOS-nonc	28	ND
2.28.5	GOS-nonc	28	ND
2.28.6	GOS-nonc	28	ND
2.28.7	GOS-nonc	28	ND
2.35.1	GOS-nonc	35	ND
2.35.2	GOS-nonc	35	ND
2.35.3	GOS-nonc	35	Group C-1
2.35.4	GOS-nonc	35	Group C-1
2.35.5	GOS-nonc	35	ND
2.35.6	GOS-nonc	35	ND
2.35.7	GOS-nonc	35	ND
3.22.1	ctl-sal	22	GroupB-2
3.22.2	ctl-sal	22	GroupB-2
3.22.3	ctl-sal	22	GroupB-2
3.22.4	ctl-sal	22	GroupB-2
3.22.5	ctl-sal	22	GroupB-1& GroupB-2
3.22.6	ctl-sal	22	GroupB-2
3.22.7	ctl-sal	22	GroupB-2 & groupC-2
3.24.1	ctl-sal	24	GroupB-2
3.24.2	ctl-sal	24	GroupB-2
3.24.3	ctl-sal	24	GroupB-2
3.24.4	ctl-sal	24	GroupB-2
3.24.5	ctl-sal	24	GroupB-2
3.24.6	ctl-sal	24	GroupB-2
3.24.7	ctl-sal	24	GroupB-2
3.28.1	ctl-sal	28	GroupB-2
3.28.2	ctl-sal	28	GroupB-2
3.28.3	ctl-sal	28	GroupB-2 & group F
3.28.4	ctl-sal	28	GroupB-2
3.28.5	ctl-sal	28	groupB-1 & GroupB-2
3.28.6	ctl-sal	28	GroupB-2
3.28.7	ctl-sal	28	GroupB-2
3.35.1	ctl-sal	35	Group C-2
3.35.2	ctl-sal	35	Group B-2 & groupC-2
3.35.3	ctl-sal	35	GroupB-2
3.35.4	ctl-sal	35	GroupB-2
3.35.5	ctl-sal	35	GroupB-2
3.35.6	ctl-sal	35	GroupB-2& groupC-1
3.35.7	ctl-sal	35	GroupB-2& Group F

Continued Table 3.3 Genetic content of GCs arrays identified by long range PCR of DNA caecal samples of birds reared in biosecure condition

Sample	feed type	age	Gene cassette
4.22.1	GOS_sal	22	Group B-2
4.22.2	GOS_sal	22	Group B-2
4.22.3	GOS_sal	22	Group B-2
4.22.4	GOS_sal	22	Group B-2
4.22.5	GOS_sal	22	Group C-1
4.22.6	GOS_sal	22	Group B-2
4.22.7	GOS_sal	22	Group B-1 & Group B-2
4.24.1	GOS_sal	24	ND
4.24.2	GOS_sal	24	Group B-2
4.24.3	GOS_sal	24	Group B-2
4.24.4	GOS_sal	24	Group B-2
4.24.5	GOS_sal	24	ND
4.24.6	GOS_sal	24	GroupB-2
4.24.7	GOS_sal	24	Group B-1& Group B-2
4.28.1	GOS_sal	28	ND
4.28.2	GOS_sal	28	ND
4.28.3	GOS_sal	28	Group C-1
4.28.4	GOS_sal	28	GroupB-2
4.28.5	GOS_sal	28	Group F
4.28.6	GOS_sal	28	ND
4.28.7	GOS_sal	28	Group B-2
4.35.1	GOS_sal	35	Group F
4.35.2	GOS_sal	35	Group B-2
4.35.3	GOS_sal	35	ND
4.35.4	GOS_sal	35	Group B-2& Group F
4.35.5	GOS_sal	35	ND
4.35.6	GOS_sal	35	Group B-2 & group C-1
4.35.7	GOS_sal	35	Group B-2 & group C-1

*Sample identifier key : ex, 1.22.1 The first digit refers to diet type (G1=1, standard control diet; G2=2, GOS supplemented diet; G3=3, standard control diet challenged by *Salmonella*; and G4 =4,GOS supplemented diet challenged by *Salmonella*). The second digit refers to sampling day (22,24,28 and 35). The third digit refers to sample number (1-7 for each feed type at one sampling point in total 28 birds / sampling point).

Table 3.4 Total numbers of GCs recovered across time of biosecure birds (7 birds per barn).

A-biosecure reared condition (*Salmonella* non-colonised birds)

GCs type	Control diet					GOS supplemented diets					Total/ gene%
	22 da	24 da	28 da	35 da	Total %	22 da	24 da	28 da	35 da	Total %	
B-1	-	-	-	-	-	-	-	-	-	-	-
B-2	5	3	1	1	10/27=37%	-	2	-	-	2/28=7.14	12/55=21.81
C-1	-	-	-	-	-	-	-	-	2	2/28=7.14	2/55=3.63%
C-2	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-
ND	2	4	6	5	17/27=62.96	7	5	7	5	24	41/55=74.54
Total/da	5	3	1	1	10/27=37%	0	2	0	2	4/28=14.28	14/55=25.45

B-biosecure reared condition (*Salmonella* colonised birds)

GCs type	Control diet with *Sal*					GOS diets with *Sal*					Total/gene %
	22 da	24 da	28 da	35 da	Total%	22 da	24 da	28 da	35 da	Total%	
B-1	1	-	1	-	2/28=7.14	1	1	-	-	2/28=7.14	4/56=7.14
B-2	7	7	7	6	27/28=96.4	6	5	2	4	17/28=60.71	44/56=78.6
C-1	-	-	-	1	1/28=3.57	1	-	1	2	4/28=14.2	5/56=8.92
C-2	1	-	-	2	3/28=10.71	-	-	-	-	-	3/56=5.35
F	-	-	1	1	2/28=7.14	-	-	1	2	3/28=10.71	5/56=8.9
ND	-	-	-	-	-	-	2	3	2	7	7/56=12.5
Total /da	9	7	9	10	35/28=125	8	6	4	8	26/28=92.85	63/56=112. 5

***ND: not detected**

******Sal*: *Salmonella***

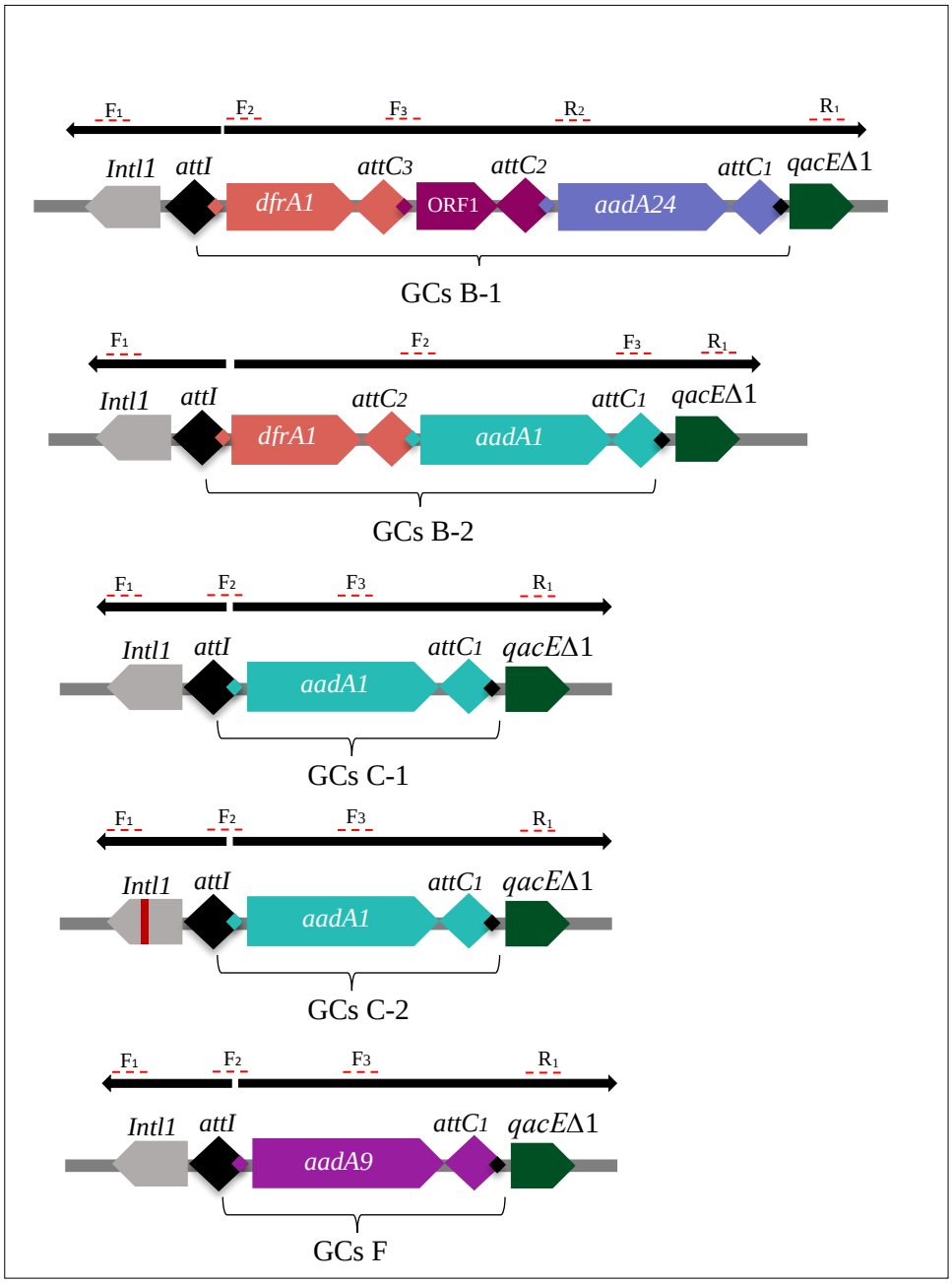


Figure 3.7 The structure of class 1 integron GCs identified from the genomic DNAs of broiler caecal samples of birds reared under biosecure conditions. In all different cassettes arrays, the grey boxes represent a partial sequence of integron (*intI1*), and the black diamond is attachment site of integron (*attI*). Orange box is trimethoprim resistant gene (*dfrA1*), green boxes are another kind of streptomycin (*aadA1*), dark pink and blue boxes are streptomycin resistance gene (*aadA9* and *aadA24*), and dark green coloured boxes are quaternary ammonium compound (*qacEΔ1*). Small, coloured diamonds represent different attachment sites for antibiotic resistance gene which are chimeric sequences between *attI* and *attC*. The location and the direction of transcription of genes are indicated by bold arrow. Primers named F_1, F_2, F_3, R_1 and R_2 are listed in Table 2.6 Chapter2. All sequence data with used oligonucleotides elucidated in Appendix 2.12.

3.2.4 Characterisation of the antibiotic resistance gene cassettes of class 1 Integrons from caecal samples of commercial chicken flocks.

The distribution of integron gene cassettes in 40 luminal caecal samples was determined by long range PCR. Three types of gene cassettes arrays were found in commercial broiler caecal samples that were categorised into three groups: GCs-A, GCs-B-2, and GCs-C-2. All resulted summarised in Table 3.5.

The most prevalent gene cassettes observed in this trial was GCs-group A, at 75% (30/40), which confers resistance to aminoglycoside 3''-adenylyltransferase (streptomycin and spectinomycin; *aadA2*) and lincosamide resistance (*linF*). The next most prevalent type was GCs-group B-2 at 22.5% (9/40), which contains *dfrA1* that confers resistance to dihydrofolate reductase (trimethoprim-resistant dihydrofolate reductase type I) and aminoglycoside 3''-adenylyltransferase (*aadA1*). This gene cassette shared a similar sequence to that obtained from biosecure broiler caecal samples, however this cassette was less prevalent than those identified in birds reared under biosecure conditions. The third type of GC was group C-2, which contains the *aadA1* gene alone (2.5%; 1/40). Results are presented in Table 3.6. Although, the GC group C-2 obtained in this trial displayed homologous sequence to GC-C-1 obtained from the controlled housing flock, the insertion of three Gs was found in the Lex A box of the integrase sequence at the position of the *Pint* promoter (Appendices 2.13.4 and 2.14), as was observed in the DNA of few samples of both biosecure and the commercial chicken flocks. PCR of the GC products resolved by agarose gel electrophoresis are presented in Figures 3.9, 3.10 and 3.11. All the GCs obtained are illustrated in Figure 3.11. The approximate position of designed primers pointed in each gene. Furthermore, all sequence reads are categorised and assembled, then consensus sequence of each GCs was created by CLC. All gene cassettes sequences data are represented in Appendix 2.13 (Figures 1-5) with used oligonucleotides and each gene highlighted by grey colour.

PCR GCs gel pictures.

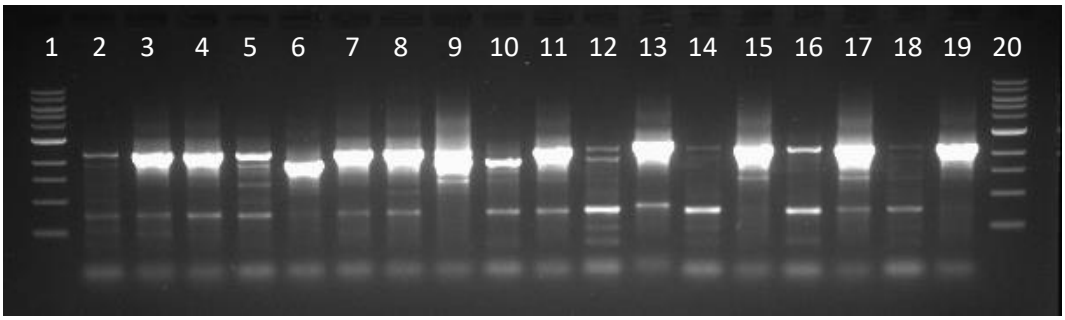


Figure 3.8 The PCR amplicon products of GCs found in chicken caecal DNA samples of commercial birds (samples 1-18). The agarose gel electrophoresis performed at 3 min extension time, 64 C° annealing temperature and primer used was F-ntf2/R-qcr2 electrophoresed on 0.8% TAE agarose gel.

Lane 1 1 kb ladder (BioLab), Lane 2 GCs sample 1, Lane 3 GCs sample 2, Lane 4 GCs sample 3, lane 5 GCs sample 4, Lane 6 GCs sample 5, Lane 7 GCs sample 6, Lane 8 GCs sample 7, Lane 9 GCs sample 8, Lane 10 GCs sample 9, Lane 11 GCs sample 10, Lane 12 GCs sample 11, Lane 13 GCs sample 12, Lane 14 GCs sample 13, Lane 15 GCs sample 14, Lane 16 GCs sample 15, Lane 17 GCs sample 16, Lane 18 GCs sample 17, Lane 19 GCs sample 18, Lane 20 1 kb ladder (BioLab).

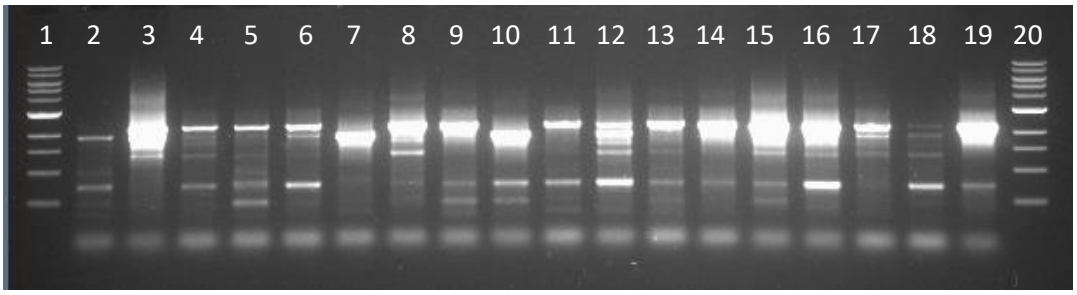


Figure 3.9 The PCR amplicon products of GCs found in chicken caecal DNA samples of commercial birds (samples 19-36). The agarose gel electrophoresis performed at 3 min extension time, 64 C° annealing temperature and primer used was F-ntf2/R-qcr2 electrophoresed on 0.8% TAE agarose gel.

Lane1 1 kb ladder (BioLab), Lane 2 GCs sample 19, Lane 3 GCs sample 20 Lane 4 GCs sample 21, Lane 5 GCs sample 22, Lane 6 GCs sample 23, Lane 7 GCs sample 24, Lane 8 GCs sample 25, Lane 9 GCs sample 26, Lane 10 GCs sample 27, Lane 11 GCs sample 28, Lane 12 GCs sample 29, Lane 13 GCs sample 30, Lane 14 GCs sample 31, Lane 15 GCs

sample 32, Lane 16 GCs sample 33, Lane 17 GCs sample 34, Lane 18 GCs sample 35, Lane 19 GCs sample 36, Lane 20 1 kb ladder (BioLab).

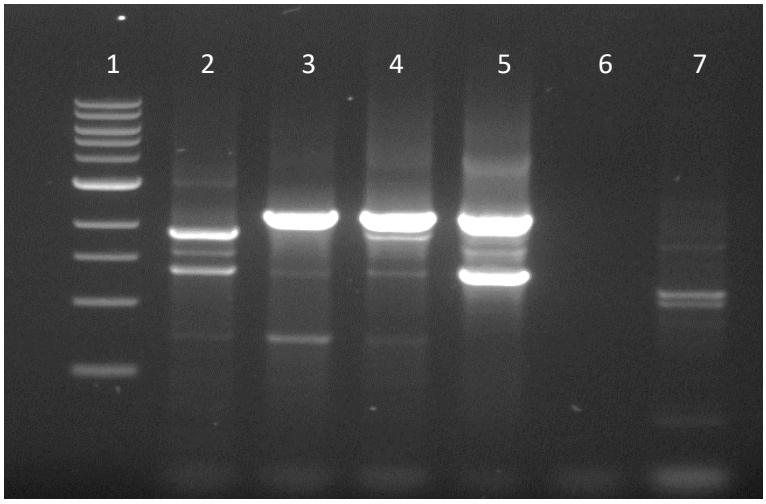


Figure 3.10 The PCR amplicon products of GCs found in chicken caecal DNA samples of commercial birds (samples 37-40). The agarose gel electrophoresis performed at 3 min extension time, 64 C° annealing temperature and primer used was F-ntf2/R-qcr2 electrophoresed on 0.8% TAE agarose gel.

Lane 1 1 kb ladder (BioLab), Lane 2 GCs sample 37, Lane 3 GCs sample 38, Lane 4 GCs sample 39, Lane 5 GCs sample 40, Lane 6 GCs sample –ve, Lane 7 +ve control.

Table 3.5 Genetic content of GCs arrays identified by long range PCR of DNA caecal samples of birds reared in commercial condition

sample	type	age	Gene cassette
mpS 1	ctl	30d	GroupA
mpS 2	ctl	30d	GroupA
mpS 3	ctl	30d	GroupA
mpS 4	ctl	30d	GroupA
mpS 5	ctl	30d	GroupB
mpS 6	ctl	30d	GroupA
mpS 7	ctl	30d	GroupA
mpS 8	ctl	30d	GroupB-2
mpS 9	ctl	30d	GroupB-2
mpS 10	ctl	30d	GroupA
mpS 11	GOS	30d	GroupA & B-2
mpS 12	GOS	30d	GroupA
mpS 13	GOS	30d	ND
mpS 14	GOS	30d	GroupA
mpS 15	GOS	30d	GroupA
mpS 16	GOS	30d	GroupA
mpS 17	GOS	30d	ND
mpS 18	GOS	30d	GroupA
mpS 19	GOS	30d	GroupB-2
mpS 20	GOS	30d	GroupA
mpS 21	GOS	37d	GroupA
mpS 22	GOS	37d	GroupA
mpS 23	GOS	37d	GroupA
mpS 24	GOS	37d	GroupB
mpS 25	GOS	37d	GroupA
mpS 26	GOS	37d	GroupA
mpS 27	GOS	37d	GroupB-2
mpS 28	GOS	37d	GroupA
mpS 29	GOS	37d	GroupA&B-2
mpS 30	GOS	37d	GroupA
mpS 31	ctl	37d	GroupA
mpS 32	ctl	37d	GroupA
mpS 33	ctl	37d	GroupA
mpS 34	ctl	37d	GroupA
mpS 35	ctl	37d	ND
mpS 36	ctl	37d	GroupA
mpS 37	ctl	37d	GroupB-2
mpS 38	ctl	37d	GroupA
mpS 39	ctl	37d	GroupA
mpS 40	ctl	37d	GroupA & GroupC-2

Table 3.6 Total numbers of GCs recovered across time of commercial birds (10 birds per barn).

GCs Type	Control diet/com			GOS diets/com			Total/gene%
	30 da	37 da	Total %	30 da	37 da	Total %	
A	7	8	15/20=75%	7	8	15/20=75%	30/40=75
B-2	3	1	4/20=20%	2	3	5/20=25%	9/40=22.5
C-2	0	1	1/20=5%	0	0	0	1/40=2.5
ND	0	1	1	2	0	2	3/40=7.5
Total	10	10	20/20=100	9	11	20/20=100	100

***ND: not detected**

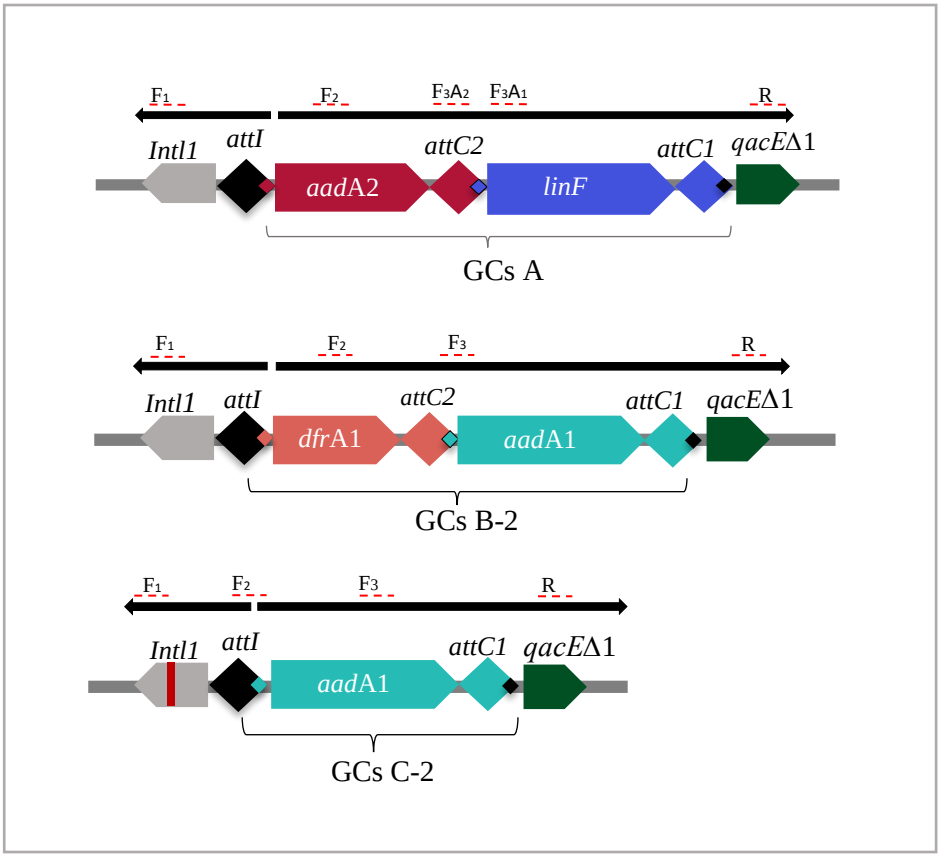


Figure 3.11 The structure of class 1 integron GCs identified from genomic DNAs of broiler caecal samples of birds reared under commercial conditions. In all the different cassettes arrangements, the grey boxes represent a partial sequence of integron (*intI1*) and the black diamond is the attachment site of integron (*attI*). Dark red boxes represent the streptomycin resistance gene (*aad*A2), orange boxes the trimethoprim resistant gene (*dfr*A1), tiffany blue boxes a second kind of streptomycin resistance (*aad*A1), dark blue boxes lincosamide resistance (*lin*F) genes and dark green coloured boxes represent the resistance determinant for quaternary ammonium compounds (*qacEΔ*1). Coloured diamonds represent different attachment sites for antibiotic resistance genes. The location and the direction of transcription of genes are indicated by bold arrows. All sequence data with used oligonucleotides elucidated in Appendix 2.13.

The findings demonstrate that all the GCs obtained from chickens' caecal contents (Tables 3.3 and 3.5) of both trials, are considered to be small GCs arrays that are likely located within mobile genetic elements (transposons and plasmids), which are possibly spread by LGT to facilitate the high prevalence observed within the broiler chicken gut microbiota. The amplicon sizes of the gene cassettes were 2218, 2394, 1911, 1332, 1335 and 1355 bp (Table 3.7). In general, by comparing the diversity of the antibiotic resistance patterns in these experiments, it is evident that each trial was dominated by one type of GC, the controlled housing birds exhibited high prevalence of GC-group B-2, and in the commercial birds the GC-group A was predominant being present in more than half of the samples.

3.2.5 Attachment site *attC* structure

All the gene cassette types (A, B-1, B-2, C-1,C-2 and F) were investigated for the presence of two GTTRRRY sequences, which act as the initiation sequence for the recombination process by integrase (*intI1*), and are located at the boundaries of each inserted gene cassette to define the entry point. Thus, GCs are categorised by the existence of essential palindromic sequences of different lengths between the RYYYAAC inverse core site and the GTTRRRY core site. In addition, the sequences were screened for integron integrase specific domains that are required in integration activity (L, R) as described in Figure 1.4 (Chapter1).

Analysis of the attachment site (*attC*) sequences of various *aadA* cassettes indicated that the *aad*A2, *aad*A1 and *aad*A24 genes share similar palindromic sequences at inverse core site (1L, 2L) and core site (2R) regions. However, the 1R core site features a substitution in the last four bases but retains the GTTRRRY consensus sequence, whereas the L2 region was different to the other *aadA* genes. The variable region shows 1% dissimilarity between *aadA1* and *aadA24.* However, 6% dissimilarity between *aadA2* genes compared to *aadA1*. For the palindrome

sequence of *attC* in the *lin*F gene, the inverse core site (GCACAAC, 1L) was located upstream the stop codon within the *lin*F gene.

However, the *dfr*A1 palindromic sequences were compatible (Biskri et al., 2005). The different sizes of *attC* length observed are shown in Table 3.7 and the imperfect inverted repeats are underlined in Table 3.8.

Table 3.7 Summary of genetic content of gene cassette arrays identified by long range PCR (F-ntf2, R-qcr2)

Group	Gene1	Gene Size bp	*attC* site size	Gene2	Gene Size bp	a*ttC* site size	Gene 3	Gene Size bp	a*ttC* site size	Total
A	*aad*A2	792	60	*linF*	821	58	-	-	-	2218
B-1	*dfr*A1	473	94	*Orf1*	519		*aad*A24	702	60	2394
B-2	*dfr*A1	473	94	*aad*A1	792	60	-	-		1911
C-1	*aad*A1	792	60	-	-	-	-	-		1332
C-2	*aad*A1	792	60	-	-	-	-	-		1335
F	*aad*A9	834	60	-	-	-	-	-		1355

Table 3.8 Summary of attachment site sequence (*attC*) of GCs obtained from broiler caecal contents showing imperfect repeats regions

Gene	*attC* Site Sequence 5'-3'	Reference
aadA1	L1 → L2 ← R2 → R1 ← >TGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGAT	(Larouche, &Roy,2011)
aadA2	>TGTCTAACAATTCGTTCAAGCCGACCGCGCTACGCGCGGCGGCTTAACTCCGGCGTTGTGC	
aadA24	>TGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCGAGCGTTAGAT	
aadA9	>CGTCTAACTATTCATTTAAGCCGAAGCCGCTTCGCGGCTCGGCTTAATTCAGGCGTTAGAT	(Tauch et al., 2002)
linF	>CGCACAACAAGTAAATCCAGCGGACGCATAAAAACGCGCCGCTGATTTTGACGTTAGAT	(Heir et al., 2004)
dfrA1	>GGTTAACAAGTGGCAGCAACGGATTCGCAAACCTGTCACGCCTTTTGTACCAAAAGCCGCGCC AGGTTTGCGATCCGCTGTGCCAGGCGTTAAACATC	(Biskri et al., 2005)
Orf1	>GCCTAACCCTTCAATCAACAGGGACAGTCCAAAGCTAGCGCTTTGTCCTGCCCCTTATTTCAA ACGTTAGAC	

3.3 Discussion

The abundance and prevalence of class 1 integrons has had a spectacular increase in bacterial communities compared to what it was estimated to be 100 years ago in clinical forms of this element (Ghaly et al., 2017;Gillings, 2017). This has meant that these DNA elements have become significant indicators for a wide range of environmental pollutants (Pruden et al., 2006;Pruden et al., 2013). The gastrointestinal tract of the chicken is highly populated and acts as a key reservoir for the transmission of foodborne disease and a fertile environment for the exchange of antibiotic resistant genes via LGT (Stalder et al., 2012;Kaakoush et al., 2014). In this regard, it is important to investigate load of antibiotic resistance genes and associated mobile elements in one of the most common food sources, which could act as a vehicle for dissemination in the human food chain and a proxy for understanding the spread of ARG amongst bacterial species.

Previous studies have shown a high abundance of integrons in *E. coli* isolates from broiler chickens (Goldstein et al., 2001;Lu et al., 2003b). In this study 92.5% of the commercial broiler caecal contents tested positive for the *IntI1* gene (5'CS) and 99% of the biosecure broiler caecal samples were positive for integrase. Therefore, there was no discrimination between two different conditions in terms of the prevalence of class 1 integrons. For clarification, four primers were used for targeting different sections of the integrase gene, the primers called *int1* Ravi and HS464 were used as the main primers. Primers for *IntI1* EB were not effective, likely due to a substitution observed in the forward primer compared to the *IntI1* sequence that might justify why this primer did not work with the majority of samples. Surprisingly, many studies have suggested that integrons were prevalent in bacterial communities that had not been directly exposed to antibiotic pressure in clinical, agricultural or environmental settings (Stalder et al., 2012). In addition, Gillings (2018) suggested that the high abundance of *IntI1* is subjected to active selection as it confers a survival advantage to the bacteria that carry it.

Escudero et al. (2015) highlighted the high frequency of integrases depending on the environment in which the integrons were found. Vásquez-Ponce et al. (2019) pointed out that this may explain how DNA segments are shared sequences across various bacterial samples obtained from different environments such as soil, fresh water, marine and pathogenic bacteria (veterinary and human). Liljebjelke et al. (2017) noted that the reservoir of antimicrobial resistance genes was larger than previously thought.

Most of the broiler chicken caecal samples were also positive for the *qacEΔ1* gene (3'CS) with 100% presence in the commercial birds and 93.7 in the biosecure caecal DNA samples. Stalder et al. (2012) pointed out that *qac* GCs are common in class 1 integrons. Rådström et al. (1994) reported that the origin of *qacE* gene in the class 1 integron could be identified as emanating from transposons Tn*402* and Tn*5090*. Moreover, the class 1 integrons containing *qac*E may act to retain antibiotic-resistant bacteria in environments polluted by quaternary ammonium compounds because the selection for *qac* resistance may lead to co-selection for antibiotic resistance (Gaze et al., 2005). Gilbert and McBain (2003) and Gillings (2014) demonstrated that this gene encodes a versatile efflux pump that confers resistance to toxic cationic molecules, and may interpreted as a role in defending cells against toxic substances presented in natural ecosystems. Therefore, the *qacE* gene has played a significant role in the emergence of class 1 integrons as it provides a survival advantage besides acting as a site for integrative mobile elements.

Similarly, the *sul1* gene was observed in almost all samples, 97.5%- 94.5% of caecal DNA samples from commercial and in biosecure conditions respectively. Although Vásquez-Ponce et al. (2019) reported that no typical structure had been found for class 1 integrons among florfenicol FB isolates in his study of multi-resistant bacterial isolates collected from the gut microbiota of extended antibiotic treated *Salmo salar* fish farms. These authors also reported the absence of the 3'-

CS of *qacEΔ1*/*sul1* genes in this group (FB), whereas 23% of oxytetracycline (OB) isolates showed these structures in the integron system. The *sul1* gene confers resistance to sulphonamides that target dihydropteroate synthase in the folic acid pathway, and it was inserted into the Tn*402*-class 1 integrons causing deletion at the end of the *qacE* to generate *qacEΔ1,* and features in clinical class 1 integrons as a 3' conserved segment (Gillings, 2014).

What is worth mentioning here is that the high stability of the class 1 integrons sequence was noted among (40+75) 115 sequence data sets of GI of broiler chicken microbiota. Dubois et al. (2007) noted high uniformity of the class 1 integrons from clinical isolates of *Shigella* spp. Furthermore, Gillings et al. (2015) found that the clinical class 1 integrons possessed homologous DNA of the integrase (*intI1*) gene, which is strong evidence that they have a single recent common ancestor. In addition, Gillings (2014) could identify a class 1 integrase in new clinical pathogens, which could also be found on the chromosomes of non-pathogenic environmental Betaproteobacteria. Thus, studying the origins of these clinical class 1 integrons may facilitate understanding of the dynamics and prevalence of antibiotic resistance. Analysis of the integrase sequence revealed that various resistance gene cassettes conserve a LexA box motif that overlaps with the –10 box of the *intI1* promoter. Cambray et al. (2011) indicated that the LexA binding motif has been found in a large proportion of database integrase sequences, suggesting that the control of integrase expression by the SOS response is conserved amongst integron systems. The in vitro assessment of LexA box functionality for class 1 integrons indicates that the SOS response increases the expression of the class 1 integrase 4.5-fold (Escudero et al., 2015).

Papagiannitsis et al. (2009) and Guérin et al. (2011) described an insertion of three G bases in the *intI1* sequence, which they claim as an activator sequence in a second promoter (P2) located downstream of Pc that increases the distance to 17 bases between the -35 and-10 hexamers. This was observed in few samples in this study recovered from genomic DNAs of both biosecure and commercial

birds. Despite this, the sequences at -35 and-10 region possessed a weak sequence promoter that may be interpreted as the reason for the abundance of the gene cassette among microbiota.

The gene cassettes obtained from both trials show discrimination for antibiotics for veterinary use. Gene cassettes encoding lincosamides (*linF*) that confer resistance to lincomycin were found to be the dominant GCs in commercial birds with total carriage 75%, (30/40), while the *linF* gene was completely absent under biosecure conditions. It is clear that the previous usage history of lincomycin in flock drinking water still enhances mobility of this gene despite the ban of using AGPs in the EU since 2006. Maxey and Page (1977) reported that lincomycin mitigated necrotic enteritis in broiler chickens caused by *Clostridium* perfringens, which may account for its sustained use. GCs B-1 was only found in the birds reared under biosecure conditions. The gene cassette was present in 4 samples, which represent atypical copies of GCs B-2 that exhibit an insertion of a gene encoding a hypothetical protein between *dfrA1* and *aadA1*. The other gene cassettes show similar sequence homology although they are reared under different conditions.

Martinez-Freijo et al. (1999) proposed that the stability of transferred variable regions (GCs) often act as part of the integron structure rather than as individual cassettes. The study findings of GCs arrays suggest that wide dissemination and uniformity among various OTUs across caecal contents of several birds may be indicative of the relative stability of these structures supporting Martinez-Freijo's hypothesis. Futher analysis of the strength or weakness of the Pc promoters is needed to help to clarify if they contribute to satability among microbiota.

Several studies have documented streptomycin resistance (*aad*A) as commonly mediated by integrons. Ebner et al., (2004) reported that GCs were detected from *Salmonella enterica* serovars isolated from animal environments

that contain the *aad*A GC at low diversity. Similarly, in this study the most prevalent gene in all cassettes was *aad*A, whether a single gene such as (*aad*A1) or in conjunction with either trimethoprim resistance (*dfr*A1) or lincosamide resistance (*lin*F, *aad*A2). The GCs of group B contained *dfr*A1 and *aad*A1 as the second most abundant configuration observed within biosecure broiler chicken caecal microbiota. Ponce-Rivas et al. (2012) pointed out that the *dfr*A1 and *aad*A1 GCs were the most prevalent genes detected within the class 1 integrons present in *Escherichia coli* isolates from poultry litter, which were located on mega-plasmids or the chromosome. Martinez-Freijo et al. (1999) also reported these resistance genes in *Enterobacteriaceae.* Although Larouche and Roy (2011) reported that *IntI1* had weak excision activity for the *dfr*A1 cassettes.

In this context the GCs of group C and F may be considered as a deletion of the *dfr*A1 gene from group B, which collectively indicate a greater genetic variation of the GCs obtained from group B, whereas the GCs of group A were more stable. However, relatively few papers discuss stability of gene cassettes as a function of time in the gut microbiome.

Although *attC* sites of the gene arrays of integrons are not considered a conserved sequence among various genes, the *attC* sites of similar gene cassettes show identical sequences in inverted repeat boxes or palindromic sequences (L1, L2, R1 and R2) in most of *attC* sites of the same resistance gene. These feature in chromosomal integrons, which show a high sequence identity of the *attC* sites suggesting a relationship between these fragments and the sequence of the recombination site in the host (Rowe-Magnus et al., 2003).

In summary, comparisons between different reared environments provide insights into the dynamics and flow of resistance genes in the absence of antibiotic use. Diversity in the lineage of class 1 integrons in these settings suggest they remain mobile and able to find their way into the genomes of zoonotic pathogens. However, the integrons present in the birds reared under biosecure condition are

likely to have arisen from the hatchery and preserved as a component of the gut microbiota in the growing chickens.

CHAPTER 4

INVESTIGATION OF ANTIBIOTIC RESISTANCE IN THE CAECAL MICROBIOTA OF BROILER CHICKENS FED STANDARD DIETS UNDER BIOSECURE AND COMMERCIAL CONDITIONS

4.1 Introduction

The microbiomes of food animals have gained antibiotic resistance genes over several decades (Allen, 2014). Antimicrobial agents have been extensively used in animal production over the past 50 years for several purposes that principally include using them as therapeutic agents for treating infection, or as prophylactics for preventing illness, and for sub-therapeutic use for improving feed efficiency and growth performance. However, as highlighted in Chapter 1 (Section 1.2.4), the use of antibiotics growth promoters (AGPs) in food producing animals has been banned in the European Union due to its contribution to accelerate the development of AR and the potential to spread to pathogens (Marshall and Levy, 2011). Hence, it is mainly the historic use of antibiotics that contribute to the continuing presence of antibiotic residues in feed and the environment (Mehdi et al., 2018).

Many studies have highlighted farmed animals and their broader environment as proxy of ARGs that can be easily acquired across species (Forsberg et al., 2012;Finley et al., 2013;Braykov et al., 2016). Animal faecal microbiota harbours a huge reservoirs of these ARGs that might be acquired by human commensals and pathogens (Allen, 2014). Antibiotic residues, resistant bacteria and ARGs may be transferred into the environment by several mechanisms such as directly from manure, leakage from holding tanks, runoff, and airborne particulates (Udikovic-Kolic et al., 2014;McEachran et al., 2015;Xiong et al., 2018a).

It was shown in the Chapter 3 that despite the difference in rearing conditions of broiler chickens, trimethoprim resistance GCs were prevalent in both environmental settings associated with other types of resistance determinants (streptomycin resistance, *aadA1*). Trimethoprim resistance was therefore selected

for investigating the AMR load in caecal contents of the microbiota of broiler chickens. So far, 30 *dfr*-genes have been identified as determinants for the developing trimethoprim resistance, and which are usually associated with integrons (Sköld, 2001). This feature results in an effective horizontal dissemination mechanism for antibiotic resistance between bacteria (White et al., 2001;Dionisio et al., 2002;Blahna et al., 2006). Limited studies have investigated the epidemiology and frequency of the different *dfr*-genes and their association with integrons or its relationship to other resistance determinants from *E. coli* or coliforms (Brolund et al., 2010).

The avian GIT microbiota rapidly develops in the early stages of life (in particular, within the first 2 weeks) since young chicks are hatched and delivered to a chicken house (typically at 1–2 da). Initially, the GI of young chick is very simple containing a small number of bacteria belonging to a few different species (Fonseca et al., 2011;Cox et al., 2012;Hiett et al., 2013;Wang et al., 2016).

Post-hatching the intestinal microbiome becomes dominated by *Enterobacteriaceae* (1 to 3 da) and then Firmicutes around 7 da (Ballou et al., 2016;Connerton et al., 2018). Through the first week of life, the abundance and taxonomic diversity of Gram-positive bacteria become dominated by the *Clostridiales* OTUs*,* causing a corresponding decrease in the proportion of Gram-negative bacteria (Ballou et al., 2016). Exogenous sources of bacteria that can be incorporated into the microbiota of the immature gut of chicks include litter materials, feed, water, and ambient air. Bacteria from these sources can easily colonise the young GI tract due to the low numbers of competitors (Wang et al., 2016). Hence, the microbiome of growing chicks undergoes a series of temporal successions (Wielen et al., 2002;Lu et al., 2003a) and the complexity and diversity of GI microbiota are increased (Wei et al., 2013), and their interactions significantly influence host physiology, immunology and nutritional status (Zhao et al., 2013). Thereby, the dynamics and flow of ARGs between farmed animals

and their environment are considered an important event in terms of shaping animal microbiota and mobilising ARG determinants.

The aim of this study was to elucidate the effect of rearing conditions on the avian GI microbiome in terms of estimating the prevalence of ARGs. In order to do this, it is important to understand how the chicken microbiome develops in chickens fed a standard diet and reared under biosecure or commercial settings. Therefore, the aim of the work described in this chapter are to evaluate trimethoprim resistance in coliform populations in chickens fed a standard diet. This includes an evaluation of the total coliform count; the trimethoprim resistance bacteria count and absolute quantification of the copy number of integrase genes with time.

4.2 Results

4.2.1 Pre-trial test optimisation of antibiotic selection method

Before starting the experiments, it was important to select the most appropriate antibiotic and the optimum concentration to add to the selective media. Randomly selected samples of chicken caecal contents were handled as described in Chapter 2, Section 2.3.3. The antibiotics chosen were trimethoprim and lincomycin. Three different concentrations were tested, these were 5, 10, 20 µg/ml for trimethoprim, and 30, 50, 70 µg/ml for lincomycin. These antibiotics were added to three different kinds of selective media (Mac-03, MRS and M-XLD). The results are shown in Figures 4.1 and 4.2.

High counts of Gram-positive bacteria resistant to lincomycin were recorded in the samples, so this antibiotic was excluded from the trials. Thus, trimethoprim at a concentration of 20 µg/ml was selected as it yielded a countable range of trimethoprim resistance bacteria. Selection for coliforms was chosen because of their role in the dissemination of antibiotics resistance genes as well as their contribution to the incidence of food borne disease.

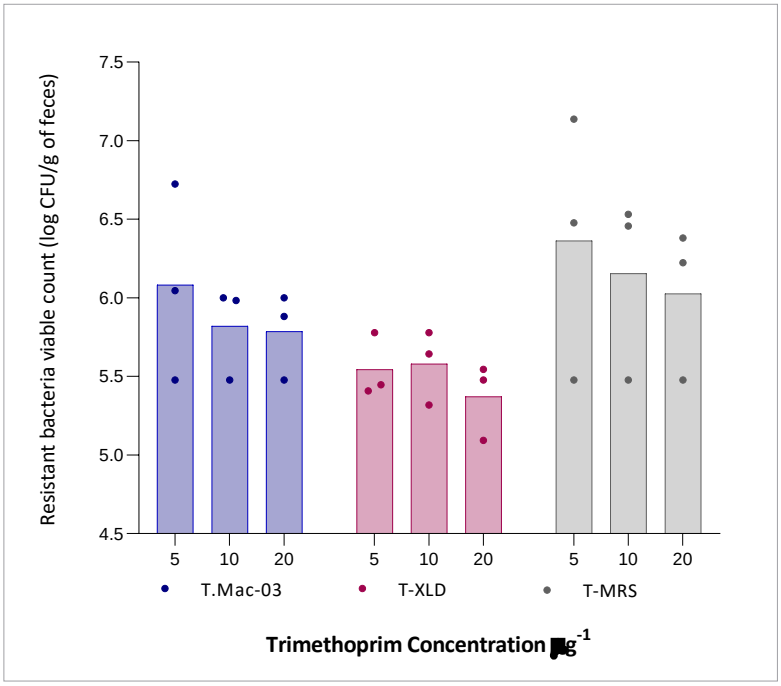


Figure 4.1 Initial test study for selecting the optimum trimethoprim concentration for plating on three types of media (MAC-03, XLD, MRS), at three different concentrations (5, 10 and 20 µg/ml) over three dilution factors (-2, -3, -4). The purple bar chart groups for enumeration coliforms, red group for *Salmonella* and grey bar chart for Lactic acid bacteria.

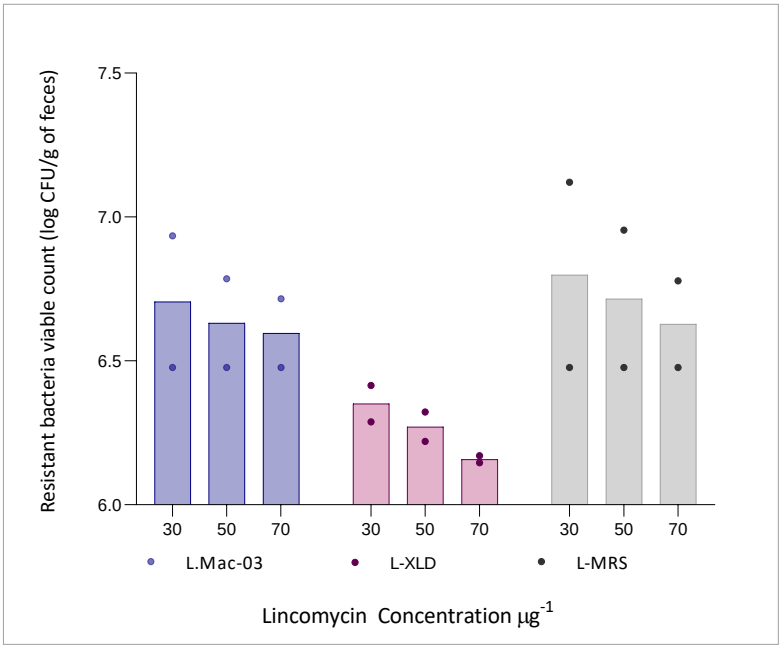


Figure 4.2 Initial test study for selecting the optimum lincomycin concentration for plating on three types of media (MAC-03, XLD, MRS), at three different concentration (30, 50 and 70 µg/ml) over two dilution factors (-3, -4). The purple

bar chart groups for enumeration coliforms, red group for *Salmonella* and grey bar chart for Lactic acid bacteria.

4.2.2 Enumeration of coliform bacteria recovered from the caecal contents of control fed birds reared under biosecure conditions.

In order to estimate the relative abundance of the caecal coliform counts (this includes all bacteria grown on the plate) with age of the chickens, enumeration of coliforms on triplicate MacConkey plates was performed as described in (Chapter 2, Section 2.4.1.1) with the control diet during 4 sampling points 22, 24, 28, 35 da (Trial1 Chapter 2, Section 2.3.4). The results are represented in Figure 4. 3.

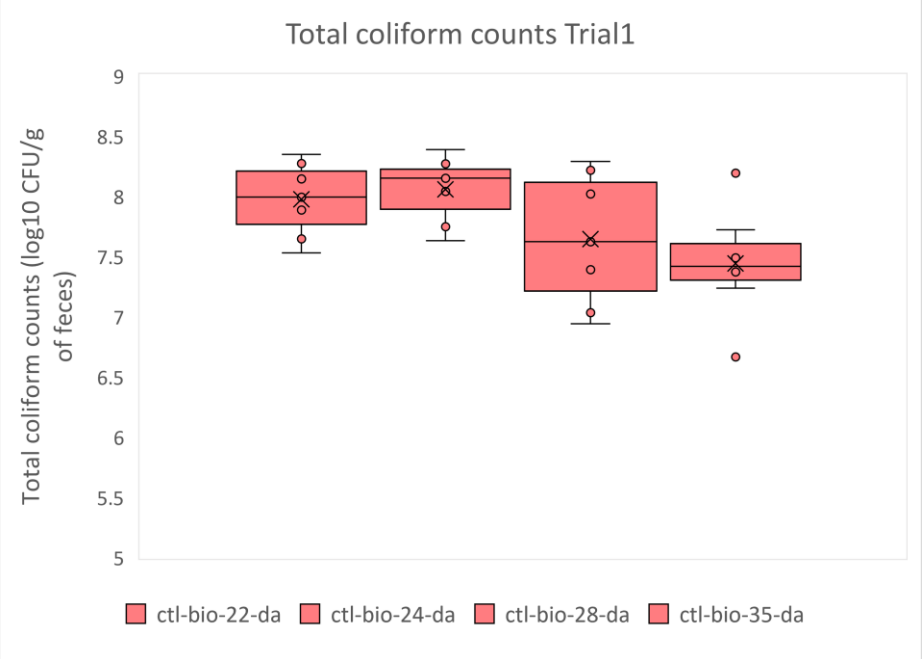


Figure 4.3 Total coliforms recovered from the caecal contents of 22, 24, 28 and 35 da old birds fed the control diet and reared in biosecure conditions (G1, trial 1). Data are expressed as standard boxplots with medians (solid black line). Outliers are represented as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x interquartile range. All orange boxes represent control fed birds. The number of birds per sampling point is 7 (total =28 birds), counted on Mac-03 media.

A significant difference was observed in total coliform counts between sampling days 22 vs 35 (p=0.026) as well as 24 vs 35 (p=0.01) with time.

Although there was no significant difference (p>0.05) found between 22 vs 24 or 28 vs 35 da. Therefore, the results show that coliform counts start to decrease at 28 da and continue to decrease to 35 da. In general, coliform numbers decrease with time.

4.2.3 Enumeration of trimethoprim resistance coliforms obtained from the caecal contents of control fed birds reared under biosecure conditions (G1, trial1).

The viable count of trimethoprim resistant bacterial were enumerated on MacConkey agar plates containing trimethoprim (20 µg /ml). The results in Figure 4.4 indicated that the viable counts of trimethoprim resistance bacteria generally remained steady across the trial with no significant difference was found (p>0.05) between different sampling days.

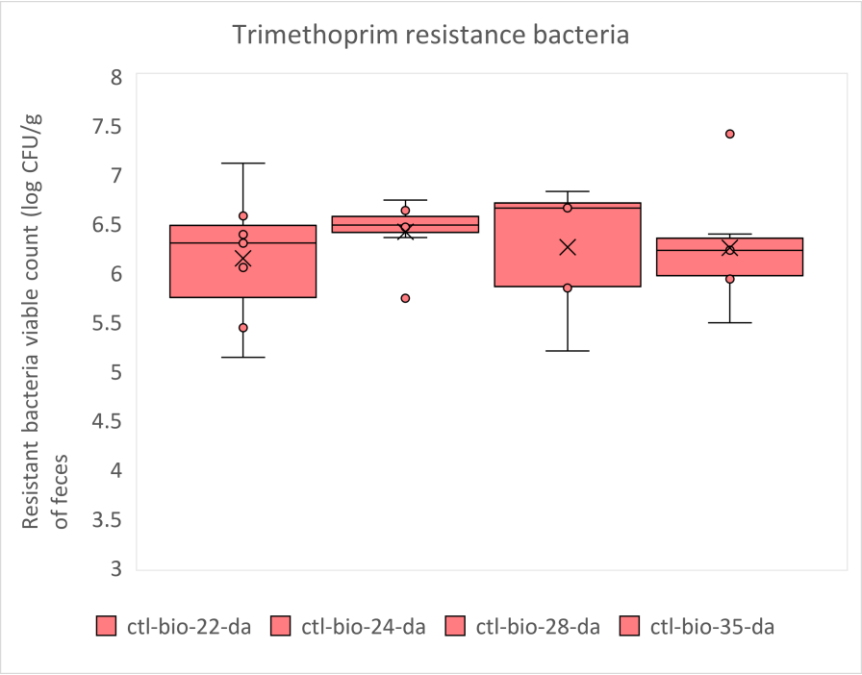


Figure 4.4 Viable counts of resistance coliforms bacteria growing on MacConkey 03 supplemented by 20 µg/ml of trimethoprim obtained from the caecal contents of 22, 24, 28 and 35 da old birds fed control diet (G1). Data are expressed as standard boxplots with medians (solid black line). Outliers are represented as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x interquartile range. All orange boxes represent control fed birds. The number of birds per

sampling point is 7 (total =28 birds), counted on Mac-03 media supplemented by 20 µg/ml of trimethoprim.

4.2.4 Ratio of coliform trimethoprim resistance bacteria from the caecal contents

The ratio of trimethoprim resistance was calculated by dividing the concentration of trimethoprim resistance coliforms by the total coliform count determined on control plates. There was no significant difference ($p > 0.05$) between the ratio of coliform trimethoprim resistant isolates in G1 when comparing between 22 vs 24 da or 28 vs 35 da. However, a significant difference was observed between 22 da vs 35 da ($p=0.014$) and between 24 vs 35 ($p=0.028$). The trend appears to be that the proportion of trimethoprim resistance bacteria increase with time.

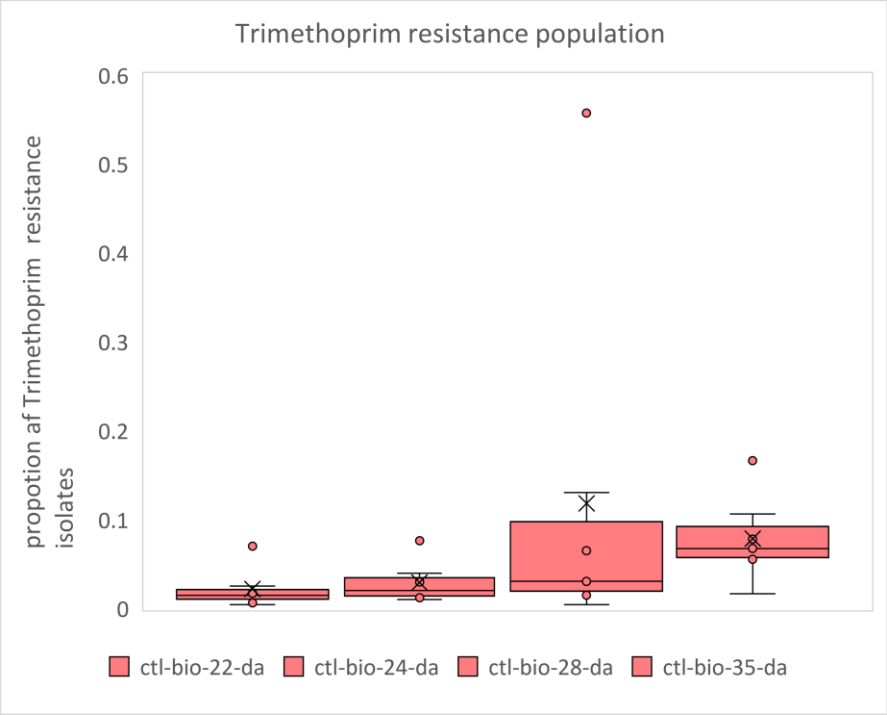


Figure 4.5 Ratio of trimethoprim resistant coliform population with time of controlled housing birds fed a conventional diet. The Figure shows the ratio of trimethoprim resistance in control diets gradually increasing with time. Specifically, the growth rate was slower at 22 and 24 da however this rate displayed a greater increase after 28 da. Data are expressed as standard boxplots with medians (solid black line). Outliers

are represented as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x interquartile range. All orange boxes represent control fed birds. The number of birds per sampling point is 7 (total =28 birds).

4.2.5 Evaluation of the class 1 integron copy number based on the integrase genes per gram of caecal content for control diet fed birds reared under biosecure conditions (ctl diet).

A second set of experiments was carried out to determine the integrase copy number for the control diet birds reared under controlled conditions. qPCR was preformed to evaluate class1 integrons signals across four sampling points (22, 24, 28, 35 da). The integrase sequence was selected because it is integral to the class 1 integron carrying ARGs and considered as an indicator of the antibiotic resistance load. Primers designed to target a small region of approximately 200 bp inside the main sequence. The established Ravi primer set was adopted for this purpose. The primary test was done by using the standard PCR for selected samples as shown in Figure 4.6, whilst the results of the qPCR assay of integrase copy number for the control diet groups are presented in Figures 4.7 and 4.8.

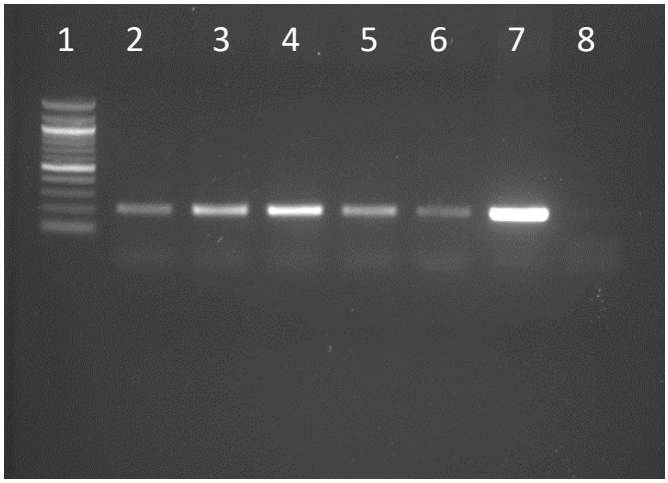


Figure 4.6 initial test for PCR amplified fragments for RT q-PCR for detecting integrase copy number (183bp). Lane 1 100 bp Ladder, Lane 2 sample1 (1.22.3), Lane 3 sample2 (1.22.7), Lane 4 sample3 (3.28.6), Lane 5 sample4 (4.24.3), Lane 6 sample 5

(4.24.6), Lane 7 *S.T.*U 288, Lane 8 negative control. Key for numbers (1=G1, ctl-bio nonc, 3=G3, ctl-bio-sal and 4=G4, gos-bio-sal).

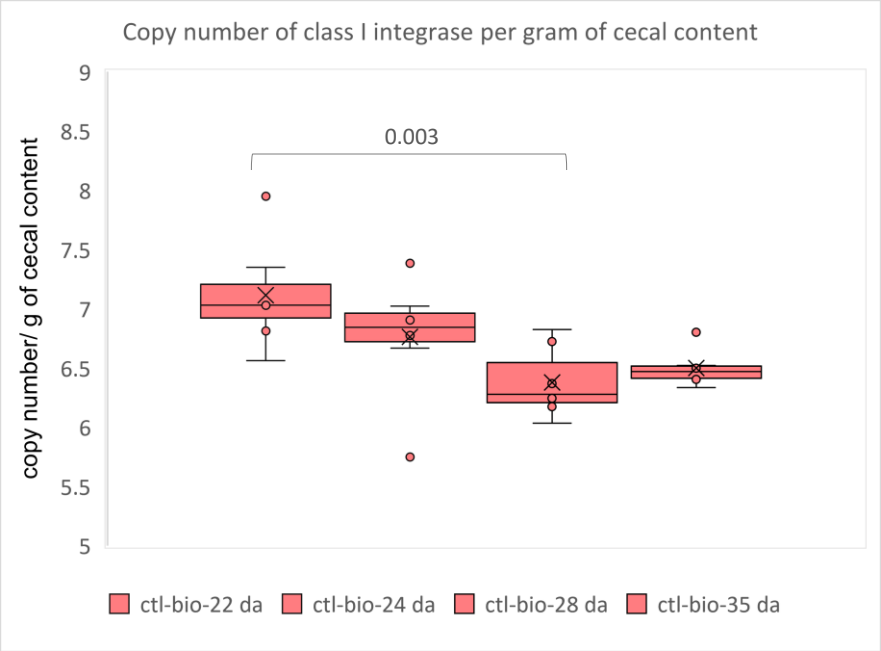


Figure 4.7 Class 1 integrase copy number quantified by qPCR per gram of caecal content for control housed birds. The specific primers were designed (Chapter 2, Section 2.5.3.3) to determine copy number of class 1 integrase gene applying *Salmone*lla typhimurium U288 as positive template to draw the standard curve (Chapter 2, Section 2.5.8.1). Comparison shows total copy number of integrases across 4 sampling points in control diets birds (Trial1). Data are expressed as standard boxplots with medians (solid black line). Outliers are represented as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x interquartile range. All orange boxes represent control fed birds. The number of birds per sampling point is 7 (total =28 birds).

Results demonstrated that there was no significant difference found in integrase copy number between the sampling points (t.test p-value= 0.19 at 22 vs 24 da, p-value 0.11 at 24 vs 28 and p-value=0.37 at 28 vs 35. However, the class 1 integrase copy number was significantly reduced at 28 da (control) compared to 22 da old birds (p-value, 0.003). Overall, the trend of integrase copy

number is to reduce with time for birds fed the ctl diet and reared under biosecure conditions.

4.2.6 Evaluation of copy number of class 1 integrase gene per gram of caecal content for commercial birds (ctl diet).

The quantification of copy number of class 1 integrase gene per gram of caecal content was carried out by using *Salmonella* Typhimurium U288 as a positive template and to create standard curve for absolute quantification copy number of the class 1 integrase gene. Results showed that there was no difference in total copy number of integrases between two sampling time in control diets of commercial birds (Trial 2).

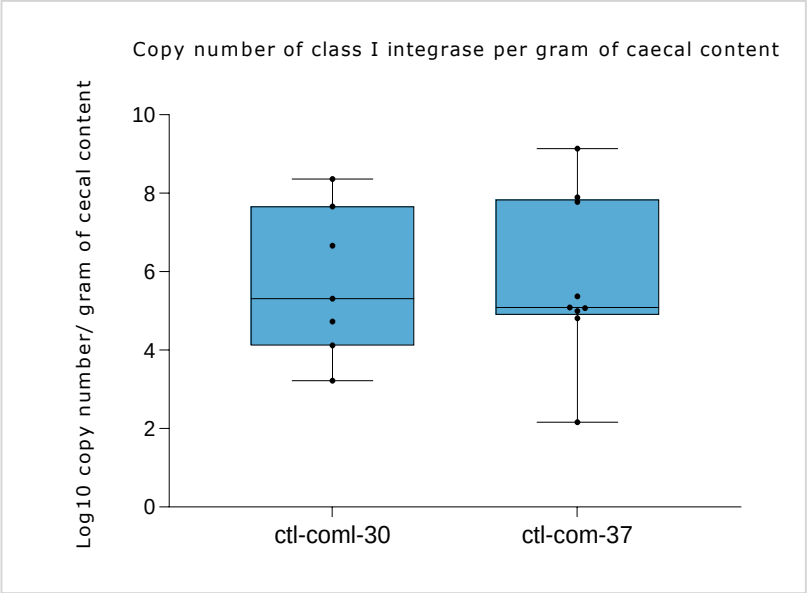


Figure 4.8 Class 1 integrase copy number quantified by qPCR per gram of caecal content of commercial birds. Data are expressed as standard boxplots with medians (solid black line). Outliers are represented as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x interquartile range. All blue boxes represent control fed birds. The number of birds per sampling point is 10 (total =20 birds).

4.2.7 Development of the caecal microbiome composition of control fed diet birds reared in controlled housing and commercial condition.

The developmental changes in the broiler chicken caecal microbiome were investigated by analysing DNA sequences of the 16S rRNA genes of the hypervariable region (V4). This allowed an assessment of both the biodiversity of caecal microbiota and the relative abundance of the community members from the two broiler chickens groups. A total of 1947973 (median: 66586, IQR: 59287.5) of quality-controlled sequence reads of 28 biosecure housed birds fed control diet, and a total of 1112207 quality-controlled sequence reads (median: 42664, IQR: 62932) obtained from 20 broiler caecal samples of commercial birds. These were classified into 4892 OTUs (distance 0.03) that split to 10 phyla. Coverage was calculated in mother (V1.39.5) by Good's coverage at 98% (range = 0.97 - 0.99%) to estimate the proportion of total OTUs present in each sample of the biosecure and commercial birds fed control diets. Rarefaction curves indicated the depth of sequencing data was sufficient for the coverage of all OTUs present in caecal samples (Appendix 3, Figure 3.1 A and B). The coverage range was deemed as sufficient for the sequence depth of all samples. Random subsampling of 3511 reads per sample, was undertaken in order to avoid bias between communities with different sampling depths for bacterial community analysis and abundance.

Generally, the dominant phyla for the microbiota of birds reared in biosecure housing were Firmicutes (*Clostridiales_unclassified*) with an overall median abundance of 93.06% ranging from 91.4%-96.6%, followed by Proteobacteria with median 3.26% (ranging from 1.78-6.34%) and smaller contributions from Bacteria_unclassified (median:1.2%) and Actinobacteria (median: 0.9%) within these phyla. Whilst the dominant phyla in the microbiomes of the commercial birds were Firmicutes with an overall median abundance 78.62% (77.96%-79.27%)

followed by Actinobacteria at median 7.52%, Bacteroidetes at median 5.70% (ranging from 3.93-7.47%), Proteobacteria median 4% (2.97-5.057%) and Bacteria_unclassified median 3.39% (4.43-2.35%). However, Bacteroidetes were only significant components of the microbiota of the birds from commercial flocks however they were absent in the caecal microbiota of birds from biosecure conditions across various sampling points.

At the class level, the results indicated that no significant difference was detected in the caecal bacterial communities by comparing the abundant OTUs at 22 da with birds at 24 da birds within the biosecure housed birds (trial 1). The exception to this was the relative abundance of Bacilli showing a decrease at 24 da (p-value 0.01, median: 7.31, 4.03, IRQ: 3.33, 0.79 at 22 vs 24 respectively). No significant change was found between the caecal bacterial communities at 24 da old birds and 28 da, or between 28 and 35 da old birds fed control diets at class level (p-value >0.05). While comparison of the sampling points (30 and 37 da) from the commercial birds indicated significant changes in the abundance of *Bacilli* (p-value 0.01, median: 8.07, 3.70%, IRQ: 6.4, 1.89 for the 30 da birds vs 37 da respectively), Negativicutes (p-value=0.0007; median: 0.00, 1.55, IRQ: 0.0, 25), Epsilonproteobacteria (p-value=0.0001; median: 0.00, 2.42%, IRQ: 0.0, 2.95).

Indeed, at the class level distinctive OTUs were evident in the caecal bacterial communities of commercial birds that were not present in caecal microbiota of controlled housed birds. These included the relative abundance of Bacteroidetes that were almost completely absent in controlled housing birds while they were found at both sampling points for the commercial birds (median: 7.47% at da 30, 3.94% at da 37, IRQ: 5.92, 3.37 in commercial birds). Betaproteobacteria represent approximately 1% (median: 1.21% at da 30, 1.0% at da 37, IRQ: 1.34, 0.62) in commercial birds whereas there was no presence of this class in controlled housed birds. Moreover, Epsilonproteobacteria (median: 0, at 30 da, 2.24% at 37 da; IRQ:0, 2.95) in the birds from commercial flocks was noted at 37 da and

absent at 30 da in the same trial. Epsilonproteobacteria were absent in trial1. The abundance of *Negativecutes* was not significantly different with less than 1% difference between all sampling points for the control housed birds. However, there was a significant difference in this class for the caecal microbiota of commercial birds at 37 da, showing the level of Negativecutes is significantly increased (p-value 0.0007).

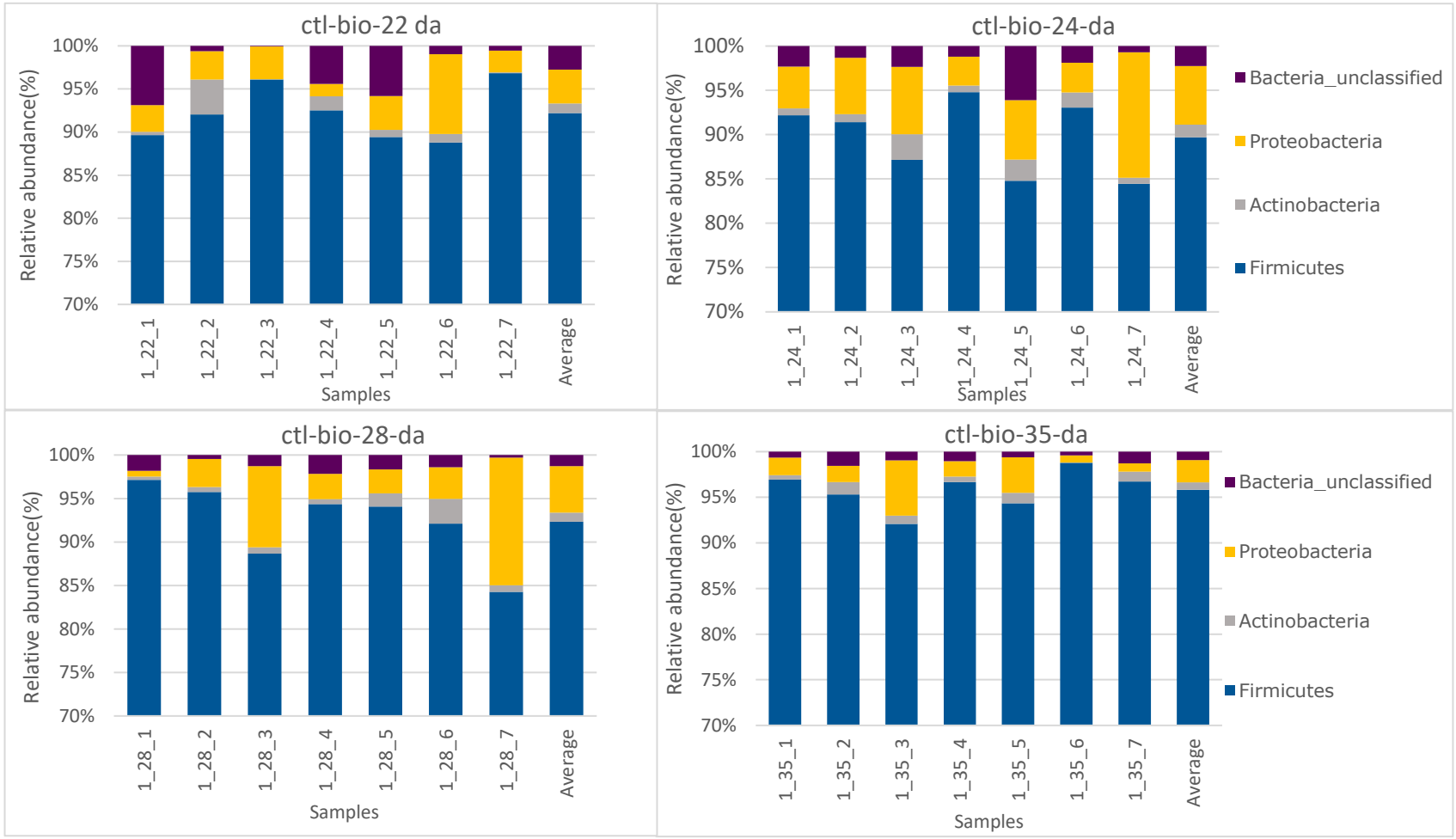


Figure 4.9 The relative abundance of dominant bacterial phyla of the chicken caecal microbiota of biosecure housed birds clustered on the basis of the Ribosomal Database Project taxonomy (phylum level). The results are presented as % and at least taxa that include 1% community was presented. For unbiased comparison all reads have been normalised.

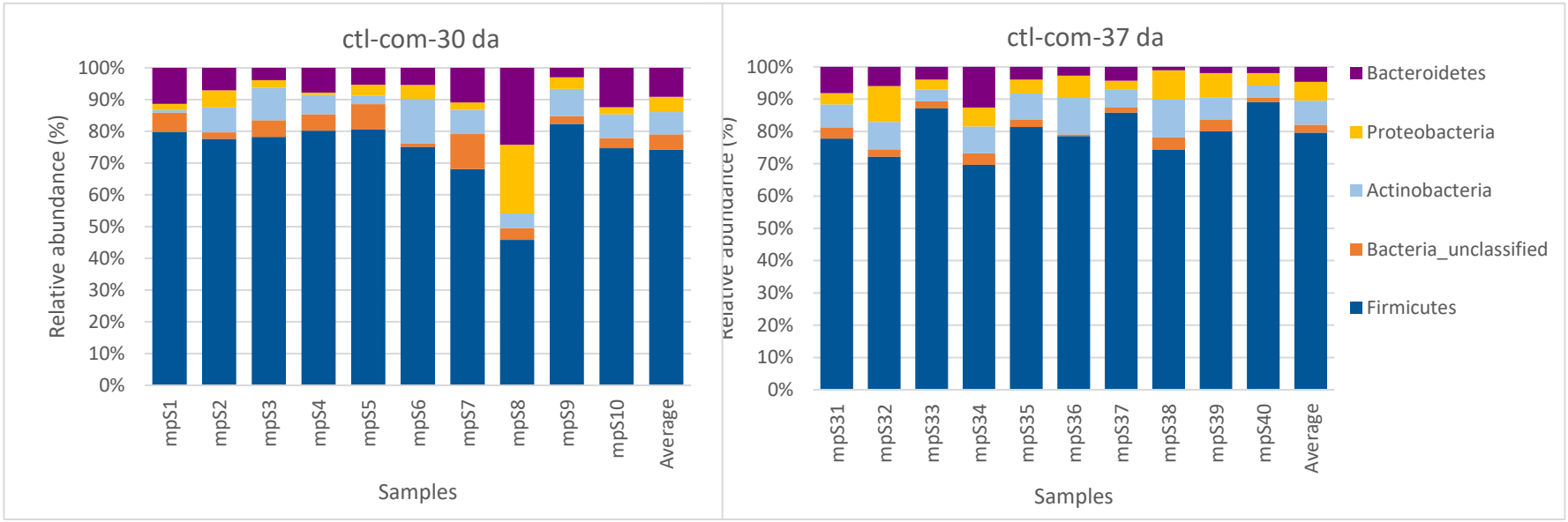


Figure 4.10 The relative abundance of dominant bacterial phyla of the chicken caecal microbiota of commercial birds clustered on the basis of the Ribosomal Database Project taxonomy (phylum level). The results are presented as % and at least taxa that include 1% community was presented. For unbiased comparison all reads have been normalised.

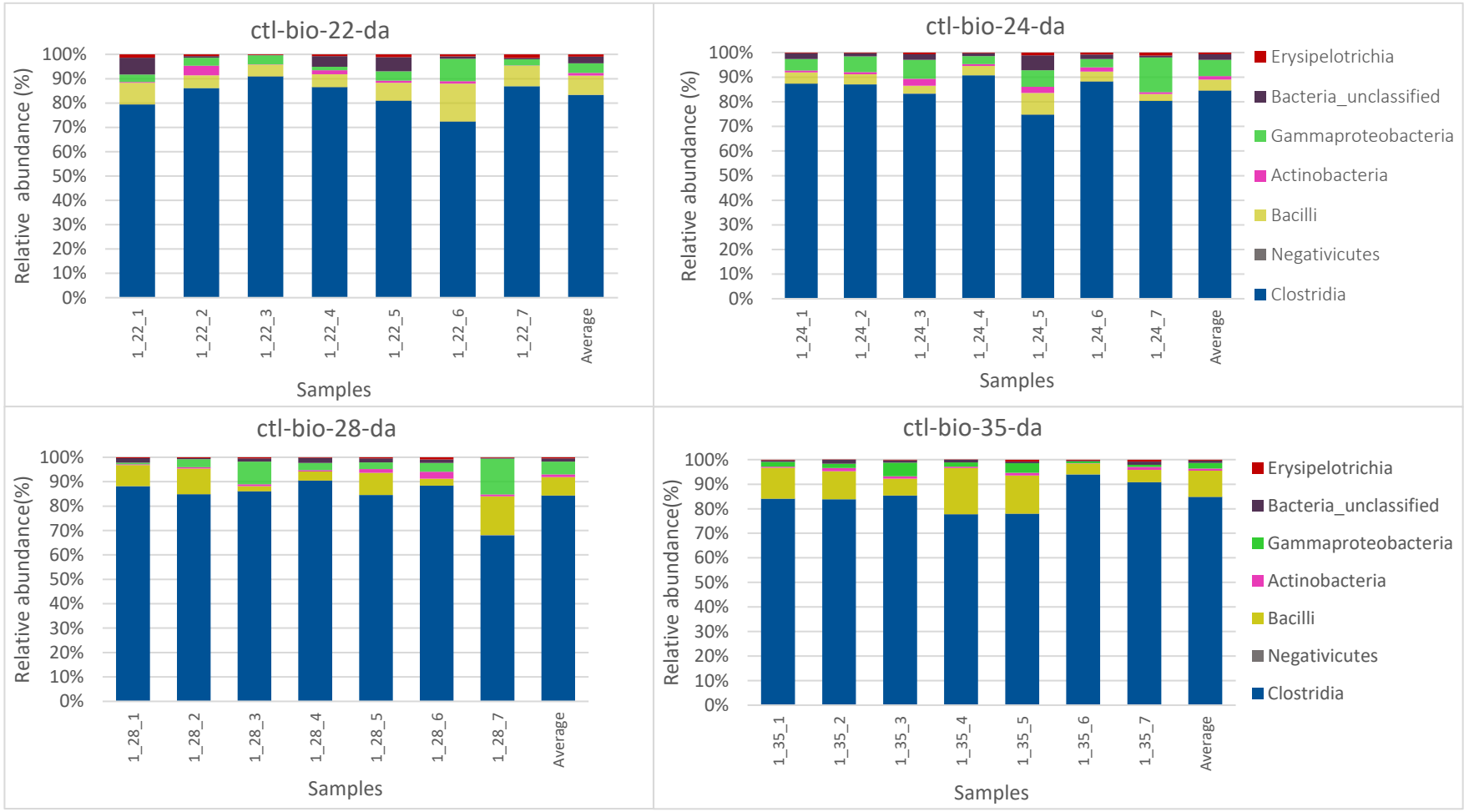


Figure 4.11 The relative abundance of dominant bacterial classes of the biosecure housed chicken caecal microbiota clustered on the basis of the Ribosomal Database Project taxonomy (class level). The results are presented as % and at least taxa that include 1% community was presented. For unbiased comparison all reads have been normalised.

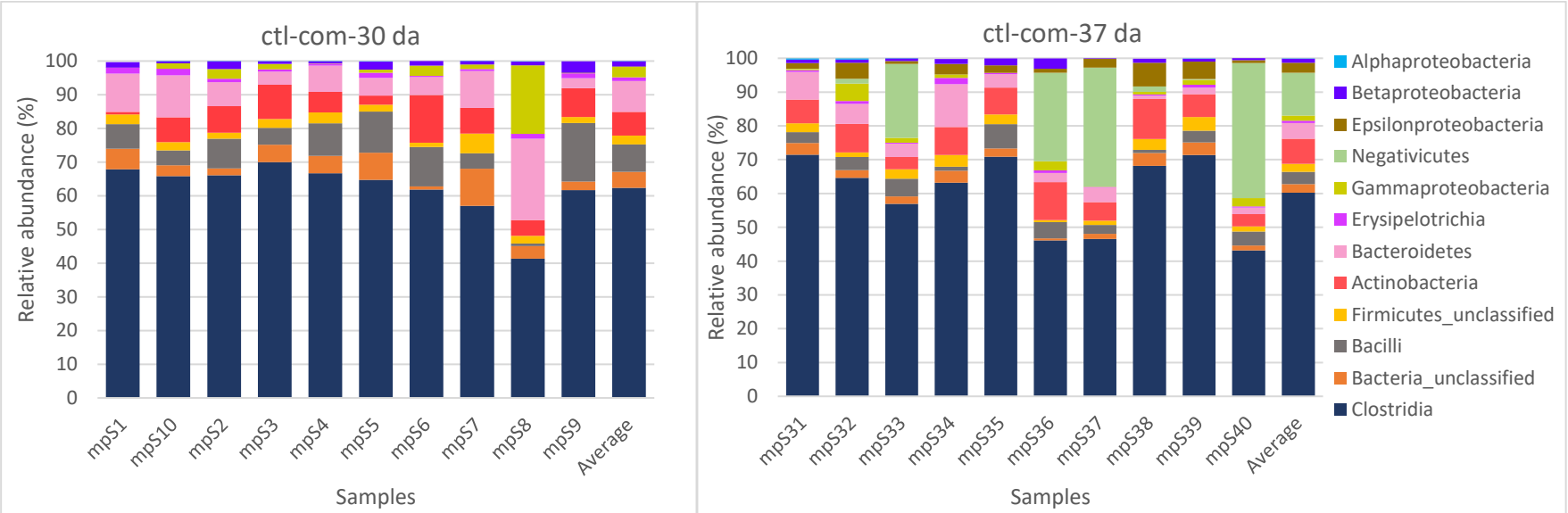


Figure 4.12 The relative abundance of the dominant bacterial classes of the commercial broiler chicken caecal microbiota clustered on the basis of the Ribosomal Database Project taxonomy (class level). The results are presented as % and at least taxa that include 1% community was presented. For unbiased comparison all reads have been normalised.

4.2.7.1 Relative abundance of Proteobacteria phylum of controlled housing (CH) birds and commercial birds fed control diets

The classification of bacterial taxa is displayed as hierarchal taxonomic groups starting with Phylum and ending with OTU (Phylum > Class > Order > Family > Genus > OTU). The median of Proteobacteria abundance (OTU003, OTU004 in trial1 and trial 2 respectively) was investigated at the phylum level in broiler caecal samples of the control fed barns indicated that there was no significant change calculated by using Mann Whitney or Wilcoxon test (Figure 4.13; p-value >0.05). The medians of the relative abundances of Proteobacteria at 22 da 3.29%, at 24 da 6.34%, at 28 da 3.23% and at 35 da 1.78% for control housed birds. There was no significant difference between the abundance of Proteobacteria (Figure 4.13) of commercial birds fed the control diet between 30 da (2.97%) and 37 da (median: 5.0 %; Wilcoxon test p-value =0.075). The results of the Shapiro-Wilk test of normality and quantile-quantile plots indicated that the distribution of OTUs of Proteobacteria phylum was not normally distributed across cohorts at 22 da in biosecure birds and at 30 da for the commercial birds (Shapiro-Wilk normality test= 0.0215 at 22 da, p-value=0.0001 at 30 da in commercial birds; Appendix 3, Figure 3.2 A and B).

There was an intra-group variability in the caecal bacterial communities at phyla level as observed in the Proteobacteria phylum for the control diet group at 30 da, (median: 3%, IQR: 1.9%). At the class level the Negativicutes, showed variation in composition with an identical bootstrap threshold for control communities at 37 da (median: 1.6%, IQR: 24.7).

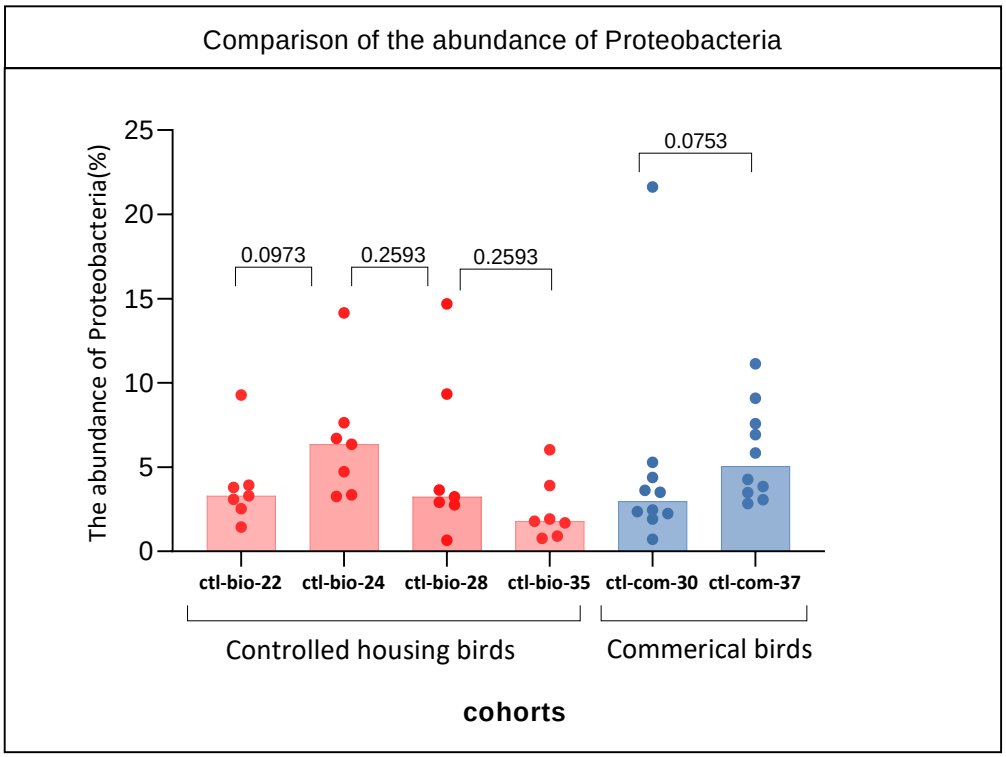


Figure 4.13 Comparison of the relative abundance of the Proteobacteria phylum of controlled housed birds during 4 sampling points and commercial ones through two sampling points both fed control diets. Bar charts indicate median of relative abundance of the Proteobacteria, Wilcoxon test *p* value was used because the relative abundance of all Proteobacteria phylum not normally distributed across cohorts (Appendix 3.2 A and B). All orange bars represent control fed birds reared in biosecure condition, while blue bars indicate to birds fed control and reared under commercial condition.

4.2.7.2 Distribution of resistance gene cassettes (GCs) arrays in Proteobacteria phylum

Phylogenetic trees of the proteobacterial OTUs identified within the chicken caecal microbiota of the biosecure housed bird and those from commercial are presented in Figures 4.14. As integron gene cassettes are frequently associated with Proteobacteria, the distribution of GCs for each bird within the Proteobacteria phylum are indicated adjacent to the identifiers of the phylogenetic trees. These show that the distribution of GCs, mainly GCs-B-2 in the CH birds, was associated with Gammaproteobacteria OTU0007 belonging to *Enterobacteriaceae_unclassified* with few contributions associated with OTU0324 which is also Gammaproteobacteria (*Enterobacteriaceae_unclassified*) and OTU0169 is Gammaproteobacteria (*Proteus*). Whilst GCs were found more prevalent among various Proteobacteria OTUs in commercial birds showing the most abundant GCs were mostly detected in OTU0007 (*Enterobacteriaceae_unclassified*) and OTU0070 (*Betaproteobacteria Parasutterella*), OTU0276 (*Proteobacteria_unclassified),* OTU0041 (*Epsilonproteobacteria Campylobacter*), OTU0176 (*Alphaproteobacteria Aestuariispira)* and OTU 0615 (*Gammaproteobacteria Enterobacteriaceae_unclassified*). Generally, A and B2 GC types were distributed among all the commercial birds featuring Proteobacteria phyla. However, the group A GCs dominated in 37 da birds which coincides with the appearance of *Campylobacter* OTU0041 at 37 da. Appendix 3, Figure 3.3 A and B exhibiting GCs distribution per sampling points for each trial. Due to the cut off value adopted ≥ 20 reads there was no Proteobacteria OTUs recorded at 28 and 35 da.

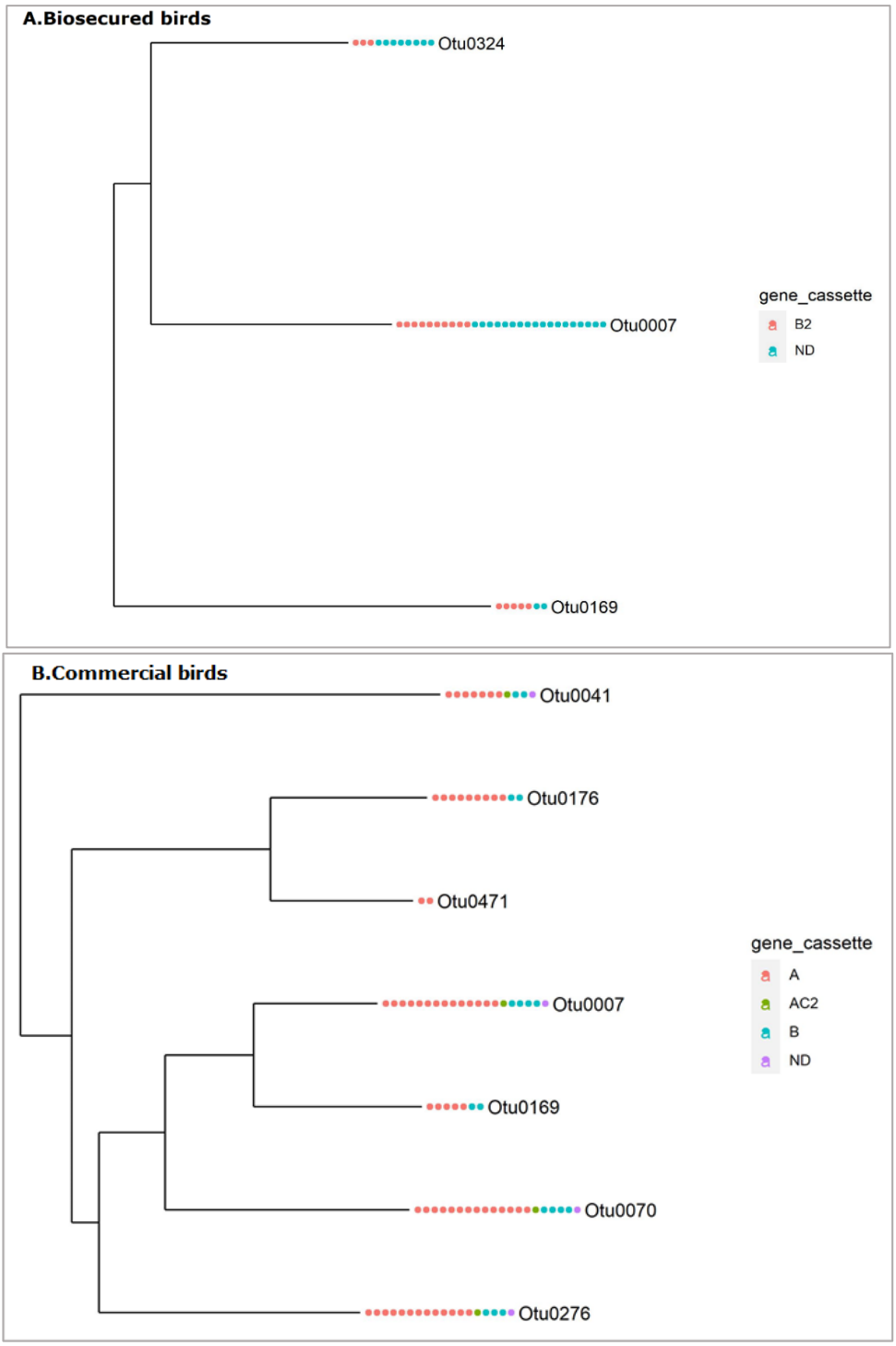


Figure 4.14. The phylogenetic tree of Proteobacteria OTUs associated with the distribution of GC groups identified in the broiler chicken microbiota. The tree was constructed by a neighbour-joining method with Bootstrap value ⩾ 20. **(A)** biosecure birds only da 22 and 24 have been presents because with applying cut-off value≥ 20 there was no Proteobacteria OTUs found at 28 and 35 da. **(B)** commercial birds age matched 30 and 37 da. The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU 0324 is Gammaproteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is

Gammaproteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gammaproteobacteria (*Proteus*), OTU0041 is Epsilonproteobacteria (*Campylobacter*), OTU0176 is Alphaproteobacteria (*Aestuariispira*), OTU0471 is Alphaproteobacteria (*Alphaproteobacteria_unclassified*), OTU0565 is Gammaproteobacteria (*Escherichia/Shigella),* OTU0070 is Betaproteobacteria (*Parasutterella*), OTU0276 is *Proteobacteria_unclassified.* ND not detected.

4.2.8 Microbial diversity analysis

To analyse the microbiome composition of the birds on the control diets of alpha (α-) and beta (β-) diversity indices were calculated from the data using Mothur (V1.39.5). For the α-diversity, the OTUs richness (observed OTUs) were calculated using the Chao1 index, while the overall diversity (evenness) was measured using the inverse Simpson's index. The Chao1 index evaluates richness (the total number) of species present in the community whereas the inverse Simpson's index gives more weight to dominant (the abundance) species. The Shapiro-Wilk test of normality was performed, which determined that inverse Simpson's index of the trial1 data were normally distributed with the exception data for 24 da (Shapiro-Wilk test p-value >0.05; except at da 24 the normality test p-value= 0.0279). Likewise, Chao index data of biosecure birds (trial 1) were normally distributed across sampling days p-value >0.05 except for 28 da the Shapiro-Wilk test p-value= 0.03780. However, the inverse Simpson's index and the Chao index were normally distributed for the commercial birds (trial 2; inverse Simpson's index p-value= 0.7348 and 0.2637 at da 30 and 37 da). The Shapiro-Wilk test of normality of the Chao index of commercial birds were p-value= 0.4431 and 0.3210 for 30 da and 37 da respectively. The fit of the data using quantile-quantile plots and frequency distribution plots (all QQ plots of normality test) are presented in Appendix 3.4 and 3.5 (A and B).

The α-diversity with respect to time are presented in Figure 4.15 for the control diets groups of biosecure and commercial birds. For the biosecure reared

birds the inverse Simpson index appears to reduce with age, but a significant change was only observed between 24 and 28 da (inverse Simpson index, p-value= 0.026). The Chao index for community richness of controlled hosing birds showed that there was no difference between the 22, 24, 28 and 35 da results as presented in Figure 4.16.

The alpha diversity of broiler chicken microbiota of the commercial birds exhibited a significant reduction in inverse Simpson index between 30 and 37 da for the control barn (Simpson index, p-value= 0.0002; Figure 4.15). While the Chao index of commercial birds indicated there was no significant change between 30 and 37 da (Chao index, p-value >0.05; Figure 4.16).

The β-diversity was estimated by Bray-Curtis dissimilarity, which demonstrated variation in species composition on the basis of age. Differences in β-diversities were visualised using principal component analysis based on the Bray-Curtis distances between each experimental group as presented in Figures 4.17 and 4.18. The results indicated that there is no significant change in caecal microbiota of the control birds (AMOVA test p-value>0.05) when comparing 22 vs 24 da (AMOVA test p-value= 0.328), da 24 vs 28 da (AMOVA p-value=0.091), However, a significant change was found between the microbiota collected at 28 vs 35 da (AMOVA test p-value=0.015) and when overall comparison of all sampling points were considered (AMOVA test p-value = 0.001). Whilst there was a significant change was found in commercial birds of microbial communities at 30 and 37 da (AMOVA test p-value = 0.001).

The linear discriminant analysis (LDA) effect size (LEfSe) method was used in order to identify OTUs with differential relative abundance between the caecal microbial communities of biosecure birds with development. The data show transitions in the beta diversity of the broiler chicken microbiota between the four sampling times (22, 24, 28 and 35) highlighting microbial succession, which is marked by the dominant order of *Clostridiales*, most OTUs belonging to the

Lachnospiraceae and *Ruminococcaceae*. AMOVA indicated a significant difference between 28 and 35 da birds for birds reared under biosecure conditions. LEfSe identified five differentially abundant OTUs at 35 da, which were *Romboutsia* (OTU0021), *Lachnospiraceae_unclassified* (OTU0012, OTU0016), *Anaerostipes* (OTU0011) and *Blautia* (OTU0034) compared to *Lachnospiraceae_unclassified* (OTU0013), *Oscillibacter* (OTU0068) and *Clostridium_IV* (OTU0125) that show greater abundance at 28 da (Figure 4.19). LEfSe was also performed to determine the differentially abundant OTUs between the microbiotas of commercial birds fed control diet collected at 30 and 37 da. OTUs identified in seven genera that appear at 37 da explain these differences (Figure 4.20): *Megamonas* (OTU0004), *Faecalibacterium* (OTU0005) *Campylobacter* (OTU0041), *Bacteroides* (OTU0115), *Subdoligranulum* (OTU0048), *Romboutsia* (OTU0021) and *Clostridium_IV* (OTU0158).

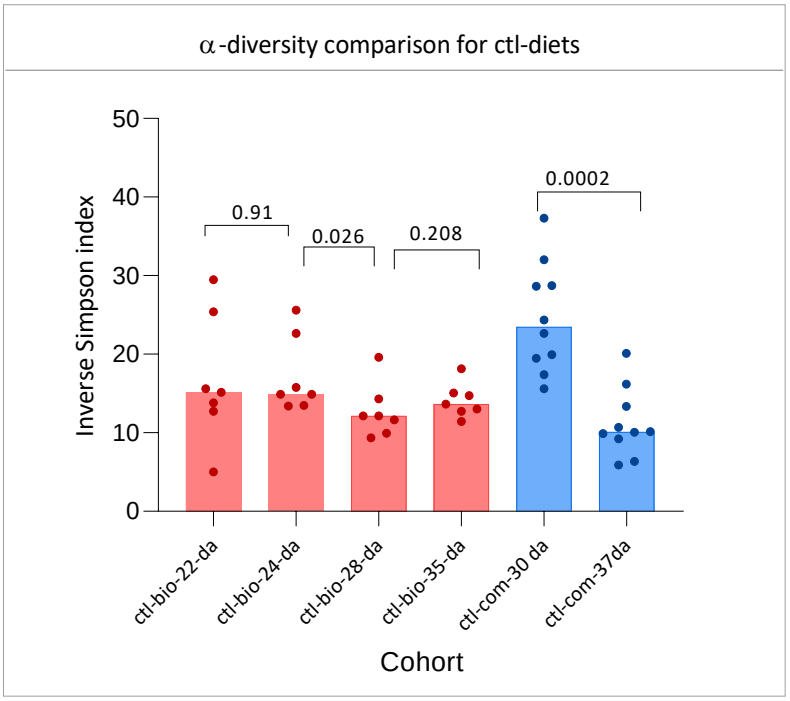


Figure 4.15 Comparison of inverse Simpson's index for alpha diversity between sampling ages for controlled housing (22, 24, 28 and 35 da) and commercial birds (30 and 37 da). Data are displayed for each control groups as bar chart with medians and outliers are represented as dots. All orange bars represent control fed birds reared in

biosecure condition, while blue bars indicate to birds fed control and reared under commercial condition.

Results showing there was no significant in alpha diversity within different sampling points of biosecure birds in trial1, however there was declining observed in inverse Simpson's index of ctl at 28 da old birds compared to ctl-24 old birds. Similarly, within trial 2 (30 vs 37 da in commercial birds) there was significant reduction in alpha diversity (inverse Simpson index, p-value 0.0002) with age. Inverse Simpson's indices were tested for normality distribution by Shapiro-Wilk test indicating data not normally distributed at 24 da old cohorts reads (Shapiro-Wilk test p-value=0.0279 at 24 da) therefore significant was calculated by Mann Whitney.

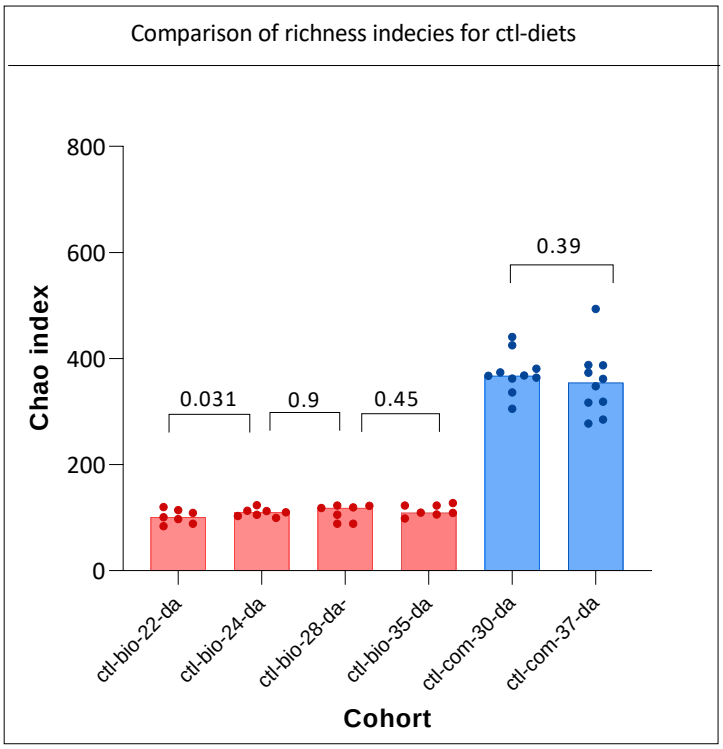


Figure 4.16 Comparison of Chao index for richness between sampling ages for controlled housing (22, 24, 28 and 35 da) and commercial birds (30 and 37 da). Results showing there was no significant difference detected between sampling points of controlled housing birds (Chao index P-value>0.05) as well as between 30 da and 37 da old birds reared in commercial condition (Chao index P-value= >0.05). Although, it indicates that richness reduced in CH birds. However, no significant difference was noted within each sampling time of each trial. The Chao indices calculated from normalised reads data are displayed for each control groups. Data are expressed median of each group. as

described above. Shapiro-Wilk test indicating that data not normally distributed at 28 da old cohorts therefor all data treated the same way by Mann whiney test.

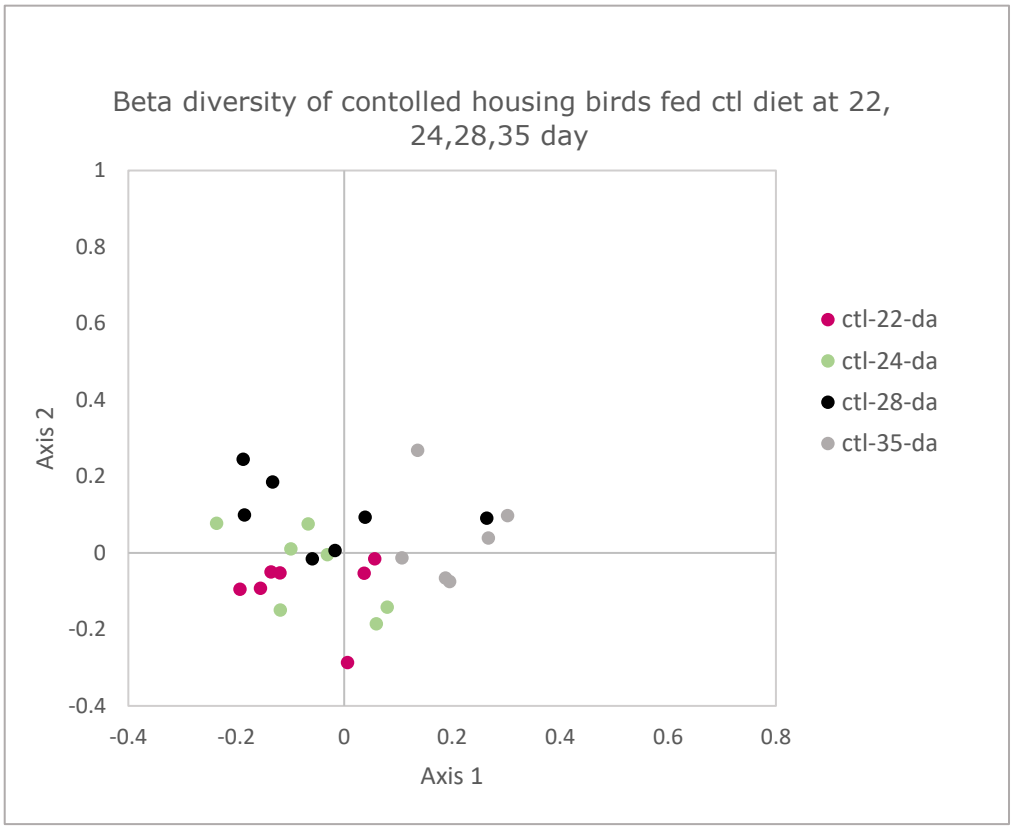


Figure 4.17 PCoA plots of Beta diversity based on Bray Curtis dissimilarity of controlled housing birds fed control diets. The control diet birds at 22 and 24 da has no significant difference between the birds (AMOVA test p-value: 0.328), There was also no significant difference between 24 and 28 da (AMOVA p-value 0.091), whilst there was a significant difference in microbiota transition between 28 and 35 da old birds (AMOVA p-value 0.015).

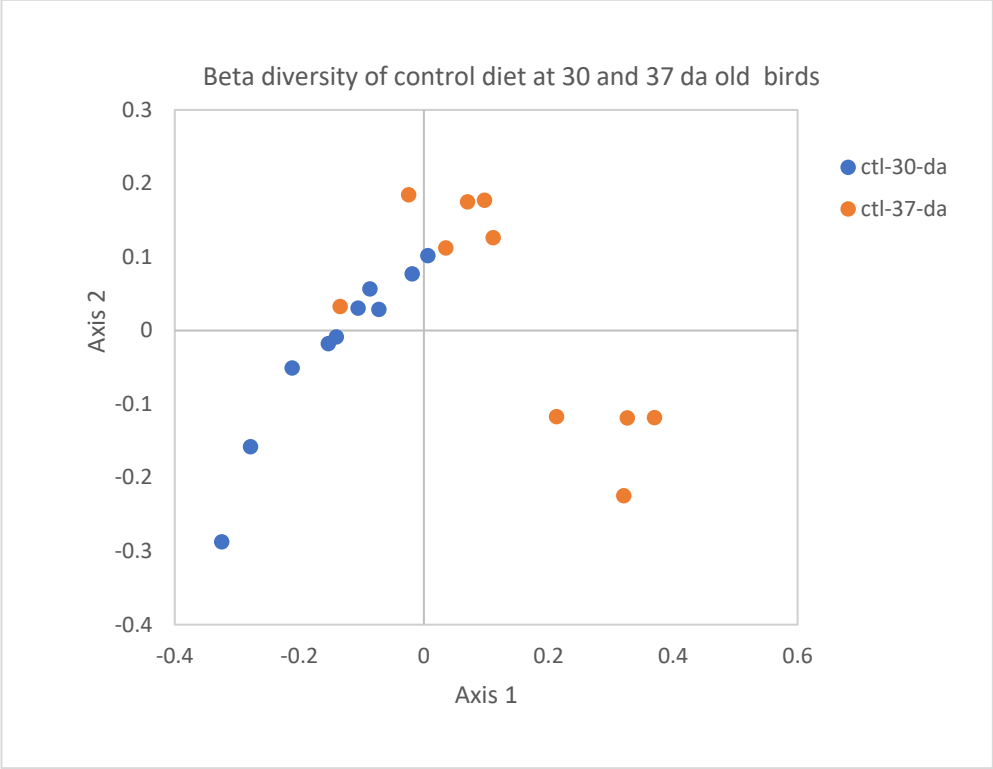


Figure 4.18 PCoA plots of Beta diversity based on Bray Curtis dissimilarity clustering data with respect to age of commercial birds fed a control diet. Comparison of caecal microbiota of commercial birds fed control diets at age 30 and 37, indicates there is a significant difference between the two barns (p-value: 0.001, AMOVA test). A clear transition in the microbiota at 37.

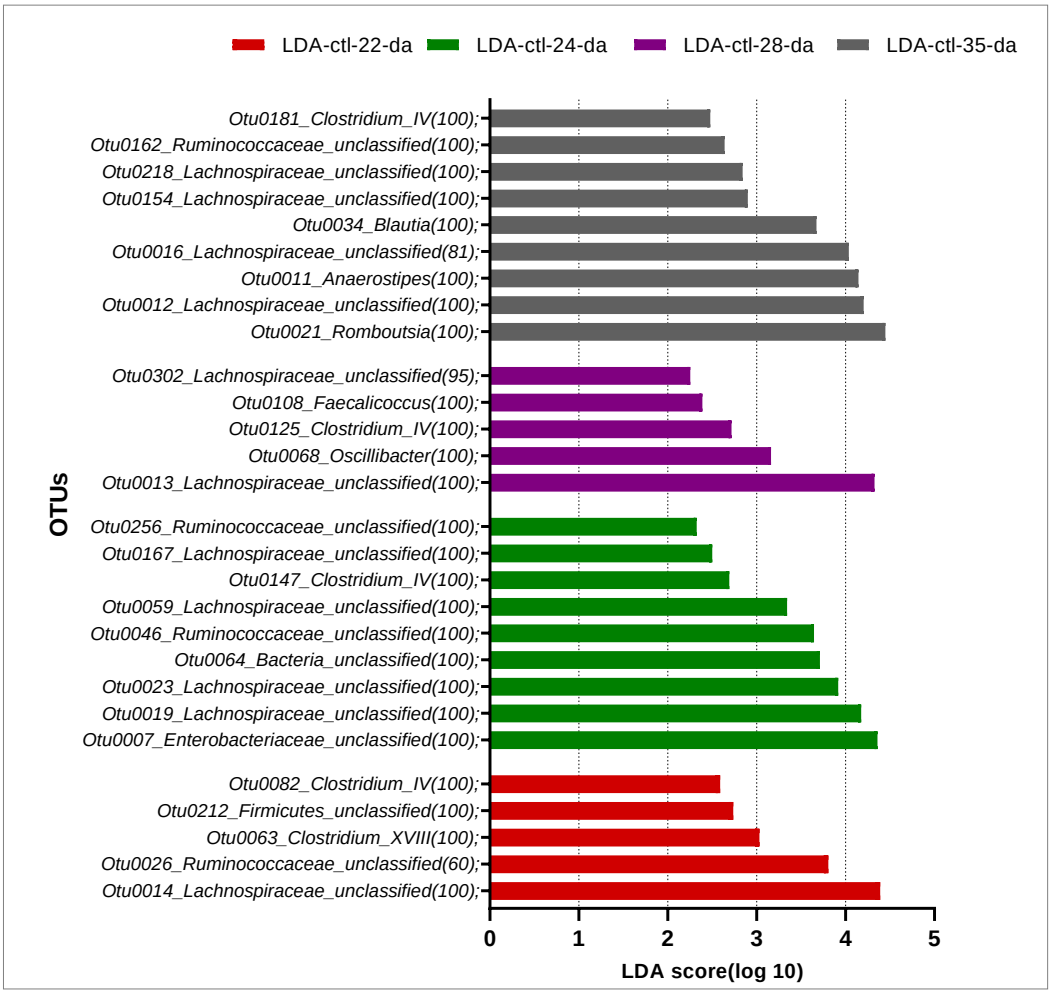


Figure 4.19 Histogram of the linear discriminant analysis (LDA) effect size (LEfSe) of control housed birds over 4 sampling times. LDA scores computed for express the differentially abundant of bacterial communities of birds fed ctl and reared in biosecure conditions at 22 da (represented by green bars), 24 da birds (represented as red bars), 28 da birds (purple bars) and 35 da (grey bars). LEfSe illustrates which clades amongst detected OTUs that are statistically explain the greatest differences between bacterial population fed the same diets and reared in similar conditions. All representative OTUs LDA≥ 2.

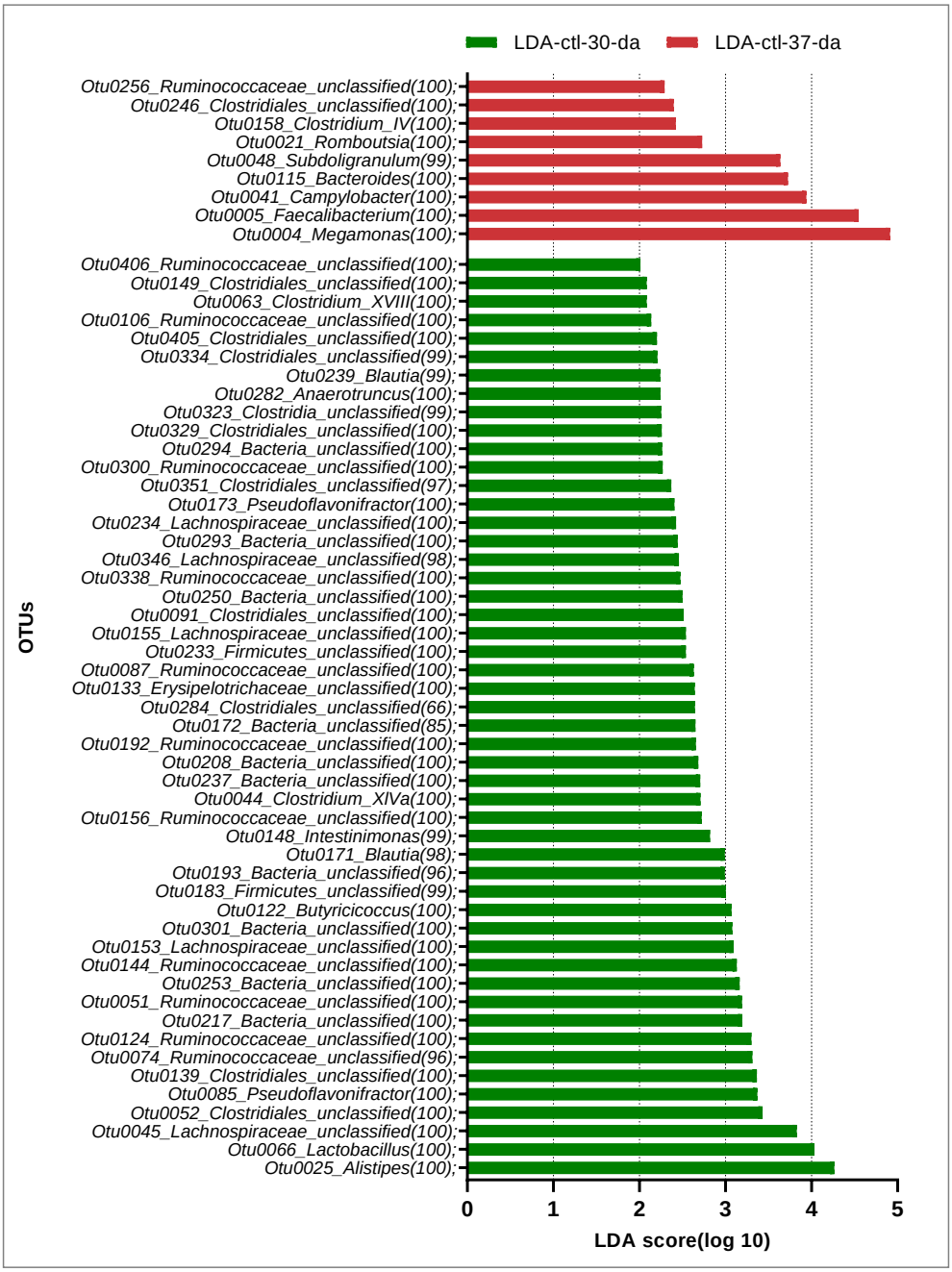


Figure 4.20 Histogram of the linear discriminant analysis (LDA) effect size (LEfSe) between commercial birds at 30 and 37 da. LDA scores computed for express the differentially abundant between commercial broiler chickens at 30 da (represented by green bars) and 37 da birds (represented as red bars). LEfSe illustrates which clades amongst detected OTUs that are statistically explain the greatest differences between bacterial population fed the same diets and reared in similar conditions.

4.3 Discussion

In Chapter 3 the relative abundance of the gene cassettes obtained from the broiler chicken caecal microbiota of birds reared in biosecure or commercial environments was generally considered. This Chapter highlights diversity in the microbiota of the birds from which the gene cassettes were identified. Under biosecure rearing conditions 37% (10/27) of the birds were identified as carrying GCs-B-2 (*dfrA1* and *aadA1*) across four sampling points but in 17 birds GCs were not detectable (see Table 3.4A). To the contrary, in commercial birds with greater stocking densities, the GCs were identified in the majority of caecal samples and showing a higher diversity in the embedded cassettes: GCs-A contains *aadA2* and *linF* resistance genes with 35% (7/20) at 30 da to 40% (8/20) at 37da with 15% GCs-B-2 at 30 da, and 5% for GCs-B-2 and GCs-C-2 at 37 da (see Chapter 3, Table 3.6). Brolund et al. (2010) researched the distribution of *dfr*-genes in *E. coli* and *K. pneumoniae* isolated from hospital patients with UTIs (frequently treated with trimethoprim), which demonstrated a disproportionate prevalence of integrons in *E. coli* and *K. pneumoniae* compared to controls. The *dfr*-genes were carried by narrow host range plasmids, suggesting the need for further studies to understand the link between gene-cassettes of plasmids, integrons, and the chromosome (Brolund et al., 2010).

As described in this Chapter, trimethoprim resistance was selected to study phenotypic antibiotic resistance as it could be discriminated in birds reared in biosecure and commercial conditions. The *dfr* gene that confers trimethoprim resistance could be identified in GCs PCR amplified from chicken caecal microbiota genomic DNA preparations. Blahna *et al.* (2006) found that most *dfr*-genes resided in gene cassettes within variable regions of integrons leading to the rapid spread of trimethoprim resistance between various bacteria. Additionally, they reported

that the most common gene present among uropathogenic *Escherichia coli* in Europe and Canada was *dfrA1*, making up 37.9% of the *dfr* containing isolates.

Similarly, the current study found *dfrA1* the predominant gene in GC-B-1 and GC-B-2 in both trials. However, several studies have reported that alternative *dfr*-genes including *dfrA5, dfrA7, dfrA12, dfrA14, dfrA17, dfrA22 and dfrA27* to be located within variable regions of class 1 integrons (Grape et al., 2005;Blahna et al., 2006;Kadlec and Schwarz, 2008;Wei et al., 2009;Šeputienė et al., 2010).

Despite the trend of trimethoprim ratios increasing with days of age, the integrase gene copy number decreased with time. This finding suggests that the resistance genes are not only embedded in class 1 integrons as measured using the integrase gene as the target. It is also plausible that there is redundancy in the bacterial populations that harbour the class 1 integrons, members of which become excluded but not those carrying the functional resistance gene. As a consequence, the proportion of the coliforms carrying trimethoprim resistance will increase while the total coliforms decline, including those carrying class 1 integrons. Yu et al. (2004) pointed out the *dfr* genes integrated within integrons seem to be more prevalent than those genes that are not associated with class 1 integrons. They suggest that studies should focus on changes in *dfr* genes associated with class 1 integrons over the time.

The analysis of 16S RNA data provided deep investigation of the diversity and abundance of the caecal luminal microbiota for the standard diets of birds reared in two different environments. Many studies have indicated that the most abundant phylum in the chicken caecal bacteriome are Firmicutes, for example a study by Yeoman et al. (2012) reported Firmicutes as the predominant taxa in the caecal microbiome. Wei et al. (2016) showed that this phylum formed 57.8% of the total bacterial sequences of caecal content samples, whereas the Bacteroidetes and Proteobacteria phyla were less abundant, comprising 5.4% and 4.3% of the total bacteria sequences respectively. However, Oakley et al. (2014) reported that

Firmicutes, Bacteroides, and Proteobacteria are the most common phyla in the chicken ceca. Likewise, the analysis of 16S RNA sequences at phyla level for the microbiota of the commercial birds was consistent with the study, while the composition of the caecal microbiota of the birds reared in controlled housing exhibit less diversity including the absence of Bacteroidetes at various sampling times. A study conducted by Oakley et al. (2014) highlighted the role of housing environment as source of diversity of microbial community in the most commercial poultry that carried over from one flock to another acting as an important inoculum for the chick gastrointestinal microbiome. Thereby variation between trials may be as consequence of rearing conditions or environmental changes that birds face but variation within each trial sampling points could be due to temporal succession. For example, van der Wielen et al. (2000) proposed a possible reason for the high prevalence of Firmicutes a consequence of the need for butyrate in the developing the intestine of young chickens but this may be reduced with increased age. While Polansky et al. (2016) observed a gradual increase in Bacteroidete numbers in the cecum after 3 weeks as consequence of the need to digest polysaccharides, which produce propionate and butyrate required for nutrient balance.

Results at class level revealed that the diversity of OTUs was higher in commercial flock than the birds reared in controlled housing. Clear variation was highlighted between two trials, most notably the emergence of *Campylobacter* OTUs (Epsilonproteobacteria) at 37 da in the commercial birds concurrent with *Negativecutes*, which were not found at 30 da. This could be an indicative to human contact via the thinning process, which took place at 30 da for the commercial poultry flock. A recent study by Connerton et al. (2018) noted that chickens can be exposed to *Campylobacter* colonization at any time during the rearing period, however the efficiency of transmission and detection of campylobacters occur after 2 weeks. The emergence of other OTUs and secondary GCs at 37 da could also be correlated with human exposure at thinning process.

The majority of previously declared *Campylobacter* free broilers can become contaminated quickly during the thinning process (Sahin et al., 2015). The European baseline study (EFSA, 2010) stated that thinning (partial depopulation) is significantly contributes to transmission of campylobacters into a poultry house considered as key risk factor for flock positivity (Newell et al., 2011). This because catchers travelling from farm to another with their own vehicles, equipment, boots, and clothing, often disregarding personal hygiene and biosecurity. This led to use these tools in depopulation process with possible contamination with campylobacters at the abattoir or other farm sites (Hutchison et al., 2004;Newell et al., 2011).

The α-diversity of the broiler chicken caecal microbiota was calculated by inverse Simpsons and used to assess differences in the bacterial populations of the developing birds. A significant reduction in the α-diversity was observed between 24 and 28 da (p-value 0.02) but otherwise the α-diversity between the sampling points for the biosecure birds were not affected. However, α-diversity in commercial birds was significantly reduced (p-value 0.0002) at 37 da, which may be for a consequence of *Campylobacter* colonization or new components of the microbiota introduced at thin. A study by Choi et al. (2015) suggested a reduction in α-diversity was indicative of an unhealthier status or a tendency towards pathogen invasion, which may have contributed to an increase in susceptibility upon decreasing α-diversity and the efficient colonisation by *Campylobacter* spp. Despite at 28 da alpha-diversity significantly reduced in CH birds there is no observation of pathogen colonization.

AMOVA significance tests of Beta diversity using Bray-Curtis indices indicated significant variation between the caecal microbiota compositions of broiler chickens collected at 30 da compared to those collected at 37 da (p-value of AMOVA 0.001). PCoA plots demonstrate partition of microbiota indices at 30 and 37 da in commercial birds. The caecal microbiota may be subject to natural

maturation with age. The composition of caecal microbiota from birds reared in controlled housing show are only different between 28 and 35 da (AMOVA p-value 0.01), which may conform with the timing of the maturation from juvenile to mature composition observed for the commercial birds. However, Connerton et al. (2018) reported a transition in microbiota of control birds aged 15 and 22 da in their study monitoring the development of the gut microbiota and innate immune responses of broiler chickens resulting from early and late challenge with *C. jejuni*. They also found that the transition observed in the broiler microbiota was less variable at 22 da for *Campylobacter* colonized birds. This finding suggest that the rearing condition may lead to delay maturation of microbiota composition. It has been well documented that biosecurity, litter management, feed availability and ambient temperature can substantially affect the GIs microbiota composition (Shang et al., 2018b).

The current study found the relative abundance of total coliform numbers to decrease with time (35 da) in CH birds (Figure 4.3). This finding corresponds to the results at phyla level that show decreases in representative members of the Proteobacteria at 35 da (Figure 4.13). This observation also coincides with the reduction in copy number of the class 1 integrons in birds reared under controlled housing conditions (Figure 4. 7). Awad et al. (2016) reported the transition in the broiler chicken caecal microbiota post 14 da coincided with an increase in the relative abundance of Firmicutes and Tenericutes at the expense of Proteobacteria. They also noted the changes in the abundance of the microbial communities' post *C. jejuni* colonisation at 14 da, and observed a reduction in *Escherichia coli* abundance at various intestinal sites whereas *Clostridium spp*. showed a significant increase.

The distribution of GCs among the Proteobacteria phylum (Figure 4.14) highlights Gammaproteobacteria (OTU0007_ *Enterobacteriaceae_unclassified)* as the main OTU that coincides with birds containing GCs for both trials. Whilst the

wide distribution of GCs observed among several Proteobacteria OTUs in commercial birds including Beta-proteobacteria (OTU0070), Epsilonproteobacteria (OTU0041_*Campylobacter*) and Alphaproteobacteria OTUs (OTU0176 and OTU0471). Gammaproteobacteria are known as a class of medically important groups of bacteria, that include many important pathogens such as the *Enterobacteriaceae* (e.g., *E. coli*), and *Salmonella* (enteritis and typhoid fever), *Vibrio cholerae* (cholera), *Pseudomonas aeruginosa* (lung infections), and *Klebsiella pneumoniae* (Broszat et al., 2014). Non-pathogenic Betaproteobacteria are thought to be a source of class 1 integrons. These environmental bacteria are found in water bodies or are associated with plants that can enter the human food chain. Therefore, presence of class 1 integrons in this bacterial group facilitate their mobilization and environmental spread (Gillings et al., 2008).

LEfSe indicated an increase in the abundance of clostridial species in the biosecure and commercial birds at 30 da. However, *Negativicutes*, and *Proteobacteria* displayed significant increases at 37da for the commercial birds. While OTU0004 (*Megamonas*) and 0041 (*Campylobacter*) were emerging OTUs in the caecal microbiota of the commercial birds at 37 da. Connerton et al (2018) showed that the caecal microbiota of control birds named TEG1 (Trial E Group 1 were birds administered with a placebo) had a greater abundance of *Enterobacteriaceae* compared to those colonized by *C. jejuni* (TEG2, Trial E Group 1 were administered with *C. jejuni*) at 2 dpi (22 da) with increases in the relative abundance of *Clostridia* in the colonized birds (TEG2). The increase of *Clostridiales* was observed in *C. jejuni*, which is attributed to the role of *Campylobacter* in hydrogen sink that could prompt growing of clostridial organisms and their competitive ability by increasing fermentation, causing increased organic acid production which can be utilized by campylobacters as an energy source (Kaakoush et al., 2014;Connerton et al., 2018).

However, variable shifts were also noted by Connerton et al. (2018) in the high abundance of several *Clostridial* OTUs in the absence of *C. jejuni*. Likewise,

the current study also found the most frequently observed OTUs of the *Clostridiales* in CH birds and commercial birds at 30 da were *Lachnospiraceae_unclassified*, *Ruminococcaceae_unclassified* and *Clostridium XIVa*, which are considered the major butyrate producing bacteria that have important roles in maintaining metabolic and immune functions in the gut. The genus *Bacteroides* (*Alistipes*), which was found to be dominant OTUs in commercial birds at 30 da, have been highlighted for their ability to improve the absorption of nutrient and provide protection for the host from pathogen colonisation. This strong metabolic activity is likely as result of the efficient fermentation of polysaccharides to SCFA (Wexler, 2007;Teng and Kim, 2018), as well as maintaining the intestinal microecological balance (Hooper et al., 2001).

4.4 Conclusion

In conclusion, this Chapter highlights the involvement of class 1 integrons as mobile genetic elements that contribute to the prevalence of antibiotic resistance in the broiler chicken microbiota, which likely become associated with the birds at hatch and remain associated regardless of the environment the birds are reared under. The results show that although there is variation between two environmental settings studied here in terms of the predominant type of antibiotic resistance (GCs), similar patterns of gene cassette determinants dominate chick gut bacterial communities. The investigation of trimethoprim resistance (*dfr*-gene) in the controlled housing environment with respect to the integrase copy number demonstrated a reduction but this did not impact the proportion of trimethoprim resistant bacteria recovered.

CHAPTER 5

GALACTO-OLIGOSACCHARIDES (GOS) AS A POTENTIAL APPROACH FOR MODULATING BROILER GUT MICROBIOME AND REDUCING ANTIBIOTIC RESISTANCE.

5.1 Introduction

The enforced withdrawal of the prophylactic use of antibiotics in livestock production is likely to negatively impact on poultry production because of the increasing of disease prevalence such as necrotic enteritis (McDevitt et al., 2006;Janardhana et al., 2009). Poultry meat is considered the second largest global food industry (Manning et al., 2007), and therefore to maintain essential poultry production and meet the global demand for reduction of antibiotic use in farming, alternative approaches to the use of antibiotics are required (Janardhana et al., 2009). Recently, prebiotics have been applied as a potential alternative to antibiotics. Modulation of the ecosystem of the gut microbiota by prebiotics is multi-faceted, which includes the promotion of alternate components of the intestinal microbiota, improvement of epithelial integrity, and to stimulate of the immune system, and regulating the interactions between the host and the intestinal microbiota (Teng and Kim, 2018). Hence, prebiotics have been extensively researched to optimise the chicken gut microbiota, notably the use of non-digestible dietary fibre products in prebiotic interventions aimed at improving poultry health and productivity (Pourabedin and Zhao, 2015). However, investigation of the influence of prebiotics to modulate gut bacterial populations may also have the potential to reduce the antibiotic resistance load in the chicken gut microbiome. In this regard, this Chapter indicates a promising role for the use of GOS as an effective approach to reduce antibiotic resistance associated bacterial communities in the chicken microbiome, and thereby to reduce the risk the prevalence of antibiotic resistant bacteria entering the human food chain from poultry sources. Furthermore, it can be used as an alternative to antibiotic growth promoters that contribute to the antibiotic resistance problem.

Evidence suggests that when bacteria carrying antibiotic resistance genes have been incorporated into an ecosystem, the prevalence and persistence of this resistance can remain even in the absence of selection pressure from the

antibiotics (Liljebjelke et al., 2017). Nevertheless, some environments act as hot-spots for genetic exchange including the animal gut microbiome because of the high density of bacterial populations, and the presence of gene mobilising elements such phages and plasmids in these settings (Kaur and Peterson, 2018).

The presence of class 1 integrons aid the mobilisation of ARGs, although they are non-mobile themselves, they are mobilised in conjugation with transmissible elements enhancing the flow of resistance genes between various members of the microbiota (Gillings, 2017;Kaur and Peterson, 2018). Thus, the integrated nature of poultry production, was chosen for the observation of antimicrobial resistance phenotypes associated with broiler chicken microbiotas, reared in either controlled housing or commercial environments in order to investigate potential control approaches for restricting resistance development through influence of the gut microbiota by dietary GOS supplementation. To assess changes in the microbiota requires high-throughput sequencing technologies such as used for 16S RNA analysis that have revolutionised microbiology in terms of understanding bacterial diversity, and have become powerful tool for analysing gut microbial composition (Pourabedin and Zhao, 2015).

Besides the established roles of prebiotics in modulation of the intestinal microbiota, they can decrease exogenous signals produced by pathogenic bacteria and resisting pathogen colonization (Kogut, 2013). This includes reduction in coliform abundance (Yang et al., 2008;Chee et al., 2010a). As reviewed in Chapter 1 (Section 1.2.10), prebiotics can be utilised for fermentation in the intestine by health-promoting bacteria, producing lactic acid, short-chain fatty acid (SCFA) and antimicrobial substances such as bacteriocins directed towards pathogenic bacteria (Bogusławska-Tryk et al., 2012). These products do not only improve the intestinal microbial structure, but also they can bring about improvements in intestinal epithelial cell integrity, which can then lead to an increase in the absorption of nutrients to support the growth performance of the animals (Lan et al., 2005). Few studies highlighted the role of prebiotics in the reduction of

coliforms in the chicken caecal microbiota, however studies in pigs have indicated that synbiotic formulations (*Bifidobacterium thermophilum* RBL67 and prebiotic FOS, GOS and MOS) show decreases in the levels of *Enterobacteriaceae* in pig faecal samples (Bomba et al., 2002) as well as the decreased adherence of *Escherichia coli* O8:K88 to the jejunal and colonic mucosa (Nemcová et al., 2007;Tanner et al., 2014). However, a synbiotic approach was not adopted for coliform inhibition in the current study.

This Chapter aimed to investigate the influence of feeding GOS on the profiles of broiler chicken microbiota from birds reared under two different conditions (controlled housing and commercial conditions). Four sampling points were examined for the controlled trial and two sampling times for the birds reared for market. In particular, the effect of GOS feeding on the reduction of antibiotic resistant-containing OTUs (*Enterobacteriaceae*), which contribute to the dissemination of antibiotic resistance in the gut microbiota. Class 1 integrons will be monitored via detection of the integrase DNA signal by qPCR.

5.2 Results

5.2.1 Enumeration of coliform bacteria recovered from the broiler chicken caecal contents of control and GOS supplemented diets (G1 and G2) reared under biosecure conditions (Trial 1)

In order to detect the impact of GOS on the relative abundance of coliform counts, the enumeration of coliform on triplicate MacConkey plates were performed as described in (Chapter 2, Section 2.4.1.1) for birds fed the control and GOS supplemented diets over 4 sampling points (Trial1 Chapter 2, Section 2.3.4). The results are represented in Figure 5. 1.

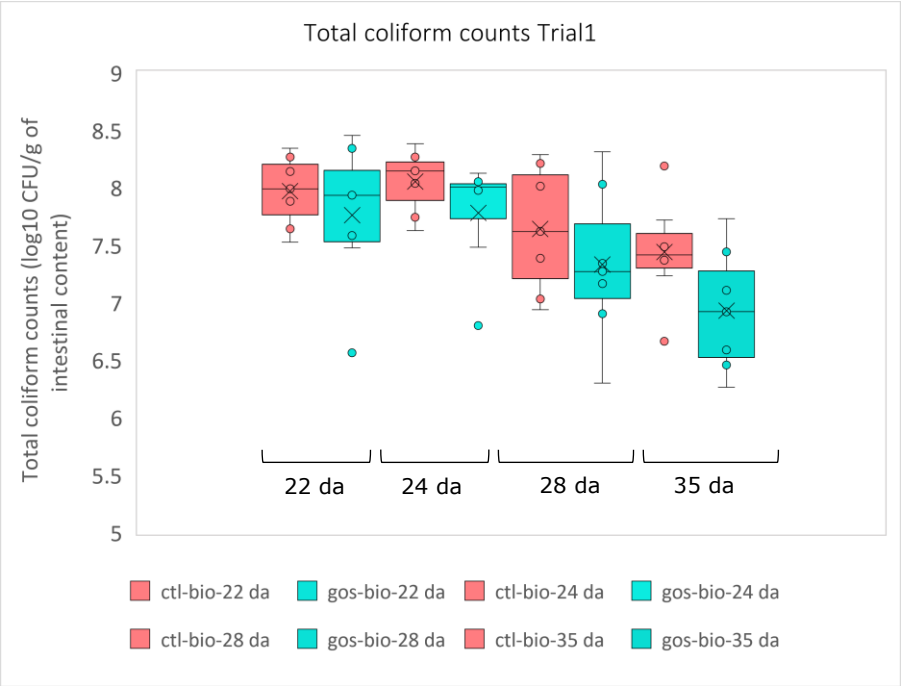


Figure 5.1 The comparison of coliform numbers recovered from the caecal contents of 22, 24, 28 and 35 da old birds fed control (G1) and GOS supplemented diets. Data are expressed as standard boxplots with medians (solid black line). The orange boxes indicate to control diet and tiffany blue boxes represent GOS diets. Outliers are represented as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x the interquartile range. n=7 birds/sampling da (total 28 birds/group).

Although there is no significant difference ($p>0.05$) observed between the two diets, a significant difference was found between ctl-22 da vs ctl-35 da ($p=0.026$) as well between GOS barn at 22 vs 35 da (0.021). The GOS supplemented diet showed a reduction in the total coliform counts compared to the control diet. In general, the results indicated that the trend line of total coliform counts in both control and GOS diet decrease with time. The levels of the total number of coliforms tend to be reduced in birds on the GOS diet compared to the control diet and this reduction is consistent until day 35.

5.2.2 Enumeration of trimethoprim resistance bacteria obtained from the caecal contents of control and GOS supplemented diets (G1 and G2) reared under biosecure conditions (Trial 1)

The viable count of trimethoprim resistant bacteria was enumerated on MacConkey-03 agar plates containing trimethoprim (20 µg /ml). The results indicated that viable counts of trimethoprim resistance bacteria generally remained steady across the trial. However, the level of trimethoprim resistant bacteria from birds on the GOS diet was lower than the control group.

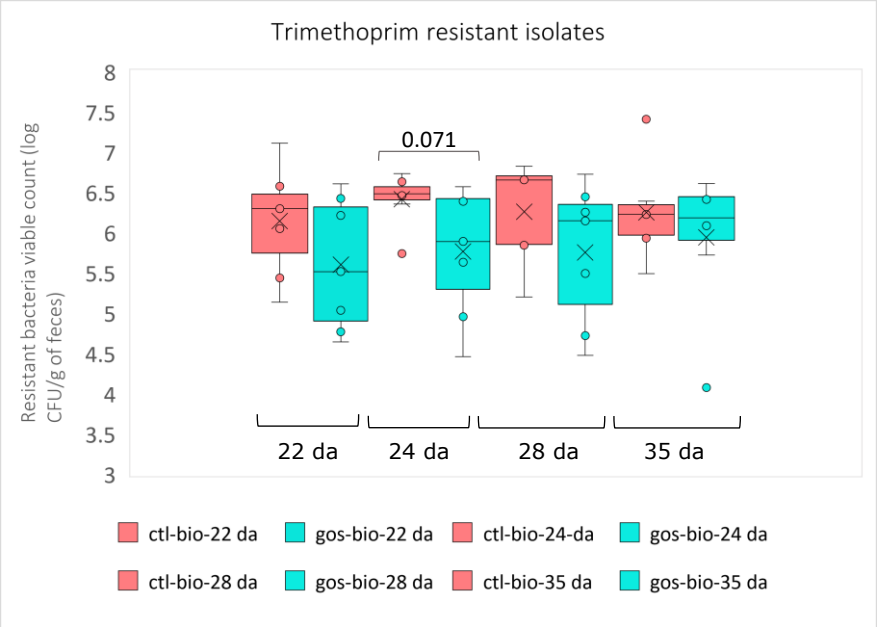


Figure 5.2 Comparison of resistance coliform bacteria growing on MacConkey-03 with trimethoprim (20 µg/ml) obtained from the caecal contents of 22, 24, 28 and 35 da old birds fed control diet (G1) and GOS supplemented diet (G2). Data are expressed as standard boxplots with medians (solid black line). The orange boxes indicate to control diet and tiffany blue boxes represent GOS diets. Outliers are represented as dots (in the style of Tukey). The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x the interquartile range. n=7 birds / sampling da (total 56 birds).

Results clearly demonstrate that the GOS supplemented diet reduced the count of the trimethoprim resistant bacteria compared to control. Therefore, although there no significant difference ($p>0.05$) found between two diets, the range of coliform counts in GOS fed birds lower than control fed birds.

5.2.3 Ratio of trimethoprim resistant coliform bacteria recovered from the caecal contents of control and GOS diets (G1 and G2)

The ratio of trimethoprim resistance calculated by dividing the number of trimethoprim resistance coliforms on total number of coliforms in control plates at selected dilution factor (usually 10^{-5} or 10^{-4} for control plates 10^{-2} or 10^{-3} for trimethoprim resistance bacteria). There was no significant difference ($p > 0.05$) between the ratio of trimethoprim coliforms resistant isolates in birds fed the control diet G1 and those fed the GOS diet G2. It seems that the trend line of trimethoprim resistance population is increased with time in both feed types. However, the growth of the resistant population in the GOS fed birds (G2) was slower or less in the 22 and 24 da old birds than in the 28 and 35 da old birds and this reduction was absent because effect of GOS disappeared in 28 da old birds thereafter. Generally, the proportion of resistant bacteria is increasing with time.

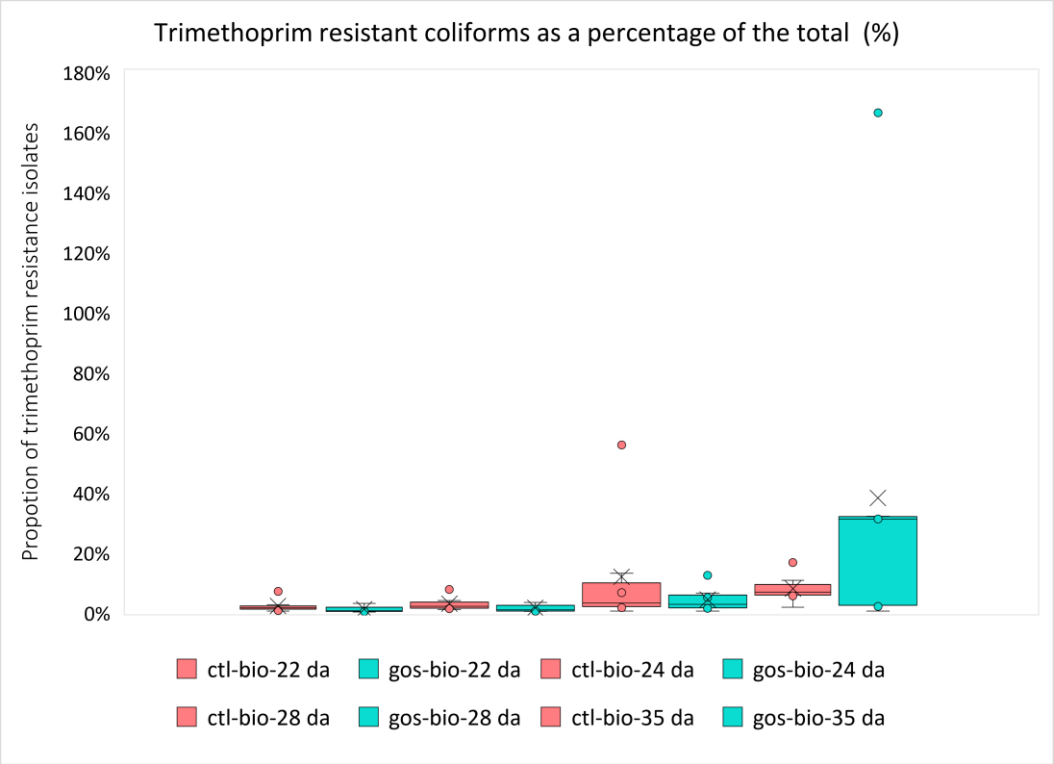


Figure 5.3 Comparison of the proportion of trimethoprim resistant isolates in the bacterial population between two different diets. Showing the proportion of coliforms exhibiting trimethoprim resistance in control and GOS diets. At the proportion remains similar at 22 and 24 da old birds, however after 28 da the proportion of trimethoprim population is increased. Data are presented as boxplot with medians (The solid black line indicates the median and the top and bottom of the shaded boxes indicate the 25th and 75th percentiles). It indicates maximum and minimum values, unless these values exceed 1.5-fold the interquartile range. Outlying data are plotted as individual markers. The orange boxes indicate to control diet and tiffany blue boxes represent GOS diets. The n=7 birds / sampling da (total 56 birds).

5.2.4 Evaluation of class 1 integron copy number of control and GOS diets obtained from biosecure housed and commercial birds

A second set of experiments were carried out to test the hypothesis that the GOS supplemented diet may restrict antibiotic resistant populations. Total genomic DNA was isolated, and qPCR was performed to evaluate the integrase copy number selected to discriminate difference between two diets in terms of the antibiotic resistance gene content arising from class 1 integrons. Similar primers used as described in Chapter 4 Section 4.2.5.

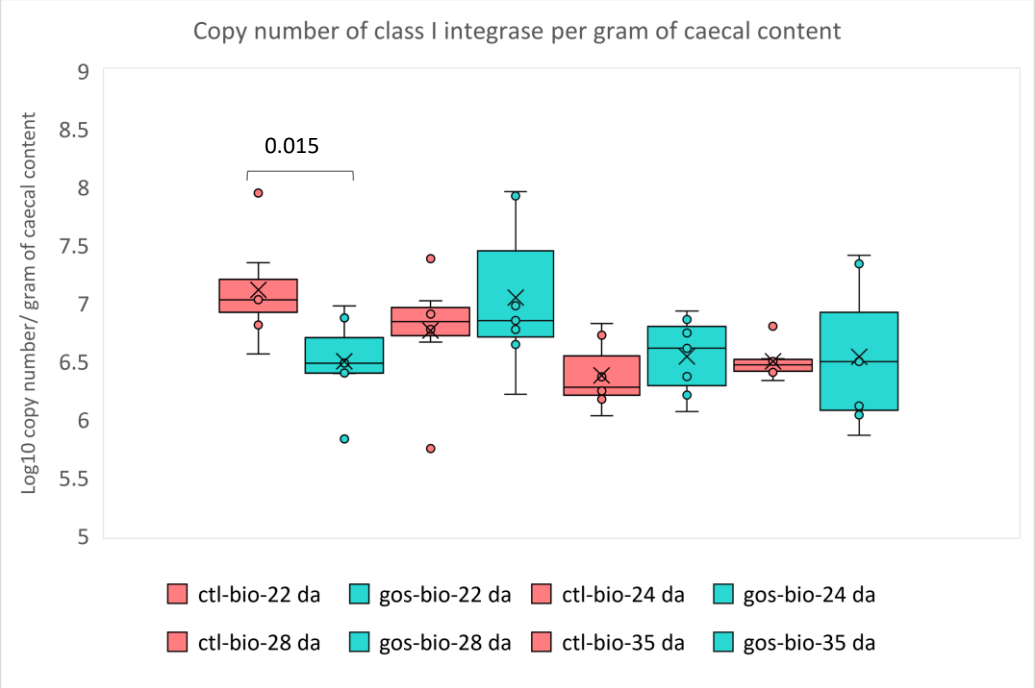


Figure 5.4 A Class 1 integrase copy number quantified by qPCR per gram of caecal content of biosecure birds. The specific primers were designed (Chapter 2, Section 2.5.3.3) to determine the copy number of class 1 integrase gene applying *Salmonella typhimurium* U288 as a positive template to generate a standard curve (Chapter 2, 2.5.8.1). Comparison shows control and GOS supplemented diets across 4 sampling points (Trial1). Data are presented as boxplot with medians (The solid black line indicates the median and the top and bottom of the shaded boxes indicate the 25th and 75th percentiles). It indicates maximum and minimum values, unless these values exceed 1.5-fold the interquartile range. Outlying data are plotted as individual markers. The orange boxes indicate to control diet and tiffany blue boxes represent GOS diets. The n=7 birds / sampling da (total 56 birds).

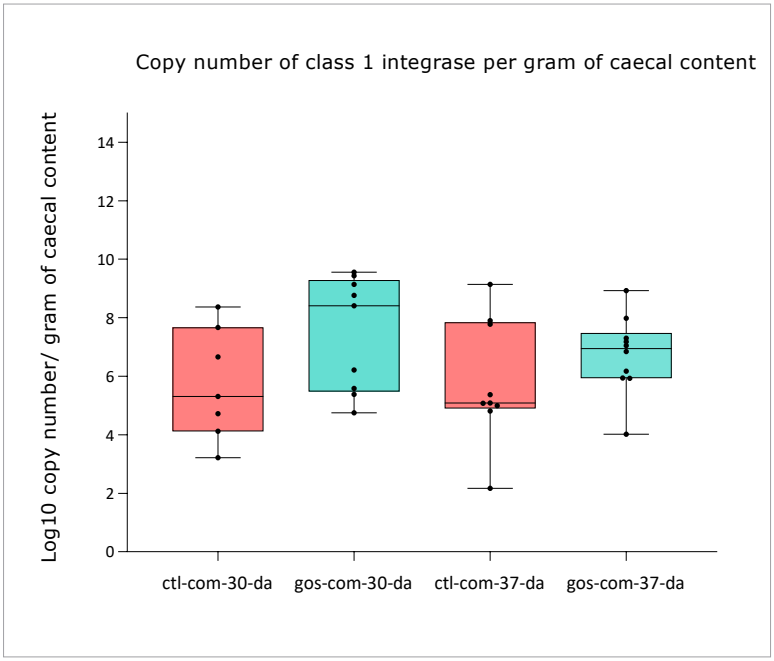


Figure 5.4 B Class 1 integrase copy number quantified by qPCR per gram of caecal content of commercial birds. As noted above a standard curve for *Salmonella typhimurium* U288 was used as positive template. Comparison shows the levels of class 1 integron was not significantly differ between control and GOS supplemented diets in commercial production birds. This may as result to high abundance of Proteobacteria in Trial 2 as it will mentation below. The orange boxes indicate to control diet and tiffany blue boxes represent GOS diets. The n=10 birds / sampling da (total 40 birds).

Figure 5.4A shows that at da 22 there is a statistically significant difference (p-value, 0.01 t-test) between the standard and GOS diets, indicating that the GOS diet contained less class 1 integrase gene copies per g of caecal content. However, this significance is not apparent for the subsequent sampling points (24, 28 and 35). What should be noted is that at day 22 feeding with GOS was stopped and the control finisher diet supplied thereafter (Chapter 2, Section 2.3.4). In addition, the trend line of the integrase copy number reduces with time in the control diet, which is consistent with the trend observed for the total coliform counts in Figure 5.1, which suggests an association between the class 1 integron

and coliform contents. The question arising is whether the GOS diet has a prolonged or legacy effect, or this effect limited to when it in use (temporary effect). However, in the commercial birds, results presented in Figure 5.4 B display variation in the integrase copy number between birds, and due to difference in trial design and sampling time it was difficult to distinguish any effect of the GOS diet one week after stopping the prebiotic feed.

5.2.5 Development of the caecal microbiome composition of birds fed control and GOS supplemented diets in biosecure housed birds

DNA sequencing of the V4 region of 16S rRNA genes was used to investigate diversity and relative abundance of the caecal luminal microbiota of broiler chickens fed two different diets and reared in two different environments. This approach allowed the study of changes in composition of the chicken gut microbiota in order to evaluate the impact of the two diets on the broiler chicken microbiome and to compare the phylogenetic relationships with the distribution of antibiotic resistance. As it mentioned in Chapter 4, the classification of bacterial taxa is displayed as hierarchal taxonomic groups starting with phylum and ending with OTU (Phylum > Class > Order > Family > Genus > OTU).

A total of 3527444 (median: 113283.5, IQR: 137021.8) quality-controlled sequence reads were obtained from 56 broilers caecal samples of control housed birds fed control (ctl) and GOS diets. These could be classified into OTUs 4892 (distance 0.03) that fall in to 11 phyla. Coverage was calculated in mothur (V1.39.5) by Good's coverage at 98% (range = 0.97 - 0.99%) for estimation the proportion of total OTUs present in each sample (Rarefaction curves Appendix 4.1A). Random subsampling of 3511 reads per sample was performed to avoid bias between communities with different sampling depths for bacterial community content and relative abundance analyses.

The control and GOS diets from biosecure housed chicken caecal samples exhibited OTUs falling in to 11 shared phyla binned at 98% similarity. In general, the results show that the dominant phyla over all sampling days were Firmicutes (*Clostridiales_unclassified*) with overall mean 92.5% in the birds fed the control diet and 92.6% in the GOS fed birds, followed by small proportion of Proteobacteria with overall mean abundance 4.56% to 3.28% in control and GOS diet respectively. Fewer contributions from the Actinobacteria (*Bifidobacterium*) with mean abundances of 1.11% to 2.46% in control to GOS diets and Bacteria_unclassified with mean abundances of 1.81% to 1.27% noted within these phyla. Bacteroidetes were notable by their absence throughout. These results are illustrated in Figures 5.5 and 5.6.

The first sampling point (at 22 da) of the caecal microbiota grouped at the phylum taxonomic level shows both diets are dominated by Firmicutes (*Clostridiales_unclassified*) at similar abundances. Firmicute abundance in the control group had a median of 92.04% (IRQ: 4.78%), and in the GOS diet the median was 92.61% (IRQ: 5.36%). The microbiota contained lesser contributions of Proteobacteria (median: 3.29% in ctl diet to 1.57% in the GOS diet; IRQ: 1% and 3.13% respectively), Bacteria_unclassified (median: 1% in ctl diet to 1.11% in the GOS diet; IRQ: 4.5% and 0.8% respectively), and Actinobacteria (*Bifidobacterium*, median:0.8, 0.7; IRQ: 1% and 0.5% respectively) in control and GOS diets as indicated. At 24 da, the abundance of Proteobacteria started to increase in both diets (median: 6.3% in ctl diet to 2% in the GOS diet; IRQ: 3.12% and 4.1% respectively) and Bacteria_unclassified (median: 1.8% in ctl diet to 1.9% in the GOS diet; IRQ: 1% and 1.4% respectively). At 28 da birds, Proteobacteria abundance slightly decreased compared to what it was at 24 da in control diet (median: 3.2%, IRQ: 3.6%), however, this abundance is still higher than that observed for the GOS diet (median: 2.66%, IRQ, 2.28%). By the end of the trial at 35 da the control diet birds were observed to be decreasing in

Proteobacteria abundance (median: 1.78%, IRQ: 1.61%), whereas for the GOS diet the abundance of Proteobacteria was increased (median: 3.71%, IRQ: 1.66%). There was no significant difference in the level of Actinobacteria (P value >0.05), which is increased in the birds on the GOS diet compared with control. Overall, no significant difference was detected at phyla level p> 0.05).

At the class level, the results showed that the most abundant bacterial classes were Clostridiales_unclassified (mean: 84.2%, 83.3% in control and GOS diets respectively), Bacilli (mean: 7.64% in control fed birds, 8.94% in GOS fed birds), Gamma-proteobacteria (mean: 4.54% to 3.28% for control vs GOS diet), Actinobacteria (mean:1.11%, 2.46% for control vs GOS diet) and Bacteria-unclassified (mean:1.81% to 1.27% for control vs GOS diet). These data are presented in Figures 5.7 and 5.8.

The level of Bacilli gradually increased in both diets with abundances at 22 da in control fed birds compared to GOS fed birds (median 7.31%, IRQ 3.33 in control birds compared to 3.35%, IRQ 0.99% in GOS fed birds, p-value Wilcoxon test= 0.0175). However, at 28 da old birds the abundance of Bacilli was higher in the GOS diet than the control diet (median of Bacilli: 15.8%, IRQ 10.21% in GOS diet compared to 8.5%, IRQ 6.48% in the control diet).

Additionally, in general the level of Gamma-proteobacteria was increased in the control birds until 24 da then this level is declined with age (median: at 22 da, 3.29%, IRQ 1; median: at 24 da, 6.34%, IRQ 3.12%; median: 3.23% at 28 da, IRQ: 3.64; median: 1.78% at 35 da, IRQ: 1.61%) but it was higher than the level for the GOS supplemented diets (median: 1.57% at 22 da, IRQ: 3.13; 2% at 24 da, IRQ: 4.13; median: at 28 da 2.65%, IRQ: 2.28; and median at 35 da, 3.7%, IRQ: 1.67).

5.2.6 Caecal microbiome composition of control and GOS supplemented diets in commercial birds.

The total quality-controlled 16S rRNA amplicon reads for the caecal contents of 40 broiler chickens on the control and GOS diets recovered from commercial birds was 1912797 (median: 63456.5, IRQ: 89409.25). These were classified into 4031 OTUs (distance 0.03) that split in to 10 phyla. The coverage was calculated in mothur (V1.39.5) by Good's coverage at 98% (range = 0.97 - 0.99%). Rarefaction curves presented in Appendix 4.1B show the proportion of the total OTUs for each sample. The coverage range was considered an acceptable level of sequence depth for all samples. Random subsampling was performed using 3511 reads per sample, in order to avoid any bias between communities with different sampling depths for the bacterial community contents and relative abundance analyses.

In the commercial birds at phyla level, the dominant phyla were Firmicutes (*Clostridiales_unclassified*) and Bacteroidetes with smaller contributions from the Actinobacteria (*Bifidobacterium*), Proteobacteria, Bacteria_unclassified (Figure 5.9). While the most abundant bacterial classes were *Clostridiales_unclassified* and *Bacteroidaceae* (Figure 5.10).

The results at phyla level showed that the was no difference between the caecal contents of birds on the two diets at da 30. However, there was an increase in the abundance of Bacteroidetes on the GOS diet compared to the control-diet birds (median of Bacteroidetes: 7.47%, IRQ: 5.92% in the control-30 da compared to 10.94%, IRQ: 4.65 for the GOS diet), and an increased level of Actinobacteria in the control compared to the GOS diet (median of Actinobacteria: 7.52%, IRQ: 3.36% for the control compared to 5.28%, IRQ 3.62 for the GOS diet). While at da 37 there was a significant shift in the abundance of Firmicutes in the GOS supplemented group (median: 79.27%, IRQ: 9.56, in the control compared to 67.15% IRQ: 5.77% for the GOS diet, p value 0.0011). The proportions of

Bacteroidetes remained significantly more abundant in broilers given the GOS-supplemented diet than the control fed birds (median: 3.9%, IRQ:3.37 for the control diet compared to 7.52%, IRQ: 7.26 for the GOS diet birds at 37 da, p-value 0.0232). Similarly, the level of Proteobacteria were increased at 37 da in GOS-fed groups compared to control groups (median: 5%, 11.6% IQR: 3.8, 11.3 for control vs GOS respectively).

At class level, the comparison between control and GOS diets displayed significant increases in the levels of Firmicutes-unclassified for the GOS diet at 30 da (median: 2.376% vs 3.971%, p-value=0.023). While the abundance of Beta-proteobacteria were detected in the control group at 30 da (median: 1.21%, compared to 0.003% for control vs GOS, p-value 0.0014). At 37 da, the only significant differences in abundance were observed for the Firmicutes-unclassified (median: 2.68% compared to 3.846 for control vs GOS; p-value 0.001) and Bacteroides (median: 3.94% to 7.523 for control vs GOS; p-value 0.023) for the GOS diet barns. While the abundance of Negativicutes was detected only in control fed birds at 37 da (median: 1.6%, vs 0.0%, IQR: 24.7 to 0.0%; p-value 0.0025 in the control vs GOS diets respectively). Whilst Epsilonproteobacteria were observed for both diets at 37 da but with higher levels in birds on the GOS diet compared to the control (median: 2.4%, 5.2%, IQR: 2.95, 13.9% in control vs GOS respectively, p-value=0.27). There was an intra-group variability in the caecal microbial communities of commercial birds at phyla level, which was determined for the Proteobacteria phylum in control diet group at 30 da, (median: 3%, IQR: 1.9%); and Actinobacteria in the GOS barn communities at 37 da, (median: 3%, IQR: 4.66%).

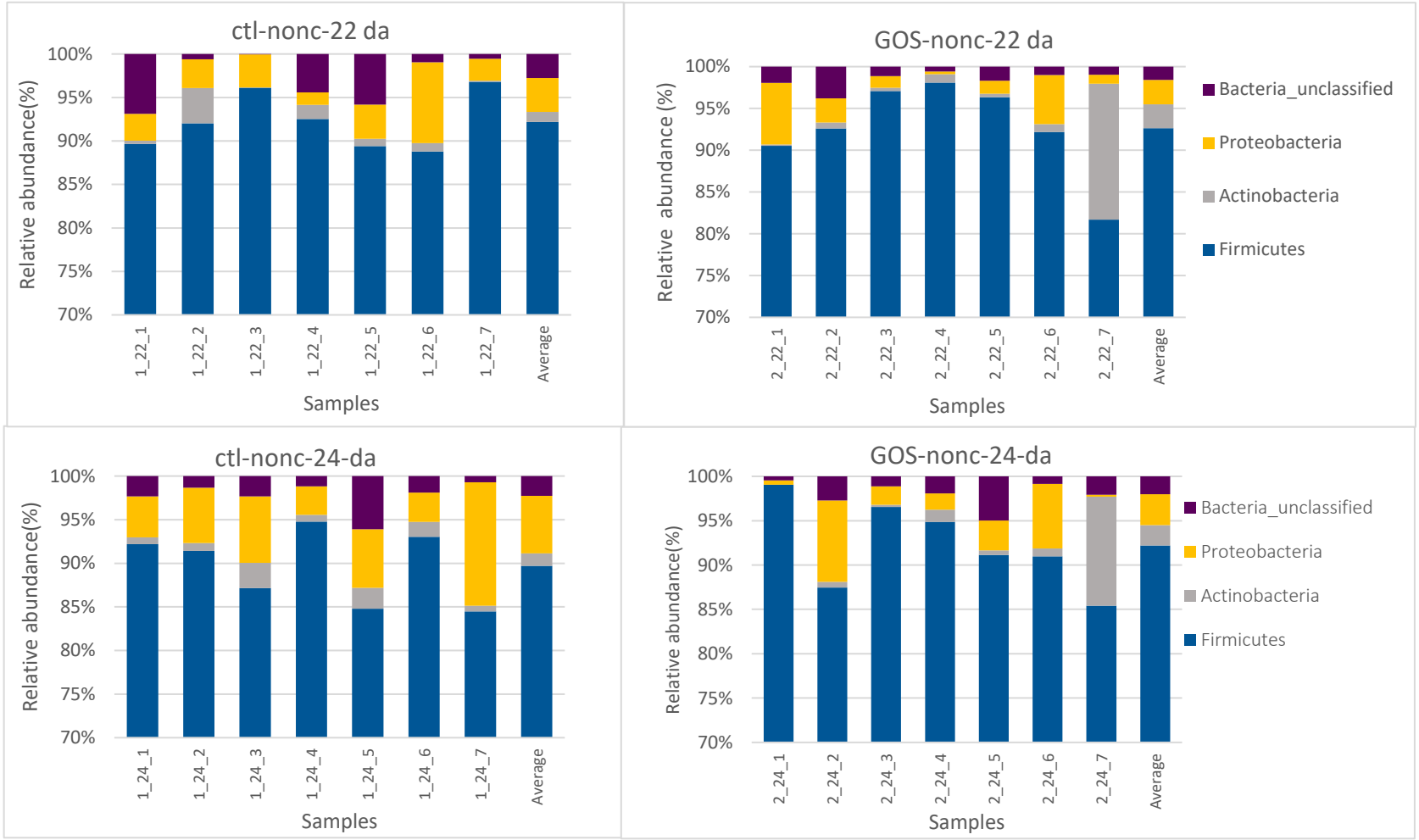


Figure 5.5 Comparison of the relative abundance of dominant bacterial phyla among biosecure housed birds gut microbiota fed control and GOS diets clustered based on Ribosomal Database Project taxonomy (phylum level). The results are presented as % and at least taxa that include 1% community was presented. For unbiased comparison all reads have been normalised.

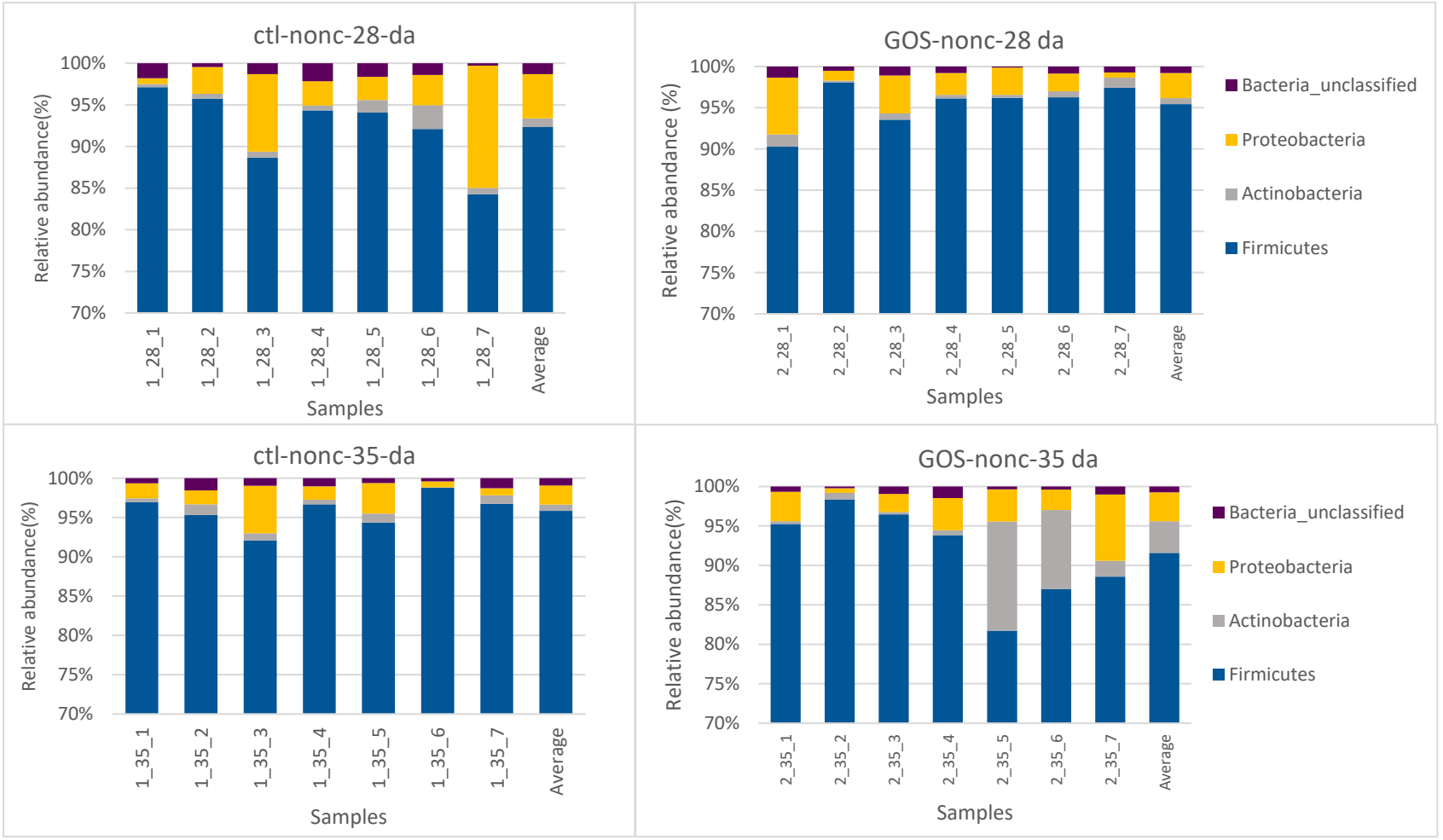


Figure 5.6 Comparison of the relative abundance of dominant bacterial phyla among biosecure housed birds gut microbiota fed control and GOS diets clustered based on Ribosomal Database Project taxonomy (phylum level). The results are presented as % and at least taxa that include 1% community was presented. For unbiased comparison all reads have been normalised.

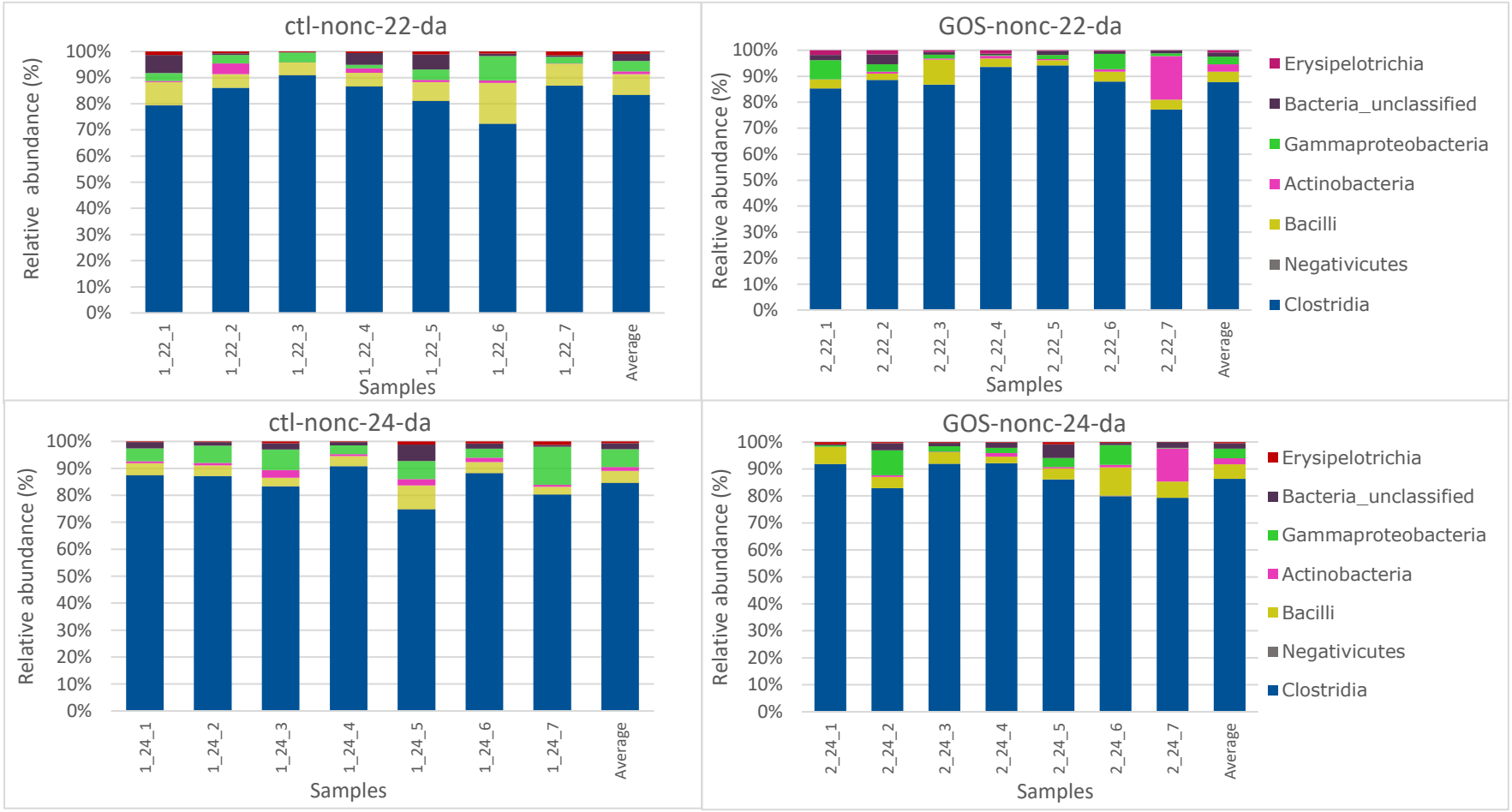


Figure 5.7 Comparison of the relative abundance of dominant bacterial classes among biosecure housed birds gut microbiota fed control and GOS diets clustered based on Ribosomal Database Project taxonomy (class level). The results are presented as %. For unbiased comparison all reads have been normalised.

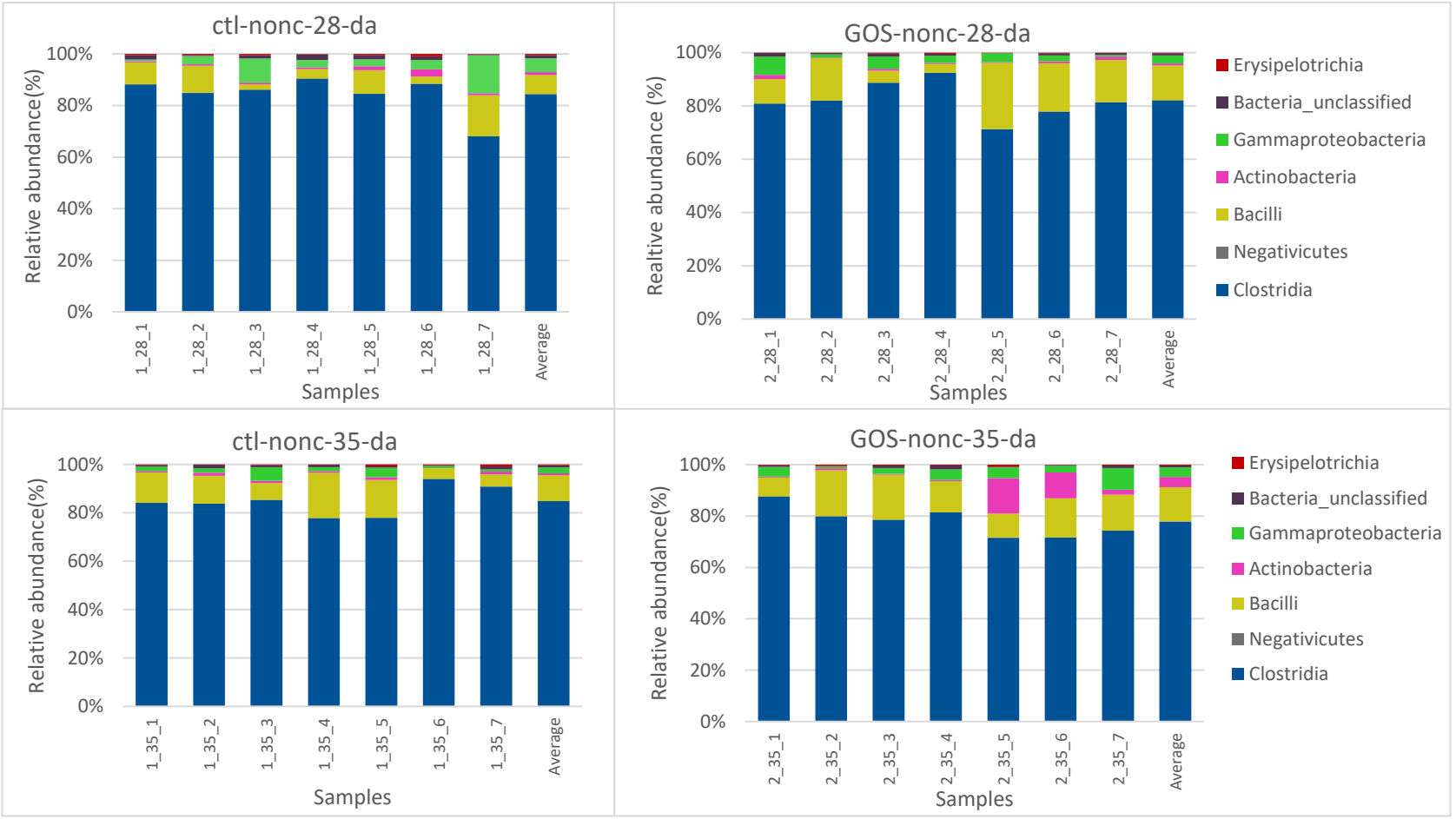


Figure 5.8 Comparison the relative abundance of dominant bacterial classes among biosecure birds gut microbiota fed control and GOS diets clustered based on Ribosomal Database Project taxonomy (class level). The results are presented as %. For unbiased comparison all reads have been normalised.

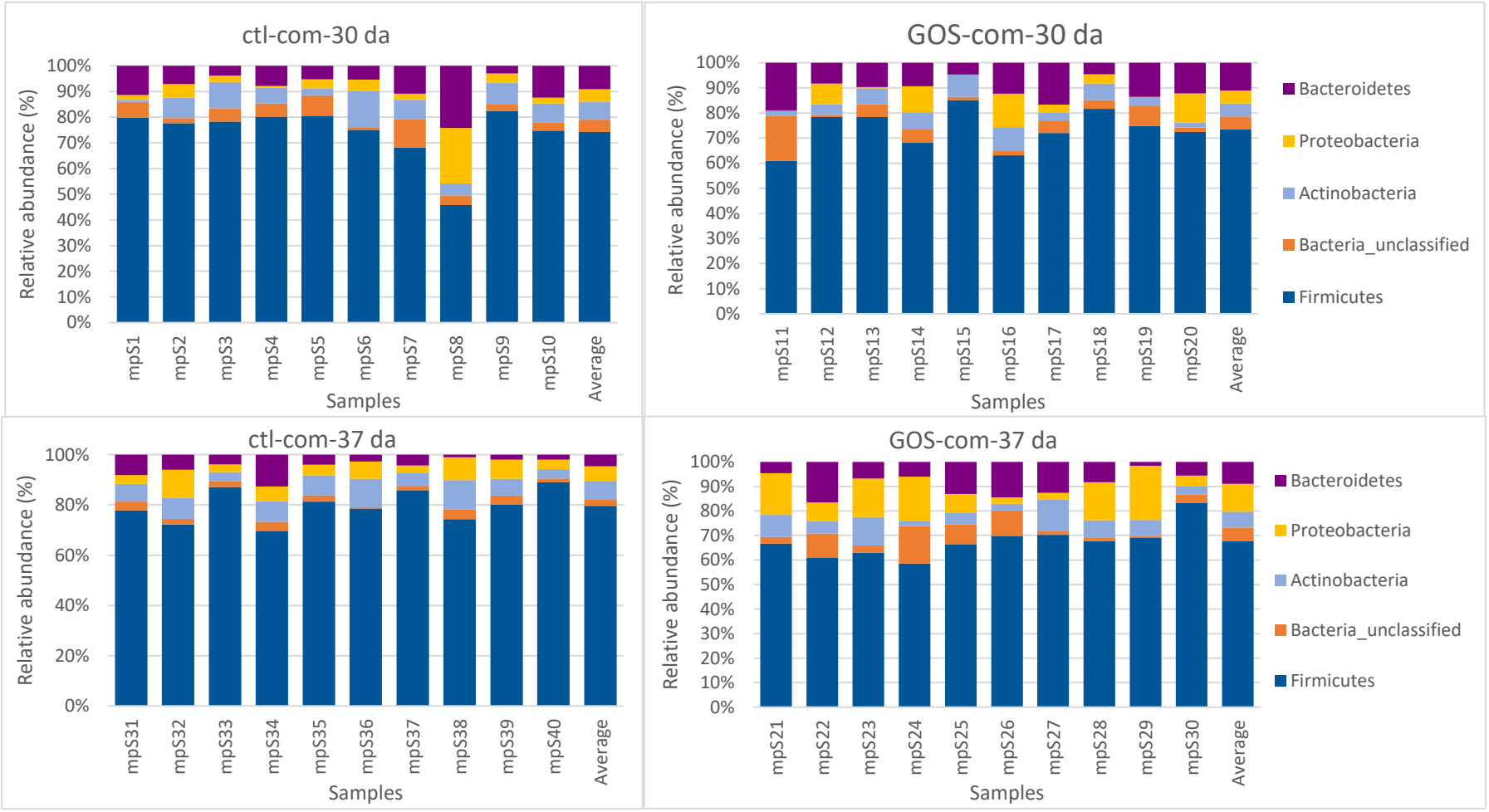


Figure 5.9 Comparison of the relative abundance of dominant bacterial phyla among commercial chicken gut microbiota fed control and GOS diets clustered based on Ribosomal Database Project taxonomy (phylum level). The results are presented as % and at least taxa that include 1% community was presented. For unbiased comparison all reads have been normalised.

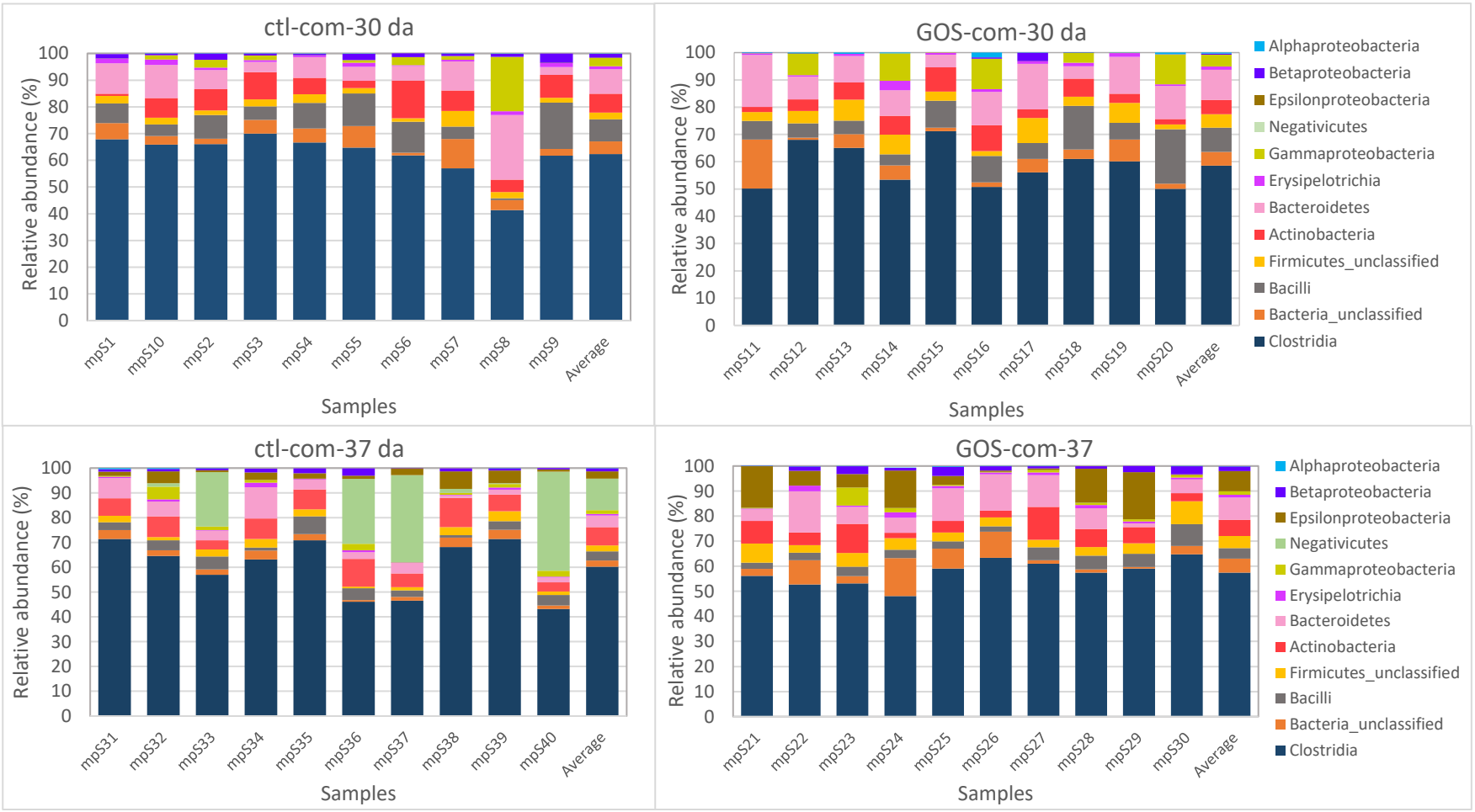


Figure 5.10 Comparison of the relative abundance of dominant bacterial phyla among commercial chicken gut microbiota fed control and GOS diets clustered based on Ribosomal Database Project taxonomy (class level). The results are presented as %. For unbiased comparison all reads have been normalised.

5.2.7 Relative abundance of the Proteobacteria phylum between GOS and control diets

The abundance of the Proteobacteria phylum between the two diets across four sampling times for birds in biosecure housing revealed that there was no significant change (p value > 0.05) in abundance of Proteobacteria in caecal contents of biosecure housed birds. The median values are presented in Figure 5.11 for control and GOS diets respectively across sampling the points: at 22 da (median: 3.29% for control and 1.57% for GOS; IRQ: 1.05, 3.13, p-value 0.2593), at 24 da (median: 6.34% for control and 2.08% for GOS, IRQ: 3.12, 4.15; p-value 0.1649) o, at 28 da (median: 3.23% for control and 2.66% for GOS, IRQ: 3.64, 2.28; p-value 0.3829) and at 35 da (median 1.78% for control and 3.71% for GOS; IRQ: 1.61, 1.66; p-value 0.2593)

Figure 5.11 shows similar results were found when investigating Proteobacteria abundance at phylum level between caecal samples of commercial birds in that there were no significant changes observed. The medians of control and GOS supplemented diets: at 30 da old bird (median: 2.97% for control and 3.41% for the GOS diet, IRQ: 1.91, 9.51; p-value 0.9118) and at 37 da (median: 5% for control and 11.68% for the GOS diet, IRQ: 3.83 and 11.36; p-value 0.123). No change was detected in control diet between the two sampling times for the control diet (median: 2.98% in ctl 30 da compared to 5.057% in ctl-37 ;IRQ: 1.9%, to 3.8%; p-value 0.075) but Proteobacteria were significantly more abundant in broilers fed the GOS-supplemented diet at 37 da compared to 30 da (median 3.41 compared to 11.68%, IRQ: 9.51 and 11.36; p -value 0.046).

The results of the Shapiro-Wilk test of normality and quantile-quantile plots indicated that the distribution of the Proteobacteria phyla was not normal for birds on the control diet at 22 da (p-value = 0.0215), while the other sampling points of both feeds indicated that the abundance *of* Proteobacteria was normally

distributed across cohorts for the biosecure birds (Appendix 4.2A). Similarly, data of Proteobacteria were not normally distributed (Shapiro-Wilk normality test= 0.0001) for control birds at 30 da, however for the other sampling point the data were normally distributed across the cohorts in the commercial flocks (p-value > 0.05, Appendix 4.2B).

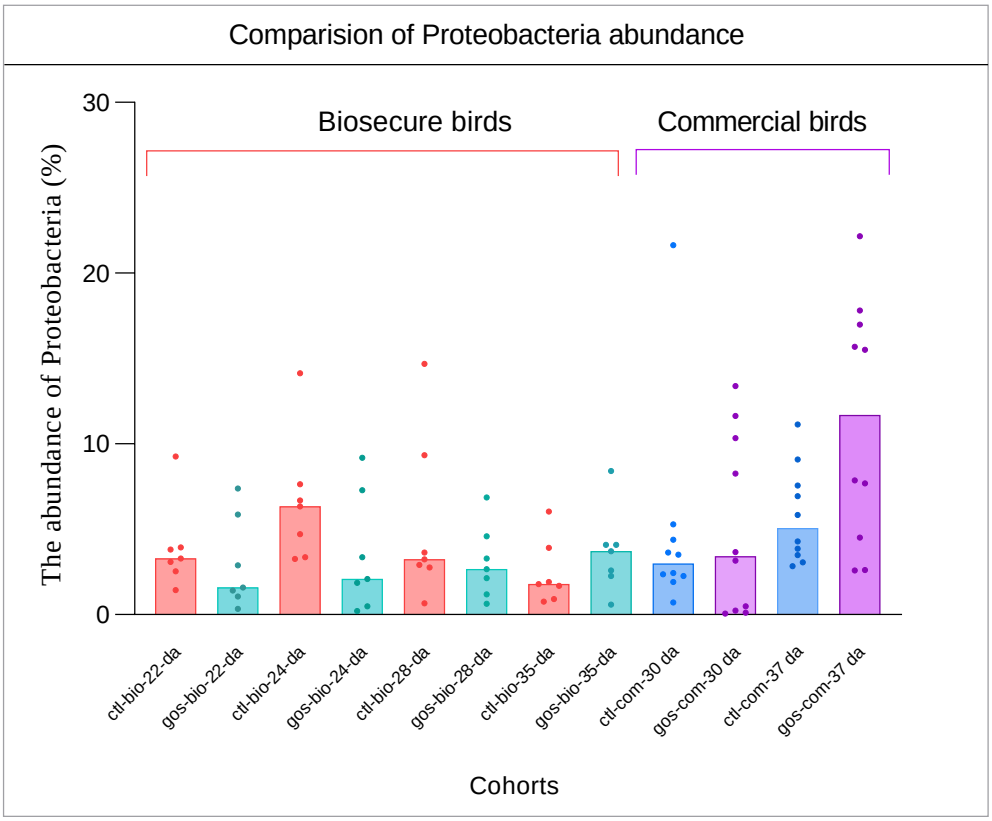


Figure 5.11 The relative abundance of the Proteobacteria phylum obtained from broiler chicken caecal contents fed control and GOS diets reared in biosecure and commercial conditions. Data are expressed as a bar chart with median for each sampling point and comparing the abundance of Proteobacteria between the two rearing conditions given either control or GOS diet. No significant change was detected within each trial. The Wilcoxon test p-value was used because the data not normally distributed, which shows no statistical difference in either trial. All orange bars represent control fed birds while tiffany blue represent GOS fed birds reared in biosecure housing condition (n=7 birds/sampling point, total /group 28 birds. The blue bars indicate to control fed birds whereas the purple bars indicate to GOS fed birds reared in commercial condition (n=10 birds/sampling point total 20 birds / group). The proportion of Proteobacteria was reduced at 22 and 24 da in GOS-fed birds, however after 28 da (6 days after stopping feeding the GOS-diet) the level

of Proteobacteria were increased in the biosecure flocks, while commercial birds showed increases in the Proteobacteria abundance at each of the sampling days in the GOS barns.

5.9 The distribution of resistance GC arrays in Proteobacteria OTUs of birds in biosecure housing conditions and commercial birds fed control and GOS diets.

Integron gene cassettes are frequently associated with Proteobacteria. Phylogenetic trees of the proteobacterial OTUs identified within the chicken caecal microbiota of the biosecure housed bird and those from commercial are presented in Figure 5.12. The Proteobacteria population in the commercial birds is clearly more diverse. The presence of GCs for each bird in which the OTU was observed are indicated with respect the diets the birds were provided. The results demonstrate that there were 4 main OTUs present within this phylum that coincided with the presence of GCs detected for biosecure birds. The most frequent associations of resistance genes were with Proteobacteria OTUs belonging to *Enterobacteriaceae_unclassified* OTUs (OTU0007, OTU0324) with lesser frequency determined for Gammaproteobacteria (*Proteus,* OTU0169) and Betaproteobacteria (OTU0097, *Sutterella)* regardless of the diet. Using the chi-squared test for all birds reared under biosecure conditions the proportion with gene cassettes on the GOS diet was not different to those on the control diet (χ^2 (1, N = 55) = 2.3, p = 0.13). However, the chi-squared test for the birds at 22 da when the GOS diet was first replaced with control indicated the proportion with gene cassettes on the GOS diet was significantly different to those on the control diet (χ^2 (1, N = 14) = 5.2, p = 0.02). For the commercial birds Proteobacteria OTUs associated with high GC frequency were OTU0007, OTU0070, OTU0176, OTU0041 and OTU0276. Comparing both trials, OTU0007 showed the greatest correlation with the presence of the GCs regardless of diet with a weaker association for OTU0169. Notably OTU0276 and OTU0471 appeared in control feed birds whilst OTU0474 emerged

only in GOS fed birds from commercial flocks. The chi-squared test for the proportion of commercial birds with gene cassettes on the GOS diet was not different to those on the control diet (χ^2 (1, N = 40) = 0.08, p = 0.77).

The diversity of the recovered GCs per sampling point and dietary type are shown in Appendix 4.3 (1-4) and 4.4 (1-2) for both trials. Results indicated that three OTUs emerged in biosecure birds at 22 da birds, which remained until the end of trial 1 (35 da). The majority of resistance genes identified arose at 22 da. In the commercial birds OTU0169 (*Proteus*) appears displaced by emerging OTU0041 (*Campylobacter*) and OTU0471 (*Alphaproteobacteria_unclassified*) which coincides with a decrease in the distribution of GC-B in the Proteobacteria OTUs at 37 da but GC-A persists. The association between the gene cassettes identified and the Proteobacterial phylogenetic trees with respect to the diet and age of the birds is presented in Figures 5.12.

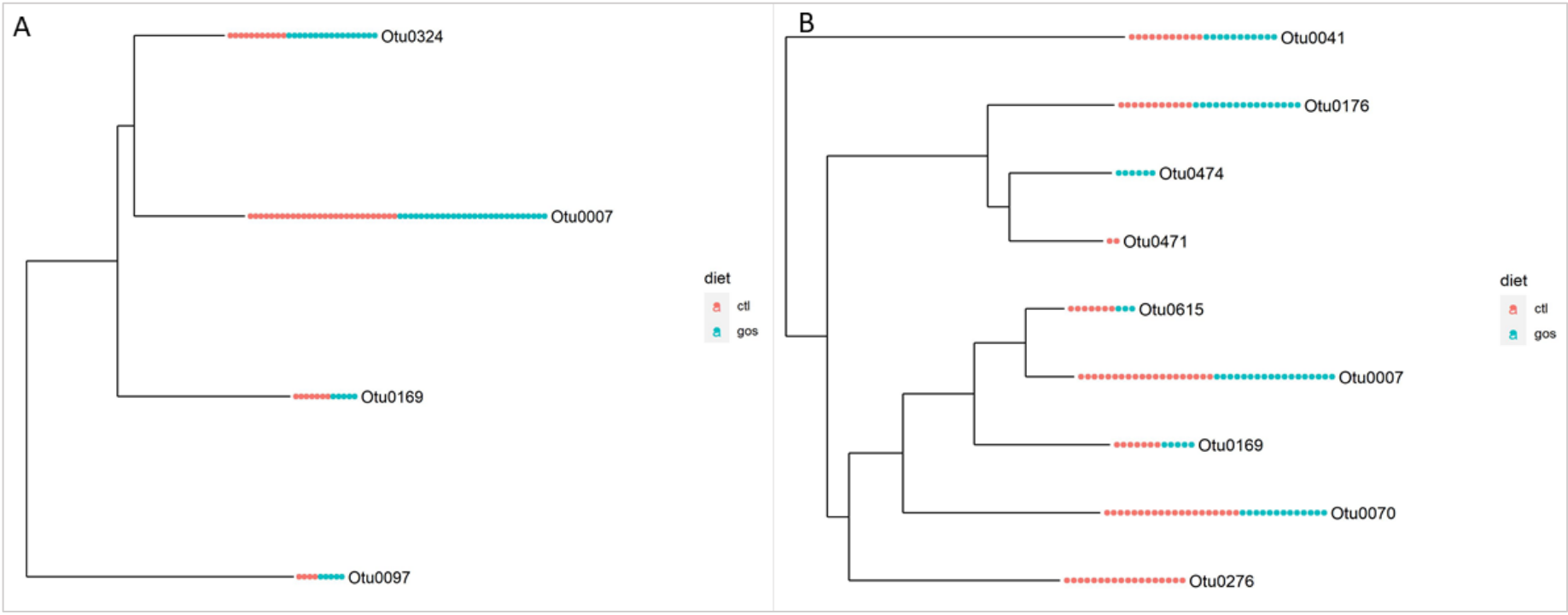


Figure 5.12 Phylogenetic tree of Proteobacteria phylum illustrates the OTUs present in broiler chicken microbiota fed control and GOS diets reared in biosecure and under commercial conditions (constructed with Bootstrap value ⩾ 20). **(A)** biosecure birds at 22 and 24 da have been presented because applying a cut-off value ≥20 reads per OTU there was no Proteobacteria OTUs found at 28 and 35 da. **(B)** commercial birds age matched 30 and 37 da. The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*), OTU0097 is (Beta-proteobacteria) *Sutterella*, OTU0041 is Epsilonproteobacteria (*Campylobacter*), OTU0176 is Alpha-proteobacteria (*Aestuariispira*), OTU0474 is *Alpha-proteobacteria_unclassified*, OTU0471 is Alpha-proteobacteria (*Alphaproteobacteria_unclassified*), OTU0615 is *Enterobacteriaceae _unclassified,* OTU0070 is Beta-proteobacteria (*Parasutterella*), OTU0276 is *Proteobacteria_unclassified.*

5.10 Microbial diversity analysis

The analysis of microbiome composition of caecal contents from controlled housed and commercial birds was estimated by using Mothur (V1.39.5). Inverse-Simpson's measure of alpha (α-) diversity and Chao index estimating the number of species richness. The beta (β-) diversity was also calculated within Mothur using Bray-Curtis dissimilarity metrics.

By comparing the inverse Simpsons index of both feed types there was no significant differences in alpha diversity detected between the different time points of control and GOS diets among biosecure birds. However, birds reared under biosecure conditions showed a reduction in alpha diversity for the control at 28 da compared to the control at 24 da (Simpson index, p-value control 24 vs control 28, Mann Whitney test=0.0262), but this was not evident in the GOS-fed birds (Simpson index, p-value GOS 24 vs GOS 28, Mann Whitney test 0.62 respectively). Overall, with time the trend showed a reduction in alpha diversity, which slightly lower in the control groups compared to the GOS supplemented groups.

The inverse Simpsons index of both control and GOS diets were tested via the Shapiro-Wilk test of normality, which indicated that the inverse Simpson's index was not normally distributed in the control diets (control 24 da) of the biosecure birds (Shapiro-Wilk test, p-value = 0.0279 at control-bio-24 da). The fit of the data in quantile-quantile plots is represented in Appendix 4.5 A. However, the normality tests of the other sampling points of both feed types for the biosecure housed birds indicated that the inverse Simpsons index was normally distributed across the cohorts for the control and GOS diets (Shapiro-Wilk test, p-value >0.05).

In contrast, the alpha diversity of the commercial barn reared birds was significantly different between control and GOS supplemented diets at 37 da, where the control birds showed a reduction. However, alpha diversity was not

affected at 30 da for either feed regime (inverse Simpson, p-value 0.8534). The comparison between two diet and trials for the inverse Simpsons is presented in Figure 5.13.

For the commercial birds, the inverse Simpsons indices were normally distributed across cohorts for both feeds. The Shapiro-Wilk test for the inverse Simpsons indices, p-value = 0.7348, 0.8189 for control and GOS respectively at 30 da birds, while p-value =0.2637, 0.1736 at 37 da. All data of quantile-quantile plots are shown in Appendix 4.6A.

The Chao index results in Figure 5.14 indicate no differentiation in richness index within controlled housed birds for the four sampling times with gradual increasing in richness in GOS diet until 24 da old birds (Chao index, p-value = 0.0728 at 24 da). At 28 da both diets show a decrease in richness (Chao index, p-value = 0.804 at 28 da). The normality test of the Chao indexes exhibited that the data was not normally distributed in the control birds at 28 da (Chao index, p-value = 0.0378), whereas all other sampling points were normally distributed (p-value >0.05) Appendix 4.5B.

For the commercial birds, no significant difference was determined for richness between the control and GOS diet at 30 da (Chao index, P-value 0.075) and no significant change in richness between the diets at 37 da birds (Chao index, p-value 0.63). Similar results were observed also in Chao index for the commercial birds, demonstrating that both diets are normally distributed at sampling times 30 and 37 da (Shapiro-Wilk test, p-value = 0.4431, 0.3884 at da 30; p-value of normality test = 0.3210, 0.7609 at 37 da for the control and GOS diets respectively). All data of quantile-quantile plots are represented in Appendix 4.6 B (Figure 5.14).

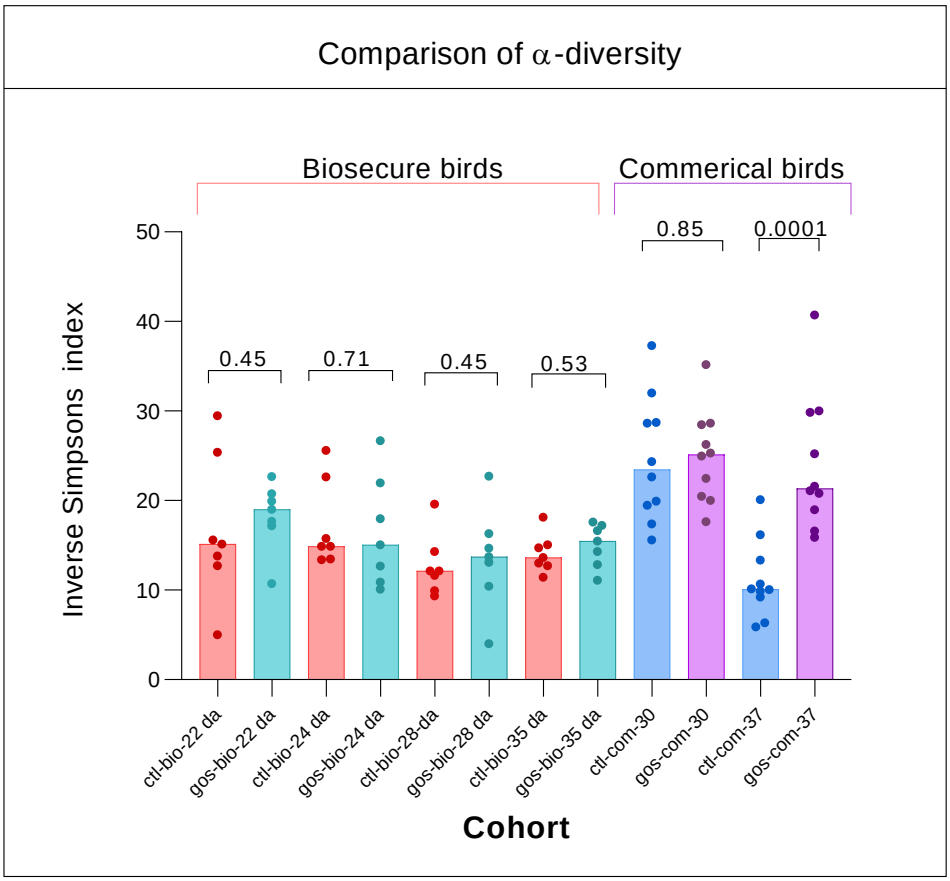


Figure 5.13 Comparison of inverse Simpson's index for alpha diversity of trial 1 and 2 during four samplings time (22, 24, 28 and 35 da) for broiler caecal communities of biosecure birds and two samplings time (30 and 37) of commercial birds fed control and GOS diets.

Data are expressed as bar chart with medians. Outliers are represented as dots. Wilcoxon test p value is used because the Shapiro-Wilk test showed the data were not normally distributed (Appendix 4.5 A and 4.6 A). There was no significant difference found in biosecure housed birds, however a significant difference in inverse Simpson index was found between two diets at 37 da for the commercial birds.

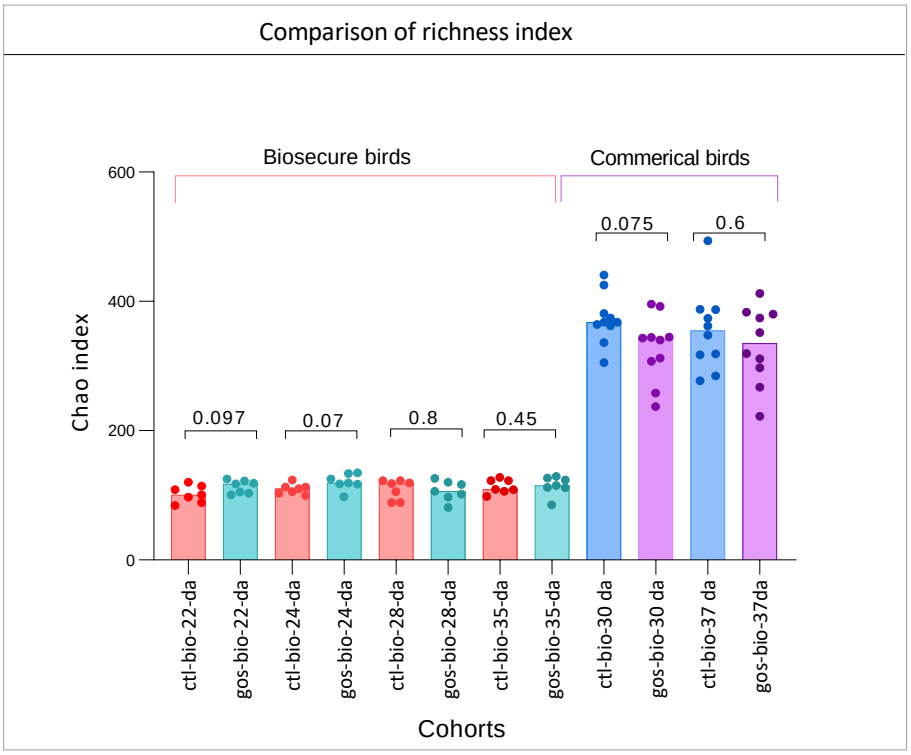


Figure 5.14 Chao index for richness between two diets through different sampling points for broilers caecal communities. The Chao indices calculated from normalised reads data are displayed for each group 22, 24, 28, and 35 for biosecure housed birds while 30 and 37 da for commercial birds. Data are expressed as described above bar charts with medians and scatter plots.

Beta diversity analysis was conducted by calculating Bray-Curtis distances using Mothur (V1.39.5). Bray-Curtis distance quantifies compositional dissimilarities between the microbiota of the birds fed control and GOS diets. Dissimilarity ordination plots were generated demonstrating comparison of dissimilarity between two trials. An AMOVA test of significance was calculated also using Mothur (V1.39.5) to detect differences between sampling times for each diet for each trial. The AMOVA test results (Figures 5.15) indicated that within controlled housed birds there was a significant shift between microbiota of the two different feeds at 22, 24 and 28 (AMOVA test, p-values: 0.003, 0.001, 0.014) but no significant difference between the two diets at 35 da (AMOVA test, p-value: 0.242).

Variation in species composition between the communities representing the two diets from commercial birds were also visualised using the Bray-Curtis distances in Figure 5.16. The AMOVA test shows a difference in the microbiota at 30 da between both control and GOS birds (AMOVA test, p-value 0.004) as well as between control and GOS birds at 37 da (p-value 0.001).

Linear discriminant analysis effect size (LEfSe) was applied to identify differentially abundant OTUs between control and GOS diets in both trials. Figure 5.17 shows the significant differentially abundant OTUs for the microbiota from all the birds reared under biosecure conditions across various samplings times. Differential abundance between the diets was dominated by members of the Firmicutes phylum in GOS fed birds at 22 da that include *Lachnospiraceae ssp* OTU0032 and OTU0030, *Enterococcus* OTU0078 and members of the *Clostridiales_unclassified* class OTU0138. Differences in the abundance of *Lachnospiraceae ssp* OTU0032 and *Enterococcus* OTU0078 were also found in GOS-fed birds at 24 da with high abundance of OTU0015_*Subdoligranulum* and other *Lachnospiraceae spp* OTUs. Data collected at 28 and 35 da showed differences in abundance of *Lachnospiraceae spp.* While for birds on the control diet, these were dominated by OTU006 *Lactobacillus* at 22 da, *Ruminococcaceae spp* OTU0020, *Clostridiales_unclassified* Otu0037, *Eggerthella* OTU0036 and OTU0043_*Blautia* at 24 da. The abundance of *Lachnospiraceae spp* was noted at 28 da control-fed birds as well as *Ruminococcaceae spp* at was observed 28 and 35 da control-fed birds.

For commercial birds, LEfSe highlights the greater differential abundance of *Parasutterella* OTU0070, *Lachnospiraceae_unclassified* OTU0045, *Butyricicocs* OTU0076, *Bacteria_unclassified* OTU0193 and *Clostridiales_unclassified* OTU0136 and *Blautia* OTU0171 in control birds at 30 da. While the GOS-fed birds at 30 da display a greater abundance of *Aestuariispira* OTU0176, *Ruminococcaceae_unclassified* OTU0107 and *Firmcutes_unclassified* OTUs

(Figures 5.18 A). At 37 da when both control and GOS diet barns were randomly colonised by *Campylobacter*, the most abundant bacterial OTUs for the GOS fed birds were *Alistipes* OTU0025 and *Bacteroides* OTU0028 and for birds on the control diet *Megamonas* OTU0004, *Faecalibacterium* OTU0005, *Lachnospiraceae_unclassified* OTU0003 and *Bacteroides* OTU0115 (Figure 5.18 B).

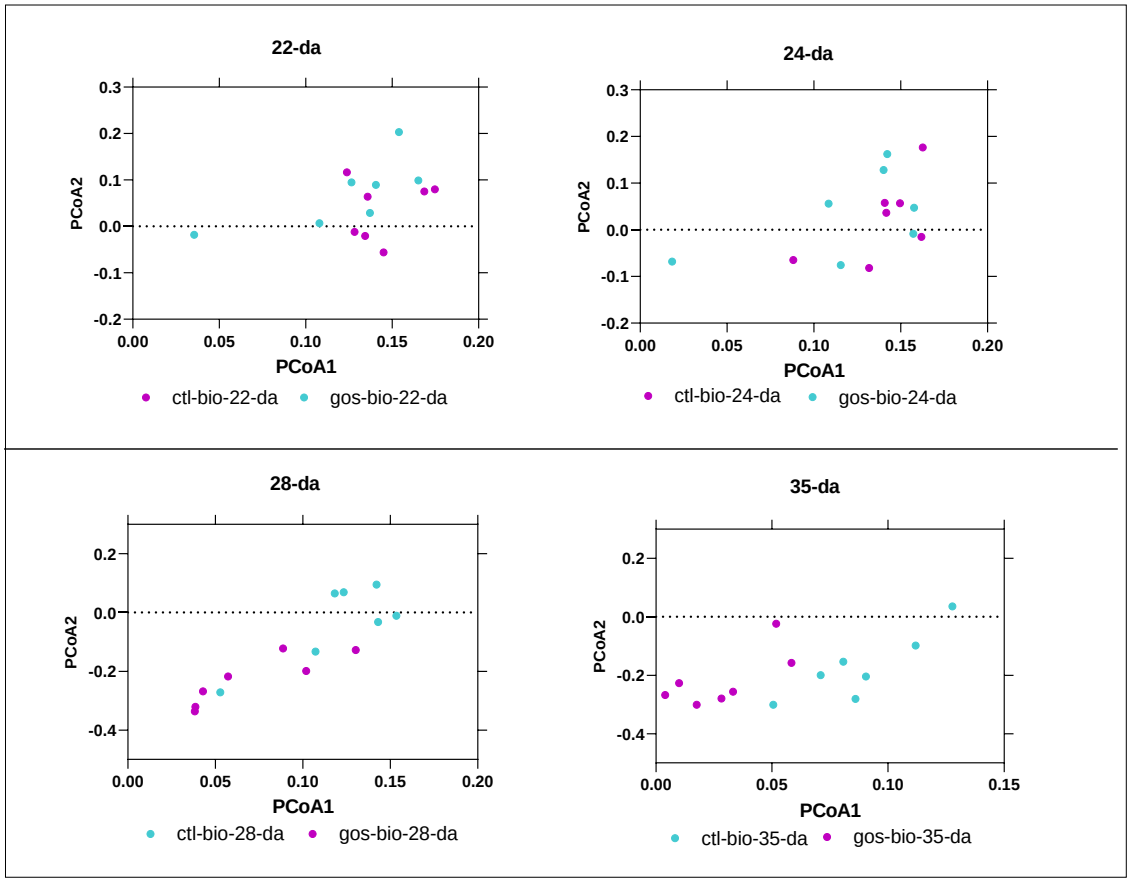


Figure 5.15 Comparison of PCoA plots of Beta diversity based on Bray-Curtis dissimilarity between control and GOS supplemented diets fed birds microbiota of biosecure birds. The comparison of AMOVA test between control and GOS diets fed birds showing that there was a significant different of clustering microbiota between the two barns at 22, 24 and 28 da birds (AMOVA test, p-values: 0.003, 0.001, 0.014) however there was no a significant difference AMOVA p-values 0.242 among comparing between control and GOS diets birds at 35 da.

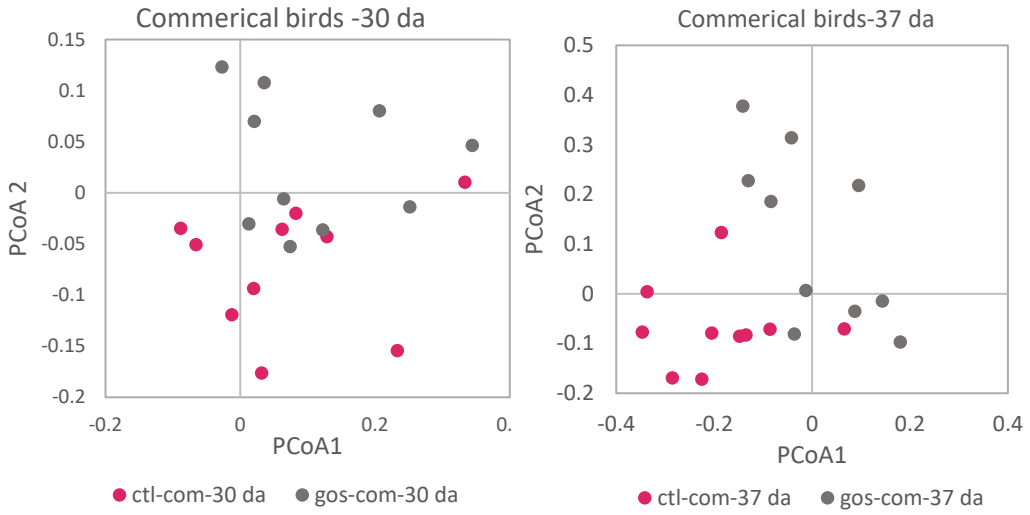


Figure 5.16 Comparison of PCoA plots of Beta diversity based on Bray-Curtis dissimilarity of control and GOS diets of commercial birds. The p-values of control diet birds at 30 da and GOS diet birds at 30 da indicates there is a significant difference between the two barns (AMOVA test, p-value: 0.004), There was also a significant difference with a AMOVA test p-value of 0.001 by comparing control diet birds and GOS diet birds at 37 da.

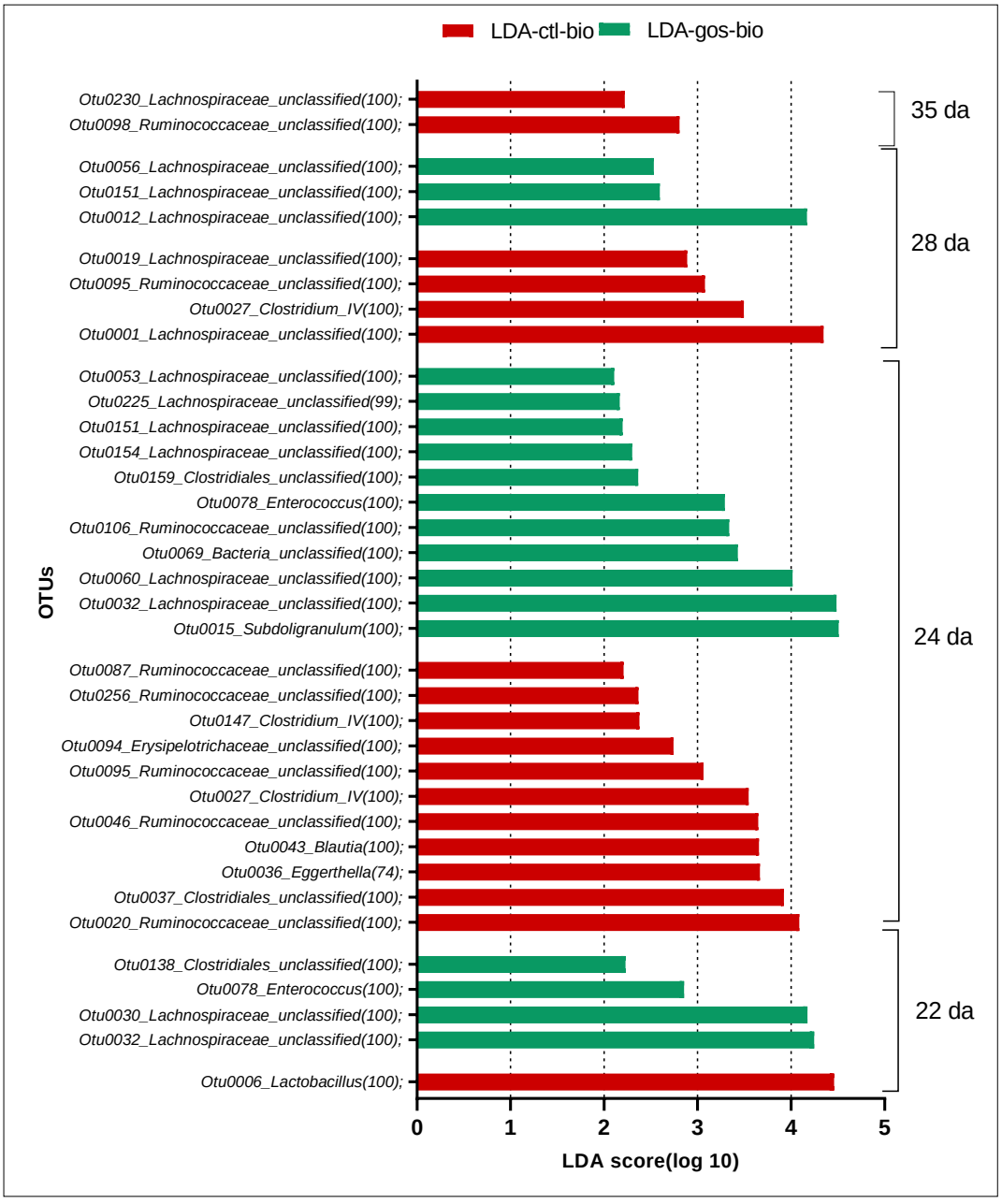


Figure 5.17 Histogram of the linear discriminant analysis (LDA) effect size (LEfSe) of biosecure housed birds fed control and GOS diets during 4 sampling times. LDA scores computed for express the differentially abundant of bacterial communities of birds fed control (represented by red bars) and GOS diets (represented by green bars) and reared in biosecure conditions. LEfSe illustrates which clades amongst detected OTUs that are statistically explain the greatest differences between bacterial population fed the different diets and reared in similar conditions. All representative OTUs subject to stringent cut off p-value 0.01 and LDA≥ 2.

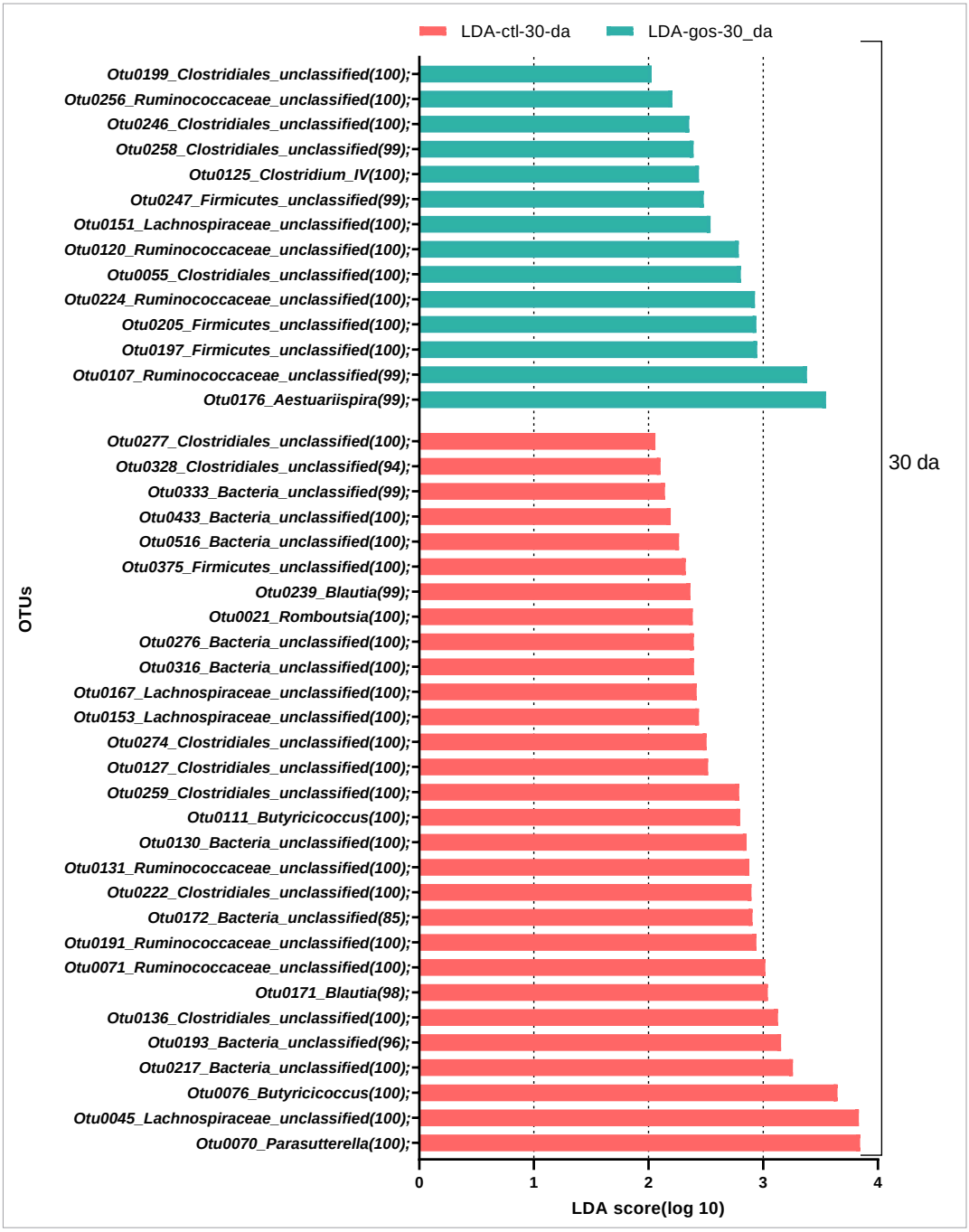


Figure 5.18 A Histogram of the linear discriminant analysis (LDA) effect size (LEfSe) of commercial birds at 30 da. LDA scores computed for express the differentially abundant between commercial broiler chickens at 30 da fed control diets (control represented by Peachy bars) and GOS diets (GOS represented as Tiffany blue bars). LEfSe illustrates which clades amongst detected OTUs that are statistically explain the greatest differences between bacterial population fed the different diets and reared in similar conditions. All representative OTUs subject to stringent cut off p-value 0.01 and LDA≥ 2.

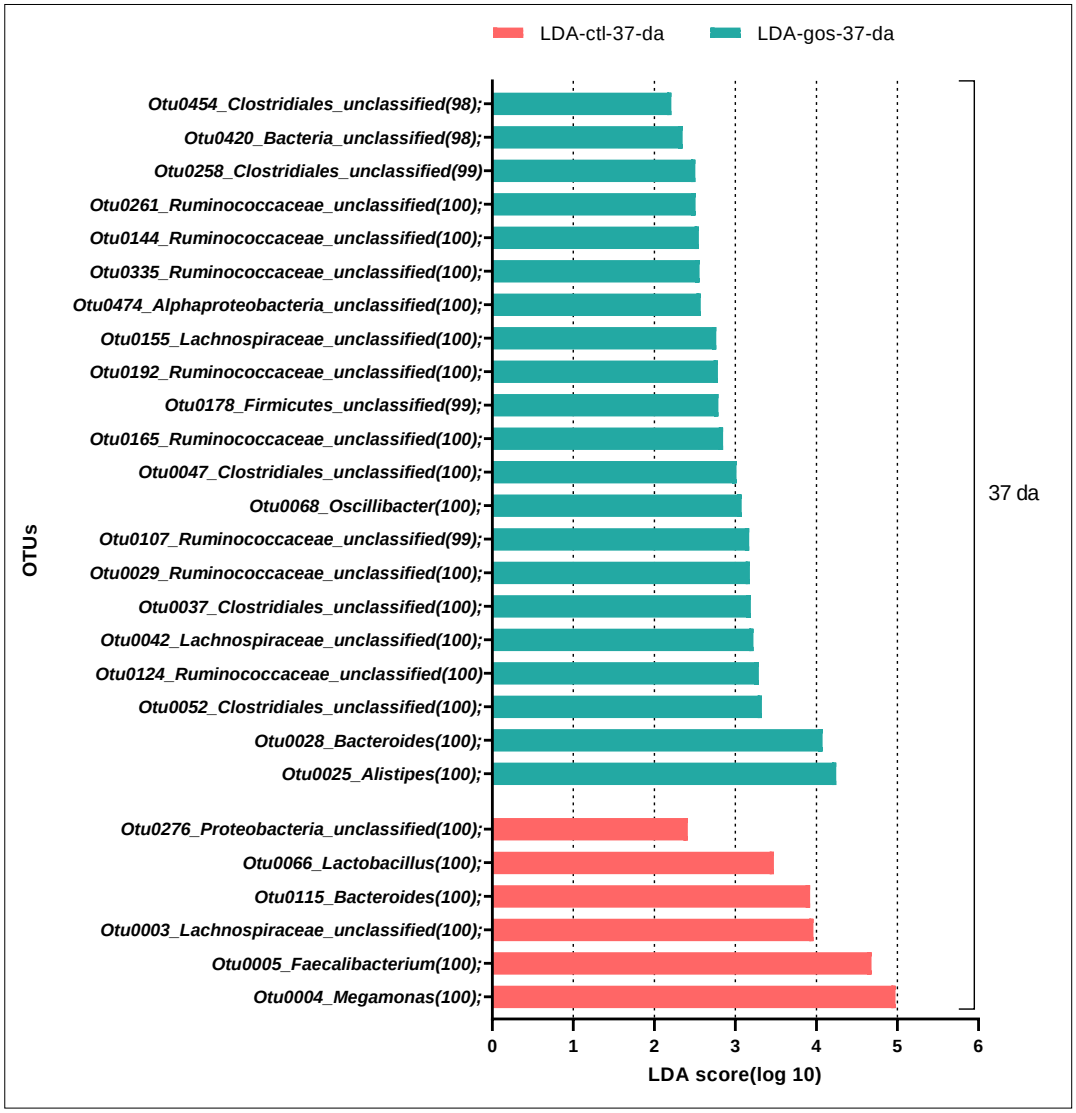


Figure 5.18 B Histogram of the linear discriminant analysis (LDA) effect size (LEfSe) of commercial birds at 37 da. LDA scores computed for express the differentially abundant between GOS and control diets in commercial broiler chickens at 37 da (control represented as orange/Peach bars) and control birds (GOS represented by tiffany blue bars). LEfSe illustrates which clades amongst detected OTUs that are statistically explain the greatest differences between bacterial population fed the different diets and reared in similar conditions. All representative OTUs subject to stringent cut off p-value 0.01 and LDA≥ 2.

5.3 Discussion

One of the most important medical issues of the twenty-first century is the problem of antibiotic resistance. Increased understanding through environmental and evolutionary studies are considered as essential to recognize the origins and destinations of resistance determinants (Wellington et al., 2013;Gillings, 2018). Most of the research on resistance has focused on diagnostics and infection control. At the same time many studies on animal feed additives have highlighted the potential for prebiotics to promote growth and immunological responses in the host. However, the influence of GOS feed on the modulation of gut bacterial populations to potentially mitigate antibiotic resistance carriage in the chicken gut microbiome has not been investigated. This Chapter attempts to address the issue of continuously emerging antibiotic resistance by maintaining a broiler microbiome that is less hospitable to the bacteria carrying resistance genes through the use of dietary GOS supplementation. Therefore, it focusses on studying the influence of GOS dietary inclusion on broiler microbiota supplied from commercial hatcheries and reared in either biosecure conditions or in a commercial environment with all the incumbent challenges that brings. To gain a greater understanding of the microbiota in which the carriers of AMR are embedded, next generation sequencing of 16S rRNA amplicons was employed and analysed with regard to the total carriage of antibiotic resistance genes (reviewed in Chapter 3) for two different diets and rearing conditions.

A recent study by Braykov et al. (2016) aimed to evaluate phenotypic patterns of antibiotic resistance between commercial (broilers and laying hens) and household birds, and the study also extended to the collection of environmental samples from the corresponding sites. They observed high levels of ARGs in both rearing conditions with a distinctive phenotypic pattern of antibiotic resistance in production birds compared to what was observed from backyard birds, and from this described a typical signature pattern of production

birds. The signature pattern was prevalent in poultry coops of production birds, but not observed in domestic environments. Similarly, to the current study, they found GCs in commercial birds are higher than birds reared in domestic locations. This may of course change as antibiotics are marketed in various countries to smallholders based on improved production without any control.

In this study approximately 37% (10/27) of the representative GCs were obtained from broiler caecal microbiota of the control diet group birds housed in biosecure conditions which were dominated by one type of antibiotic resistance configuration (GCs-B-2 containing the genes *dfrA1* and *aadA1* that confer resistance to trimethoprim and streptomycin respectively). Half of them were collected at da 22 after which the number decreased with increasing age (Table 3.4A, Chapter 3). However, only four of 28 samples (14.2%) contained the GCs from GOS-fed birds, two of them obtained at 24 da showing similar pattern of ARGs detected in the control feed birds (GCs-B-2), while the other two GCs were recovered at 35 da and belong to GCs C-1 (*aadA1*), which is considered a deletion of GCs-B (Table 3.4A, Chapter 3).

The microbiota of all the commercial birds fed control and GOS diets contained GCs (20/20) with 75% (15/20) of the ctl birds containing GC-A, 20% (4/20) containing GCs-B-2 and 5% (1/20) containing GCs-C-2. Similarly, 75% (15/20) of the GOS fed birds carried GCs-A, and 25% (5/20) carried GCs-B-2 (Table 3.6, Chapter 3). These findings indicate that the environment in which the birds are reared plays an important role in determining the ARG load and dissemination in poultry. Although two trials birds were given similar diets the level of resistance was higher in commercial birds than those reared in biosecure conditions. As Connerton et al. (2018) justified high densities of birds in commercial production facilitate the dispersion of microorganisms throughout the flock and creating a source of microbiota for other flocks on the farm. Hence, in commercial production, young chicks are exposed to several exogenous sources

of bacteria that can readily colonise the immature gut of young chicks such as litter materials, feed, water, and ambient air shaping the composition of gut microbiota (Wang et al., 2016). Thereby, the association of antibiotic resistant strains with poultry production is likely to be originated post hatch through environmental sources, suggesting that it might be better to control widespread AR from rearing environments. Furthermore, it is noted that the GOS feed showed an impact on the GCs present from biosecure birds denoting both factors (GOS and biosecure rearing conditions) reduce the carriage of AR genes.

In order to confirm the effect of GOS diet in reducing the ARG load, first the total coliform counts and trimethoprim resistance strains were enumerated to emphasise if the GOS diet has influence on trimethoprim resistance or not as trimethoprim resistance was more prevalent in biosecure housed birds. The experimental findings indicated that the total coliform counts were reduced with time, and this reduction was higher in the GOS diet birds than those fed the control diet. Over the rearing period the recovery of trimethoprim resistance isolates was lower from the GOS diet groups than the control diet. However, the ratio of trimethoprim resistance was shown to increase over time, suggesting that the trimethoprim resistant coliform bacteria were not displaced to the same extent as the general coliform population.

Erdoğan et al. (2010) reported dietary supplements of synbiotic and phytobiotic either alone or in combination significantly decreased the caecal coliform count ($p < 0.01$) in broiler chicken caecal samples. They postulated that this effect was due to probiotics and prebiotics providing a balance on the microecosystem of the GI by controlling pathogenic bacterial populations via competitive exclusion. Furthermore, studies have reported that the administration of dietary prebiotics might support the intestinal microbiota of young broiler chicks by boosting the abundance of *Lactobacilli* and *Bifidobacteria* and reducing the counts of coliforms (Yang et al., 2008;Chee et al., 2010a;Shang et al., 2018b).

Braykov et al. (2016) reported that the prevalence of resistant phenotypes tended to decrease with bird age for all antibiotics tested in the study except for the highest resistance levels to the drugs sulfisoxazole, trimethoprim-sulfamethoxazole, and tetracycline. They suggest that since birds are purchased from commercial hatcheries, and resistant strains to some drugs are already in the systems of these birds, which may explain why they observe resistance carriage to decline with age. Consequently, it suggested that feeding broiler chicks GOS could reduce coliform numbers, which will simultaneously reduce the load of antibiotic resistance genes carried by Gram-negative bacteria.

Additional information was sought in support of the suppressive role of dietary GOS under biosecure conditions in reducing the spread of ARGs by determining the copy numbers of class 1 integrase using q-PCR (Figure 5.4A). Interestingly, a significant reduction was found (p-value, 0.015) for the integrase copy number at 22 da in the GOS supplemented diet compared to the control diet, proposing that GOS can restrict ARGs, and that this effect is eroded in the absence of the GOS diet. Generally, the integrase copy number decreased with time in the control diet. This finding is consistent with study conducted by Diarra et al. (2007), which demonstrated that the prevalence of AR obtained from 197 broilers isolates exposed to different antibiotic regimens in a large case-control study in Canada showed decreasing resistance levels between 7 and 35 da that correlated with a decrease in the carriage of class 1 integrons and the *tet* resistance gene. Thus, the level of AR was higher among younger birds and was associated with the conditions of rearing and source of the chicks. Commercial considerations mean that GOS is likely to be used in young birds to establish a productive juvenile microbiota whist avoiding the increased cost of feeding mature birds (Richards et al., 2020). In this context the suppressive role of GOS was at an early stage of chick's life that is more likely to be enriched in bacteria carrying antibiotic resistance. The collective effect of the hatch and rearing environments with early

GOS feed could potentially reduce the dissemination of antibiotic resistance and reduce the health risk of chickens to be reservoirs for resistance genes. Apajalahti et al. (2004) pointed out that both the diet and the environments can manipulate the microbial status of the gastrointestinal tract of chickens, which may explain why effect of GOS supplemented diets was absent in commercial birds compared to the biosecure housed birds. Although the study design differed in both experiments, bird to bird variation in integrase copy number even in GOS supplemented birds was evident. Several studies have reported that the small intestine of chickens can be effected by many factors including the age of the birds, the diet and the surrounding environment (Knarreborg et al., 2002;Lu et al., 2003a;Apajalahti et al., 2004;Rehman et al., 2007;Danzeisen et al., 2011;Torok et al., 2011;Yeoman et al., 2012;Ballou et al., 2016;Shang et al., 2018b).

Analysis of 16S rRNA gene sequences at phyla level indicated that Firmicutes remained the most predominant phylum in the caecum for all sampling points for both diets in the biosecure and commercial birds. The mean abundance data for this phylum was similar for the GOS diet compared to the control diet in biosecure housed (mean: 92.5% in control vs 92.9% in GOS diets), whereas the control diet for the commercial birds recorded a higher percentage than the GOS diet (mean: 76.92% in control; 70.53% in GOS diets). At phyla level there was variation in the abundance of Proteobacteria between two diets for biosecure housed birds (mean: 3.66% in control compared to 2.46% in GOS), Actinobacteria 0.84% compared to 3.28% and Bacteria_unclassified 1.3% compared to 1.27%. The second most abundant phylum for the commerical birds was the Bacteroidetes, with a mean of 6.89% in the control birds and 9.98% in the birds given the GOS supplemented diet. The Bacteroidetes were followed by Proteobacteria (mean: 5.31% in the control and 8.23% in the GOS diet); Actinobacteria (mean: 7.22% in control; 5.89% in GOS) and Bacteria_unclassified (mean: 3.64% in control, 5.29% in

GOS). This agrees with previous reports that Firmicutes, Proteobacteria and Bacteroidetes from the dominant phyla of the chicken caecal microbiota (Wei et al., 2013;Yan et al., 2017;Shang et al., 2018c). Furthermore, Mesa et al. (2017) found similar results in their study of broiler chicken caecal microbiota in birds fed either a control diet or supplemented with prebiotics (MOS) and nucleotides, and notably only detected the Bacteroidetes phylum at 35 da. However, the current study on biosecure housed birds ended by day 35 with less than 1% of Bacteroidetes present. Polansky et al. (2016) proposed a role for Bacteroides in the metabolism of oligosaccharides and formation of short-chain fatty acids in that they expressed xylose isomerase required for polysaccharide degradation and the enzymes leading to propionate and butyrate production. However, Jumpertz et al. (2011) stated that Bacteroidetes were linked to a decrease in nutrient absorption. Kers et al. (2018), proposed that the presence or absence of Bacteroidetes in various studies is likely to be caused by differences in diet, experimental conditions, age or sampling time, variation in sequencing technology and the differences in the primers used.

Analysis at class level of the control and GOS diets from commercial birds indicated an increase in abundance of Epsilonproteobacteria (*Campylobacter* OTUs) that seems to have emerged post 30 da and which coincides with increase the abundance of Negativecutes in control fed birds only. This suggests contamination of both barns followed thinning, and it highlights a possible role for humans influencing the composition of the microbiota (discussed in Chapter 4).

Wielen et al. (2002) stated that each bird has variations in the relative abundance of members of the intestinal bacterial community. Previous studies have indicated that successional changes within the intestinal microbiota at 5 to 20 days post hatch can be amended by the provision of exogenous microbial communities and suppressed by antimicrobial dietary additives (Hume et al., 2003;Yin et al., 2010;Torok et al., 2011). Hume et al. (2003) showed shifts in the caecal microbiota at 2 days and 5 to 20 days of age. The change in composition

of the broiler caecal microbiota was reported with age as well as the complexities of bacterial communities in microbiome (Wielen et al., 2002;Hume et al., 2003;Gong et al., 2008). These age-related alterations in the gut microbiota tend to be partially associated to the physiological changes that occur in the chick's GI tract post hatch. The GI tract exhibits a rapid increase in the size in early development and is reported to reach maximal relative size of the digestive organs at 3 to 8 days post hatch with the intestine continuing to increase in the length and diameter until 14 days post hatch (Noy and Sklan, 1997). Therefore, different environments settings that bird's early exposure to with the host's physiology have a direct influence on the development of the gut microbiota in the newly hatched chick.

Analysis of alpha diversity of the inverse Simpson index for biosecure housed birds showed a reduction at 28 da old in both diets. This decrease in alpha diversity was lower in GOS-fed birds. In the commercial birds a significant decrease was observed in the control diet (discussed in Chapter 4) compared to the GOS diet. This could be a residual consequence of the GOS supplemented diet to retain microbial diversity. This finding suggests that GOS promotes diversity in the intestinal microbiota. Gao et al. (2017) studied the influence of feed additives including probiotics and antibiotics on the maturation of the intestinal microbiota in broiler chickens to conclude that probiotic feed provides a great acceleration in the maturation of the intestinal microbiota by 15 days. Contrary, they revealed a significant retardation with eventual delay (10 day) on intestinal maturation of the broiler chicken microbiota using antibiotic supplemented feed. Therefore, it is possible that the structural and functional dynamics of intestinal microbiota can be used as a signature to characterize, compare, and evaluate the feeding regimens in the poultry industry.

Estimates of beta diversity using Bray-Curtis dissimilarity indicates that significant differences exist between the control and GOS supplemented diet in biosecure housed birds at 22, 24 and 28 da but losing this significance at 35 da.

Additionally, significant differences in beta diversity were found between control and GOS birds at 30 and 37 da in commercial birds with increasing age. Moreover, it is illustrated that despite the halting the provision of GOS at day 20, the effect of the prebiotic feed persists in the gut microbiota since elements of the microbiota become established. Thus, it seems to be GOS retain diversity of gut population even though the absence of GOS feeding, which may explain the reason why α-diversity not affected in GOS 37 da diet.

The phylum of Proteobacteria have been documented as a reservoir of class 1 integrons. The association of class 1 integrons with *Enterobacteriaceae* has been extensively reported since 1973 in various geographical locations of the globe (Kaushik et al., 2018). This includes the intestinal microbiota of farm animals such as poultry, swine, cattle, and aquatic animals that have been widely associated with various classes of integrons (Cocchi et al., 2007;Zhang et al., 2009). Especially, when animals are reared for commercial purposes are they frequently associated with class 1 integrons, likely due to their exposure to antimicrobial agents more frequently than other animals (Cocchi et al., 2007). Primary analysis by the phylogenetic tree highlights the distribution of embedded GCs in the Proteobacteria of the two rearing environments and the diets provided. The most prevalent GCs (GC-B2 and GC-A) in birds from the biosecure and commercial housing show higher abundance in the control feed birds over the GOS-fed birds (Appendix 3.3 A and B). Resistance to trimethoprim by the *dfrA* gene (GC-B2) was observed to reduce in the biosecure birds with time through the loss of the specific gene cassette to form GC-C1 (*aadA1*). Although, resistance load of commercial birds did not decrease with time, it showed an increase in GC diversity. A study by Braykov et al. (2016) reported similar observation compatible with findings for the biosecure birds. They proposed that control should be exerted in poultry hatcheries and sources along the distribution chain because young birds have potential to act as reservoirs of AR bacteria. They also called for monitoring to

control the dissemination of AR bacteria in poultry production and to assess AR bacteria emerging from external sources. The rearing environment has a role in the acquisition and dissemination of ARGs. Wellington et al. (2013) pointed out the role of the natural environment in the mobilization of genetic elements from environmental bacteria to Gram-negative bacteria, as well as the mobility of these elements being associated with responses to selective pressures, induced by exposure to antibiotics and pollutants in the environment.

The distribution of GCs among Proteobacteria OTUs demonstrates high frequency of recovered resistance load (GCs) in Gamma-proteobacteria (OTU0007) in both trials. Zhang et al. (2018) investigated the phylogenetic distribution of integrons using the bacterial whole genome database (WGD) and plasmid database to demonstrate out of 2440 integrons, 2295 were identified as harboured by the phylum Proteobacteria, mainly by Gamma-proteobacteria (93%). They also detected a few examples of integrons in Alpha-proteobacteria (5 in 2879 of the available genomes) and Beta-proteobacteria (109 in 3401 genomes). Several studies have reported that the most frequently identified integrons (90%) were obtained from Gamma-Proteobacteria (Schmitz et al. (1999);Zhao et al. (2001);Moura et al. (2009). Similarly, the current study findings also highlight high contributions of GCs in Beta-Proteobacteria OTUs in both trials, while alpha-Proteobacteria OTUs were only observed in the commercial birds. The results show high frequency of GCs-A was found to coincide with α-Proteobacteria OTU0176 with fewer dissemination was detected in both OTU0474 and in the two feed of commercial birds (Appendix 4.4, Figures 1 and 2). Cury et al. (2016) studied the taxonomic distribution of integrons in 243 bacterial genomes to find that GCs were absent in *α*-Proteobacteria if they encoded tyrosine recombinases that contributed to the integration of GCs, however they detected 20% GCs in gamma-proteobacterial and 10 % in beta-proteobacteria.

LEfSe identified a relative increase in the abundance of *Lachnospiraceae ssp* (OTU0032 and OTU0030) and *Clostridiales_unclassified* (OTU0138) as differentially abundant OTUs for GOS fed birds compared to those on the control diet at 22 da, which could be acting as competitors to decrease the expansion of GCs in the GOS fed birds. LEfSe analysis also shows that although commercial birds fed control and GOS diets became colonised by *Campylobacter* at 37 da, the abundance of *Megamonas* coincides with *Campylobacter* colonisation in control-fed birds but not in the GOS-fed birds. This may be an indicative of the suppressive role of the GOS established microbiota (it could be attributed to *Alistipes* OTU0025 and *Bacteroides* OTU0028) that are competing with the growth of *Megamonas*. Duggett (2016) showed that the abundance of *Megamonas* was determined in chicken's microbiome by feeding a wheat rich diet as the most responsive genus with over five times in the number of 16S rRNA gene sequences.

5.4 Conclusion

To summarise the previous findings, the involvement of a prebiotic GOS has been investigated by modulating the broiler caecal microbiota using prebiotic dietary intervention. This study used class 1 integrons as an indicator of the distribution of antibiotic resistance in the caecal microbiota of broiler chickens reared under biosecure or commercial conditions and fed either a control or GOS supplemented diet. Based on the study findings, at 22 da when the birds were on GOS feed there was a significant decrease in the integrase copy number, a low percentage of recovered GCs, and a reduction in trimethoprim resistance compared to the control diet. GOS feed shows promise in mitigating the resistance load in juvenile chickens. At this age the microbiota of the bird shows a dependence on the major Proteobacterial OTU007 (*Enterobacteriaceae_unclassified*).

The resistance load was high in young birds independent of the rearing regime; hence they have the potential to act as reservoirs of AR bacteria. Therefore, management practice should focus on first forms of contact with birds (hatcheries and the distribution chain) responsible for spreading AR genes in poultry production. Hence, GOS not only can positively prevent pathogen colonization but also has the potential to mitigate AR containing OTUs. This suggests that dietary GOS supplementation combined with biosecurity can restrict the expansion ARGs in the early rearing period, where control could reduce the dissemination of these genes. However, this restriction is eroded in the absence of the GOS diet.

CHAPTER 6

IMPACT OF DIETARY GALACTO-OLIGOSACCHARIDE (GOS) ON ANTIBIOTIC RESISTANCE IN BROILER CHICKEN CAECAL MICROBIOTA COLONISED BY *SALMONELLA* ENTERITIDIS

6.1 Introduction

As reviewed in Chapter 1, *Salmonella* represents one of the leading causes of foodborne illness that is often associated with poultry and poultry products, which are considered as a major source of human infection (Hughes et al., 2017). Therefore, it is important to control *Salmonella* within poultry production (meat and eggs) to reduce the risk of human consumption. Poultry are a frequently symptomatic carriers of *Salmonella* infection (Kogut and Arsenault, 2017), and are therefore a target for research to understand how birds respond to *Salmonella* colonisation and how interventions can reduce *Salmonella* carriage without compromising bird health or production (Hughes et al., 2017). Research in this chapter investigates the impact of dietary GOS supplementation to prevent or support the removal of *Salmonella* from broiler chickens and how intervention could reduce the dissemination of ARGs.

The two most common serotypes responsible for approximately 40 to 60% of all *Salmonella* infections associated with foodborne disease emanating from poultry are: *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (Altekruse et al., 2006;Boore et al., 2015;Azcarate-Peril et al., 2018). *Salmonella* can be detected in various concentrations in all regions of the gastrointestinal tract (GIT) of challenged chickens (Fanelli et al., 1971;Snoeyenbos et al., 1982;Micciche et al., 2018). However, the caecum remains the most frequently investigated part of GIT of poultry for *Salmonella* (Soerjadi et al., 1981;Hargis et al., 1995;Heres et al., 2003;Huang et al., 2006). The caecum, as the favoured environment for colonisation, harbours relatively high densities of bacterial counts with up to 10^{11} cells/g of digesta at three days post-hatching (Apajalahti et al., 2004;Stanley et al., 2014). *Salmonella* could be detected by culture, in the duodenum and the small intestines of 5–45% of young birds, 1 day post-infection, when challenged with high levels of *Salmonella* (Fanelli et al., 1971) but not with lower levels (Micciche et al., 2018).

Recent data propose that *Salmonella* colonisation factors promote horizontal gene transfer of antimicrobial resistance genes by increasing the local density of *Salmonella* in colonised intestines (Yue and Schifferli, 2014). It is proposed there is a link between the acquisition of adhesins of *Salmonella* and AMR as HGT drives the acquisition of the elements. This is because ARGs in enterobacteria including *Salmonella,* are frequently harboured on mobile genetic elements such as integrons, transposons, plasmids, and integrative conjugative elements (Vo et al., 2007;Su et al., 2008;Ajiboye et al., 2009;Call et al., 2010;Yue and Schifferli, 2014). Mobilisation of these DNA elements by HGT occurs in the favourable environment of the intestines (Nijsten et al., 1995;Lester et al., 2004;Rowe-Magnus and Mazel, 2006;Schjørring et al., 2008;Trobos et al., 2009;Faure et al., 2010). The local inflammatory response of the intestinal mucosa caused by *Salmonella* adhesion and invasion can provoke HGT events and is illustrated in Figure 6.1 (Stecher et al., 2012). The cyclical colonisation process in the intestines leads to intestinal persistence (Figure 6.1 B) that create favourable conditions for HGT events (Figure 6.1 C). This enhances the antibiotic resistance gene pool which is further stabilised by clonal expansion and selection if antibiotics are administered. Moreover, specific ileum colonisation can increase the rate of intra-intestinal conjugation (García-Quintanilla et al., 2008). HGT of antibiotic resistance genes can promote expression of some adhesins (Sahly et al., 2008), which suggests a positive mechanism between intestinal colonisation and HGT leading to the accumulation of antimicrobial resistance genes in such strains.

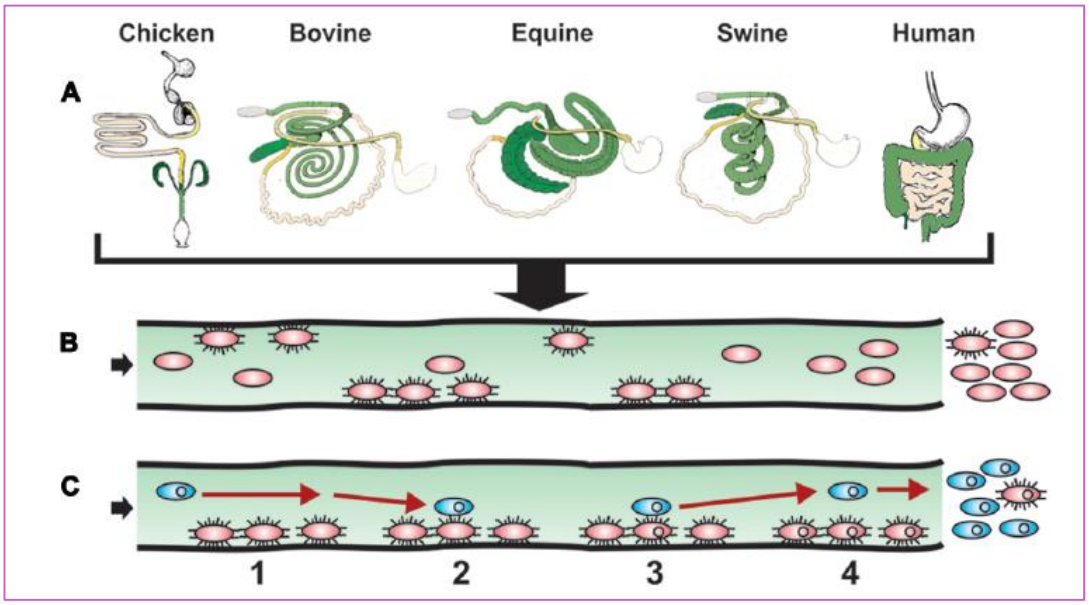


Figure 6.1 Intestinal surface colonisation and HGT model (Yue and Schifferli, 2014). **(A)** Wide adaptation of *Salmonella* to multitude of environments and hosts with distinctly different anatomies. **(B)** *S*. enterica possessing specific adhesins for recognising the cognate host intestinal receptors and cellular targets to initiate successful colonisation.**(C)** During intestinal colonisation, *Salmonella* (red ovals) optimises contact (event numbers and time span) with a constant flow of new bacteria (blue ovals) via specific allelic adhesins, some encoding ARGs on conjugative or mobilizable elements (small circles in ovals), leading to an increase in HGT efficiency and antibiotic resistant.

Farm environments can serve as reservoirs of pathogens that carry antibiotic-resistant genes (Kelley et al., 1998;Chen and Jiang, 2014). Recently, MDR *Salmonella* isolates resistant to streptomycin (30.9%), gentamicin (12.6%), sulfadimethoxine (20.9%), tetracycline (13.9%), and trimethoprim-sulfamethoxazole combination (8.6%) were recovered from broiler farms (Nair et al., 2018). High prevalence rates of *S*. Enteritidis were observed in feed, hatching eggs, litter, drinkers, bird rinse, and caeca, with 88% of *S.* Enteritidis found to be resistant to multiple antimicrobials including ampicillin, nalidixic acid, and tetracycline (Al-Zenki et al., 2007). AMR strains of *Salmonella* serovars are frequently isolated from broiler carcasses including *S*. Enteritidis, *S.* Infantis, *S.* Typhimurium, and *S.* Heidelberg (Medeiros et al., 2011).

Sustainable intervention strategies are in development to control antibiotic-resistant *Salmonella* in poultry at the farm level and its dissemination to carcasses during processing but progress is slow. Prebiotic applications are a potential control strategy for controlling intestinal *Salmonella* infection. These may be added to feed and/or water without any modification required from current production procedures (Hughes et al., 2017). Prebiotic diets have been shown to modulate the intestinal microbiota, modify transit time, luminal pH, and microbial metabolites products in humans and in animal models (Chambers and Lu, 2002;Kogut et al., 2012;Park et al., 2017b). Prebiotics are proposed to act as soluble decoy receptors to prevent the attachment of pathogens to mucosal surfaces (Shoaf et al., 2006;Quintero et al., 2011;Azcarate-Peril et al., 2018).

The prebiotics inulin, fructo-oligosaccharides (FOS), and mannan-oligosaccharides (MOS) have been ascribed protective roles in chicks during the first few days post-*Salmonella* infection, with a reduction in shedding of the colonising pathogen (Fukata et al., 1999;Patterson and Burkholder, 2003;Baurhoo et al., 2007). Prebiotic GOS fed to chickens resulted in an increase in the abundance of beneficial *Bifidobacteria* and *Lactobacillus* (Jung et al., 2008). GOS is also reported to reduce the adherence and invasion of *Salmonella* in human enterocytes (Searle et al., 2010). Inclusion of prebiotics in broiler diets therefore offer the prospect of reducing *Salmonella* colonisation through modification of the hosts' immune response and the gut microbiome. It may also reduce the expansion of ARGs in co-affected *Enterobacteriaceae.*

The work described in this chapter aimed to understand the effectiveness of dietary GOS supplementation on the elimination of *Salmonella enterica* serovar Enteritidis 125109 colonising broiler chickens. These studies were also planned to determine the effect of GOS on the carriage of AR genes in the presence of *Salmonella* by monitoring the ARG load embedded in class 1 integrons within the caecal microbiota. An attempt was also made to detect a colonising *Salmonella*

that might have acquired a trimethoprim gene cassette (*dfrA*) to assess the mobility of AR genes in the microbiome of the chicken caecum.

6.2 Results

6.2.1 Enumeration of *Salmonella* in the caecal contents of challenged birds fed control and GOS diets

The enumeration of *Salmonella* (the strain is trimethoprim sensitive, and it does not carry a class 1 integron) from the caecal contents of challenged birds fed control diet (G3, named ctl-sal) and GOS diet (G4, named gos-sal) were carried out at 2, 4, 8, 15 da post infection (dpi) and the results are presented in Figure 6.2. Birds infected with *Salmonella enterica* serovar Enteritidis 125109 at 20 days became caecal colonised within 2 days of exposure with median counts of 3.43 (SD $\pm$ 0.66) and 3.51 (SD $\pm$ 0.84) $\log_{10}$ CFU/g for control and GOS fed birds. The level of detection for these experiments was 1.8 $\log_{10}$ CFU/g and by 4 dpi *Salmonella* were only detectable by enrichment from the caeca of specific birds <1.8>1.0 $\log_{10}$ CFU/g, and not at all for two members of the GOS-fed group. For this reason, the colonisation data for each group were ranked and analysed using the Wilcoxon signed-rank test as a non-parametric statistical test. The Wilcoxon test revealed that *Salmonella* counts significantly reduced by 8 dpi in GOS fed birds compared to the control diet (p-value = 0.0476). The significance level increased by 15 dpi (p = 0.0075). No *Salmonella* were isolated from the mock infected birds (Groups 1 and 2).

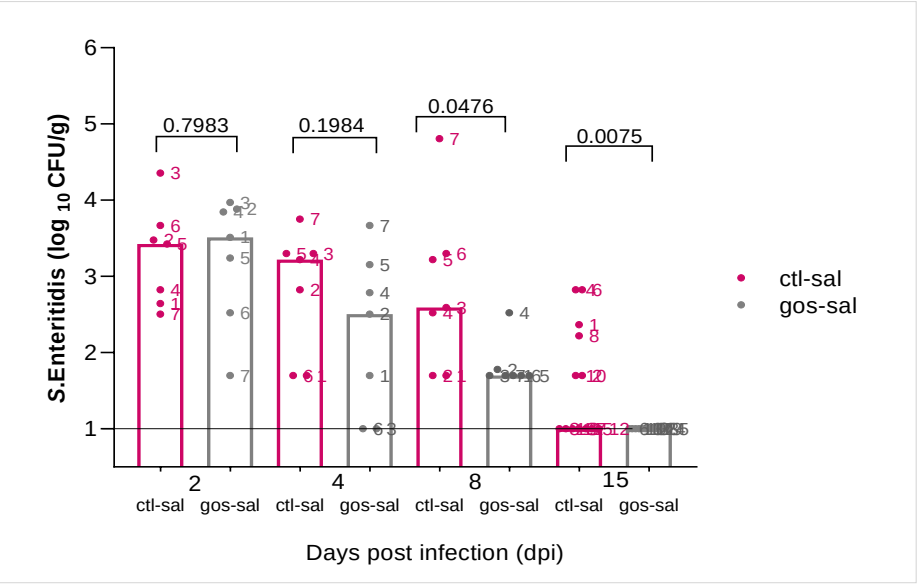


Figure 6.2 Comparison of *Salmonella* Enteritidis 125109 numbers recovered from the caecal contents of birds fed control diet or GOS diet. Dietary GOS reduced the level of *Salmonella* at 8 days post infection. At 2 dpi GOS feeding was stopped. Bars indicate median *Salmonella* concentration. Each individual bird is marked with corresponding ID. A non-parametric Wilcoxon test was used to determine significant differences for each sampling time with the level of significance $p<0.05$.

6.2.2 Screening for *Salmonella* in the livers and spleens of challenged birds fed control and GOS diets

Detection of *Salmonella* in the liver and spleen of the challenged groups (G3 and G4) was carried out at 2, 4, 8, 15 dpi by direct plating of homogenised tissue on selective agar out of 7 birds per treatment and by enrichment. The results showed that *Salmonella* was detected in the livers of three birds in ctl-sal group at 2 dpi whereas all seven birds were positive in the gos-sal group. At 4 dpi, all livers sampled from both diets were positive. While at 8 dpi, both diets displaying a decrease in *Salmonella* detection (6/7). At the last sampling day (15 dpi) both control and GOS diet birds recorded a lower number of *Salmonella* positive livers (28.5%). The results are presented in Figure 6.3.

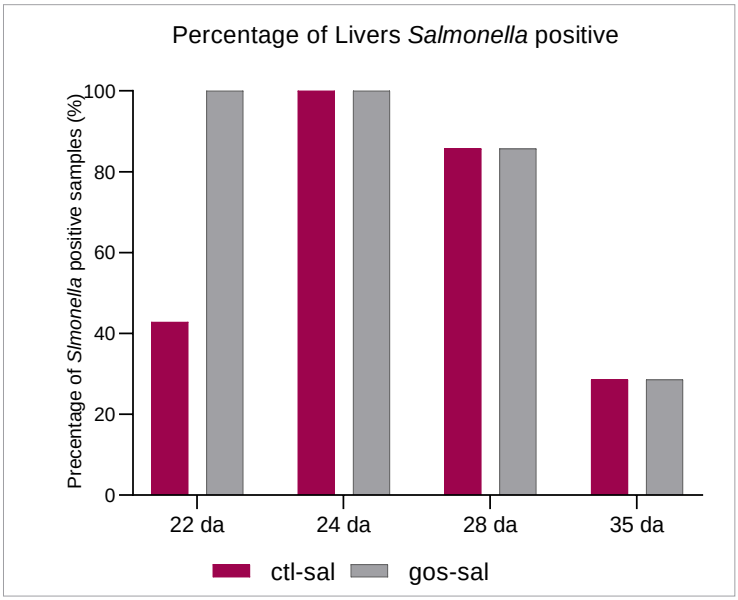


Figure 6.3 The comparison of livers *Salmonella* Enteritidis 125109 positive birds fed the control diet or the GOS diet. Percentage indicating that no significant difference was found among two diets. Data expresed as bar charts with red bars represented ctl-sal groups and gray bars represented gos-sal groups.

Salmonella was recovered from the spleens of the infected birds with five samples positive in the ctl-sal group compared to 6 positive samples in the gos-sal group at 2 dpi. All spleen samples were positive at 4 dpi from both groups and 6/7 for the ctl-sal birds compared to 7/7 samples for gos-sal at 8 dpi. However, a 15 dpi the number of positive spleen samples in both diets declined (three positive samples in ctl and two positives in GOS diets). The results are displayed in Figure 6.4. No *Salmonella* were detected in the liver and spleen of birds from Groups 1 and 2 (non-infected controls).

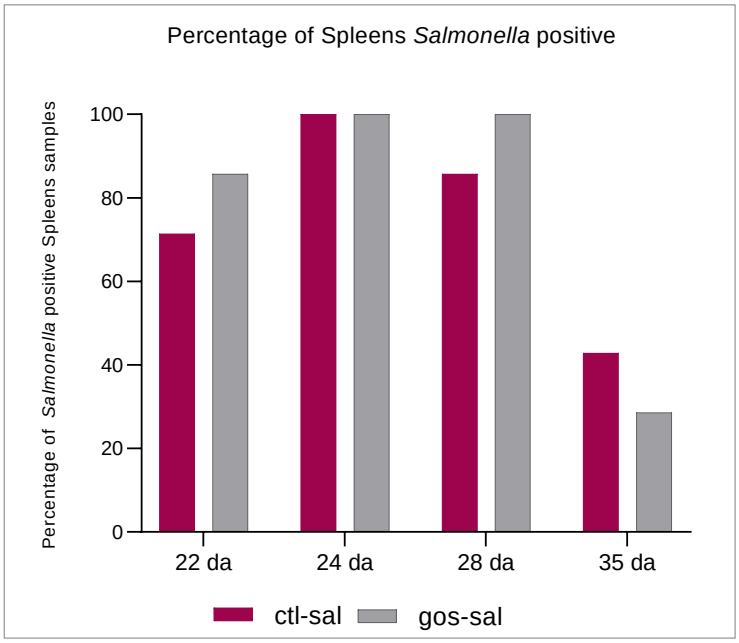


Figure 6.4 Comparison of *Salmonella* positive spleen samples of birds fed control diet and the GOS diet (Late challenge). Showing no significant difference in positive samples number between two diets colonised by *Salmonella* (G3 and G4). However, the percentage decreased with age in both feeds.

6.2.3 Enumeration of coliform bacteria recovered from the caecal contents of *Salmonella* challenged groups fed control and GOS diets

Experiments were carried out to investigate the influence of GOS supplemented diet on the reduction of coliform total counts and trimethoprim resistant bacteria. In this set of experiments, the normal broiler chicken gut microbiota was disrupted by *Salmonella* Enteritidis infection to analyse the effect if any on coliform numbers and persistence. As described previously the challenge with *Salmonella* was carried out at 20 da while feeding with GOS was halted at 22 da (2 dpi).

The results presented in Figure 6.5 show the total coliform viable count from caecal contents of birds sacrificed fed control and GOS diets, infected and non-infected by *Salmonella* at 4 sampling ages. A trend of declining coliform counts

with age was observed for all groups. The decline in coliform count was greater in the GOS-fed birds independent of *Salmonella* infection but only reached significance ($p>0.017$) at 4 dpi (24 da) comparing the two diets for the *Salmonella* infected birds (G3 and G4). The general observation of reduced coliform counts in the GOS-fed birds supports the hypothesis that prebiotic GOS has an impact on total coliform counts that could reduce the load of ARGs. Thereby GOS supplemented diet retained its ability to reduce total coliform number even when challenged with *Salmonella* Enteritidis.

The total number of coliform in control diet fed non-colonised birds ranged from 7.5 to 8.3 $\log_{10}$ CFU/g (G1), whilst the *Salmonella* challenged fed control diet ranged from 6.5 to 8 $\log_{10}$ CFU/g (G3) with no significant difference was detected p value> 0.05. The GOS-diet cohort colonised with *Salmonella* (G4) ranging from 5.8 to 8 $\log_{10}$ CFU/g and non-colonised birds ranged between 6.2 and 8.4 $\log_{10}$ CFU/g (G2) with no significant difference was detected p value> 0.05.

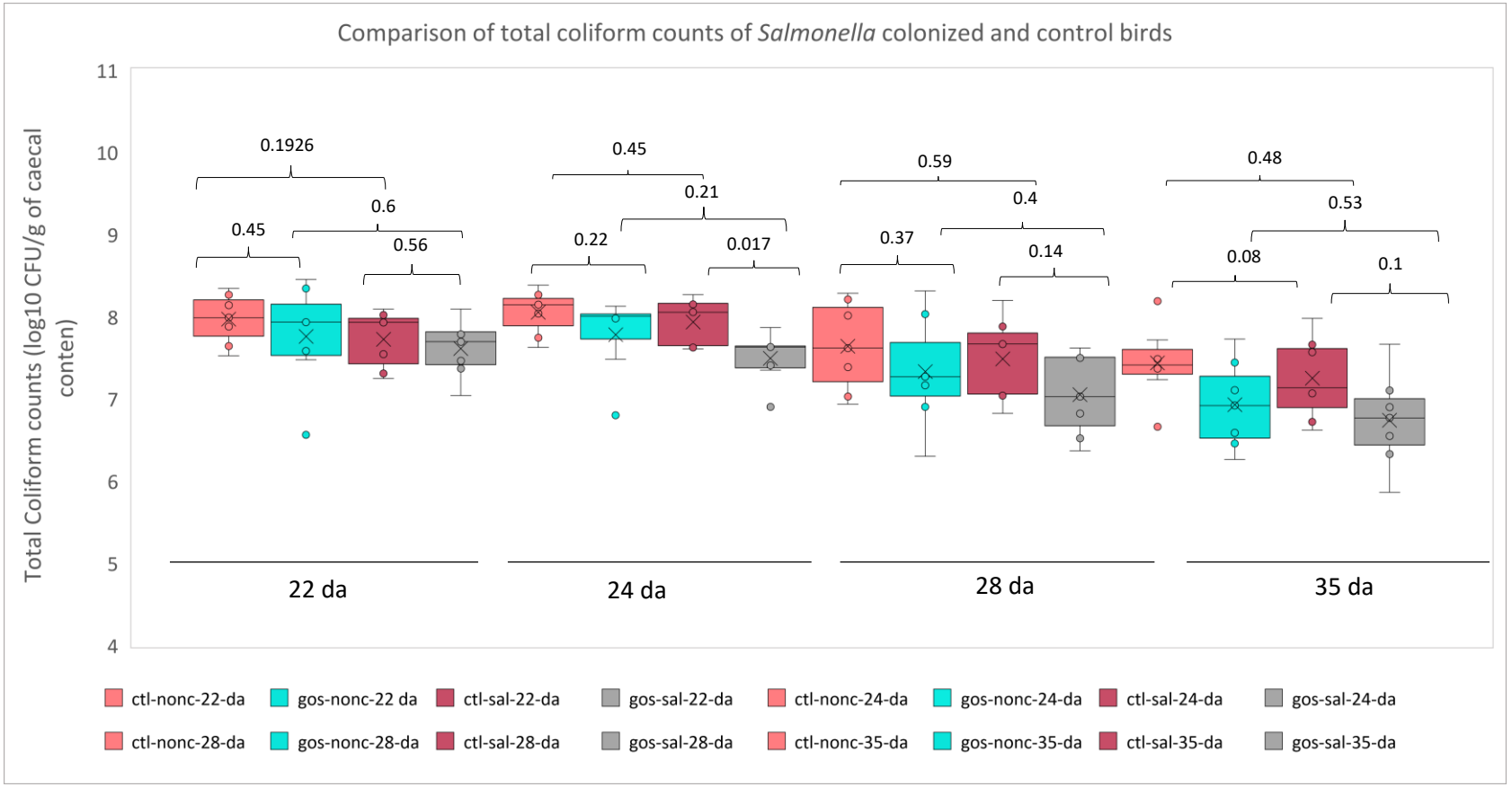


Figure 6.5 Comparison of coliform numbers recovered from the caecal contents at 22, 24, 28 and 35 da of birds non-infected and infected by *Salmonella* Enteritidis 125109, fed control diet (G1) or GOS supplemented diet (G2) or control diet colonised with *Salmonella* (G3) or GOS diet challenged with *Salmonella* (G4). Data are expressed as standard boxplots with medians (solid black line), where n=7.

6.2.4 Enumeration of trimethoprim resistant coliforms from caecal contents of control and GOS fed birds challenged with *Salmonella.*

The viable counts of trimethoprim resistant bacteria were enumerated on MacConkey agar containing trimethoprim (20 µg /ml). Figure 6.6 shows the counts for the various groups ranging from $\log_{10}$ 4-7 CFU/g. The results indicate that viable counts of trimethoprim resistance coliforms were significantly reduced at 2 and 4 dpi (p-value = 0.04 and 0.0009 respectively) in the GOS supplemented diet birds challenged with *Salmonella* (G4) compared to control diet fed *Salmonella* infected birds. Although, this significance disappeared at 8 dpi the counts of trimethoprim resistance coliforms were still lower in *Salmonella*-colonised GOS diet birds than *Salmonella*-colonised control diet birds. At 4 dpi the trimethoprim resistance counts were reduced in the presence of *Salmonella* for birds fed GOS diet (p-value = 0.045). Generally, the counts of trimethoprim resistant bacteria were lower in the GOS groups throughout.

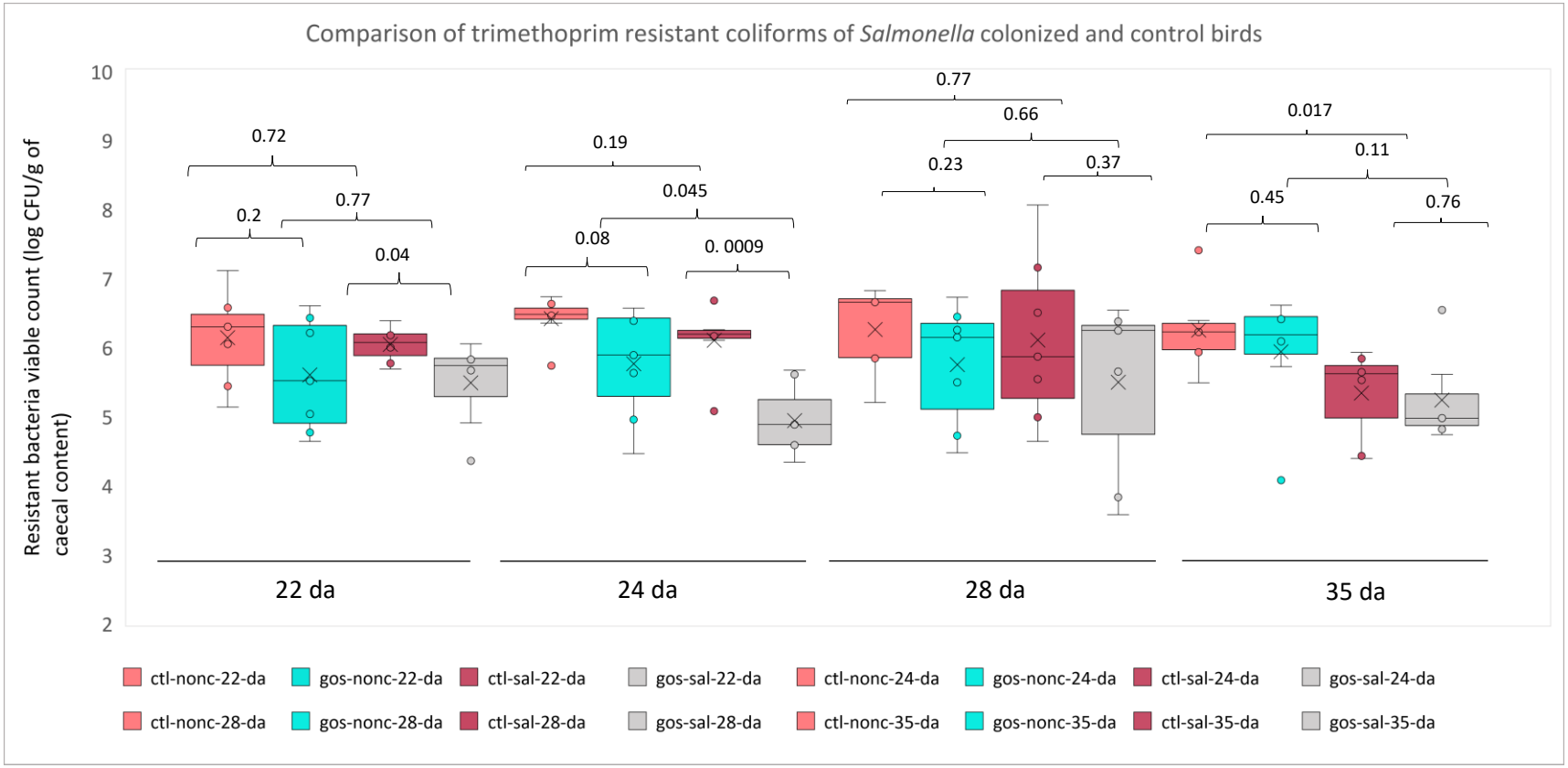


Figure 6.6 Comparison of trimethoprim resistance bacteria collected from the caecal contents at 22, 24, 28 and 35 da of birds non-infected and infected by *Salmonella* Enteritidis 125109, fed control diet (G1) or GOS supplemented diet (G2) or control diet colonised with *Salmonella* (G3) or GOS diet challenged with *Salmonella*. Data are expressed as standard boxplots with medians (solid black line), where n=7. The general trend shows trimethoprim resistant bacteria were lower in G4 than G3 and it seems to be restricted in GOS diet.

6.2.5 Ratio of coliform trimethoprim resistance bacteria obtained from the caecal contents of birds reared in biosecure conditions fed control and GOS diets, challenged by *Salmonella*

The proportion of trimethoprim resistant coliforms was calculated by dividing total number of trimethoprim resistant coliform bacteria by the total number of coliforms. Figure 6.7 shows that the proportion of the trimethoprim resistant bacteria in the total population increases with age. Consistent with the observation that the trimethoprim resistant coliforms were reduced in birds fed GOS diet (Figure 6.6), a reduced proportion of trimethoprim resistant bacteria at 2 and 4 dpi in birds fed GOS diet was observed. At 8 dpi the proportion of trimethoprim resistant bacteria increased in both GOS diet groups. The ratio of trimethoprim resistant bacteria reduced at 22 and 24 da after withdrawing GOS at 22 da but this effect did not persist at 28 and 35 da and that may relate to changes in abundance of specific members of the microbiota supported by GOS supplementation.

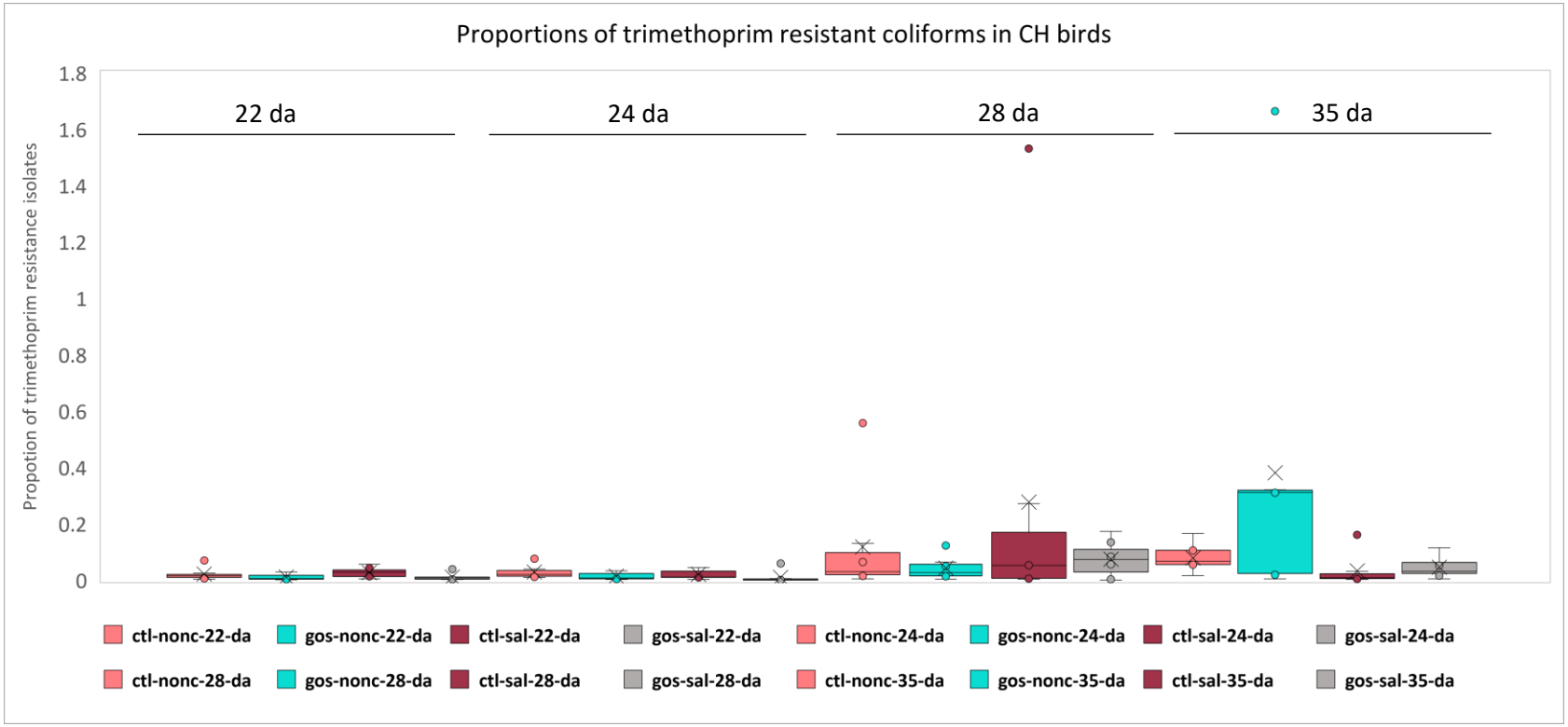


Figure 6.7 Ratios of trimethoprim resistant coliform population in birds fed control and GOS diets, challenged and non-challenged by *Salmonella*. Data are expressed as standard boxplots with medians (solid black line), where n=7. The orange boxes indicate to control-non colonised birds, tiffany blue boxes represent GOS non-colonised birds, dark red boxes represent control *Salmonella* colonised birds and grey boxes represent GOS *Salmonella* colonised birds. Outliers are expressed as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x the interquartile range.

6.2.6 Evaluation of class 1 integron integrase copy number of *Salmonella* colonised birds fed control and GOS diets reared in biosecure conditions.

To extend the observations regarding the enumeration the total coliform counts and trimethoprim resistant coliform population for the *Salmonella* colonised birds (G3 and G4) and to enable comparison with the non-colonised groups (G1 and G2, Chapter 4) fed either control or GOS diets, q-PCR experiments was preformed to determine the class 1 integron copy number using the integrase gene primer described in Chapter4 (Section 4.2.5). The *S*.Typhimurium U288 was used as positive control to quantify the absolute quantification of integrase gene copy number per gram of intestinal contents. The results presented in Figure 6.8 show at 2 dpi the birds on the GOS diet had significantly less class 1 integrase that the control diet with *Salmonella* colonisation (p-value = 0.004) or without (p-value = 0.015). Therefore, this finding supports the hypothesis that the GOS supplemented diet leads to a class 1 integron population and the incumbent ARGs but this effect is lost once the GOS supplementation is halted.

6.2.7 Comparison of the antimicrobial gene cassettes in the caecal microbiota of birds with and without *Salmonella*.

Table 6.1 shows the class 1 integron GC types present in the caecal microbiota of the birds fed control and GOS diets with and without *Salmonella* colonisation across all sampling points. Notably the challenge strain *Salmonella* Enteritidis P125109 does not contain a PCR amplifiable class 1 integron. The total representative GC types from broiler caecal microbiota of birds on the control diet colonised by *Salmonella* was 37 from 28 birds (G3), such that all birds contained at least one GC. Almost all (27/28) carried the GC with the antibiotic resistance type GCs-B-2 (containing the resistance genes *dfrA1* and *aadA1*), followed by GCs-

C-2 (3/28; characterised by the insertion of the trinucleotide GGG in the integrase gene), and 2/28 for GCs-B-1, GCs-F and 1/28 GCs-C-1. Whereas 26 of 28 birds carried class 1 integron GCs for the GOS supplemented diet challenged by *Salmonella* (G4). The most frequent GC type detected was GCs-B-2 (17/28), with lesser contributions from GCs-C-1 (4/28), GCs-B-1 (2/28) and GCs-F (3/28). Using the chi-squared test for all birds colonised by *Salmonella* the proportion exhibiting gene cassettes on the GOS diet was significantly different to those on the control diet (χ^2 (1, N = 56) = 8.00, p = 0.004). For the non-colonised groups, the proportion exhibiting gene cassettes on the GOS diet (G2) compared to those on the control diet (G1) marginally failed to meet significance (χ^2 (1, N = 55) = 2.3, p = 0.13). Comparison for all birds exhibiting gene cassettes on the GOS diet with control shows a significant difference (χ^2 (1, N = 111) = 6.75, p = 0.009). Comparison of the proportion of all birds exhibiting gene cassettes with or without *Salmonella* shows a significant difference (χ^2 (1, N = 111) = 43.52, p = 0.001).

Table 6.1 Gene cassettes present in the caecal microbiota of chickens reared under biosecure conditions.

Type of GC	Control diet non-colonised (G1)					GOS diet non-colonised (G2)				
	22 da	24 da	28 da	35 da	total	22 da	24 da	28 da	35 da	total
B-1	-	-	-	-	-	-	-	-	-	0
B-2	5	3	1	1	10/27=37%	-	2	-	-	2/28=7.14
C-1	-	-	-	-	-	-	-	-	2	2/28=7.14
C-2	-	-	-	-	-	-	-	-	-	0
F	-	-	-	-	-	-	-	-	-	0
ND	2	4	6	5	17	7	5	7	5	24
Total	5	3	1	1	10/27=37%	0	2	0	2	4/28=14.28
	Control diet with Salmonella (G3)					**GOS diets with Salmonella (G4)**				
B-1	1	-	1	-	2/28=7.14%	1	1	-	-	2/28=7.14
B-2	7	7	7	6	27/28=96.4%	6	5	2	4	17/28=60.71
C-1	-	-	-	1	1/28=3.57%	1	-	1	2	4/28=14.2%
C-2	1	-	-	2	3/28=10.71%	-	-	-	-	2/28=7.14%
F	-	-	1	1	2/28=7.14%	-	-	1	2	3/28=10.71%
ND	-	-	-	-	0	-	2	3	2	7
Total	9	7	9	10	35/28=125%	8	6	4	8	26/28=92.85

6.2.8 *Salmonella* Enteritidis antimicrobial resistance mobility test

An attempt was made to detect if the colonising *Salmonella* strain had acquired resistance to trimethoprim (*dfrA*) during the experiment. Caecal contents of *Salmonella* colonised groups were serially decimally diluted and plated on trimethoprim XLD plates. No *Salmonella* isolates resistant to trimethoprim were recovered for any of the sampling days.

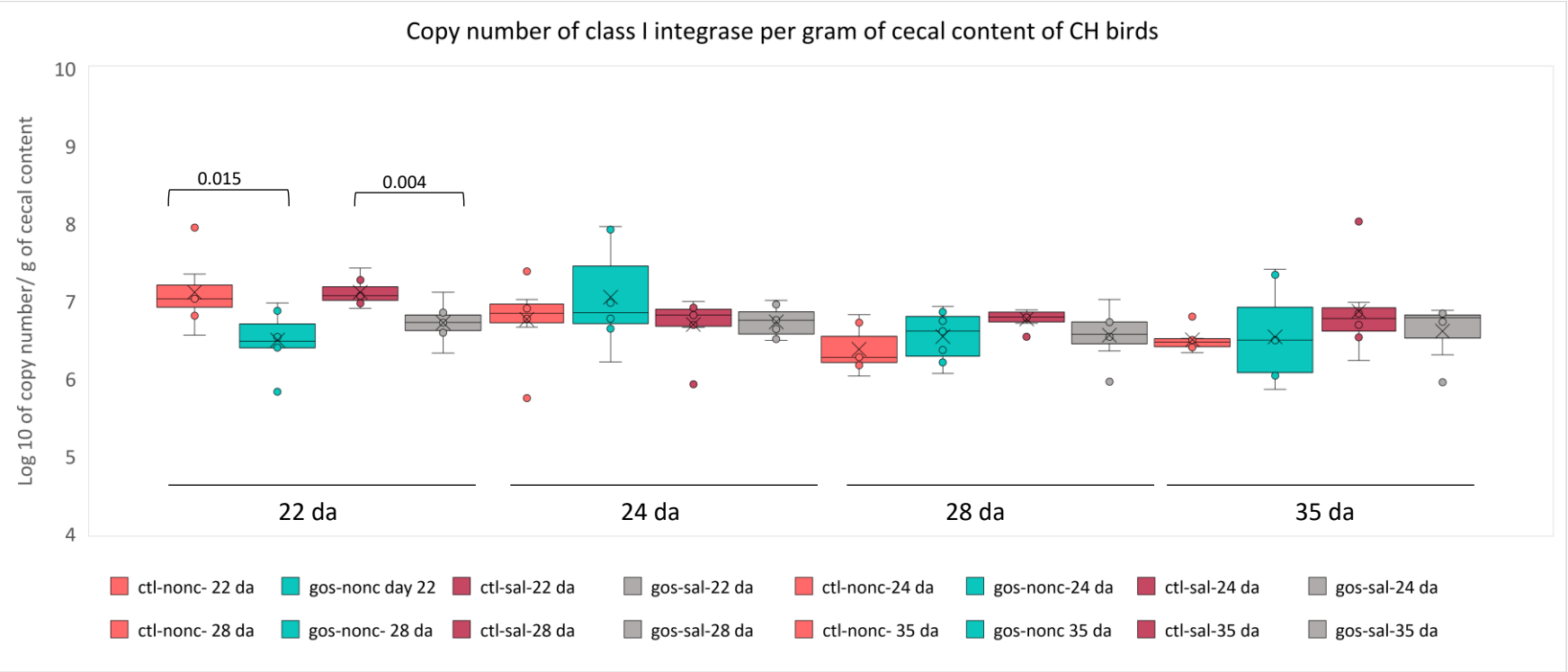


Figure 6.8 Comparison of class 1 integrase copy number quantified by qPCR per gram of caecal contents of birds fed both diets challenged and nonchallenged by *Salmonella*. Data are expressed as standard boxplots with medians (solid black line), where n=7. The orange boxes indicate to control-non colonised birds, tiffany blue boxes represent GOS non-colonised birds, dark red boxes represent control *Salmonella* colonised birds and grey boxes represent GOS *Salmonella* colonised birds. Outliers are expressed as dots. The top and bottom Whiskers indicate maximum and minimum values, unless these values exceed 1.5x the interquartile range.

6.2.9 Development of the caecal microbiota composition fed control diets and GOS supplemented diets colonised by *Salmonella*

A similar approach to that described earlier was adopted to profile the caecal bacterial community by PCR-amplifying the V4 regions of the bacterial 16S rRNA genes. All sequences data of the 16S rRNA gene were quality filtered and then clustered into operational taxonomic units (OTUs) by Mothur (Schloss et al., 2009) using the Schloss lab. MiSeq SOP2 (Kozich et al., 2013). Rarefaction curves were plotted to evaluate sampling effort covered sufficient depth (Appendix 5.1 for ctl-sal and gos-sal birds, for non-colonised 3.1 A).

A taxonomy-based comparison was performed to determine the differences between the microbiota of control fed and GOS supplemented given birds. Figure 6.9 A and B shows that the dominant phyla, at 2 dpi (22 da) were Firmicutes (*Clostridiales_unclassified)* with similar medians of 89.97% for G3 and 89.73% for G4 and an inter quartile range (IQR) of 4.81, 6.43 respectively (Wilcoxon p-value =0.7104). The second most numerous phyla were Proteobacteria for both diets with a median of 3.90% for G3 and 2.20% for G4 (IQR, 2.61, 0.44; Wilcoxon p-value= 0.3829). The abundance of the group described as Bacteria_unclassified was higher in G3 than G4 with a median of 3.28% and 1.44% respectively (IQR, 2.23%, 2.63%, Wilcoxon p-value = 0.3829). The abundance of Actinobacteria was less than 1% in G3 (median: 0.994%; IQR, 3.18), the abundance for G4 was approximately 2% (median: 1.9%; IQR, 3.78). The abundance of Bacteroidetes was noted at 22 da olds birds in both diets as variable between individual samples with a mean of 3.23% for G3 and 3.85% for G4. Figure 6.9 C and D shows that at 4 days post-infection (24 da birds), the phyla observed were similar with no significant differences between the phyla abundances for G3 and G4. Bird to bird variation in the abundance of Bacteroidetes remained a feature of the data.

Figure 6.10 A and B show the abundances of phyla at 8 da post *Salmonella* colonisation (28 da old birds), with the control-sal group (G3) displaying an increase in abundance of Firmicutes (median: 93.44%, IQR, 8.0) while for the GOS diet (G4) caecal abundance of Firmicutes was reduced (83.29%, IQR, 11.24; Wilcoxon p-value =0.0973). In contrast, there was a significant increase in the abundance of Actinobacteria in gos-sal group (G4) with a median of 10.76% and IQR of 9.28, whereas the abundance of Actinobacteria was deceased in control-sal birds with a median of 0.37% and an IQR of 2.94 (Wilcoxon p-value =0.0262). The abundance of Proteobacteria was similar between the two diet groups with medians of 3.05%, and 3.22% for G3 and G4 respectively (IQR of 3.88 and 2.79; Wilcoxon p-value =0.9015). Figure 6.10 C and D show that at 15 dpi (35 da) the abundance of the Firmicutes was reduced in the control-sal (G3) with a median of 88.38% and IQR of 11.40% but was not significantly different to that recorded for the gos-sal group (G4) with a median of 90.14% and an IQR of 3.11% (Wilcoxon p-value =0.4557). The proportion of Proteobacteria was reduced in caecal contents from birds fed either diet, however its abundance was lower in G3 with a median of 0.98% and IQR of 0.52, compared to a median of 2.25% in G4 with and IQR of 1.35 (Wilcoxon p-value= 0.0728).

Figures 6.11 A and B show the class level abundance at 2 dpi where there was one significant difference detected between the two diets in conjunction with *Salmonella* colonisation. This was the OTU corresponding to Erysipelotrichia with a median of 1.76% and 0.398%, for G3 and G4 respectively (Wilcoxon p-value 0.0111). There was no significant change detected at 4 dpi (Figure 6.11 C and D). At 8 dpi (Figure 6.12 A and B) significant differences were found in the class level abundance of Clostridia with a median of 78.07% for G3 and 51.94% for G4 (IQR, 9.78 and 13.95; Wilcoxon p-value= 0.0262). The abundance of Negativicutes, were found to be greater in G4 with a median of 34.33% compared to 9.55% for G3 and IQR values of 29.09%, 2.19%, respectively (p-value Wilcoxon

test=0.0973). Actinobacteria also showed a significantly greater class level abundance in the GOS diet (G4) with a median of 10.76% and IQR of 9.29 compared to control diet (G3) with a median of 0.37% and IQR of 2.94 (p-value= 0.0262). At 15 dpi (Figure 6.12 C and D) the abundance of Clostridia was reduced in G4 with a median of 27.52% and IQR of 5.61 compared to G3 with a median of 67.09% and IQR of 11.70 (p-value 0.0012). Negativicutes OTUs displayed a significantly greater class level abundance at 15 dpi for the GOS diet (G4) with a median of 61.87% and IQR of 17.17 compared to control diet (G3) with a median of 9.11% and IQR of 12.30 (p-value Wilcoxon test = 0.0012).

A group-wise class level comparison combining the individual bird data and showing the effects of diet and *Salmonella* colonisation is shown in Figure 6.13 for each sample day. This indicated a significantly lower relative abundance of Clostridia in the *Salmonella* colonised control diet group (G3) compared to non-colonised control diet (G1) with Wilcoxon p-values of 0.0070, 0.0070, 0.0262, 0.0023 for 2dpi, 4dpi, 8dpi and 15 dpi respectively. Negativicutes emerged in the microbiota of birds fed control diet colonised by *Salmonella* (G3) at 2 dpi (22 da). With a median abundance of 17.81% and IQR of 16.45. In contrast, Negativicutes which were all but absent in the non-infected control diet (G1) with a median of 0.0016% for G1 and IQR of 0.001 (p-value = 0.0006). The difference in Negativicutes persisted until the end of the study at 15 dpi with a median of 0.00% and IQR of 0.0 compared to a median of 9.118% and IQR of 12.3 for G3, (p-value = 0.0006). The relative abundance of the OTU corresponding to Bacilli at 2 dpi was significantly higher in G1 with a median of 7.311% and IQR of 3.33 compared to G3 with a median of 3.597% and IQR of 2.57 (p-value = 0.0041). The relative abundance of Actinobacteria was significantly greater in G3 than G1 at 15 dpi (median for Actinobacteria 0.905% for G1 and 7.197% for G3; IQR: 0.57, 5.03; p-value = 0.0175) and for Bacteroidia (median for Bacteroidia 0.00% for G1 and 3.85% for G3; IQR: 0.0, 7.11;p-value = 0.0373).

Comparison between the non-*Salmonella* colonised birds on the GOS diet (gos-nonc, G2) and *Salmonella* colonised birds (gos-sal, G4) at class level (Figure 6.13) demonstrated a significant shift (decrease) in the relative abundance of the Clostridia recorded at 22 da in GOS-*Salmonella* cohorts with a median of 87.88% and IQR of 4.93 for G2 and a median of 66.67% and IQR of 17.75 for G4 (p-value = 0.0175). Negativicutes emerged in the microbiota of birds fed GOS and colonised by *Salmonella* (G4) with a median of 12.35% and IQR of 21.12. In contrast, Negativicutes had a low abundance in the microbiota of non-colonised GOS diet birds (G2) with a median of 0.002% and IQR of 0.0 (p-value = 0.0012). These differences also persisted until the end of the trial at 35 da but with the abundance of Negativicutes significantly greater in *Salmonella* colonised birds with a median 61.87% and IQR of 17.1 for G4 compared to a median of 0.04% and IQR of 0.02 (p-value = 0.0006). This difference appeared to be largely at the expense of Clostridia (median: 78.44% for G2 and 27.53% for G4; IQR: 7.63 and 11.70; p-value = 0.0006) and Bacilli (median: 13.86% for G2 and 3.63% for G4; IQR: 5.81 and 1.7; p-value = 0.0023). Actinobacteria showed significant increases in the relative abundance observed for the GOS-challenged birds from 28 da (median: 0.72% for G2 and 10.77% for G4; IQR: 0.60 and 9.29; p-value 0.0006).

No significant change was detected in the proportion of Gammaproteobacteria for the GOS diet groups in the presence or absence of *Salmonella* until 15 dpi when Gammaproteobacteria reduced in G4 compared to G2 (median: 3.71% for G2 and 0.68 % for G4; IQR: 1.67 and 0.5; p-value = 0.0530). Similarly, no significant difference was found among Gammaproteobacteria in birds fed standard diet colonised or non-colonised by *Salmonella* until 15 dpi (median:1.78% for G1 and 0.98% % for G3;IRQ: 1.6 and 0.5; p-value =0.12).

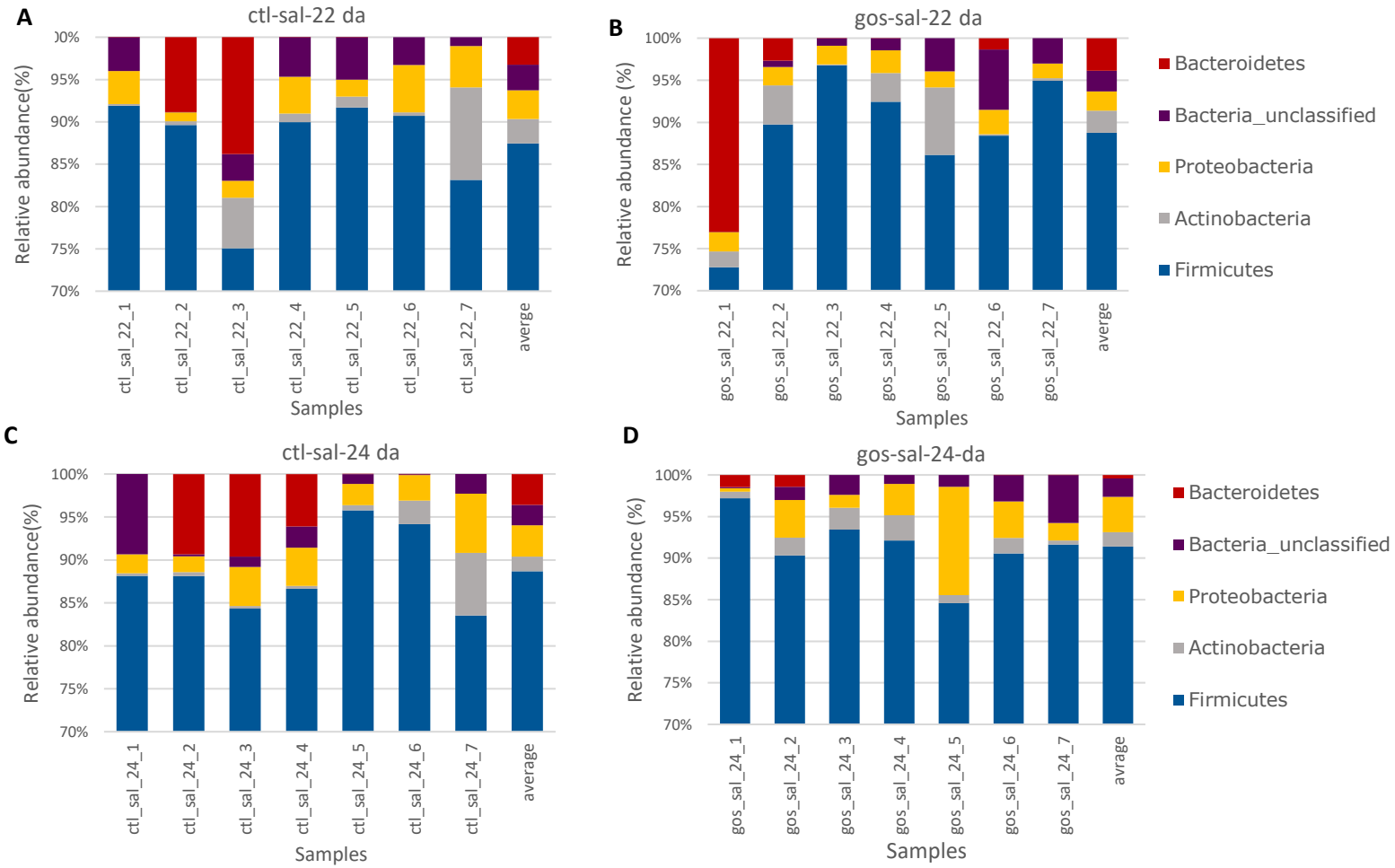


Figure 6.9. Comparison of the relative abundance of dominant bacterial phyla of gut microbiota of birds fed control and GOS diets challenged with *Salmonella*. The data were clustered based on Ribosomal Database Project taxonomy (phylum level). The results are presented as a normalised percentage of the total abundance where the taxa representing at least 1% of the caecal community. A) 22 da control diet; B) 22 da GOS diet; C) 24 da control diet; D) 24 da GOS diet

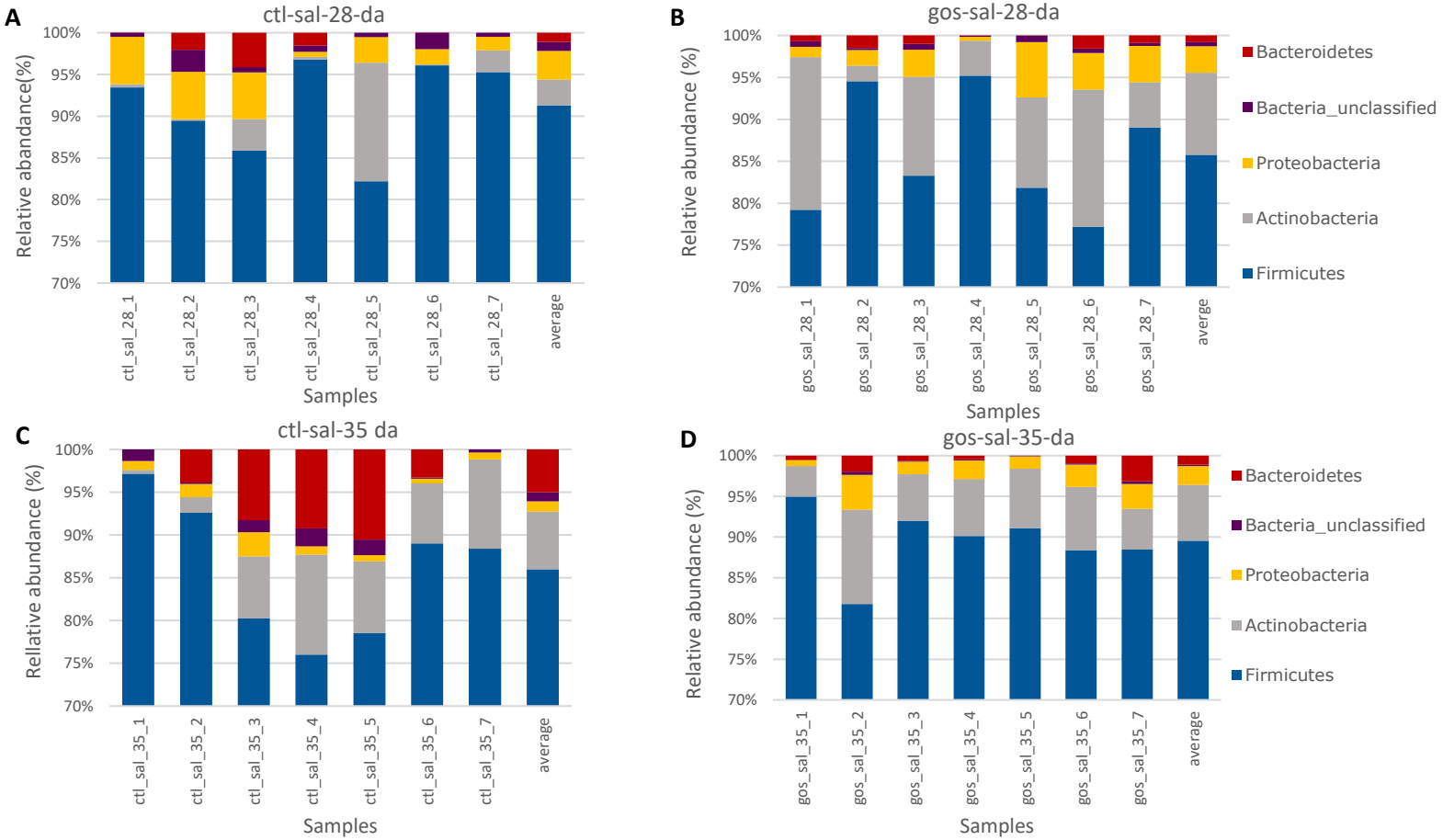


Figure 6.10 Comparison of the relative abundance of dominant bacterial phyla of gut microbiota of CH birds fed control and GOS diets challenged with *Salmonella*. The data were clustered based on Ribosomal Database Project taxonomy (phylum level). The results are presented as a normalised percentage of the total abundance where the taxa represent at least 1% of the caecal community. A) 28 da control diet; B) 28 da GOS diet; C) 35 da control diet; D) 35 da GOS diet.

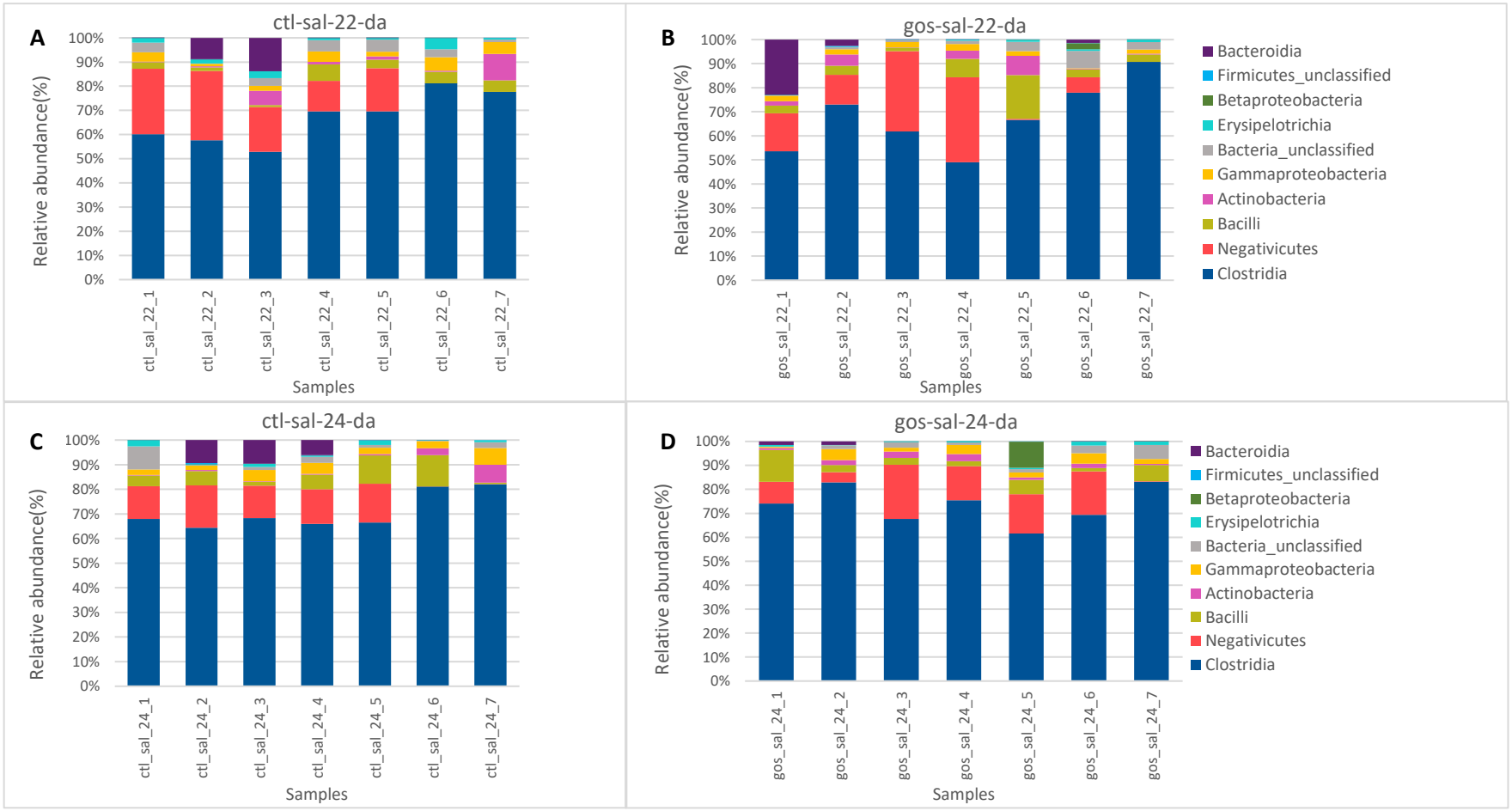


Figure 6.11 Comparison of the relative abundance of dominant bacterial classes among CH chicken gut microbiota fed control and GOS diets challenged with *Salmonella*. The data were clustered based on Ribosomal Database Project taxonomy (class level). The results are presented as a normalised percentage of the total abundance where the taxa represent at least 1% of the caecal community. A) 22 da control diet; B) 22 da GOS diet; C) 24 da control diet; D) 24 da GOS diet.

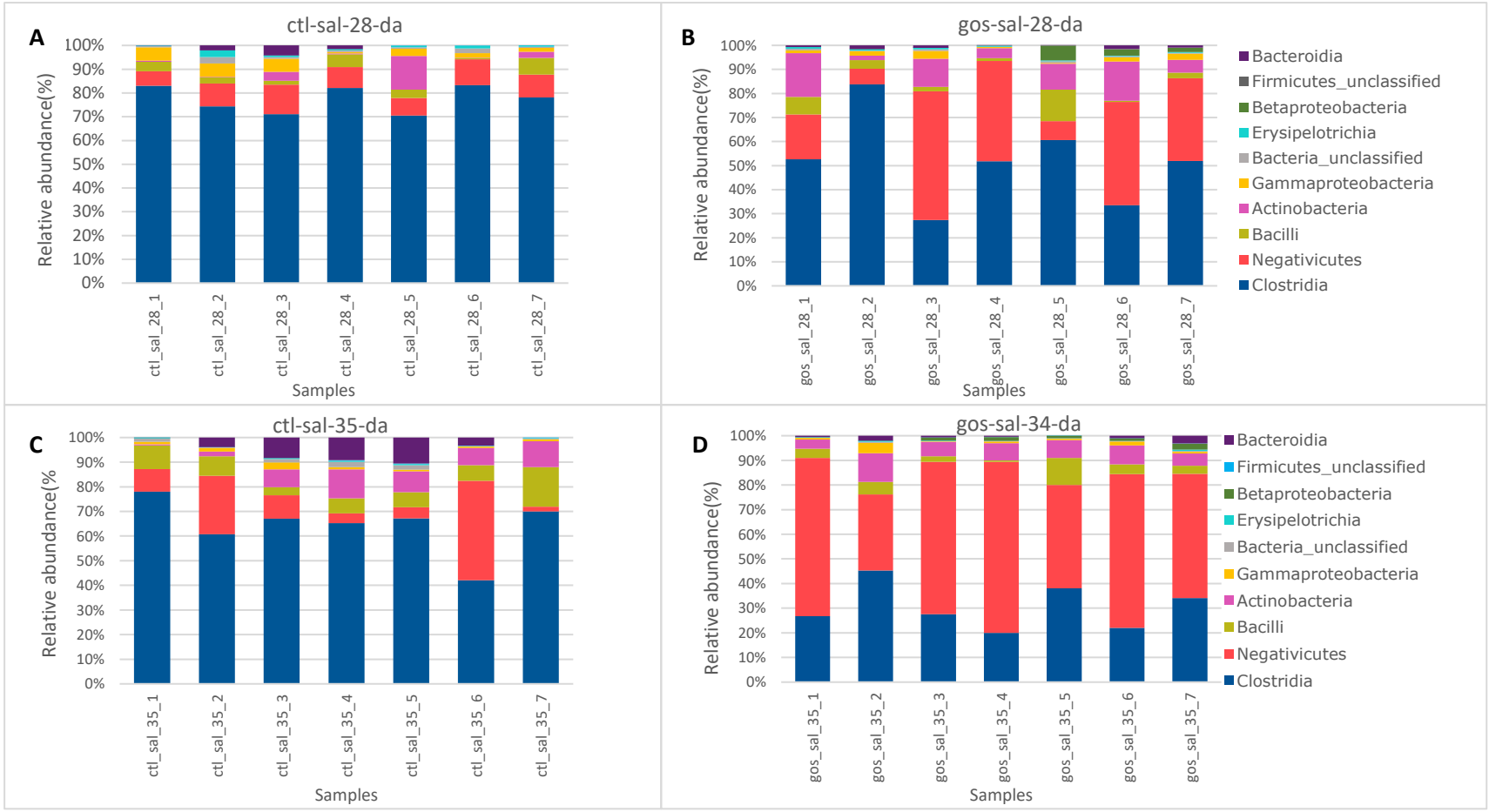


Figure 6.12 Comparison of the relative abundance of dominant bacterial classes among CH chicken gut microbiota fed control and GOS diets challenged with *Salmonella*. The data were clustered based on Ribosomal Database Project taxonomy (class level). The results are presented as a percentage of the normalised total abundance where the taxa represent at least 1% of the caecal community. A) 28 da control diet; B) 28 da GOS diet; C) 35 da control diet; D) 35 da GOS diet.

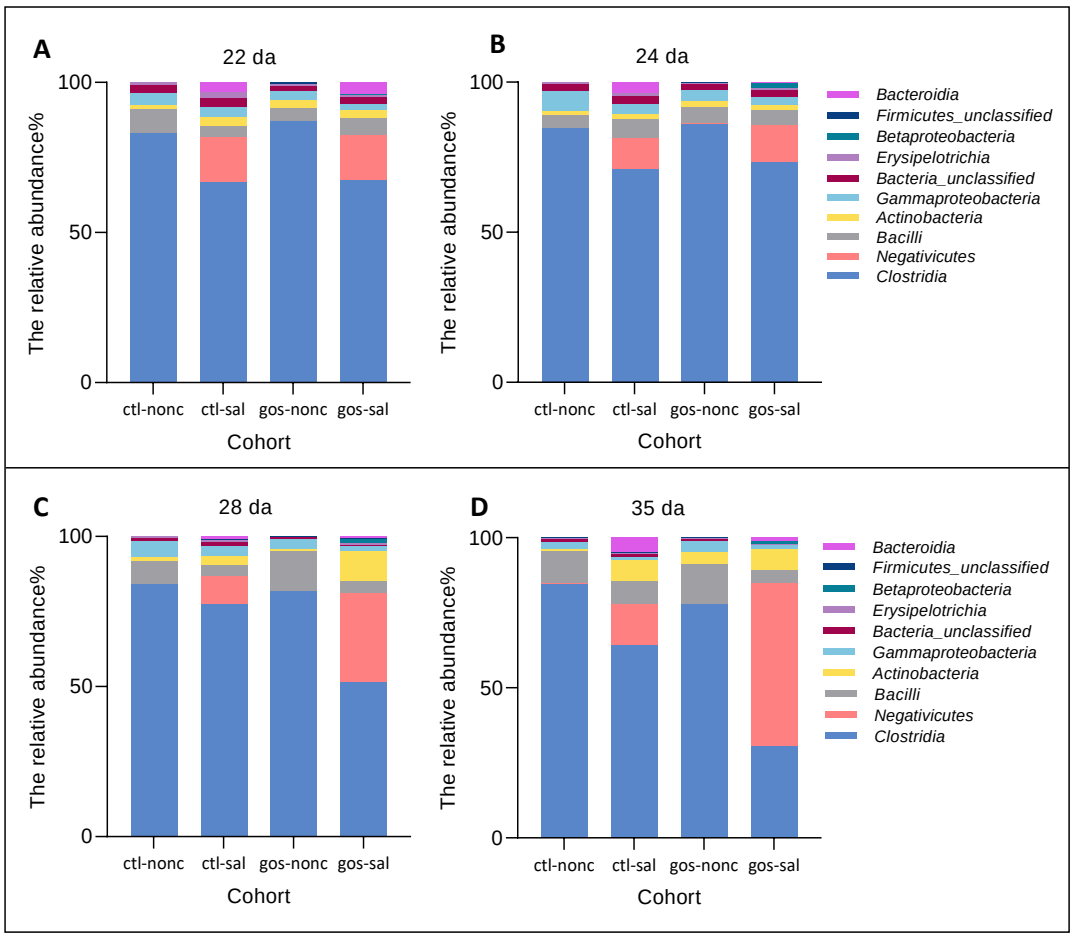


Figure 6.13 Summary of the relative abundance of dominant bacterial classes for chicken caecal microbiota challenged with *Salmonella* comparing control and GOS diets. The data were clustered based on Ribosomal Database Project taxonomy (class level). The results are mean values for each OTU presented as normalised % abundance for control and GOS feeds with and without *Salmonella* challenge. A) 22 da; B) 24 da; C) 28 da; D) 35 da.

6.2.10 Relative abundance of Proteobacteria among *Salmonella* challenged and non-challenged birds fed control and GOS diets

The relative abundance of Proteobacteria was investigated at phylum level to evaluate if there is any difference between the four groups fed control or GOS diets and colonised or non-colonised by *Salmonella*. The abundance of each group was tested by Shapiro–Wilk test for normality indicating that the relative abundance of Proteobacteria was not normally distributed at 4 dpi in gos-sal birds (Shapiro–Wilk test, p-value= 0.035) as well as for ctl-sal at 15 dpi old birds (Shapiro–Wilk test, p-value = 0.030). The relative abundance of Proteobacteria was otherwise normally distributed across various sampling days in both groups (Appendix 5.2). Therefore, the Mann–Whitney-Wilcoxon test for unpaired data was used for determining the significance between groups. Results in Figure 6.14 show no significant change was detected between the non-infected control (ctl-nonc) and GOS feed groups (gos-nonc) or between the *Salmonella* colonised groups (ctl-sal vs gos-sal) across Proteobacteria cohorts through all sampling points (p-value > 0.05). Moreover, comparison between control diets with and without pathogen (G1 vs G3) at 22, 28 and 35 showed no difference (p> 0.05). However, a significant difference was indicated at 24 da (median: 6.34% for G1 and 2.98% for G3; p-value = 0.053). No difference was observed between the GOS feed groups (G2 vs G4) for the abundance of Proteobacteria at any sampling time.

An attempt was made to investigate the correlation between the relative abundance of the Proteobacteria present and the ratio of trimethoprim resistant/sensitive coliform populations through four sampling points for G1-G4 (ctl-nonc, gos-nonc, ctl-sal and gos-sal). The results presented in Appendix 5.3 indicate a significant negative correlation between the Proteobacteria abundance and the proportion of trimethoprim resistant coliforms for the control diet *Salmonella* colonised group (G2) at 22 da (r= -0.78, p = 0.038) and the GOS diet *Salmonella* colonised group (G4) at 28 da (r= -0.83, p = 0.020).

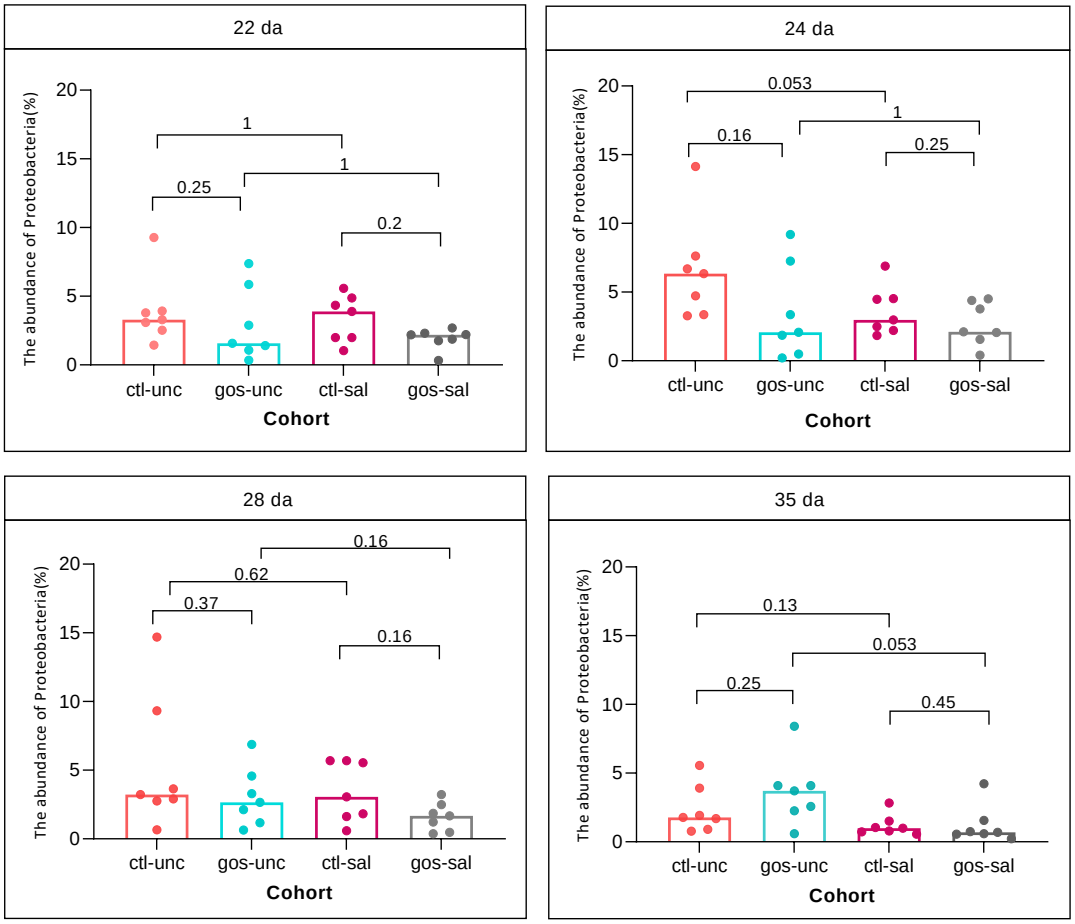


Figure 6.14 Comparison of the relative abundance median of the Gammaproteobacteria from 4 sampling points for non-infected control and GOS diet birds (G1 and G2) and infected control and GOS diets groups (G3 and G4). Wilcoxon test *p* value was used as all Proteobacteria phylum not normally distributed across cohorts: ctl-nonc at 22 da, gos-sal at 24 da and ctl-sal at 35 da (Normality test, Appendix 5.2 and 4.2A) otherwise all data normally distributed p>0.05.

6.2.11 The distribution of resistance GC arrays within the gut microbiota of birds fed control and GOS diets colonised and non-colonised by *Salmonella*

Correlations between integron gene cassettes within broiler caecal microbiota was examined with respect to Proteobacteria OTUs. The phylogenetic trees presented in Figures 6.15 A, B, C and D show the distribution of different type GCs among birds carrying the Proteobacteria OTUs indicated. The results demonstrated that at the first sampling point (2 dpi) in control fed birds there were 4 main OTUs detected within the phylum whether they were *Salmonella* challenged or not. Birds featuring *Enterobacteriaceae_unclassified* (OTU007 and OTU0324) also frequently feature GCs-B2. Proteobacterial OTUs corresponding to Betaproteobacteria_*Sutterella* (OTU0097) and Gammaproteobacteria_*Proteus* (OTU0169) are less frequently associated with the presence of GCs. Fewer of the GOS fed birds contained GCs in their caecal microbiota throughout the rearing period but those that did, possessed the most common OTU corresponding to *Enterobacteriaceae_unclassified* (OTU007). At 4 dpi the birds fed the control diet displayed a similar pattern of resistance dominated only by GCs-B2 associated with OTU0007 and OTU0324. The Betaproteobacteria_*Sutterella* OTU0097 is lost from the control diet birds at 4 dpi and does not feature thereafter, but was present throughout for the GOS-fed birds. At 8 dpi (28 da) fewer control birds contained GCs, which included those featuring OTU007. A new GC designated GCs-F containing the *aadA* gene appeared at 8 dpi, and at 15 dpi (35da). The expansion of GCs may be correlated with the mechanism of excising *dfrA1* to create the GCs-B2C1 and B2F types that are deletions of GCs-B2. The diversity of recovered GCs per sampling point and diets type are shown in Appendix 5.4 (A-H) for either feed.

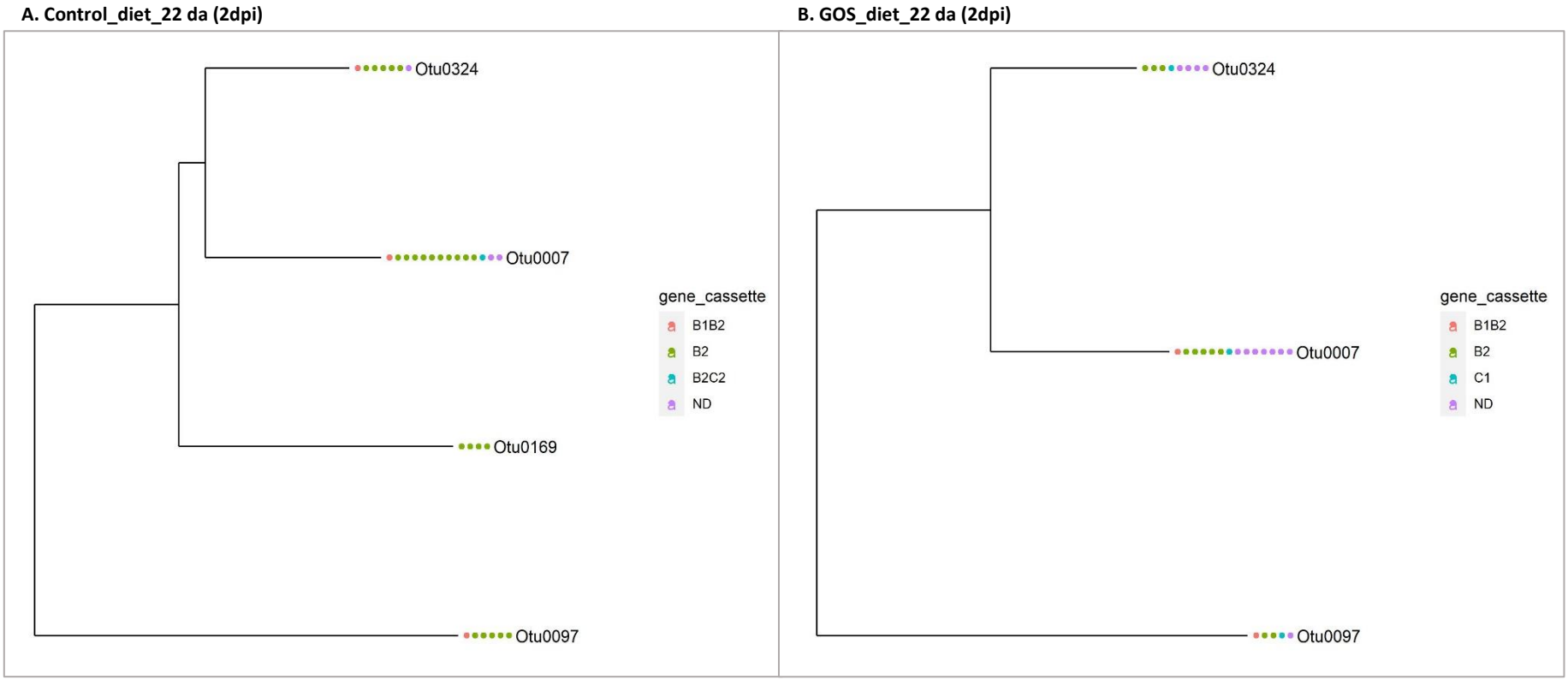


Figure 6.15 Phylogenetic tree of Proteobacteria phylum illustrates the OTUs associated with the distribution of GC groups in broiler chicken microbiota fed control and GOS diets challenged and non-challenged by *Salmonella.* All birds were reared under biosecure conditions. The phylogenetic tree was constructed with Bootstrap value ≥ 20 reads. **(A)** Control fed birds at 22 da *Salmonella* colonised and non-colonised **(B)** GOS fed birds at 22 da *Salmonella* colonised and non-colonised. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*), OTU0097 is (Beta-proteobacteria) *Sutterella.* The GCs are named as in Chapter 3 where ND is none detected.

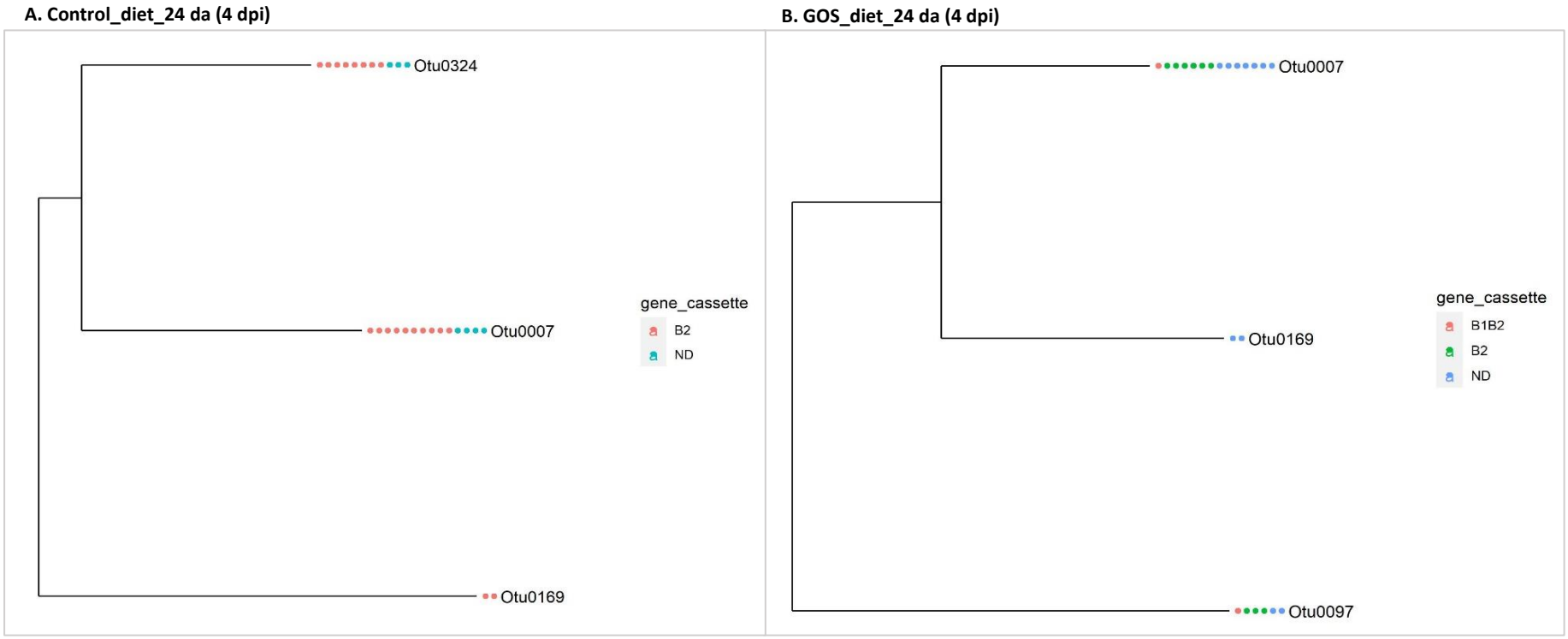


Figure 6.16 Phylogenetic tree of Proteobacteria phylum illustrates the OTUs associated with the distribution of GC groups in broiler chicken microbiota fed control or GOS diets challenged and non-challenged by *Salmonella*. All birds were reared under biosecure conditions. The phylogenetic tree was constructed with Bootstrap value ≥ 20 reads. **(A)** Control fed birds at 24 da *Salmonella* colonised and non-colonised **(B)** GOS fed birds at 24 da *Salmonella* colonised and non-colonised. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*), OTU0097 is (Beta-proteobacteria) *Sutterella.* The GCs are named as in Chapter 3 where ND is none detected.

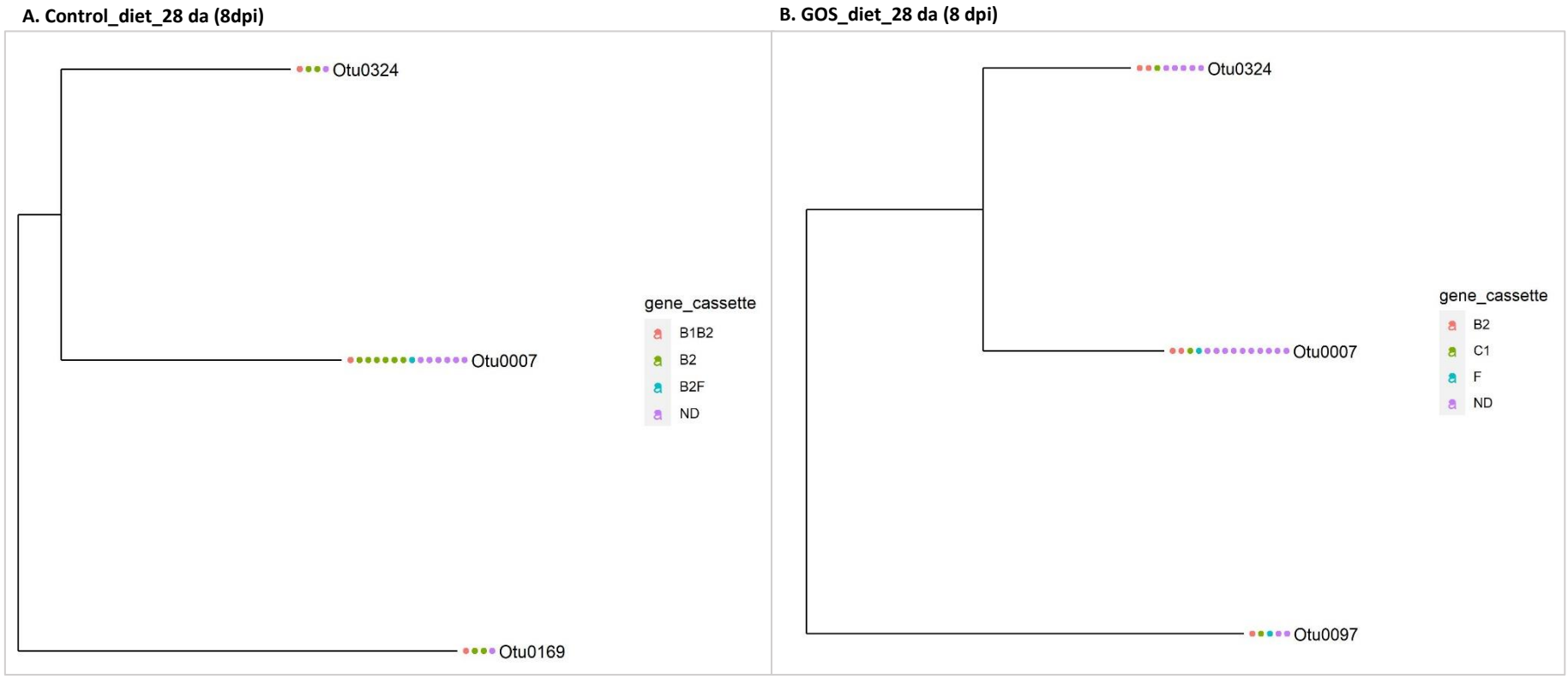


Figure 6.17 Phylogenetic tree of Proteobacteria phylum illustrates the OTUs associated with the distribution of GC groups in broiler chicken microbiota fed control or GOS diets challenged and non-challenged by *Salmonella*. All birds were reared under biosecure conditions. The phylogenetic tree constructed with Bootstrap value ≥ 20 reads. **(A)** Control fed birds at 28 da *Salmonella* colonised and non-colonised **(B)** GOS fed birds at 28 da *Salmonella* colonised and non-colonised. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*), OTU0097 is (Beta-proteobacteria) *Sutterella.* The GCs are named as in Chapter 3 where ND is none detected.

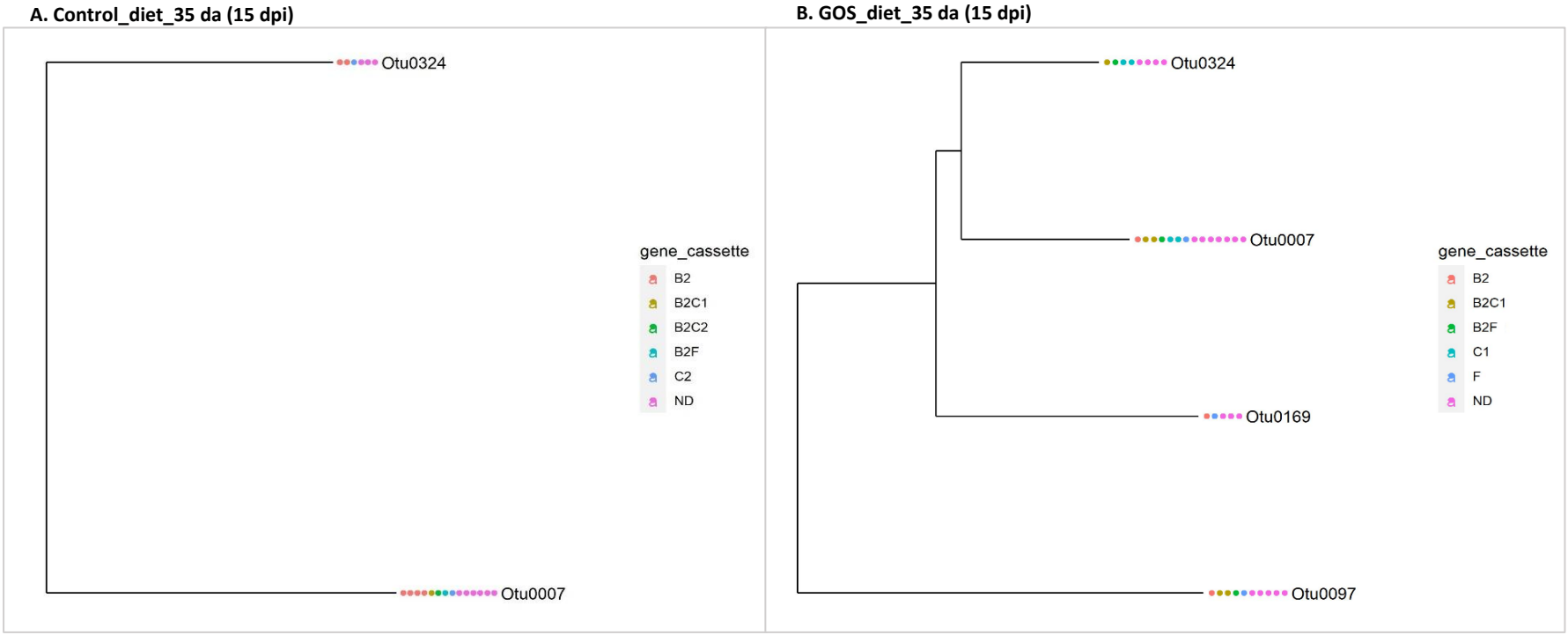


Figure 6.18 The phylogenetic tree of Proteobacteria phylum illustrates the OTUs associated with the distribution of GC groups in broiler chicken microbiota fed control or GOS diets challenged and non-challenged by *Salmonella*. All birds were reared under biosecure conditions. The phylogenetic tree constructed with Bootstrap value ≥ 20. **(A)** Control fed birds at 35 da *Salmonella* colonised and non-colonised **(B)** GOS fed birds at 35 da *Salmonella* colonised and non-colonised ones. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*), OTU0097 is (Beta-proteobacteria) *Sutterella.* The GCs are named as in Chapter 3 where ND is none detected.

6.2.12 Microbial diversity analysis

Analysis of the composition of caecal microbiome contents was performed by using Mothur (V1.39.5) and for comparison of the diversity indices (alpha and beta diversity). As noted previously, the inverse Simpson's index is used for the α-diversity or the overall diversity (evenness), whereas the observed OTUs or the OTUs richness was calculated using the Chao index. The complete comparison of the medians for both indices are presented in Figure 6.19 (A) for inverse Simpson's index and (B) for Chao index.

Normality tests were performed using the Shapiro-Wilks test for all groups (ctl-nonc G1, ctl-sal G3, gos-nonc G2, and gos-sal G4) indicating that the inverse Simpsons indices was not normally distributed at 24 da for the ctl-nonc group (Shapiro-Wilk test, p-value = 0.0279, Appendix 4.5A, Chapter 5) and 15 dpi in gos-sal birds (Shapiro-Wilk test, p-value = 0.0064), while all other sampling times show normal distributions for the Simpsons indices (p-value >0.05). The fit of the data in quantile-quantile plots is represented in Appendix 5.5A for only ctl-sal and gos-sal.

The majority of Chao indices calculated were normally distributed among various sampling times for both control and GOS diet cohorts either *Salmonella* challenged or not. The exceptions were gos-sal at 22 da (Shapiro-Wilk test, p-value = 0.0116), ctl-nonc at 28 da (Shapiro-Wilk test, p-value = 0.0378, Appendix 4.5B), and ctl-sal birds at 28 da (Shapiro-Wilk test, p-value = 0.0163). The fit of the data in quantile-quantile plots are illustrated in Appendix 5.5 B.

Based on calculations of alpha diversity and Wilcoxon tests for unpaired data there was no significant difference observed between the microbiome in caecal contents from birds fed control and GOS diets following *Salmonella* colonisation (G3 and G4) on the overall diversity (evenness) after 2, 4 and 8 dpi (p-value> 0.05). However, a significant change was noted in alpha diversity at 15 dpi between G3 and G4 (p-value = 0.0023). In addition, comparison between ctl-nonc birds and control-sal groups (G1 and G3) during four sampling times revealed that

no significant difference was evident in the evenness of the OTUs whether these groups were colonised or not by *Salmonella* (p> 0.05). However, there were statistically significant differences detected at 2 and 15 dpi in the *Salmonella* colonisation in gos-sal birds (G4) compared to gos-nonc G2 (inverse Simpson, p-value= 0.026 at 2 dpi, p-value= 0.001 at 15 dpi). These results are presented in Figure 6.19 A.

There was no significant difference in Chao richness of the two diets (G3 and G4) at 2,4, and 8 dpi of *Salmonella* infection, however at 15 dpi, a significant reduction in the Chao index was observed for G4 compared to G3 (p-value = 0.011). Comparison between the caecal microbiota of control diet groups with and without *Salmonella* colonisation (G1 and G3) indicated that the only significant change was detected at the first sampling day (p-value = 0.016) in G1 compared to G3, whilst no differences were observed for other sampling points (p >0.05). Likewise, no significant changes in richness were determined by comparing G2 with G4 (p>0.05). All results are presented in Figure 6.19 B.

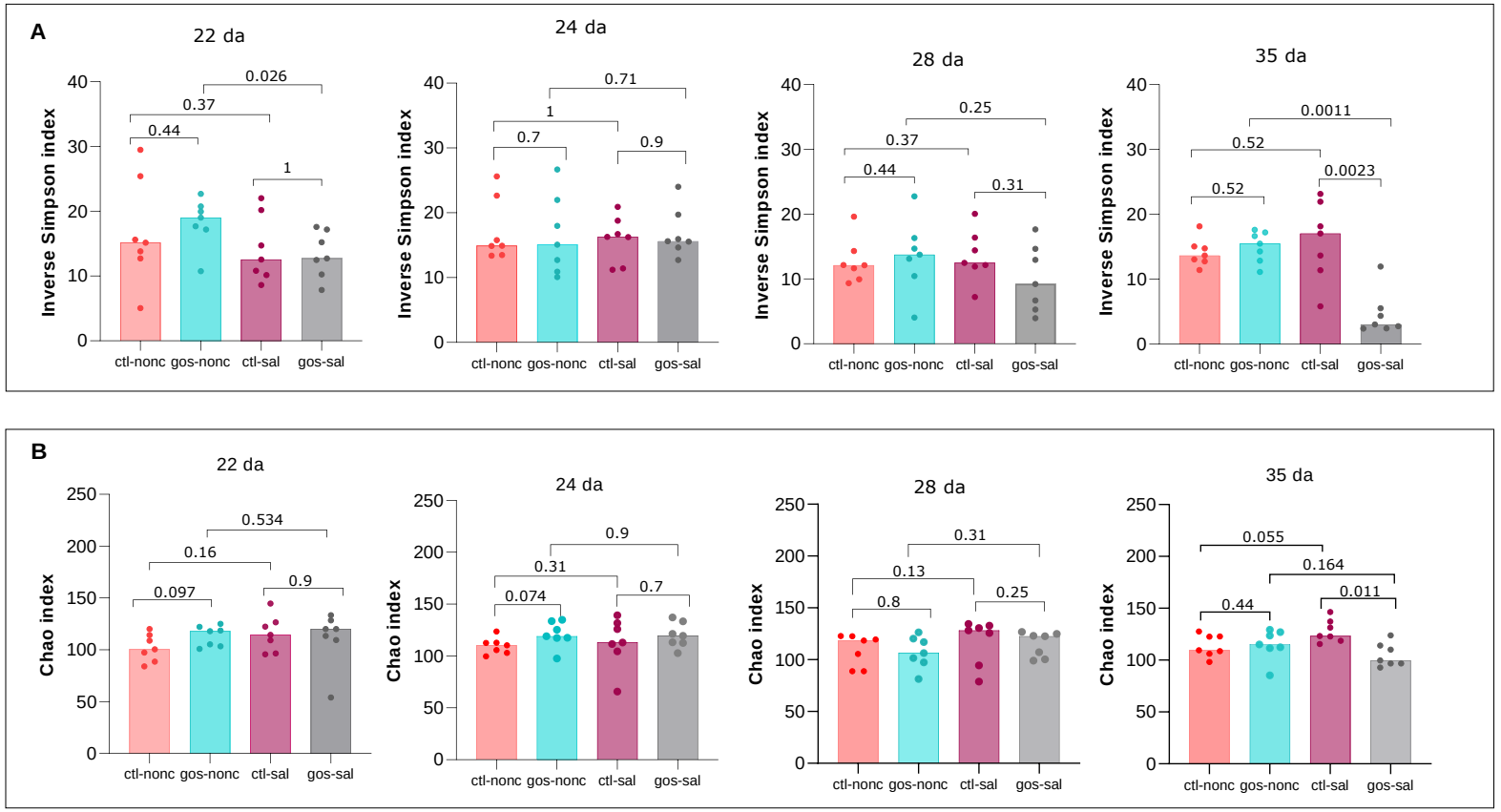


Figure 6.19 (A) Inverse Simpson indices, (B) Chao indices of caecal microbiota for control and GOS diets with or without *Salmonella* colonisation. Wilcoxon rank significance tests were used to calculate p-values used for identifying significant differences because the inverse Simpson indices were not normally distributed at ctl-nonc at 24 da and gos-sal al 35 da (Appendix 5.5A and 3.5A). Whilst for the Chao index data the indices were not normally distributed at 22 da for gos-sal and at 28 in ctl-sal groups (Appendix 5.5 B)

In order to examine the effect of diet and infection interactions, the relationship between the compositions of the bacterial communities were analysed by calculating Bray-Curtis dissimilarity. Ordination plots were generated that included comparisons of dissimilarity between the caecal microbiota for the two diets for birds infected or non-infected by *Salmonella* (Figure 6.20). AMOVA tests were also calculated using Mothur (V1.39.5) to detect significant differences between groups for the four sampling times. AMOVA test results denoted that no significant shift was detected between G3 and G4 at 2 and 4 dpi following *Salmonella* colonisation, however, significant shifts in microbiota composition were observed at 8 and 15 dpi between G3 and G4 (AMOVA test, p-value = 0.004 and 0.001 respectively). However, by comparing microbial composition of the control diet cohorts with and without pathogen colonisation (G1 and G3), the AMOVA test showed significant changes between bacterial communities at all sampling points (AMOVA test, at 22 da p-value = 0.004, while at 24, 28 and 35 da p-value = 0.001). Similarly, the GOS supplemented diet cohorts displayed significant shifts across the sampling times whether they were challenged or not (AMOVA test, at 22 da p-value = 0.004, at 24 da p-value = 0.002 while at 28 and 35 da p-value = 0.001).

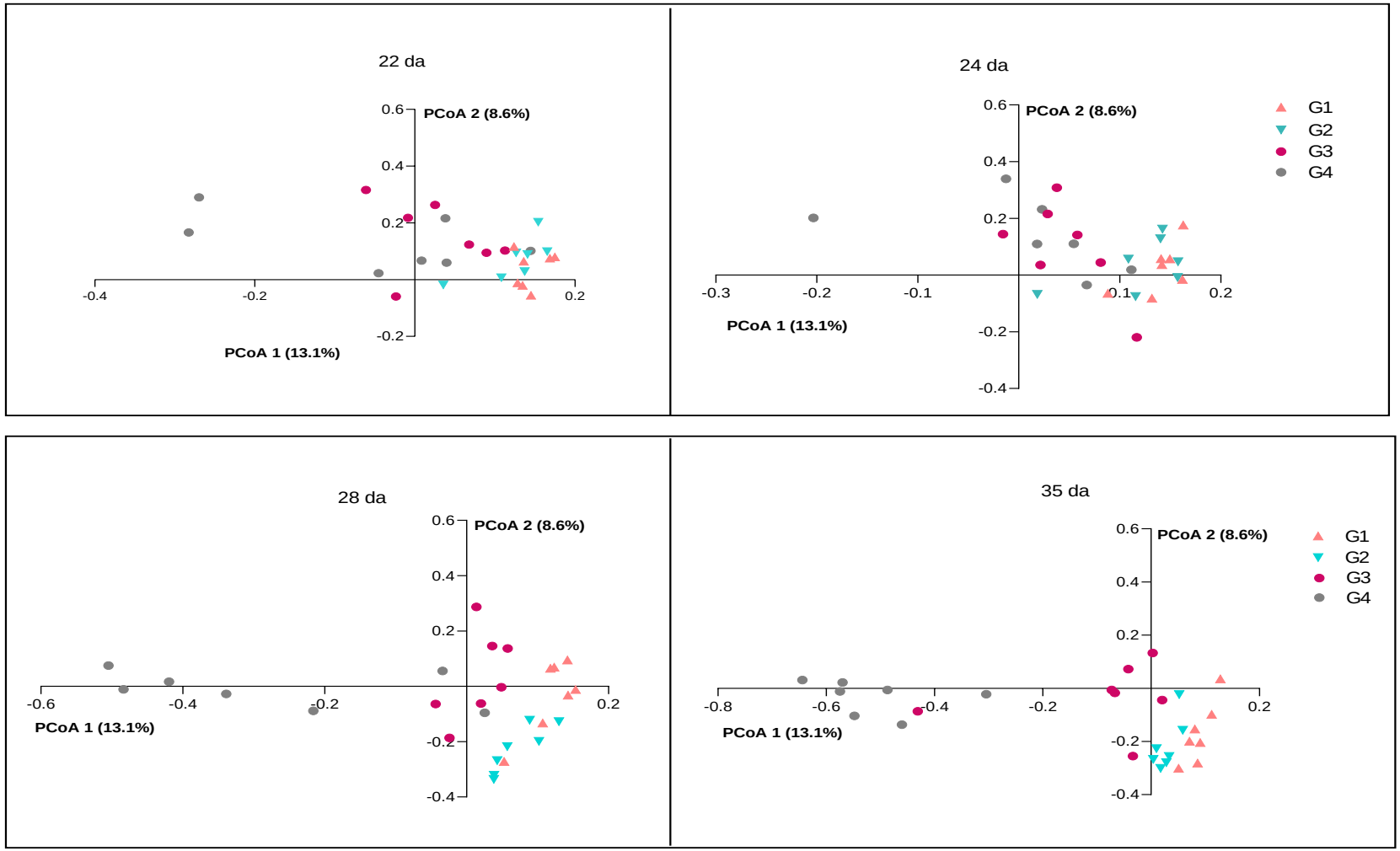


Figure 6.20 Relationship between bacterial communities' composition is affected by diet/infection interaction. Communities subset by age. R2= 0.56. AMOVA test results showed that no significant shift was detected between G3 and G4 at 2 and 4 dpi following *Salmonella* colonisation, however, significant shifts in microbiota composition were observed at 8 and 15 dpi between G3 and G4 (AMOVA test, p-value = 0.004 and 0.001 respectively).

LEfSe identified the most differentially abundant taxons between control and GOS diets for *Salmonella* colonised or non-colonised caecal microbiota. The results are shown in Figures 6.21 (22 and 24 da) and 6.22 (28 and 35 da). At 22 da the non-colonised control diet birds (G1) were enriched with a high abundance of *Lactobacillus* (OTU0006, data not shown due to cut-off p-value <0.01 applied) and *Lachnospiraceae_unclassified* (mainly OTU0168). While ctl-sal groups were significantly enriched by *Acidaminococcaceae_unclassified* (OTU0009), *Clostridiales_unclassified* (OTU0081), *Oscillibacter* (OTU0068) *Ruminococcaceae_unclassified* (OTU0126), *Megamonas* (OTU0004), *Lachnospiraceae_unclassified* (OTU0230), *Olsenella* (OTU0109) and *Bifidobacterium* (Otu0010).

Similarly, GOS diet (nonc) was dominated by a high abundance of *Lachnospiraceae_unclassified* (OTU0012, OTU0030, OTU0031), *Eggerthella* (Otu0036) and *Clostridiales_unclassified* (Otu0054). However, gos-sal birds were dominated by abundance of *Acidaminococcaceae_unclassified* (OTU0009) and *Lactobacillus* (OTU0039).

At 24 da old, non-colonised birds fed control diet (nonc) displayed high abundance of *Lachnospiraceae_unclassified* (OTU0042), *Clostridiales_unclassified* (OTU0037) and *Blautia* (OTU0043), whereas for *Salmonella* colonised birds fed control diet *Acidaminococcaceae_unclassified* (OTU0009) still remained dominant with the emergence of *Bacteroides* (OTU0033), *Ruminococcaceae_unclassified* (OTU0038, OTU0126) and *Clostridiales_unclassified* (OTU0047, OTU0267). The GOS fed birds (nonc) were dominated by *Subdoligranulum* (OTU0015); *Lachnospiraceae_unclassified* (OTU0059, OTU0225), while the gos-sal birds were enriched by *Acidaminococcaceae_unclassified* (OTU0009), *Ruminococcaceae_unclassified* (OTU0038), *Oscillibacter* (OTU0035); *Lactobacillus* (OTU0039) and *Lachnospiraceae_unclassified* (OTU0102).

At 28 da the microbiota of non-colonised control diet (ctl-nonc) birds showed a high abundance of *Lachnospiraceae_unclassified* (OTU0080) whilst the microbiota of *Salmonella* colonised birds fed control diet (ctl-sal) was dominated by *Acidaminococcaceae_unclassified* (OTU0009), *Clostridiales_unclassified* (OTU0054), *Ruminococcaceae_unclassified* (OTU0126) and *Clostridium_XlVb* (OTU0252). The microbiota of birds fed GOS diet exhibited a high abundance of *Lactobacillus* (OTU0022 not shown due to p-value >0.01) and *Lachnospiraceae_unclassified* (OTU0002) while *Salmonella* colonised GOS diet birds (gos-sal) groups recorded a higher abundance of *Megamonas* (OTU0004) *Acidaminococcaceae_unclassified* (OTU0009), *Bifidobacterium* (OTU0010), *Bacteroids* (OTU0033) and *Faecalicoccus* (OTU0141).

At 35 da the caecal microbiota of non-colonised control diet (ctl-nonc) birds was dominated by *Lachnospiraceae_unclassified* (OTU0002, OTU0230, OTU0243), *Clostridiales_unclassified* (OTU0024), *Clostridium_XlVb* (OTU0067) and *Clostridium_IV* (OTU0181), whilst the microbiota of *Salmonella* colonised birds fed control diet (ctl-sal) demonstrated a high abundance of *Acidaminococcaceae_unclassified* (OTU0009) and *Bacteroides* (OTU0033). Several OTUs showed increases in abundance in the caecal microbiota among the non-colonised GOS fed birds (nonc), including *Anaerostipes* (OTU0011), *Clostridiales_unclassified* (OTU0024) and *Blautia* (OTU0034), while the caecal microbiota of *Salmonella* challenged birds fed GOS diet showed persistence of the OTUs detected at 28 da noted above in addition to *Sutterella* (OTU0097) and *Eubacteriaceae_unclassified* (OTU0444).

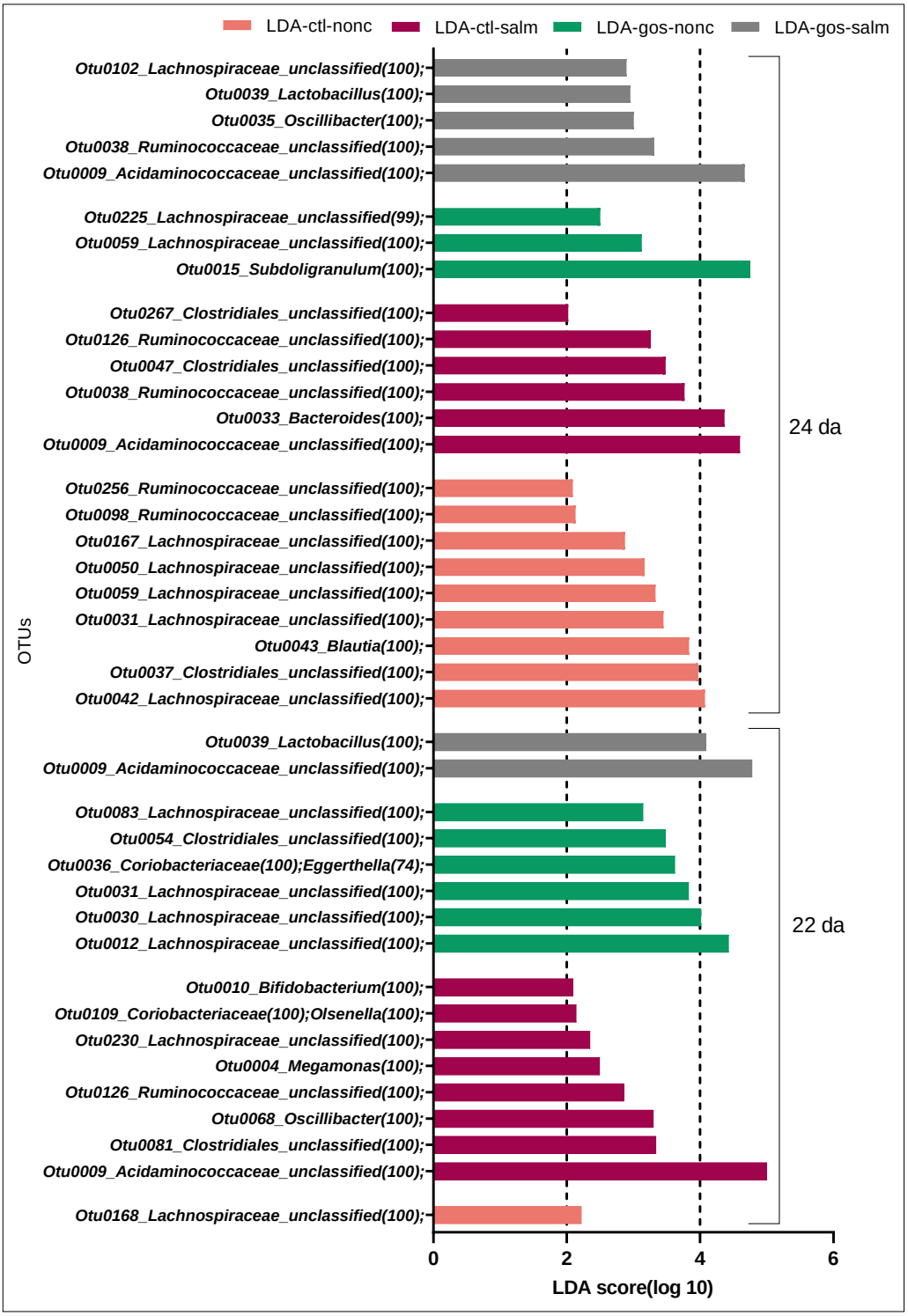


Figure 6.21 Comparison of microbial variations at the genus level, using the LEfSe for CH birds fed ctl and GOS diets *Salmonella* challenged and non-challenged birds at 22 and 24 da. LDA scores computed for express the differentially abundant of bacterial communities of birds fed ctl-nonc (orange bars), ctl-sal (red bars), GOS-nonc (green bars) and GOS-sal (grey bars). LEfSe illustrates which clades amongst detected OTUs that are statistically explain the greatest differences between bacterial population fed the different diets and reared in similar conditions. All representative OTUs subject to stringent cut off p-value 0.01 and LDA≥ 2.

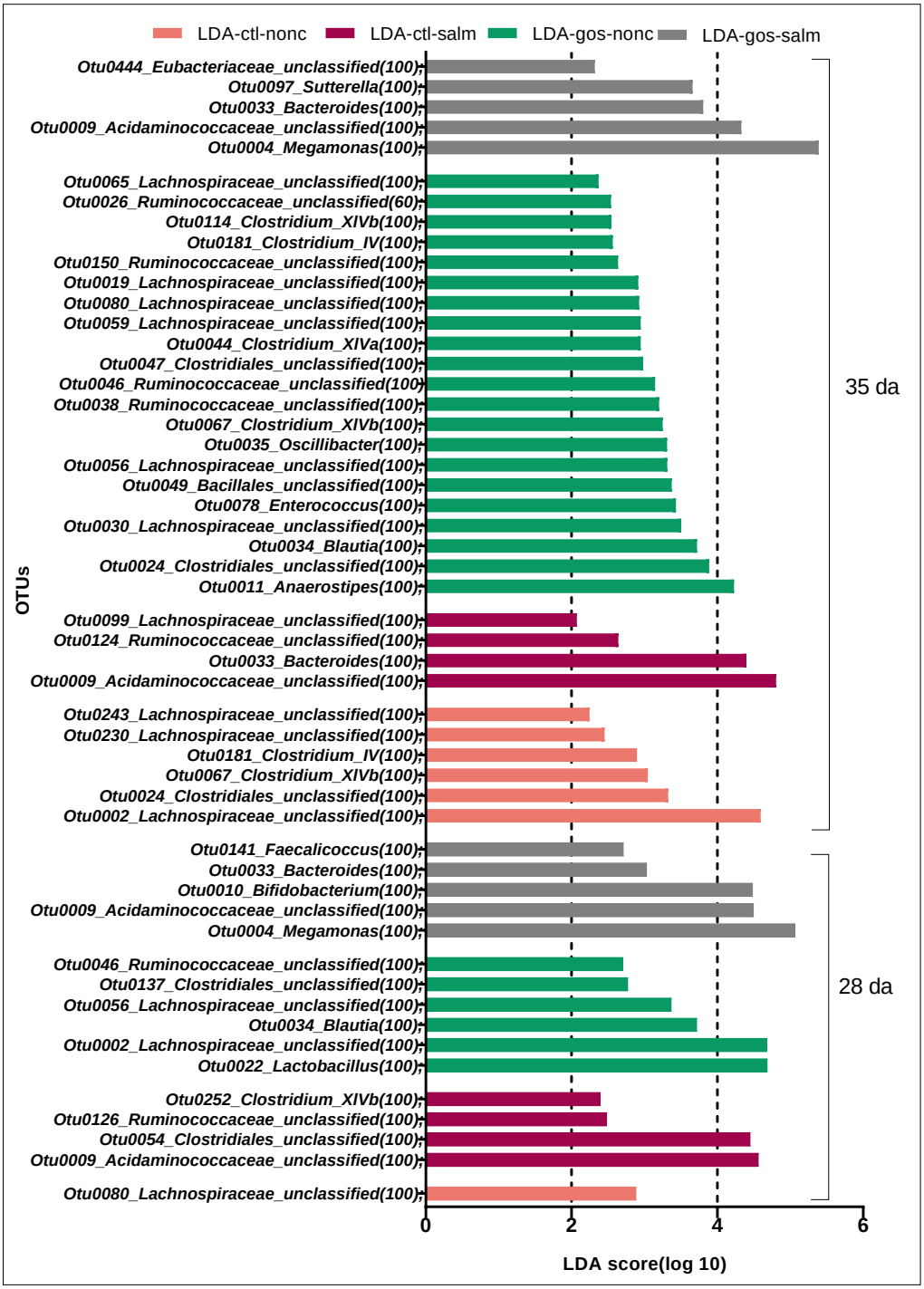


Figure 6.22 Comparison of microbial variation at the genus level, using the LEfSe for CH birds fed ctl and GOS diets *Salmonella* challenged and non-challenged birds at 28 and 35 da. LDA scores computed for express the differentially abundant of bacterial communities of birds fed ctl-nonc (orange bars), ctl-sal (red bars), GOS-nonc (green bars) and GOS-sal (grey bars). LEfSe illustrates which clades amongst detected OTUs that are statistically explain the greatest differences between bacterial population fed the different diets and reared in similar conditions. All representative OTUs subject to stringent cut off p-value 0.01 and LDA≥ 2.

6.3 Discussion

The prevalence of antibiotic resistance in foodborne pathogens like *Salmonella* is a major concern for public health (Nair et al., 2018). Therefore, more attention is required to control this pathogen in the animal food chain. The elimination of *Salmonella* from its hosts and food animals is difficult because they often serve as reservoirs of the pathogen, frequently without obvious pathogenic effects on the animal (Nair et al., 2018). Thus, preventing early colonisation of chicks by *Salmonella* is a key factor for reducing the incidence of Salmonellosis acquired from poultry (Hughes et al., 2017).

This Chapter aimed to investigate the response of the caecal microbiota of birds fed prebiotic GOS to the carriage of *Salmonella* and antibiotic resistance cassettes embedded in class 1 integrons. This study may provide insights into using GOS for controlling *Salmonella* infection by modifying the structure of the gut microbiome of the bird and thereby the innate immune response which collectively could reduce the load of antibiotic resistance genes.

It has been demonstrated that the GOS supplemented diet accelerates the clearance rate of *Salmonella* recovered from the caecal contents of birds at 8 days post colonisation compared to birds on a matched control diet that had not cleared (Figure 6.2). Azcarate-Peril et al. (2018) found that GOS can promote host resistance to *Salmonella* colonisation by promoting an advantageous microbiome structure by increasing the abundance of beneficial bacteria that accelerate clearance. Pourabedin et al. (2017) reported that diets supplemented with prebiotics like mannan-oligosaccharides (MOSs) and xylo-oligosaccharides (XOSs) significantly reduced (1.6 and 1.0 $\log_{10}$ CFU/g, respectively) chicken caecal colonisation by *S*. Enteritidis. Another study by Tanner et al. (2014) also found that GOS and fructo-oligosaccharides (FOS) reduced *S.* Typhimurium numbers *in vitro* when cultured in proximal colon conditions of pigs (38 C, pH 6.0, retention

time 9 h, and anaerobiosis) in combination with the probiotic *Bifidobacterium thermophilum* RBL67.

Translocation of *Salmonella* from the intestine to the liver and spleen is an established occurrence in chickens (Turnbull and Snoeyenbos, 1974). The results show translocation of *Salmonella* started by 2 dpi for both diets and its level was reduced by 8 dpi in liver and spleen samples with a greater percentage in spleen compared to liver. Hughes et al. (2017) demonstrated that *S*. Typhimurium (*S*.T) was present in chicken liver of a few chickens sampled at 4 and 12 dpi on a GOS supplemented diet, with no significant difference (p= 0.05) in the counts of *S*.T positive liver samples between the birds fed the control diet and those fed the prebiotic. However, the *S*. Enteritidis was detected in 7/8 birds at 1 dpi, 3/8 at 4 dpi, and was undetectable by 7 dpi in birds fed prebiotic GOS, whilst for birds fed the control diet *S*.E was determined in 8/8 birds at 1 dpi, 4/8 at 4 dpi, 2/8 at 7 dpi, and then undetectable by 12 dpi.

Relman and Lipsitch (2018) demonstrated that ARGs tend to be more transferable than others across ecological niches and habitats, with increasing likelihood of sharing these ARGs recovered from animal farms and human food with human isolates. In this context, the carriage of AR genes recovered from caecal contents of *Salmonella* colonised birds is significantly greater than non-colonised birds reared under similar conditions (χ^2 p = 0.001). The inclusion of dietary GOS significantly reduced the presence of ARGs in the *Salmonella* challenged birds (χ^2 p = 0.004).

HGT adds an important dimension to the transfer of antibiotic resistance genes (ARGs) to multiple unrelated pathogens (Lerminiaux and Cameron, 2019) elucidated that both transmissible elements and HGT have contributed to the emergence of antibiotic resistance originating in the 1960s. Based on model laboratory experiments on the dissemination of ARGs it has become clear that commensals can become donors, recipients, and reservoirs of ARGs (Shoemaker et al., 2001). It was apparent the presence of *Salmonella* induced the prevalence

of ARGs although the colonising *Salmonella* did not possess the class 1 integron or the integrase.

Trimethoprim resistance coliforms displayed a significant reduction at 2 and 4 dpi in GOS-sal group (G4) compared to the control *Salmonella* colonised group (G3). This finding was in accordance with the integrase copy number, which exhibited a significant reduction at 2 dpi in GOS-*Salmonella* groups compared to the ctl-*Salmonella* (p-value 0.004). Prebiotic GOS brought about a significant reduction in integrase copy number, which was compatible with decrease expansion of trimethoprim resistance bacteria achieved at 2 and 4 dpi but these differences were not apparent thereafter, likely as a result of removing the GOS supplementation at 22 da.

Surveys suggest 88% of poultry sourced *S.* Enteritidis are resistant to multiple antimicrobials such as ampicillin, nalidixic acid, and tetracycline, with high prevalence rates observed in hatching eggs, litter, feed, drinkers, bird rinse and caeca (Al-Zenki et al., 2007). However, in the work described here we did not observe the trimethoprim resistance gene (*dfrA*) to be transferred to colonising *Salmonella* despite an increase in the ARG load. There are two possible hypotheses that may explain why the trimethoprim resistance gene (*dfrA*) carried on a class 1 integron was not transferred to *Salmonella*. The first hypothesis is simply that as most studies report that young birds carry high loads of AR genes and this load decreases with age (Diarra et al., 2007;Braykov et al., 2016) so in this study the infection occurred too late at 20 da, for lateral gene exchange to occur. Nevertheless, at 2 dpi the integrase copy number in the control birds challenged with *Salmonella* was high but declined with age in support of the hypothesis that ARGs decrease as the microbiota matures. Ijaz et al. (2018) reported that the intestinal contents of early-stage birds were dominated by the Proteobacteria phylum (3 da) and showed a general reduction up to 7 da. This finding also could suggest that there is a possible association between high level of ARGs in young birds and high abundance of Proteobacteria phylum at this stage. Furthermore,

Rychlik (2020) elucidated that *E. coli* is a ubiquitously distributed facultative anaerobic bacteria which can readily survive in the environment to colonise newly hatched chicks. Hence, the environmental resistome is likely to be involved in the dissemination of resistance. In this experiment birds were kept under biosecure conditions so environmental exposure was minimised.

The second possible explanation is based on the observations of Bythwood et al. (2019) who explained that antimicrobial resistance in *Salmonella* relies on resistance genes acquired from the environment. Therefore, transfer of antimicrobial resistance genes is dependent on cell contact (Sieckmann et al., 1969) with another cell provided by a conjugative genetic element with a donor because *Salmonella* are not naturally transformable (Lorenz and Wackernagel, 1994). Additionally, Bythwood et al. (2019) stated that the antibiotic resistance donor's population size is an important factor, affecting antimicrobial resistance in *Salmonella*. Moreover, they studied the impact of antimicrobial administration on prevalence of antimicrobial resistant *Salmonella* in chickens by colonising chickens with *Salmonella* and *E. coli* carrying a ceftiofur resistance plasmid for determining how the administration antibiotics impacted resistance in *E. coli* and *Salmonella*. The group found increasing levels of streptomycin resistance in *E. coli*. However, the impact was minimal on *Salmonella*, leading to the conclusion that other factors may significantly govern the prevalence of AMR *Salmonella* in chickens. Nevertheless *Salmonella* have been isolated from the poultry environment with tendency to harbour class 1 integrons (Bass et al., 1999;Goldstein et al., 2001), which are associated with diverse resistances genes including those conferring resistance to β-lactams, phenicols, sulfonamides, aminoglycosides, and quaternary ammonium compounds (Lu et al., 2003b;Nandi et al., 2004;Smith et al., 2007). It is possible that the host immune response directed at *Salmonella* and donor organisms may reduce the frequency of integrase mobility in *Salmonella* and reduce the rate of HGT.

The metagenomic analysis of 16S RNA at class level revealed that non-challenged groups were characterised by a relatively high abundance of Clostridia, with overall mean of 84.18% in ctl-nonc birds (G1) and 83.27% in GOS-nonc group (G2) (Figure 6.13), where most are unclassified species of the family Lachnospiraceae. While the *Salmonella* challenged groups are characterised by decline in abundance of Clostridia, with overall mean 69.92% for ctl-sal (G3) and 55.79% in GOS-sal (G4). The groups feature a high abundance of Negativicutes (*Megamonas*), with an overall mean of 12% for ctl-sal compared to 27.68% for GOS-sal. It is hypothesised that the clearance/reduction of *Salmonella* in GOS-sal is associated with the expansion of the Negativicutes, which only emerge in the *Salmonella* challenged groups. The interaction with GOS in the *Salmonella* colonised birds appears to boost Negativicutes abundance and Actinobacteria by 8 dpi compared to (ctl-sal) control diet challenged by *Salmonella*. These significant increases arise at the expense of Clostridia and Gamma-Proteobacteria, where a reduction in the latter may reduce the transfer of ARGs. Polansky et al. (2016) pointed out that *Megamonas* was one of the main propionate producing microorganisms in the Firmicute phylum and could encode enzymes that contribute to melibiose and alanine metabolism. *Megamonas* expressing alanine dehydrogenase can produce ammonia leading to a rise in the pH of the caecal contents and affect the metabolism of host epithelial cells and other members of the microbiota (Davila et al., 2013;Polansky et al., 2016). Oakley et al. (2014) attributed a high abundance of *Megamonas*, *Helicobacter*, and *Campylobacter* bacteria to the presence of hydrogenase, which seems to stimulate the production of SCFAs in the caecum. Consequently, SCFAs raise osmosis in the lumen of the intestine and indirectly stimulate the response of macrophages to modulate the immune system (Patel and Goyal, 2012). Furthermore, Polansky et al. (2016) noted that SCFAs negatively influence the expression of the virulence factors of bacterial pathogens.

Megamonas may decrease the availability of oxygen in the GOS-sal birds which will have an effect on the growth of *Salmonella* and accelerate clearance in GOS-sal over ctl-sal. The avian gut plays vital role in the control of the innate immune system that can disrupt cell membranes of various enteric pathogens and eliminate infection by producing antimicrobial peptides (β-defensins). This interaction between gut microbial communities and host innate immune system can stimulate adaptive immune response via either be B-cell dependent or T-cell dependent (Pan and Yu, 2014), and properly involves in the development and homeostasis of immune system (Oakley et al., 2014) that are negatively affected by antibiotic growth promotors (Kumar et al., 2018). Consequently, the gut microbial community contributes in exclude pathogenic taxa, ferment complex polysaccharides, and provide the host energy in the form of metabolizable volatile fatty acids.

Analysis of the α-diversity of the caecal microbiome demonstrates that α-diversity was significantly reduced at 35 da in gos-sal birds compared to ctl-sal (Inverse Simpson index: p-value =0.0023; Figure 6.19A), and similarly compared to gos-nonc at 22 and 35 da (Inverse Simpson index: $p < 0.026$, 0.0011 respectively; Figure 6.19A). Community richness (Chao index) was not significantly different through to 28 da between the infected groups fed control or GOS diet. However, at 35 da the richness was reduced in GOS-sal group (Chao index p-value 0.011, Figure 6.19B). This observation could be accomplishing by interaction between GOS and *Salmonella* that changing in the intestinal microbiota due to overgrowth of Negativicutes and reduction of Clostridia.

Analysis of microbiota composition among the different groups using LEfSe demonstrated a high abundance of *Acidaminococcaceae_unclassified* (OTU0009) across all sampling points in ctl-sal birds with *Clostridiales _unclassified* OTU and *Bacteroides* (OTU0033). *Acidaminococcaceae _unclassified* dominated at 2 and 4 dpi while *Megamonas* dominated at 8 and 15 dpi. *Veillonellaceae* (*Megamonas*)

and *Acidaminococcaceae* (*Acidaminococcaceae _unclassified*) belong to the class Negativicutes. These families belong to Gram-positive Firmicutes but have acquired genes responsible for the biosynthesis of cell wall components similar to Gram-negative bacteria, and are strict anaerobes non-spore forming bacteria (Rychlik, 2020). Therefore, the predominant growth of representative members of the Negativicutes maintains the environment strictly anaerobic free of alternative electron acceptors like nitrogen or sulfate, which could prevent the overgrowth of *E. coli* or *Salmonella* by limiting the availability of the substrates for effective anaerobic metabolism (Rychlik, 2020).

6.4 Conclusion

By investigating the conditions that produce mobility and horizontal transfer of ARGs in the gut microbiota, new approaches could be adopted aimed at reducing AMR (Hegde et al., 2016). However, the dynamics of transferring genes that confer antibiotic resistance are still not well understood (Lerminiaux and Cameron, 2019). Manipulation of gut microbiota by administration or selection of probiotic organisms may reduce antibiotic resistant populations via displacement or exclusion of the resistant organisms. In this context, galacto-oligosaccharides (GOS) may modify the structure of the gut microbiome and contribute to control the impact of pathogen (*Salmonella*) colonisation and mitigate the spread ARGs.

At 2 and 4 dpi the count of trimethoprim resistant coliforms recovered from the microbiota of gos-sal (G4) were significantly reduced compared to ctl-sal (G3). This reduction did not persist in birds older than 24 da since the GOS supplemented feed was halted at 20 da due to the relative commercial cost of feeding larger birds. The integrase copy number for gos-sal also exhibited a significant reduction at 2 dpi suggesting at least one mechanism by which ARGs are disseminated. Examination of the GCs recovered from the caecal contents of *Salmonella* colonised birds demonstrated a significantly greater prevalence than

non-colonised birds. Dietary GOS significantly reduced the presence of ARGs in the *Salmonella* challenged birds

The study has demonstrated that both *Salmonella* and GOS diets influence the structure of the gut microbiome. Prebiotic GOS enhances the abundance of specific taxonomic OTUs. These important changes result in faster clearance of *Salmonella* infection in GOS-fed birds over control birds and in doing so displaces Proteobacteria that carry class 1 integrons and have the potential to disseminate ARGs. Specifically, treatment with the GOS resulted in a significant increase of Negativicutes on expense of Clostridiales thereby restricting the abundance of Proteobacteria as result of oxygen depletion.

CHAPTER 7

IDENTIFICATION OF CLASS I INTGRON PLASMIDS

7.1 introduction

Recently, developments in next-generation DNA sequencing technologies have facilitated the tracking of outbreak resistant organisms and revolutionised the number of bacterial whole-genome sequences. Analysing these data has enabled the fundamental characterisation of bacterial pathogens and provided opportunities to study the evolution of resistance. Molecular analyses of multi-drug resistant bacteria indicate the spread of resistance can be attributed to the acquisition of pre-existing determinants followed by amplification as a response to selection. Furthermore, sequences of many mobile genetic elements (MGEs) and plasmid backbone segments are highly conserved with low sequence errors. This feature has enabled the identification of variation between analogous plasmids that are epidemiologically or functionally important (Partridge et al., 2018).

Horizontal gene transfer plays vital role in the acquisition of new features, such as pathogenicity, antibiotic resistance and support the tremendous adaptive potential of bacteria (Partridge et al., 2018). The horizontal transfer of resistance genes can arise from plasmids or from within the bacterial chromosome. Resistance gene transfer mediated by plasmids is the most effective type of transmission that can occur at high frequency and involve the simultaneous transfer of multiple resistance genes (Carattoli, 2003). The process of capturing, accumulating, and disseminating resistance genes often features mobile genetic elements (MGE) in promoting intracellular DNA mobility such as mobility from the chromosome to a plasmid or between plasmids.

Insertion sequences (IS) and transposons (Tn) are discrete DNA modules that are capable of randomly migrating themselves (and associated resistance genes) to new locations in the same or different DNA segments within a single cell. Integrons use site-specific recombination to transfer resistance genes between defined sites. The frequent presence of these MGE types in many copies and at various positions in genomes, can facilitate homologous recombination.

Thus, interactions between the different types of MGE promoting the rapid evolution of different multi-resistant pathogens that the encounter of antimicrobial chemotherapy (Partridge et al., 2018).

Metagenomics have helped to understand the drivers behind the functional selection of ARGs in genetic exchanges between various microbial species (De la Cruz and Davies, 2000;Shoemaker et al., 2001;Lester et al., 2006) and have also recognized that ARGs exist in bacteria obtained from the environment that have not been exposed to antibiotics (D'Costa et al., 2011;Moore et al., 2013). Ongoing work has revealed the mechanistic features of emerging antibiotic resistance in bacteria, and their reservoirs from diverse microbial communities (Danzeisen et al., 2011;Durso et al., 2011;Andersson and Hughes, 2012). These studies have facilitated tracking of new antimicrobial resistance from their origins to pathogenic bacteria (Solberg et al., 2006;Van Hoek et al., 2011;Hegde et al., 2016).

The aim of this Chapter is to characterise the types of mobile genetic elements as vehicles of class 1 integrons, and to associate OTUs observed in the chicken gut microbiota with the prevalence of the integrons. Finally, to compare the observations made from plasmid sequences with pure bacterial cultures of Gram-negative bacteria that have acquired antibiotic resistance.

7.2 Results

In order to study the boundaries of class 1 integrons and the types of mobile genetic elements they are located in, individual bacterial isolates carrying class 1 integrons with an embedded *dfrA1* gene (GCs-B) were selected. The process of selecting single cell colonies from trimethoprim resistance plates was carried out during viable counting of trimethoprim resistant bacterial populations in Trial 1 throughout all sampling points (22, 24, 28 and 35 da). Approximately 200 bacterial isolates were collected from biosecure housed birds that were infected and non-infected by *Salmonella* and fed either the control or GOS diet. From these 35 isolates were randomly selected (Table 7.1), single colony purified and then the DNA extracted using the GenElute bacterial Genomic DNA kit (Chapter 2, section 2.5.2.1). Three different PCRs were performed to characterise the isolates: first, 16S rRNA gene amplification and sequencing was carried out to avoid picking similar bacteria (Figure7.1), second the detection of class 1 integrons (Figure 7.2), and third long range PCR aimed to detect the GC sequence environment (Figure 7.3). Based on these results 6 isolates were selected and subjected to whole genome sequencing using the illumina MiSeq platform. Plasmid DNA sequences were identified from isolate numbers 19 and 24 carrying GC-B-2 (dfrA1 and aadA1), while bacterial isolate 38 encoded GC-B-1 (dfrA1, hypothetical protein and aadA1). Isolates AA_2 and 6 contained different GCs. The results are presented in Figures 7.1 to 7.3 and summarised in Table 7.1.

Table 7.1 Selected trimethoprim resistance bacterial isolates collected for WGS of biosecure birds

Sample No	ID	Trial	*IntI1*	GCs
1	G4 pooled samples	biosecure	positive	negative
2	G4-01	biosecure	positive	negative
3	G3-04	biosecure	positive	negative
4	G4-12	biosecure	positive	negative
5	G4-13	biosecure	negative	negative
6	G3-03	biosecure	positive	negative
7	G4-06	biosecure	positive	negative
8	G1-26	biosecure	negative	negative
9	G3-21	biosecure	negative	negative
10	G4-22	biosecure	negative	negative
12	G4-21	biosecure	negative	negative
13	G1-14	biosecure	positive	negative
14-01	G3-01	biosecure	negative	negative
14-02	G3-01	biosecure	negative	negative
16	G2-12-1	biosecure	negative	negative
17	G2-12-2	biosecure	positive	negative
18	G4-22-2	biosecure	positive	positive
19	G1-22-4	biosecure	positive	positive
20	G2-35-1	biosecure	negative	negative
21	G1-35-4	biosecure	negative	negative
22	G4-22-4	biosecure	positive	positive
23	G3-35-4	biosecure	positive	negative
24	G3-24-6	biosecure	positive	positive
25	G3-28-6	biosecure	negative	negative
26	G1-35-3	biosecure	negative	negative
27	G3-24-1	biosecure	positive	positive
28	G3-35-6	biosecure	negative	negative
29	G4-35-6	biosecure	negative	negative
30	G3-28-1	biosecure	negative	negative
31	G3-28-3	biosecure	negative	negative
32	G4-24-5	biosecure	negative	negative
36	G3-22-3	biosecure	positive	negative
37	G2-28-4	biosecure	negative	negative
38	G4-28-6	biosecure	positive	positive
39	G2-22-6	biosecure	negative	negative

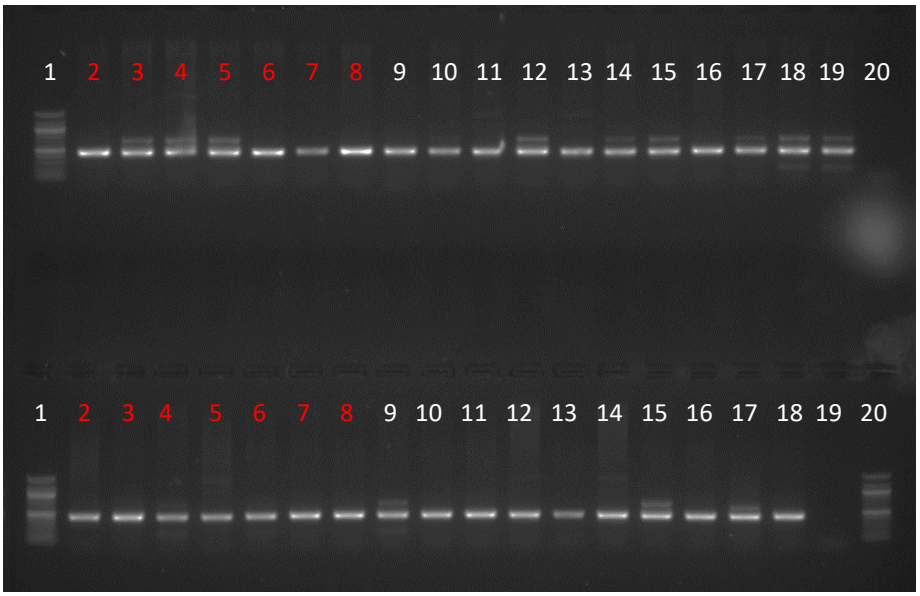


Figure 7.1 PCR amplicon products of 16S RNA genes obtained from trimethoprim resistant bacterial isolates. Specific primers (Hugerth et al., 2014) were used to target V3-V4 region in chicken caecal DNA samples electrophoresed on 1% TAE agarose gel.

Row 1: Lane 1 100 bp ladder (BioLab), Lane 2 int1 Ravi sample 01, Lane 3 16S RNA sample 02, Lane 4 16S RNA sample 03, Lane 5 16S RNA sample 04, Lane 6 16S RNA sample 05, Lane 7 16S RNA sample 06, Lane 8 16S RNA sample 07, Lane 9 16S RNA sample 08, Lane 10 16S RNA sample 09, Lane 11 16S RNA sample 10, Lane 12 16S RNA sample 12, Lane 13 16S RNA sample 13, Lane 14 int1 Ravi sample 14-1, Lane 15 16S RNA sample 14-2, Lane 16 16S RNA 16, Lane 17 16S RNA sample 17, Lane 18 16S RNA sample 18, Lane 19 16S RNA sample 19, Lane 20 16S RNA -ve control.

Row 2: Lane 100 bp ladder (BioLab), Lane 2 16S RNA sample 20, Lane 3 16S RNA sample 21, Lane 4 16S RNA sample 22, Lane 5 16S RNA sample 23, Lane 6 16S RNA sample 24, Lane 7 16S RNA sample 25, Lane 8 16S RNA sample 26, Lane 9 16S RNA sample 27, Lane 10 16S RNA sample 28, Lane 11 16S RNA sample 29, Lane 12 16S RNA sample 30, Lane 13 16S RNA sample 31, Lane 14 16S RNA sample 32, Lane 15 16S RNA sample 36, Lane 16 16S RNA 37, Lane 17 16S RNA sample 38, Lane 18 16S RNA sample 39, Lane 19 16S RNA +ve control, Lane 20 int1 Ravi -ve control.

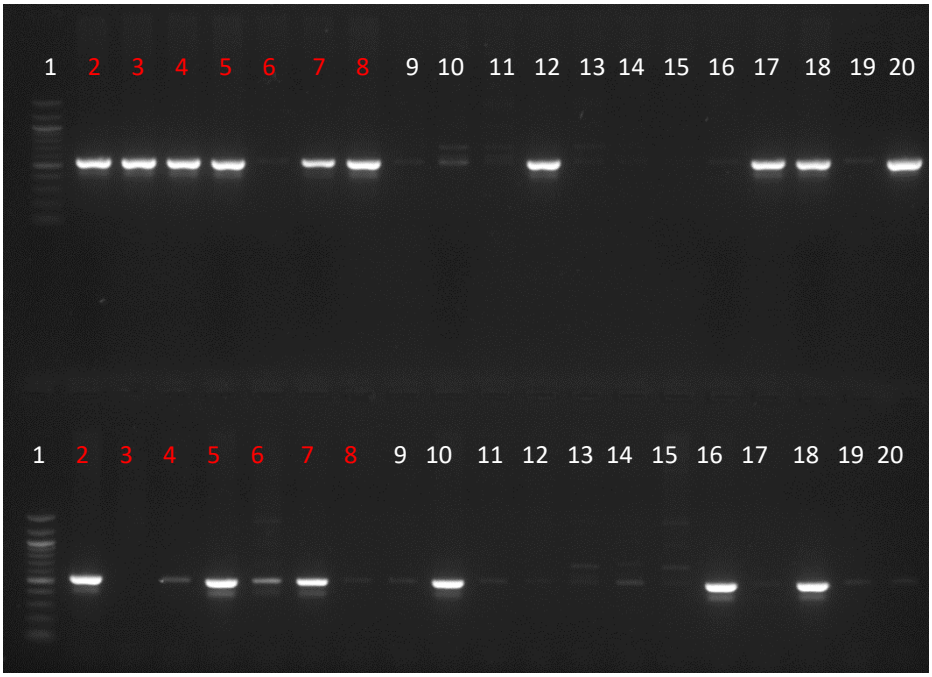


Figure 7.2 Agarose gel of amplification products of PCRs using Ravi primers. The Figure shows PCRs products of the 546 bp target genes of class 1 integrase gene (*intl1* Ravi) obtained from DNA of selected coliforms colonies that picked from broiler caecal DNA samples reared in bio-secure condition and electrophoresed on a 1 % TAE gel.

Row 1: Lane 100 bp ladder (BioLab), Lane 2 *int1* Ravi sample 01, Lane 3 *int1* Ravi sample 02, Lane 4 *int1* Ravi sample 03, Lane 5 *int1* Ravi sample 04, Lane 6 *int1* Ravi sample 05, Lane 7 *int1* Ravi sample 06, Lane 8 *int1* Ravi sample 07, Lane 9 *int1* Ravi sample 08, Lane 10 *int1* Ravi sample 09, Lane 11 *int1* Ravi sample 10, Lane 12 *int1* Ravi sample 13, Lane 13 *int1* Ravi sample 13, Lane 14 *int1* Ravi sample 14-1, Lane 15 *int1* Ravi sample 14-2, Lane 16 *int1* Ravi 16, Lane 17 *int1* Ravi sample 17, Lane 18 *int1* Ravi sample 18, Lane 19-ve control, Lane 20 *int1* Ravi +ve control.

Row 2: Lane 100 bp ladder (BioLab), Lane 2 *int1* Ravi sample 19, Lane 3 *int1* Ravi sample 20, Lane 4 *int1* Ravi sample 21, Lane 5 *int1* Ravi sample 22, Lane 6 *int1* Ravi sample 23, Lane 7 *int1* Ravi sample 24, Lane 8 *int1* Ravi sample 25, Lane 9 *int1* Ravi sample 26, Lane 10 *int1* Ravi sample 27, Lane 11 *int1* Ravi sample 28, Lane 12 *int1* Ravi sample 29, Lane 13 *int1* Ravi sample 30, Lane 14 *int1* Ravi sample 31, Lane 15 *int1* Ravi sample 32, Lane 16 *int1* Ravi 36, Lane 17 *int1* Ravi sample 37, Lane 18 *int1* Ravi sample 38, Lane 19 *int1* Ravi sample 39, Lane 20 *int1* Ravi -ve control.

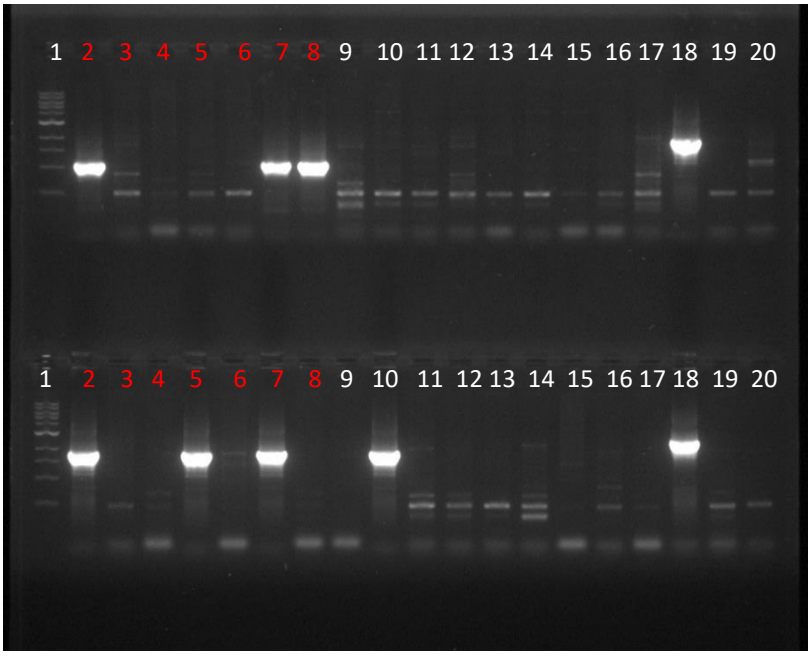


Figure 7.3 Agarose gel of PCR amplification of GCs using primer ntf2/qcr2. The primer pair targets gene cassettes isolated from DNA of selected coliforms colonies picked from biosecure condition chicken caecal DNA samples electrophoresed on 0.8% TAE agarose gel with 3 min annealing time.

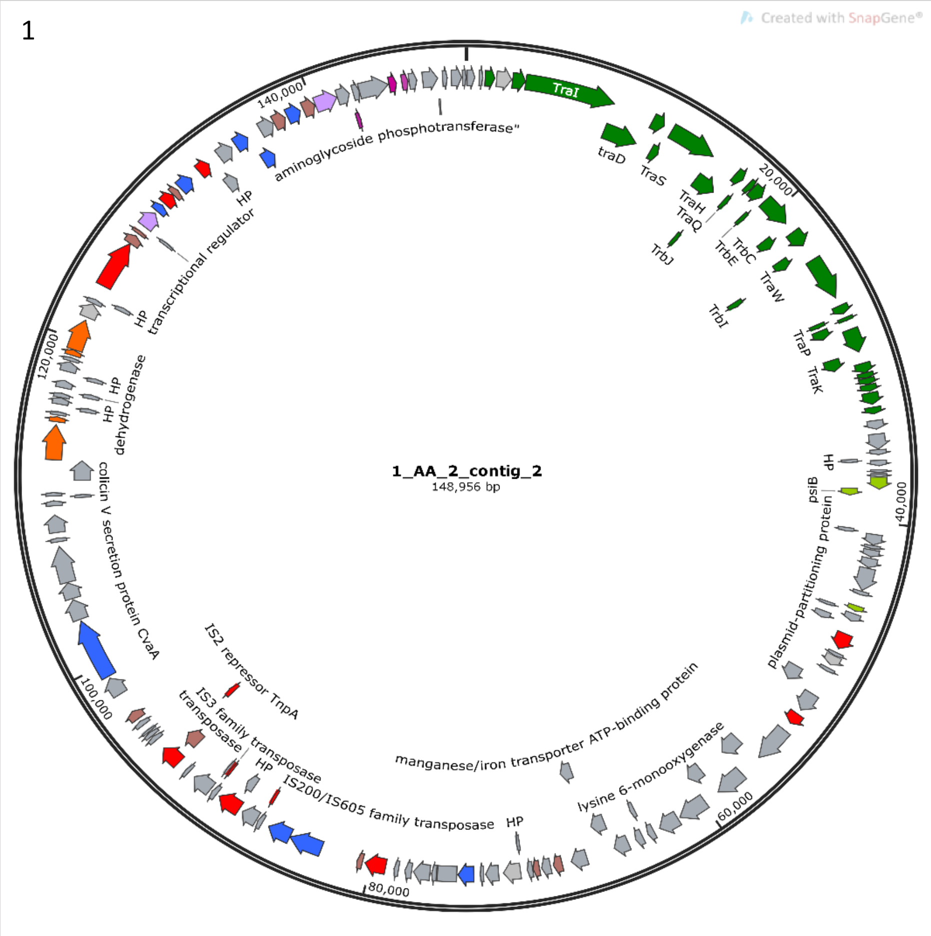
Row 1: Lane 1 KB bp ladder (BioLab), Lane 2 GCs sample 01, Lane 3 GCs sample 02, Lane 4 GCs sample 03, Lane 5 GCs sample 04, Lane 6 GCs sample 05, Lane 7 GCs sample 06, Lane 8 GCs sample 07, Lane 9 GCs sample 08, Lane 10 GCs sample 09, Lane 11 GCs sample 10, Lane 12 GCs sample 13, Lane 13 GCs sample 13, Lane 14 GCs sample 14-1, Lane 15 GCs sample 14-2, Lane 16 GCs 16, Lane 17 GCs sample 17, Lane 18 sample 18, Lane 19- ve control, Lane 20+ve control.

Row 2: Lane 1 KB bp ladder (BioLab), Lane 2 GCs sample 19, Lane 3 GCs sample 20, Lane 4 GCs sample 21, Lane 5 GCs sample 22, Lane 6 GCs sample 23, Lane 7 GCs sample 24, Lane 8 GCs sample 25, Lane 9 GCs sample 26, Lane 10 GCs sample 27, Lane 11 GCs sample 28, Lane 12 GCs sample 29, Lane 13 GCs sample 30, Lane 14 GCs sample 31, Lane 15 GCs sample 32, Lane 16 GCs 36, Lane 17 GCs sample 37, Lane 18 sample 38, Lane 19 sample 39, Lane 20 -ve control.

BlastN analysis of the plasmid sequence data revealed that a 148,956 bp plasmid of isolate AA_2_contig_2 showed 99.99% sequence identity to *Escherichia coli* strain G749 plasmid pG749_1 (Genbank Acc. No. CP014489.1) and 100.00% sequence identity to *E. coli* strain 2009-52 plasmid pSDJ2009-52F (Genbank Acc. No. MH195200.1). While a 45,609 bp plasmid of isolate AA_6 exhibited 99.89% sequence identity to plasmids from multiple *Salmonella* Typhi and the chromosomes of *Proteus mirabilis* (Genbank Acc. No. CP015347.1) and *E. coli* (Genbank Acc. No. CP057176.1). The 139,294bp plasmid of AA_19 _contig_103 showed 100.00% sequence identity to *E. coli* strain CFS3273 plasmid pCFS3273-1(Genbank Acc. No. CP026933.2). Similarly, the 175,545 bp of isolate AA_24_contig_1 showed high sequence similarity (99.99%) to *E. coli* isolate 2-101 plasmid p2-101(Genbank Acc. No. CP053786.1) and *E. coli* plasmid pESBL20150178 (Genbank Acc. No.MK181568.1), as well as the 105,518 bp of the bacterial isolate AA_25_contig_35 also displayed sequence similarity 99.99% and 100% to E*. coli* strain 13KWH46 plasmid p13KWH46-2 and *Escherichia coli* strain CFS3313 plasmid pCFS3313-2 respectively (Genbank Acc. No. CP019252.1 and CP026941.2). Whilst the 49,958 bp plasmid of isolate AA_38 contig_5 is showing high similarity to *Klebsiella pneumoniae* TUM14373 pMTY14343_IncN DNA, with 99.92% sequence identity (Genbank Acc. No. AP018557.1) and *Klebsiella pneumoniae* strain C16KP0065 plasmid pC16KP0065-1 (Genbank Acc. No. CP052452) with 99.96% sequence identity. These data are summarised in Table 7.2.

Annotated circular maps of all the plasmids found in the six bacterial isolates are illustrated in Figure 7.4 (parts 1-6).

1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TraK
HP
psiB
plasmid-partitioning protein
manganese/iron transporter ATP-binding protein
lysine 6-monooxygenase
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IS200/IS605 family transposase
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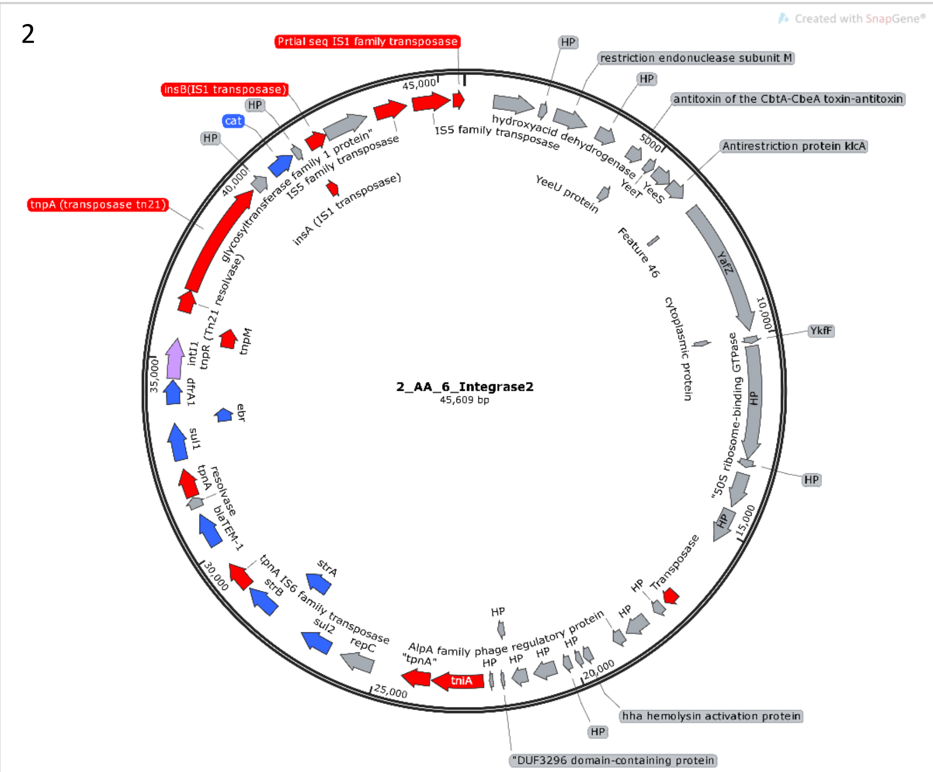
2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hydroxyacid dehydrogenase
YeeU protein
YeeT
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Feature 46
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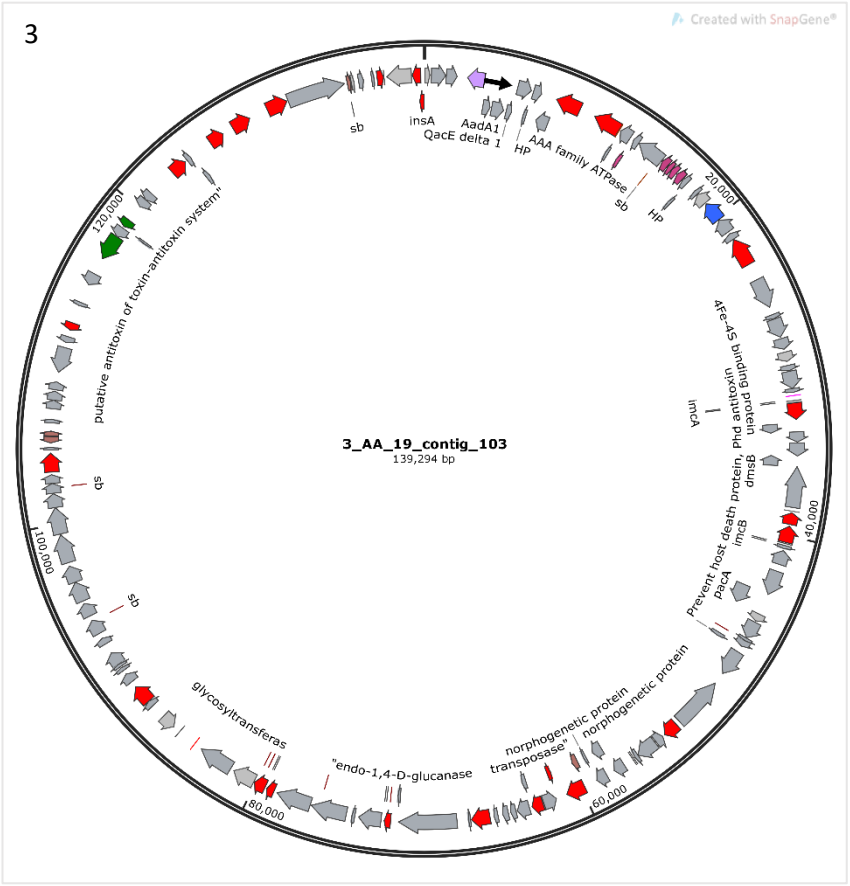
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dmsB
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Prevent host death protein, PhD
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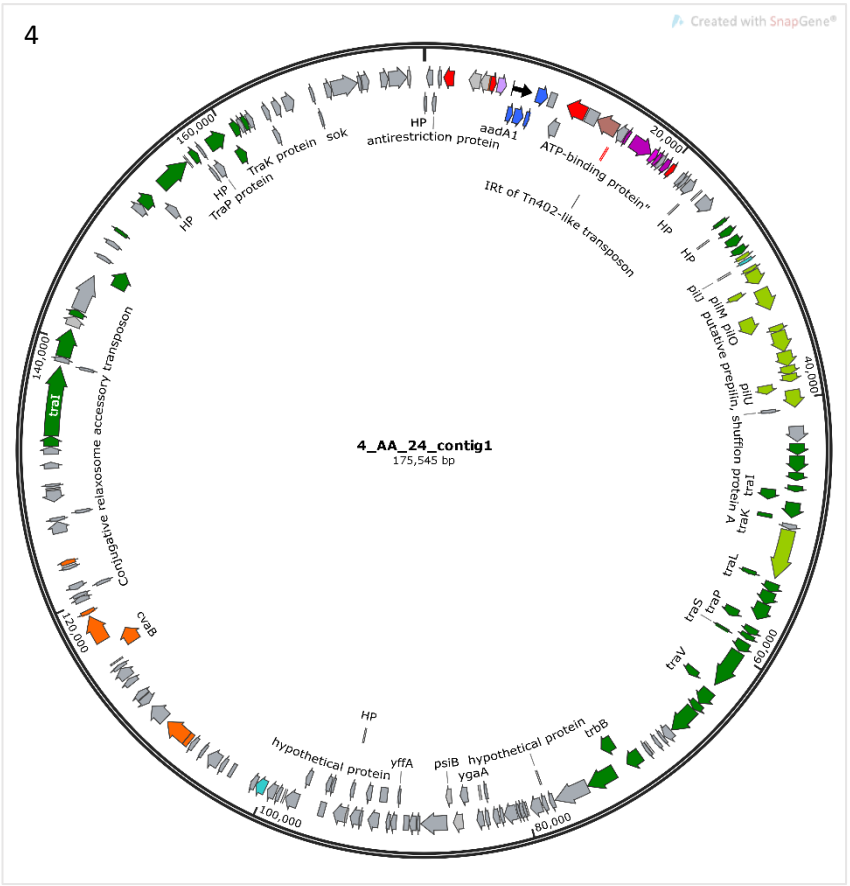
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HP
pilJ
pilM
pilO
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pilU
40,000
traA
traK
traL
traS
traP
traV
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cvaB
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HP
TraP protein
HP
TraK protein
sok
160,000

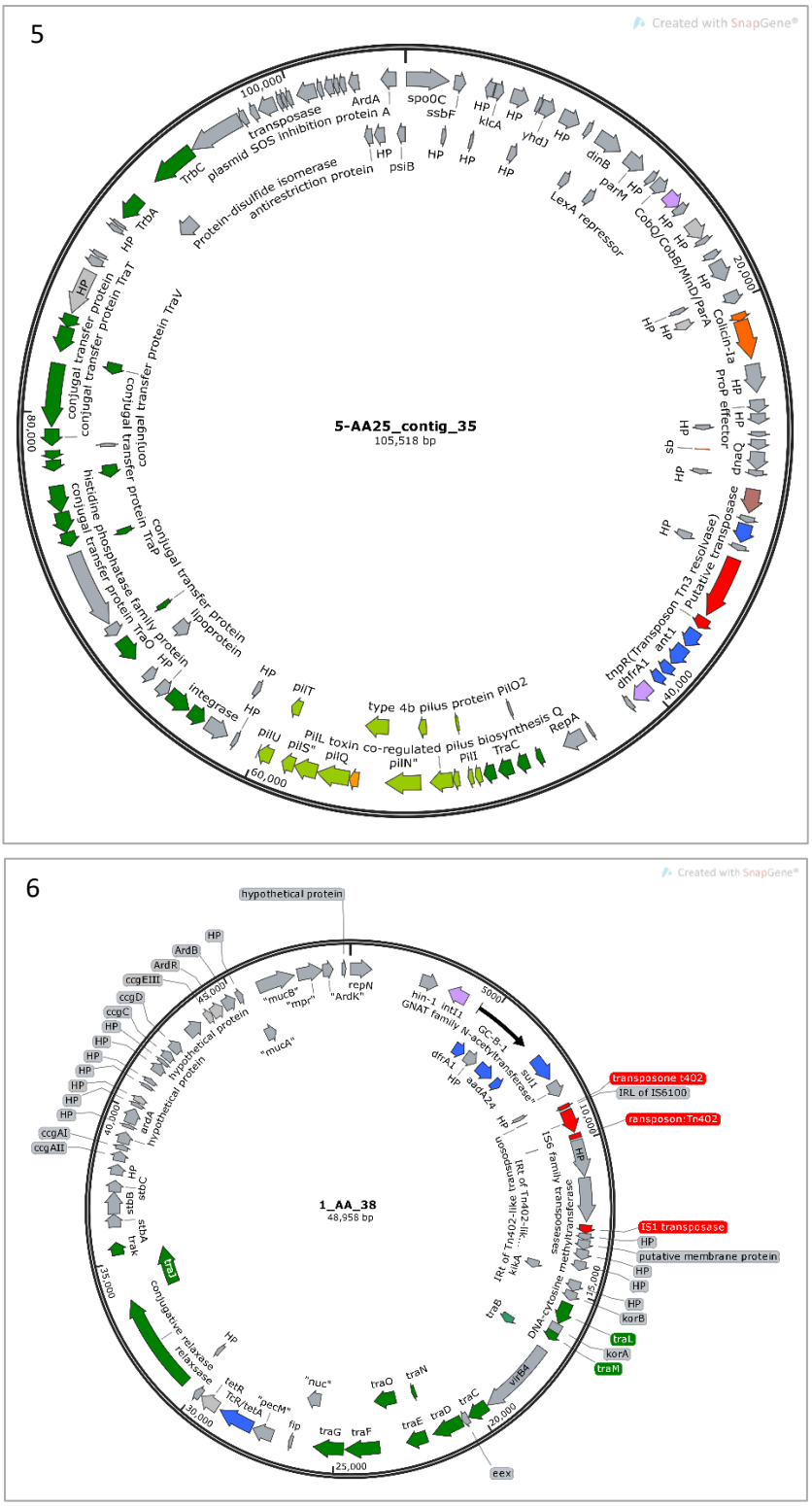

Figure 7.4 A schematic circular representation map of plasmids found in six bacterial isolates carrying class 1 integrons. Dark green arrow highlighting conjugative genes, light green arrows *pil* genes, dark pink arrows mercury resistance genes, red and brown arrows transposons, light purple arrows class 1 integron gene, dark blue arrows antibiotic resistance genes, orange arrows colicin resistance gene. All grey arrows hypothetical protein (HP) and other DNA maintenance genes. GCs are indicated by black arrows.

The plasmid isolated from *E. coli* (AA_2) encodes two class1 integrase sequences at different locations (127,769-128,782 and 140,116-141,420). Upstream of the first integrase gene there is a member of the Tn*3* transposase family and colicin transporter. While downstream of this integrase gene there are trimethoprim resistance dfrA5 gene, two IS6 family transposases flanking class A beta-lactamase resistance, and two aminoglycoside resistance genes. These are followed by the second integrase sequence and several virulence associated genes including mercury resistance genes, *tra* and pili genes involved in the conjugal transfer of resistance genes, and antimicrobial resistance Mig_14 genes, macrolide transporter and resistance-nodulation-division (RND) transporter. This plasmid belongs to the IncFIB group (Genbank Acc. No. AP001918) with 98.39% identity identified by plasmid finder 2.1.

BlastN analysis of the 45,609bp plasmid isolated from *Proteus mirabilis* (AA_6) contains one copy of a class 1 integron. Upstream of this structure there are two IS6 family transposases flanked by beta lactamase resistance, *strA* and *strB* (streptomycin resistance) and *sul2*. Downstream of *intI1* there are genes for *dfrA1*, *emrE* (Ethidium bromide-methyl viologen resistance) and *sul1.* Followed by many mobilizable elements such as Tn*21* module (transposon Tn*21* modulator protein, Tn*21* resolvase and transposase Tn*21*), IS1 transposase and IS5 transposase and *cat* gene (chloramphenicol resistance). However, the plasmid does not carry conjugative apparatus and belongs to IncQ1 with 100% sequence identity (Genbank Acc. No. M28829).

BlastN analysis of the plasmid sequence from AA_19 revealed a class 1 integron (2597/3610) containing GC-B-2 is embedded between IS1 transposase family upstream and IS21 family transposase (IS1326) downstream of the integrase system. This is followed by a mercury resistance module, *tetA* gene (tetracycline resistance) and Tn*3* family transposase. The plasmid encodes several transposase genes, however only two conjugative genes were identified (traH and incomplete gene encoding a putative conjugative transfer protein). The plasmid

could be categorised as p0111 with 98.53% identity (Genbank Acc. No. AP010962) by plasmid finder 2.1.

The boundaries of the class 1 integron that encodes GC-B-2 from AA_24_conting_1 feature a Tn*21* modulator protein and IS*110*-like element ISEc32 family transposase upstream and IS*21*-like element IS*1326* family transposase downstream. The integrase system is followed by mercury resistance genes and several conjugative (tra and trb operons) and pili encoding genes. This plasmid also possesses colicin 1B and V encoding genes (cvaA, cvaB and cvaC) and belongs to IncFII (Genbank Acc. No. AY458016).

While the plasmid isolated from AA_25 carries a class 2 integron (39,959-40,936 bp) with a different arrangement in the gene cassettes. This GC contains *dfrA1* (trimethoprim resistance), *sat2*, *aadA* (streptomycin resistance) and *bla_1* (Beta-lactamase) genes. Upstream of this structure are a Tn*3* transposase (32,613-35,618) and sul2 gene (30,885-31,781). This plasmid contains colicin E1 and *tra* and *trb* conjugative genes. The plasmid also features a phage integrase *XerC* gene with no imbedded GCs (14,017-14,796). The plasmid is IncI1-I(Gamma) with 99.3% sequence identity (Genbank Acc. No. AP005147).

Klebsiella pneumonia AA38 contains a plasmid of 49,958 bp, which encodes GCsB-1(*dfrA1*, ORF1, *aadA24*). Downstream of the class 1 integron is IRT of Tn*402*-like transposon tni module inserted in a IS*6100* family transposase. The IRT ends of Tn *402*-like transposons were identified in KY020154.1 (100% identity). The 765 bp at 8946-9710 bp of contig 5 shown similarity to transposable element IS6100 that was identified in BlastN with 100% coverage in CP052359.1 and KY020154.1. Moreover, there was another 267 bp nucleotide sequence of the transposable element IS*1* (12,757-13,023) with 100% identity in CP052359.1. This conjugative plasmid encodes cluster of conjugative elements and can be ascribed to the IncN plasmid group based on with 99.81% identity (Genbank Acc. No. AY046276). The plasmid also encodes tetracycline resistance and several tra genes. All results summarized in Table 7.2. and Figure 7.5.

Table 7.2 Summary and comparative plasmid sequence analysis by BlastN of bacterial isolates picked from broiler caecal content reared in biosecurity condition.

Isolate ID	Bacterial ID	Plasmid size bp	Plasmid name	Plasmid type	GCs type/resistance genes	Integrase copy	Acc. No.	Sequence identities
AA_2	*E. coli*	148,956	IncFIB	conjugative	*dfrA5/bla_1/ /sul1/aadA/merC*	2 *intI1*	CP014489.1	99.99%
AA_6	*P. mirabilis*	45,609	IncQ1	mobilizable	*bla/strA/strB/sul2/dfrA1/EmrE/ sul1/cat*	1 *intI1*	CP015347.1	99.89%
AA_19	*E. coli*	139,294	p0111	mobilizable	GC_B2(*dfrA1/aadA1/qacEΔ1/sul1*) *merC/tetA*	1 *intI1*	CP026933.2	100%
AA_24	*E. coli*	175,545	IncFII	conjugative	GC_B2(*dfrA1/aadA1/qacEΔ1/sul1*)/*merC*	1 *intI1*	CP053786.1	99.99%
AA_25	*E. coli*	105,518	IncI1-I	conjugative	*dfrA1/sat2/aadA/bla_1/sul2*	*IntI2* and phage integrase	CP019252.1	100%
AA_38	*K.pneumonia*	49,958	IncN	conjugative	GC-B-1 (*dfrA1*/HP/*aadA1/qacEΔ1/sul1*)/*tetA*	1 *intI1*	CP052452	99.96%

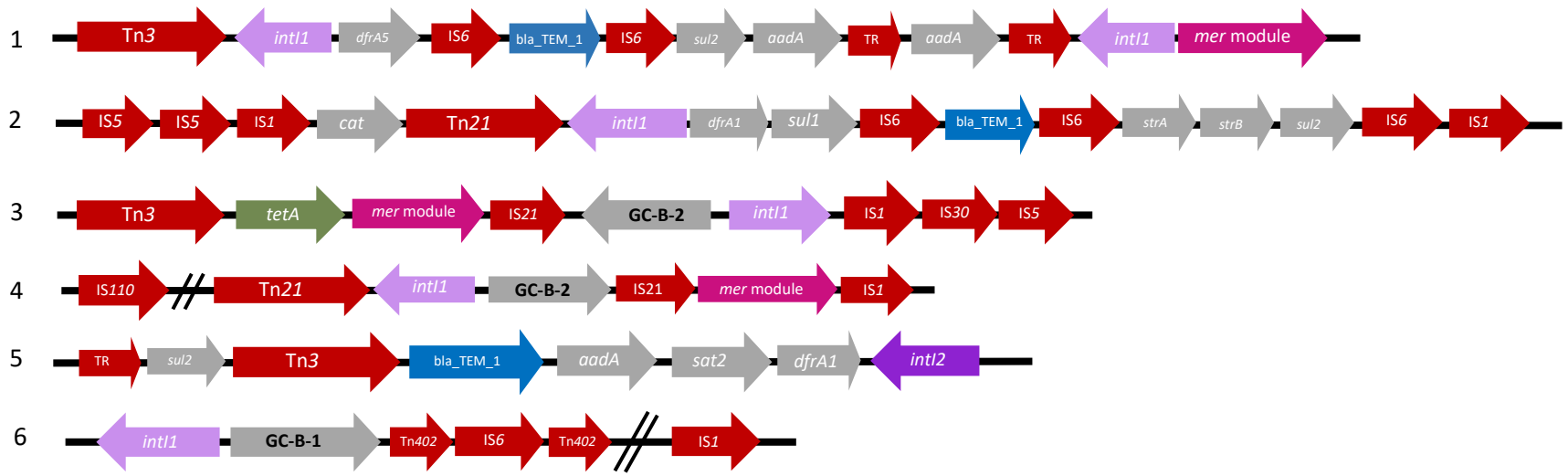

Figure 7.5 Boundaries of integrase genes detected in plasmids from *E.coli*, *P.mirabilis* and *K.pneumonia*.

Red arrows are transposons and IS sequences and transposon like elements, light purple class 1 integron while dark purple is class 2 integron, blue arrows are beta-lactamase resistance, light green tetracycline resistance, grey arrows are resistance genes embed in gene cassettes and dark pink are mercury resistance genes. Plasmid order 1- IncFIB (*E. coli*), 2- IncQ1 (*P. mirabilis*),3- p0111 (*E. coli*), 4- IncFII (*E. coli*), 5- IncI1-I (*E. coli*), 6- IncN (*K. pneumonia*).

7. 3 Discussion

The vital role of plasmids has been highlighted in the evolution and dissemination of bacterial ARGs among the most clinically important pathogens (Alekshun and Levy, 2007;Carattoli, 2013;San Millan, 2018). The main drivers of the spread ARGs in bacterial families such as *Enterobacteriaceae* and *Enterococcaceae* including some of the most important nosocomial pathogens, are conjugative plasmids (Vincent, 2003;Boucher et al., 2009;Rozwandowicz et al., 2018). Analysis using BlastN of the six plasmids from caecal contents cultured on Mac-03 medium supplemented with 20 mg/ml of trimethoprim revealed that four plasmids (AA_2, AA_24, AA_25, AA_38) that possessed a set of common genes that are predicted to play a role in plasmid replication, conjugative transfer, stability, and partition control clustered in functional modules. While two plasmids isolated from AA_6 and AA_19 are likely to be mobilizable (non-conjugative) plasmids as they do not encode conjugative genes however they do encode mobilizable and mobile genetic elements (transposase and IS). All plasmids carry genes encoding replication initiation protein (RepA or RepB or RepC or RepN) required for plasmid replication. Conjugative plasmids isolated from AA_2 and AA_24 contain more than 20 *tra* genes with *trb*, *pil* genes and feature more than one rep genes per replicon. These plasmid are likely belonging to IncF plasmid group. Rozwandowicz et al. (2018) described IncF plasmid as the most common plasmid type from human and animal that it is frequently found in *E. coli*. Similarly, the current study found both plasmids were isolated from *E.coli.* They are also highlighted global association of IncF plasmid with the spread of ESBL genes (blaCTX-M-15) in human *E. coli* isolates of the ST131 and ST405 groups which was observed in the AA_2 plasmid.

While AA_25 encodes 17 *tra* genes, 2 *trb* and 12 *pil* genes, AA_38 encodes only 11 conjugative *tra* genes. Plasmid AA_25 does not carry a class 1 integron, however it encodes dfrA1 embedded in a class 2 integron that belongs to the IncI1-

I incompatibility group. This plasmid group are described by Carattoli (2009) as plasmids that produce type I pili making them susceptible to phage. Garcillán-Barcia et al. (2011) stated that this group consist of low copy number, narrow-host-range, and conjugative plasmids. The IncI1 plasmids are commonly detected in enteric bacteria from food animal sources and are linked to clinically relevant strains (Kaldhone et al., 2019). They are also known for their potential carriage and dissemination of ARGs among enteric pathogens (Wong et al., 2016;Mo et al., 2017). For instance, several of the plasmids that showed association with the dissemination of resistance to ceftriaxone (an antimicrobial agent used for the control of severe *Salmonella* infections) have been reported to be IncI1 plasmids (Smith et al., 2015).

Plasmid AA_38 found in *K. pneumoniae* could be placed in the IncN group and was found to encode GC-B-1 in addition to the tetA gene. A study conducted by Eikmeyer et al. (2012) isolated four novel plasmids, pRSB201, pRSB203, pRSB205 and pRSB206 from the final effluent of a municipal wastewater treatment plant, which were demonstrated to belong to the IncN incompatibility group. Plasmid AA_38 shows sequence similarity to the plasmid module present in pRSB201. Eikmeyer et al. (2012) indicated that IncN plasmids although possessing a limited host range are widespread. They pointed out that IncN plasmids need to be considered as important genetic elements involved in the dissemination of clinically relevant ARGs. They further highlighted the large variety of antibiotic resistance determinants associated with IncN plasmids include fluoroquinolones (nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin, moxifloxacin), cephalosporins (cefotiam, cefotaxime, ceftazidime, cefepim), carbapenems (imipenem, meropenem), monobactams (aztreonam), fosfomycin, chloramphenicol and aminoglycosides (amikacin, tobramycin, netilmicin, gentamicin), indicating the plasmids of this incompatibility group are suitable vehicles for the incorporation of various resistance transposons, insertion sequences and integron gene cassettes containing resistance genes. However, Garcillán-Barcia et al. (2011) noted that

IncN plasmids are known as a broad-host range plasmids and that their copy number is controlled by iterons. Although IncN plasmids have ability to replicate in various *Enterobacteriaceae*, they are most frequently found in *E. coli* and *Klebsiella pneumonia*, where they are involve in the dissemination of cephalosporin and carbapenem resistance (Carattoli, 2013;Blau et al., 2018). In the current study it found in *Klebsiella pneumoniae* with an identical GCs-B-1 that emerged from *Salmonella* infected birds.

Plasmid AA_6 belongs to IncQ and for which conjugative genes are absent within the sequence. Rozwandowicz et al. (2018) described IncQ plasmid as a group of mobilizable elements with a medium-range copy number (4–12 copies/cell). Even though, IncQ plasmids do not possess conjugation genes, they can be transmitted at a high frequency in the presence of a helper plasmid and have been successfully mobilized to a large number of gram-negative bacterial hosts (Rozwandowicz et al., 2018). Similarly, plasmid isolate AA_19 seems to be mobilizable plasmid.

The association of antibiotic resistance and mercury resistance has been previously reported (Wireman et al., 1997). The current study indicated that plasmids AA_2, AA_19 and AA_24 isolated from *E. coli* carried mercury resistance loci with antibiotic resistance gene. Nguyen et al. (2019) stated that *Pseudomonas aeruginosa* and *E. coli* were the most frequently studied bacteria that showed co-occurrence of resistance to many heavy metals and antibiotic classes. They also, proposed that the prevalence of heavy metals in the environment is likely to increase resistance to both heavy metals and antibiotics through co-resistance and cross-resistance mechanism.

Sunde et al. (2015) indicated that class 1 integrons are usually considered as a component of transposons that allows them to move between different plasmids and between plasmids and the chromosome. Transposition into various plasmids groups may contribute to a further dissemination of the integron structure in the bacterial mapping that flanking DNA of integrons in the isolates.

The analysis of class 1 integrons boundaries show that three plasmids (IncFIB, p0111 and IncI1-I) carried different gene cassettes harboured integrase genes flaked by the Tn*3* transposon family, while the integrase gene in the IncQ1 and IncFII plasmids were integrated in to a Tn*21* transposon family member. Pal et al. (2017) described Tn*21* as a Tn*3* subfamily of transposable element embedded between the mercury resistance genes and the transposition genes (Pal et al., 2017). Therefore, the association between the Tn*3* transposon family and mercury resistance (often Tn*21* or Tn*1696* family), and the carriage of integrons has been widely detected (Gaze et al., 2011;Gillings et al., 2015). There are several reports of mercury-resistant Tn*21* or Tn*1696*-related transposons which encode integrons, that themselves carry antibiotic and biocide resistance genes (Partridge et al., 2001;Rosewarne et al., 2010;Pal et al., 2017).

However, the integrase gene found in the IncN plasmid showed the presence of an IS6 transposon that flanked by IRt of the Tn*402* module downstream of the integrase structure. Additionally, downstream of this structure another IS1 transposon is present. It seems that the structure of Tn*402* is incomplete. Stokes et al. (2006) reported that the presence of a complete structure is relatively rare, however, most of most class 1 integrons are associated with an incomplete transposition (tni) module. Many studies on the origin of class 1 integrons highlight the possible early association between the ancestor of the Tn*402* transposon with a class 1 integrase and an *attI1* site. This early association was raised because of the observation of the majority of class 1 integrons carry the 5'-CS region at the same position (Toleman and Walsh, 2011;Domingues et al., 2012). While the 3'-CS region has been proposed to be as consequence of a fusion of the *qacE* gene of the Tn*402* transposon with the *sul1* gene leading to partial deletion of the *qacE* gene; which happened at the same time as a deletion event in the transposition functions of the Tn*402* transposon, causing a loss in self-mobilization for this structure (Toleman and Walsh, 2011). Therefore, most class 1 integrons are defective transposons (Brown et al., 1996;Domingues et al.,

2012). Additionally, insertion sequences also play an important role in the dissemination of antibiotic resistance genes (Domingues et al., 2012). Several studies have reported class 1 integrons bounded on each side by an IS, which are mainly elements of the IS*6* family (Domingues et al., 2012). In this study ISs were present in the plasmids featuring IS*6*, IS*5*, IS*1* and IS*3*, as well as IS*110* and IS*21*-like elements. The configuration of IS6 surrounding the blaTEM_1 was found in plasmids of incompatibility groups IncFIB and IncQ1 that were isolated from similar trial. Hendrickx et al. (2020) stated that the IS*1* and IS*3* transposase families are commonly detected in the *K. pneumoniae* plasmids. They found that IS*1* family transposase was the most predominant among the plasmids and in multiple copies within plasmids. Likewise, IS*1* was the most frequently detect in the current study.

7.4 Conclusion

Plasmids carry multidrug resistance determinants and the machineries required to transfer genes between bacteria. Therefore, the identification of plasmid characteristics in different bacterial hosts may provide fundamental knowledge regarding the historical and potential for transmission of ARGs. Molecular identification of plasmid and strain genotypes can distinguish whether the spread of ARGs are driven by an epidemic plasmid to different bacterial hosts or by clonal spread of bacterial organisms harbouring these plasmids with ARGs (Rozwandowicz et al., 2018).

This study showed that all the plasmids recovered from broiler chickens harboured various GC types with *E. coli* a frequent carrier of plasmids could be categorised to the IncFIB, IncFII, IncI1-I, IncQ1, pO111, and IncN compatibility groups. The most representative GCs (B-2) type in identified in two isolates from biosecure housed. Most of the plasmids belong to incompatibly groups that have the potential to be major contributors to the propagation of ARGs within enteric

bacteria. Transposases and IS sequences are likely to play a major role in of the transfer of ARGs whether the plasmid was conjugative or non-conjugative.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE WORK

8.1 General discussion

The aims of this study, outlined in Chapter 1, were to increase knowledge of the prevalence and type of antibiotic-resistant bacteria in broiler chicken intestinal contents and to understand the effects of the prebiotic GOS on these populations.

8.1.1 Prevalence of ARGs in during rearing in biosecure or commercial conditions

The work described in Chapter 3 was to evaluate the prevalence of ARGs in broiler chickens reared under biosecure conditions and compare this to the prevalence in broiler chickens reared under commercial production conditions. Class 1 integrons were used as biomarkers for this purpose. The types of GCs present in the microbiota were determined by obtaining sequences of the conserved genes in the integron-integrase system. This showed that 99% of the bacterial populations in caecal contents samples from birds reared under biosecure conditions were positive for the *IntI1* gene while 92.5% were positive for the same gene in birds reared under commercial conditions. Similar results were obtained by Che et al. (2019) who reported a 97% prevalence for the *IntI1* gene in faeces from commercially farmed birds. For the *qacEΔ1* gene that generally marks the opposite end of the integron to *IntI1,* 93.7% of birds reared under biosecure conditions were positive while 100% of birds reared under commercial conditions were positive. A high prevalence of this gene (100%) was reported by Enany et al. (2019) in *E. coli* isolates from diseased commercial birds and from environmental samples in Egypt. In this study 94.5% of the DNA samples from caecal microbiota was positive for *sul1* resistance in biosecure housed birds to 97.5% of commercial birds. Sulfonamide resistance genes (*sul1* and *sul2*) are common in *E. coli* and *Salmonella* isolated from commercial pig and poultry sources (Kozak et al., 2009).

Having established a high prevalence of AMR genes in both groups of birds, the second stage of the work described in chapter 3 was the molecular characterisation of the GCs. Five different GC arrangements were discovered among birds reared under biosecure conditions whilst three GC arrangements were identified from commercial birds (Figure 3.7 and 3.11). Comparing the patterns between the two types of rearing conditions revealed that only two GCs shared a similar pattern of resistance (GCs-B-2 and GCs-C-1). The predominant discriminatory resistance GC in commercial birds was GCs-A (lincosamides, *linF*) with 75% prevalence, where no difference was observed in prevalence between the GOS and control diets. Interestingly the *linF* resistance gene was completely absent in the microbiota of birds reared under biosecure conditions. This finding suggests that mobility of this gene could potentially be attributed to selective pressure of antibiotics used for veterinary purposes in the commercial environment that is not encountered by birds reared under biosecure conditions. The use of lincosamides may be historic but resistance determinant persistent due to carriage in highly adapted bacteria colonising successive flocks. The GC B-2 (*dfrA1* and *aadA1*) type was frequently detected in the microbiota of birds reared under biosecure conditions (21.81%). Birds fed prebiotic GOS and infected with *Salmonella* were less likely to have the GC B-2 type in their microbiota (7.14%) than birds infected with *Salmonella* and fed control diet (96.4%). Non-infected birds also showed a difference in this type between GOS diet (60%) and standard diet (37%). The concept that prebiotic diets could diminish the gut "resistome" in humans is a relatively recent idea (Wu et al., 2016). The work described in this chapter highlights the potential of applying this strategy to chickens. However, the pattern for this particular GC type was not observed in commercially reared birds with the GC B-2 type being more prevalent in GOS fed birds (25%) than birds fed standard diet (20%). It was clear that the role of prebiotics in influencing the type and frequency of GCs was complex and required further study. Other differences

in GC types found between different diet and rearing conditions are discussed in Chapter 3.

8.1.2 To what extend can the prebiotic GOS mitigate the load of ARGs present in the broiler chicken microbiome?

To answer of this question, it was first necessary to estimate the base-line resistance of broiler caecal microbiota from birds fed standard diets. This was achieved using with trimethoprim resistance as a marker in populations of coliforms and the relative copy number of integrase genes in the population across time. Metagenomics was also applied to identify changes in the overall caecal microbiota composition with time. The results detailed in Chapter 4 follow the developmental changes of the broiler chicken caecal microbiota from birds fed standard control diet and reared under biosecure or commercial conditions. This revealed that resistance load was highest in young biosecure housed birds. Integrase copy number decreased with time. However, the ratio of trimethoprim resistance to sensitive members of the population increased with age. This suggests that *dfrA* gene may or may not embedded in integrase system. The diversity of representative OTUs in the caecal microbiota was higher in commercial birds than biosecure reared birds, which was probably due to their greater exposure to the environment.

The work described in Chapter 5 focussed on the impact on antibiotic resistance by feeding prebiotic GOS diet. This was assessed using metagenomics to compare the microbiota of birds fed prebiotic with control birds fed a standard control diet. To focus on prevalence of antibiotic resistance, the abundance of Proteobacteria (*Enterobacteriaceae*) was specifically targeted. The effect of rearing system (biosecure or commercial) was also assessed. In addition to the metagenomic approach, trimethoprim resistance and total coliforms were enumerated, and the ratio of resistance in the population estimated. Integrase

copy number was also assessed and showed a significant reduction at 22 da (p value = 0.015) in birds fed GOS diet compared to those fed control diet when the direct impact of GOS supplementation would be most effective as the GOS supplementation was halted at 20 da.

The abundance of Proteobacteria in birds fed GOS diet and reared under biosecure conditions was reduced compared to those fed the control diet at 22-24 da. However, this effect did not continue in later sampling points. It suggests that dietary GOS supplementation in a biosecure environment may suppress AR in early life, which may reduce dissemination of these genes even though the effect did not persist. Metagenomic analysis of the microbiota of GOS fed birds compared to that of birds fed the control diet at 22 da displayed differential abundance dominated by members of the Firmicutes phylum that included *Lachnospiraceae ssp* OTU0032 and OTU0030, *Enterococcus* OTU0078 and members of the *Clostridiales_unclassified* OTU0138. The increased abundance of these OTUs may contribute to the decrease in Proteobacteria, and as a consequence the prevalence of GCs. Further work would be required to identify exactly which members of the population were responsible for the reduction in GC at 22 da. Yang et al. (2008) and Chee et al. (2010) reported that administration of dietary prebiotics might support young broilers intestinal microbiota by boosting the abundance of *Lactobacilli* and *Bifidobacteria* and reducing the coliform titres. The work described here supports the idea that targeting young birds with prebiotic could be an effective measure for reducing AMR.

The proportion of Proteobacteria was higher in commercial birds than biosecure birds. Comparison of alpha-diversity showed that both inverse-Simpsons and richness indices were higher in commercial than biosecure housed birds in both diets probably due to greater environmental exposure.

8.1.3 Does the beneficial effect of GOS persist if the gut is colonised by a pathogen such as *Salmonella*?

The work in Chapter 6 described the impact of *Salmonella* colonisation on the composition of the broiler gut microbiota. It also reports the prevalence ARGs carried by caecal community members from birds fed the GOS diet compared to control diet for Salmonella colonised and non-colonised birds.

The data collected indicated that the GOS diet accelerates the clearance rate of *Salmonella* from the caecal contents of birds by 8 days post colonisation compared to control diets. Correlation between abundance of Proteobacteria and ratio of trimethoprim resistance indicated that the decreasing abundance of Proteobacteria coincided with increased resistance to trimethoprim. Interestingly, there was a significant reduction in class 1 integron copy number (p-value = 0.004) at 2 dpi in GOS supplemented diet for birds challenged with *Salmonella* or mock challenged. The integrase copy number remained relatively stable until the end of the study at 15 dpi in the *Salmonella* infected GOS diet group but not for the non-colonised GOS diet group. This finding coincided with day that the GOS prebiotic was removed from the diet. Comparing viable counts of trimethoprim resistant isolates in caecal contents between *Salmonella* infected birds fed GOS diet and those *Salmonella* infected and fed the control diet indicated a significant reduction at 2 and 4 dpi associated with the GOS diet. Total coliform viable counts showed a gradual decline with age in the caecal contents from GOS diet *Salmonella* infected birds compared to *Salmonella* infected birds on the control diet. The results of metagenomic analysis indicated that the presence of *Salmonella* increased the proportions of Negativicutes OTUs in both diet groups. The effect was more pronounced in the GOS diet group. There was an increase in the abundance of Negativicutes (*Megamonas*) with age at the expense of *Clostridia* (*Lachnospiraceae*) that was potentially restricting abundance of Proteobacteria. It is possible that the expansion of this OTU influenced oxygen availability leading to

accelerated clearance of *Salmonella*. Colonisation of chickens with *Salmonella* has been shown to require a virulence-factor-dependent increase in gut epithelial oxygenation (Litvak et al., 2019). Both *Veillonellaceae* (*Megamonas*) and *Acidaminococcaceae* (*Acidaminococcaceae _unclassified*) belong to class Negativicutes which includes strictly anaerobic and non-spore forming bacteria that maintain an anaerobic environment free of alternative electron acceptors (Rychlik, 2020). Other possible explanations for the reduction in *Salmonella* numbers coinciding with increased Negativicutes in birds fed GOS diet include changes in competition for certain nutrients or production of organic acids which decrease the expression of virulence factors from pathogens like Salmonella(Van Immerseel et al., 2003). LEfSe analysis highlighted the high abundance of *Acidaminococcaceae_unclassified* (OTU0009) within microbiota compositions of *Salmonella* colonised groups which have the potential to produce butyrate (Rychlik, 2020). A combined effect of the prebiotic GOS feed and invasion by *Salmonella* may have stimulated the avian immune system to promote the elimination of *Salmonella*.

The presence of *Salmonella* increased the proportion of birds carrying class 1 integron ARGs unrelated to the colonising organism in the caecal microbiota of the chickens reared under biosecure conditions (χ^2 p = 0.001). However, the provision of dietary GOS up to day 20 reduced ARG carriage in the *Salmonella* colonised birds (χ^2 p = 0.004). Zhang et al. (2018) reported the host species of class 1 integrons were highly conserved with 96% of the database class 1 integrons lodged in Gammaproteobacteria with the family *Enterobacteriaceae* dominant. Stecher et al. (2012) indicated that the normal gut harboured low densities ($<10^8$ cfu/g) of *Enterobacteriaceae*. Having low densities of both donor and recipient bacteria may reduce the frequency of direct bacterial encounters and thus decrease the chance of conjugation-mediated HGT. Stecher et al. (2012) also showed that conjugative HGT of the colicin-plasmid p2 from *Salmonella enterica* serovar Typhimurium to *E. coli* happened at unprecedented rates when the gut

(mouse model) was colonised by the pathogen. The mechanism was suggested to be driven by inflammatory responses that increased transient enterobacterial blooms. This transconjugation efficiency of approximately 100% *in vivo,* were attributed to the high intrinsic p2-transfer rates. Yue and Schifferli (2014) demonstrated that *Salmonella* colonisation factors promote horizontal gene transfer of antimicrobial resistance genes by increasing the local density of *Salmonella* in colonised intestines (reviewed in Chapter 6). Types of mobile genetic elements that act as vehicles of class 1 integrons and associated OTUs were investigated in the chicken gut microbiota in Chapter7. Six trimethoprim resistance isolates selected for the whole genome sequence analysis showed that the most frequent carrier of plasmids carry multidrug resistance determinants and the machineries required to transfer resistance genes between bacteria is *E.coli*. Plasmids observed could be categorised to the IncFIB, IncFII, IncI1-I, IncQ1, pO111, and IncN compatibility groups discussed in more details in Chapter 7. The most representative GCs (B-2) type in identified in two isolates from biosecure housed birds. The presence of this plasmid in this commensal bacterial isolate in the chicken intestine indicates how easily the GCs can spread in the Proteobacterial population by HGT including transmission to human pathogens such as *Salmonella*.

8.2 Conclusion

This study has highlighted broiler chickens as one of the most important food animal reservoirs involved in transmission of antibiotic resistance genes to human pathogens and the wider environment. A possible control strategy by manipulating the broiler chicken microbiota via dietary intervention using a prebiotic GOS diet was investigated. This resulted in early elimination of *Salmonella* colonization and modification of caecal bacterial communities.

The involvement of class 1 integrons in the spread of antibiotic resistance was assessed. It was shown that acquisition of GCs was influenced by the rearing environment and that GCs can acquire or lose ARGs with time. Moreover, regardless of antibiotic pressure, they can persist without selection pressure as result of robustness of the integration mechanism via i*ntI1* and a*ttC*. Hence, they can act as reservoirs for disseminating various resistance genes in every environment.

Metagenomic analysis of the microbiomes of birds fed prebiotic GOS or standard diets and either colonised or non-colonised with *Salmonella* has increased our understanding of how prebiotic compounds such as GOS achieve the beneficial effects that have been observed empirically. Studying resistance in these same birds has increased our understanding of the way in which AMR spreads at the molecular level.

Collectively, the results obtained in this thesis confirm the study hypothesis that feeding GOS to chickens is a possible approach for restricting expansion of *Salmonella* and other Proteobacteria that are known to be associated with high prevalent of ARGs in Gram-ve bacteria belonging to the *Enterobacteriaceae* family.

8.3 Future work

Besides breeding and selection, nutritional optimisation of broiler chickens is a fundamental component of efficient poultry production. Hence, effective control strategies are needed to make the cecum less hospitable for zoonotic pathogens colonisation. Prebiotics are a promising approach for mitigating antibiotic resistance gene loads, and by optimising the avian microbiome, improvements in zootechnical performance with a reduction in the risk of transmitting pathogenic species. The model experiments established here could be applied to different prebiotics and synbiotics. The study could be extended to include other classes of integrons and gram-positive bacteria to investigate the impact of GOS. Correlation

of resistance patterns fond in the broiler gut microbiota with their surrounding environment could give insights into the sources of the GCs. Extending the work to include laying hens would provide more valuable information because of their longer life and resistance patterns may different as the microbiome matures. Collectively, it may provide insights into important players in the dissemination of antimicrobial resistance within this production system.

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Appendices

Appendix 1: preparation of media

1.1 Media preparation

The suppliers for all media used and chemicals substances in this thesis are listed in Table 2.1. All media used in this thesis were prepared using reverse osmosis (RO) water followed by sterilization cycle by autoclaving at 121 °C for 15 min, in case if the components of media were heat labile the frequent agitation was used until the medium boiled. The antibiotics were added to the molten agar after sterilizing, once it had cooled to approximately 50 °C. The media was poured into sterile Petri dishes (Sarstedt, Leicester, UK) in a laminar flow cabinet. Liquid media was generally stored at room temperature (unless specified) and prepared agar plates was stored at 4 °C.

All media used in this thesis for culturing and storing bacterial growth were prepared in reverse osmosis (RO) water and then sterilised via autoclaving on liquid cycle at 121°C and 15 psi for twenty minutes. Required antibiotics were added to agar media after it cooled to 50°C in a water bath, and then poured into sterile Petri dishes (Sarstedt, Leicester, UK) in a laminar flow cabinet. Broth media were stored at room temperature whereas agar plates were stored at 4°C for a maximum of four weeks.

1.1.1 Modified Xylose-Lysine-Desoxycholate (mXLD) agar

The media was prepared by dissolving 53 g of XLD medium (Oxoid; CM0469; UK) into 1 litre of RO water and then, heated by stirrer hotplates (Stuart; CB162; UK) with frequent agitation until the medium boiled (over boiled should be avoided). It was cooled to 50 °C in water bath before addition of 1 µg/ml of Novobiocin (Sigma-Aldrich) and 12.5 µg/ml Nalidixic acid (Sigma-Aldrich), mixed well then poured into sterile Petri dishes (Sarstedt, Leicester, UK) in a laminar flow cabinet and agar media were stored at 4°C for a maximum of four weeks.

1.1.2 Xylose-Lysine-Desoxycholate (XLD) agar

XLD medium was prepared as above without antibiotics.

1.1.3 Modified Semi-solid Rappaport Vassiliadis (MSRV) agar

Approximately 15.8 g of MSRV Medium Base (Oxoid; CM1112; UK) was suspended in 1 litre of RO water, heated with frequent agitation until the medium boiled and then It was cooled to 50°C before addition of Novobiocin (20 µg/ml) Mix well and poured into sterile Petri dishes. The plates were air dried at room temperature for at least one hour and kept right way up because it is semi-solid media.

1.1.4 MacConkey agar No 3 (Mac_03)

51.5g of MacConkey no 3 (Oxoid; CM115; UK) was suspended in 1 litre of RO water and sterilised by autoclaving at 121°C for 15 minutes, cooled in water bath and then poured into sterile Petri dishes for control plates whereas trimethoprim plates were prepared by inoculated medium with 20 ug/ml of trimethoprim after medium was tempered to 50 °C, mixed well and poured into marked Petri dishes.

1.1.5 De man, Rogosa and Sharpe (MRS) agar

MRS medium (31 g; Oxoid; CM0361) was suspended in 1 litre of RO water and sterilised by autoclaving at 121°C for 15 minutes, cooled then poured into sterile Petri dishes.

1.1.6 Luria-Bertani (LB) agar

LB medium was prepared by adding 10 g tryptone (BiTek™; USA), 5 g yeast extract (BiTek™; USA), 5 g sodium chloride (Sigma-Aldrich; UK) and 15 g bacteriological agar No. 1 (Oxoid; LP0011 UK) was prepped in 1 L of RO water. The medium was autoclaved for sterility at 15 psi, 121 °C for 15-20 minutes. Sterile LB Petri dishes were stored at room temperature or 4°C until required.

1.1.7 LB broth

LB broth was prepared as mentioned previously in 1.1.6 without adding bacteriological agar no.1 autoclaved and LB broth was stored at room temperature for a maximum of eight weeks.

1.1.8 Nutrient Agar (NA)

Nutrient agar (Oxoid; CM0003; UK) was prepared by dissolving 28g of Nutrient agar into 1 litre of RO water. After autoclaving, Nutrient agar was cooled in water bath, poured in Petri dishes and stored at room temperature for a maximum of eight weeks.

1.1.9 Nutrient broth No. 2

Nutrient broth No. 2 (Oxoid; CM0067; UK) was prepared by dissolving 25g of Nutrient broth No. 2 into 1 litre of RO water. After autoclaving, Nutrient Broth No. 2 broth was stored at room temperature for a maximum of eight weeks.

1.1.10 YT broth

YT broth was prepared by adding 0.5% Difco Bacto yeast extract, 0.5% NaCl, 0.8% Difco Bacto tryptone and was adjusted to pH 7.5. YT broth was autoclaved for sterility at 15 psi, 121 °C for 15-20 minutes and stored at room temperature until required.

1.2 Buffers and solutions

1.2.1 Buffered peptone water

Buffered Peptone Water (20 g; Oxoid; CM509) was suspended in 1 litre of RO water and sterilised by autoclaving at 121°C for 15 minutes.

1.2.2 Maximum recovery diluent (MRD)

MRD was prepared by adding 9.5g of maximum recovery diluent (Oxoid; CM0733; UK) in 1 litre of RO water. After autoclaving, MRD was stored at room temperature for a maximum of eight weeks.

1.2.3 Phosphate buffered saline (PBS)

To prepare PBS, one phosphate buffered saline tablet (ThermoFisher) was dissolved in 200 ml of RO water. After autoclaving, PBS buffer was stored at room temperature.

1.2.4 Long-term bacterial storage medium

Glycerol stock was prepared to maintain and store bacterial growth by adding 300 µl of sterile glycerol (50 % v v) to 700 µl nutrient broth No.2 (1.1.9) to a final concentration of 30% v/v. After autoclaving, the bacterial storage medium was stored at room temperature for a maximum of eight weeks.

1.2.5 Antibiotics

Selected antibiotics were prepared in appropriate solvent according to manufacturer's instructions. After preparation, antibiotics were sterilised by 0.22 µm filter (Sartorius Stedim Biotech; Germany) and aliquoted as 1ml stock solutions and stored at -20°C. Stock concentrations for antibiotics used in this thesis are listed in Table 1.1.

Table 1. 1 Antibiotics used in this thesis.

Antibiotic	Supplier	Solvent	Stock concentration	Working Concentration
Trimethoprim	Sigma-Aldrich	100% Ethanol /DMSO	20 mg/ml	20 µg/ml
Novobiocin	Sigma-Aldrich	RO water	1 mg/ml	1 µg/ml
Nalidixic acid	Sigma-Aldrich	RO water	12.5 mg/ml	12.5 µg/ml
Lincomycin	Sigma-Aldrich	RO water		

1.1.13 Proteinase kinase

Approximately 0.1 g of proteinase K (Sigma-Aldrich) was dissolved in 5 ml of RO water (20 mg/ml) for GenElute Bacterial Genomic DNA kit, sterilised by filter (0.22 µm) then aliquoted as 60 µl stock solutions and stored at -20°C. It commonly used to digest protein and remove contamination from DNA because it inactivates nucleases that might degrade the DNA or RNA during purification.

Appendix 2.1 Table 2.1.1 The PCR ampilicon product results of three conserved genes of class 1 integrons of broiler caecal samples biosecure birds

Sample	feed type	age	*intI1* Ravi	*intI1* EB	*intI1* H	*intI1* C	*sul1*	*qacEΔ1*
1.22.1	ctl-nonc	22	+	neq	+	+	+	+
1.22.2	ctl-nonc	22	+	neq	+	+	+	+
1.22.3	ctl-nonc	22	+	neq	+	+	+	+
1.22.4	ctl-nonc	22	+	neq	+	+	+	+
1.22.5	ctl-nonc	22	+	neq	+	+	+	+
1.22.6	ctl-nonc	22	+	neq	+	+	+	+
1.22.7	ctl-nonc	22	+	neq	+	+	+	+
1.24.1	ctl-nonc	24	+	neq	+	+	+	+
1.24.2	ctl-nonc	24	+	neq	+	+	+	+
1.24.3	ctl-nonc	24	+	neq	+	+	+	+
1.24.4	ctl-nonc	24	+	neq	+	+	+	+
1.24.5	ctl-nonc	24	+	neq	neg	neg	neg	+
1.24.6	ctl-nonc	24	+	neq	+	+	+	+
1.24.7	ctl-nonc	24	+	neq	+	+	+	+
1.28.1	ctl-nonc	28	+	neq	+	+	+	+
1.28.2	ctl-nonc	28	+	neq	+	+	neg	+
1.28.3	ctl-nonc	28	+	neq	+	+	+	+
1.28.4	ctl-nonc	28	+	neq	+	neg	+	+
1.28.5	ctl-nonc	28	+	neq	+	+	+	+
1.28.6	ctl-nonc	28	+	neq	+	neg	neg	+
1.28.7	ctl-nonc	28	+	neq	+	+	+	+
1.35.1	ctl-nonc	35	+	neq	+	+	+	+
1.35.2	ctl-nonc	35	+	neq	+	+	+	+
1.35.4	ctl-nonc	35	+	neq	+	+	+	+
1.35.5	ctl-nonc	35	+	neq	+	+	+	+
1.35.6	ctl-nonc	35	+	neq	+	+	+	+
1.35.7	ctl-nonc	35	+	neq	+	neg	+	+
2.22.1	GOS-nonc	22	+	neq	+	+	+	+
2.22.2	GOS-nonc	22	+	neq	+	+	+	+
2.22.3	GOS-nonc	22	+	neq	+	neg	+	neg
2.22.4	GOS-nonc	22	+	neq	+	+	+	neg
2.22.5	GOS-nonc	22	+	neq	+	+	+	neg
2.22.6	GOS-nonc	22	+	neq	+	+	+	+
2.22.7	GOS-nonc	22	+	neq	+	+	+	+
2.24.1	GOS-nonc	24	+	neq	+	+	+	+
2.24.2	GOS-nonc	24	+	neq	+	+	+	+
2.24.3	GOS-nonc	24	+	neq	+	+	+	+
2.24.4	GOS-nonc	24	+	neq	+	+	+	+
2.24.5	GOS-nonc	24	+	neq	+	+	neg	neg
2.24.6	GOS-nonc	24	+	neq	+	+	+	+
2.24.7	GOS-nonc	24	+	neq	+	+	neg	+

Continued. Table 2.1 The PCR ampilicon product results of three conserved genes of class 1 integrons of broiler caecal samples biosecure birds

Sample	feed type	age	*intI1* Ravi	*intI1* EB	*intI1* H	*intI1* C	*sul1*	*qacEΔ1*
2.28.1	GOS-nonc	28	+	neq	+	+	+	+
2.28.2	GOS-nonc	28	+	neq	+	+	+	+
2.28.3	GOS-nonc	28	+	neq	+	+	+	+
2.28.4	GOS-nonc	28	+	neq	+	+	+	+
2.28.5	GOS-nonc	28	+	neq	+	+	+	+
2.28.6	GOS-nonc	28	+	neq	+	+	+	+
2.28.7	GOS-nonc	28	+	neq	+	+	+	+
2.35.1	GOS-nonc	35	+	neq	+	+	+	+
2.35.2	GOS-nonc	35	+	neq	+	+	+	+
2.35.3	GOS-nonc	35	+	neq	+	+	+	+
2.35.4	GOS-nonc	35	+	neq	+	+	+	+
2.35.5	GOS-nonc	35	+	neq	+	+	+	+
2.35.6	GOS-nonc	35	+	neq	+	neg	neg	neg
2.35.7	GOS-nonc	35	+	neq	+	+	+	+
3.22.1	ctl-sal	22	+	neq	+	+	+	+
3.22.2	ctl-sal	22	+	neq	+	+	+	+
3.22.3	ctl-sal	22	+	neq	+	+	+	+
3.22.4	ctl-sal	22	+	neq	+	+	+	+
3.22.5	ctl-sal	22	+	neq	+	+	+	+
3.22.6	ctl-sal	22	+	neq	+	+	+	+
3.22.7	ctl-sal	22	+	neq	+	+	+	+
3.24.1	ctl-sal	24	+	neq	+	neg	+	+
3.24.2	ctl-sal	24	+	neq	+	+	+	+
3.24.3	ctl-sal	24	+	neq	+	+	+	+
3.24.4	ctl-sal	24	+	neq	+	+	+	+
3.24.5	ctl-sal	24	+	neq	+	+	+	+
3.24.6	ctl-sal	24	+	neq	+	+	+	+
3.24.7	ctl-sal	24	+	neq	+	+	+	+
3.28.1	ctl-sal	28	+	neq	+	+	+	+
3.28.2	ctl-sal	28	+	neq	+	+	+	+
3.28.3	ctl-sal	28	+	neq	+	+	+	+
3.28.4	ctl-sal	28	+	neq	+	+	+	+
3.28.5	ctl-sal	28	+	neq	+	+	+	+
3.28.6	ctl-sal	28	+	neq	+	+	+	+
3.28.7	ctl-sal	28	+	neq	+	+	+	+
3.35.1	ctl-sal	35	+	neq	+	neg	+	+
3.35.2	ctl-sal	35	+	neq	+	+	+	+
3.35.3	ctl-sal	35	+	neq	+	+	+	+
3.35.4	ctl-sal	35	+	neq	+	+	+	+
3.35.5	ctl-sal	35	+	neq	+	+	+	+
3.35.6	ctl-sal	35	+	neq	+	+	+	neg
3.35.7	ctl-sal	35	+	neq	+	neg	+	+

Continued. Table 2.1 The PCR ampilicon product results of three conserved genes of class 1 integrons of broiler caecal samples biosecure birds

Sample	feed type	age	*intI1* Ravi	*intI1* EB	*intI1* H	*intl1* C	*sul1*	*qacEΔ1*
4..22.1	GOS-sal	22	+	neq	+	neg	+	+
4..22.2	GOS-sal	22	+	neq	+	neg	+	+
4..22.3	GOS-sal	22	+	neq	+	neg	+	+
4..22.4	GOS-sal	22	+	neq	+	+	+	+
4..22.5	GOS-sal	22	+	neq	+	+	+	+
4..22.6	GOS-sal	22	+	neq	+	+	+	+
4..22.7	GOS-sal	22	+	neq	+	+	+	+
4.24.1	GOS-sal	24	+	neq	+	neg	+	+
4.24.2	GOS-sal	24	+	neq	+	neg	+	+
4.24.3	GOS-sal	24	+	neq	+	neg	+	+
4.24.4	GOS-sal	24	+	neq	+	+	+	+
4.24.5	GOS-sal	24	neg	neq	+	neg	+	neg
4.24.6	GOS-sal	24	+	neq	+	neg	+	+
4.24.7	GOS-sal	24	+	neq	+	+	+	+
4.28.1	GOS-sal	28	+	neq	+	neg	+	+
4.28.2	GOS-sal	28	+	neq	+	neg	+	+
4.28.3	GOS-sal	28	+	neq	+	neg	+	+
4.28.4	GOS-sal	28	+	neq	+	+	+	+
4.28.5	GOS-sal	28	+	neq	+	neg	+	+
4.28.6	GOS-sal	28	+	neq	+	+	+	+
4.28.7	GOS-sal	28	+	neq	+	+	+	+
4.35.1	GOS-sal	35	+	neq	+	neg	+	+
4.35.2	GOS-sal	35	+	neq	+	+	+	+
4.35.3	GOS-sal	35	+	neq	+	neg	+	+
4.35.4	GOS-sal	35	+	neq	+	+	+	+
4.35.5	GOS-sal	35	+	neq	+	+	+	+
4.35.6	GOS-sal	35	+	neq	+	+	+	+
4.35.7	GOS-sal	35	+	neq	+	+	+	+

Appendix 2.2 The PCR ampilicon product of class 1 integron (*intI1* Ravi primer) of biosecure birds

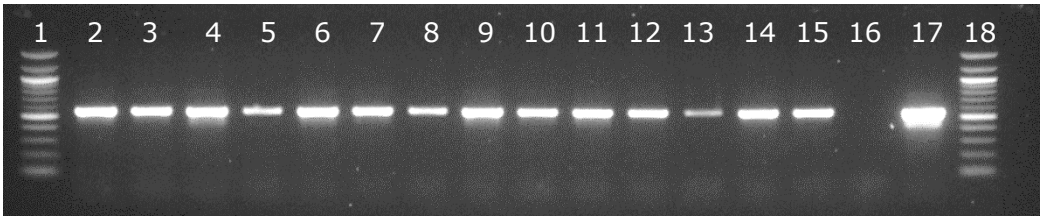


Figure 2.2.1 Agarose gels amplification products of PCRs by using Ravi primers showing target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of control group (G1) electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 1.22.1, Lane 3 *IntI1* Ravi sample 1.22.2, Lane 4 *IntI1* Ravi sample 1.22.3, Lane 5 *IntI1* Ravi sample 1.22.4, Lane 6 *IntI1* Ravi sample 1.22.5, Lane 7 *IntI1* Ravi sample 1.22.6, Lane 8 *IntI1* Ravi sample 1.22.7, Lane 9 *IntI1* Ravi sample 1.24.1, Lane 10 *IntI1* Ravi sample 1.24.2, Lane 11 *IntI1* Ravi sample 1.24.3, Lane 12 *IntI1* Ravi sample 1.24.4, Lane 13 *IntI1* Ravi sample 1.24.5, Lane 14 *IntI1* Ravi sample 1.24.6, Lane 15 *IntI1* Ravi sample 1.24.7, Lane 16 *IntI1* Ravi -ve control Lane 17 *IntI1* Ravi +ve control, Lane 18 100 bp ladder (BioLab).

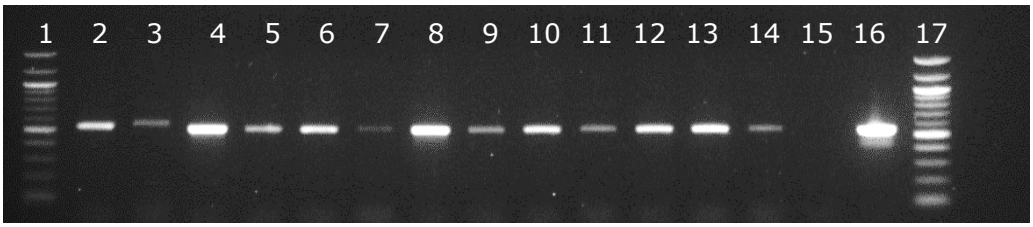


Figure 2.2.2 Agarose gels amplification products of PCRs by using Ravi primers showing target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of G1 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 1.28.1, Lane 3 *IntI1* Ravi sample 1.28.2, Lane 4 *IntI1* Ravi sample 1.28.3, Lane 5 *IntI1* Ravi sample 1.28.4, Lane 6 *IntI1* Ravi sample 1.28.5, Lane 7 *IntI1* Ravi sample 1.28.6, Lane 8 *IntI1* Ravi sample 1.28.7, Lane 9 *IntI1* Ravi sample 1.35.1, Lane 10 *IntI1* Ravi sample 1.35.2, Lane 11 *IntI1* Ravi sample 1.35.4, Lane 12 *IntI1* Ravi sample 1.35.5, Lane 13 *IntI1* Ravi sample 1.35.6, Lane 14 *IntI1* Ravi sample 1.35.7. Lane 15 *IntI1* Ravi -ve control, Lane 16 *IntI1* Ravi +ve control, Lane 17 100 bp ladder (BioLab).

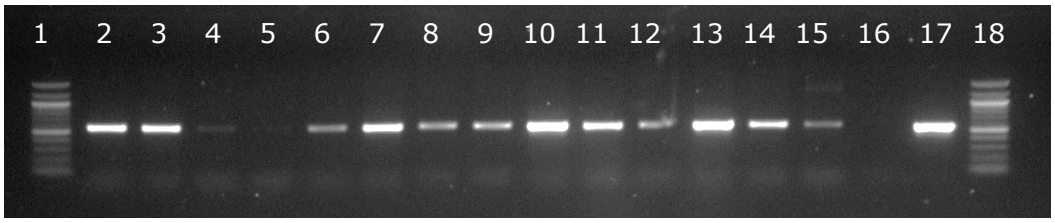


Figure 2.2.3 Agarose gels amplification products of PCRs by using Ravi primers showing target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of GOS supplemented diet (G2) electrophoresed on a 1 % TAE gel

Lane 1 100 bp ladder (BioLab), Lane 2 *intI1* Ravi sample 2.22.1, Lane 3 IntI1 Ravi sample 2.22.2 , Lane 4 *intI1* Ravi sample 2.22.3, Lane 5 *IntI1* Ravi sample 2.22.4, Lane 6 *intI1* Ravi sample 2.22.5, Lane 7 IntI1 Ravi sample 2.22.6, Lane 8 *intI1* Ravi sample 2.22.7, Lane 9 IntI1 Ravi sample 2.24.1, Lane 10 *IntI1* Ravi sample 2.24.2, Lane 11 IntI1 Ravi sample 2.24.3, Lane 12 *IntI1* Ravi sample 2.24.4, Lane 13 IntI1 Ravi sample 2.24.5, Lane 14 *intI1* Ravi sample 2.24.6, Lane 15 IntI1 Ravi sample 2.24.7, Lane 16 *intI1* Ravi -ve control, Lane 17 IntI1 Ravi +ve control, Lane 18 100 bp ladder (BioLab).

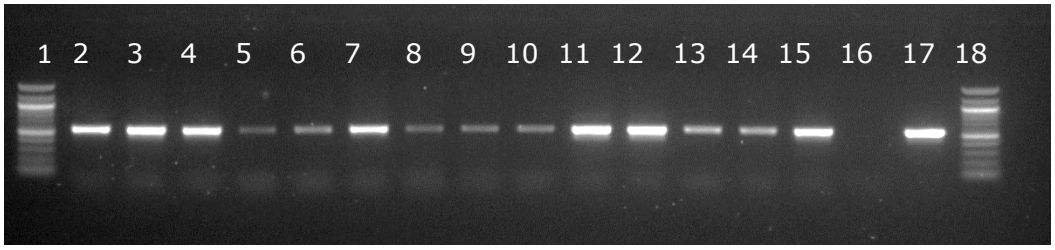


Figure 2.2.4 Agarose gels amplification products of PCRs by using Ravi primers showing target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of G2 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 2.28.1, Lane 3 *IntI1* Ravi sample 2.28.2, Lane 4 *IntI1* Ravi sample 2.28.3, Lane 5 *IntI1* Ravi sample 2.28.4, Lane 6 *IntI1* Ravi sample 2.28.5, Lane 7 *IntI1* Ravi sample 2.28.6, Lane 8 *IntI1* Ravi sample 2.28.7, Lane 9 *IntI1* Ravi sample 2.35.1, Lane 10 *IntI1* Ravi sample 2.35.2, Lane 11 *IntI1* Ravi sample 2.35.3, Lane 12 *IntI1* Ravi sample 2.35.4, Lane 13 *IntI1* Ravi sample 2.35.5, Lane 14 *IntI1* Ravi sample 2.35.6, Lane 15 *IntI1* Ravi sample 2.35.7, Lane 17 IntI1 Ravi -ve control, Lane 18 100 bp ladder (BioLab).

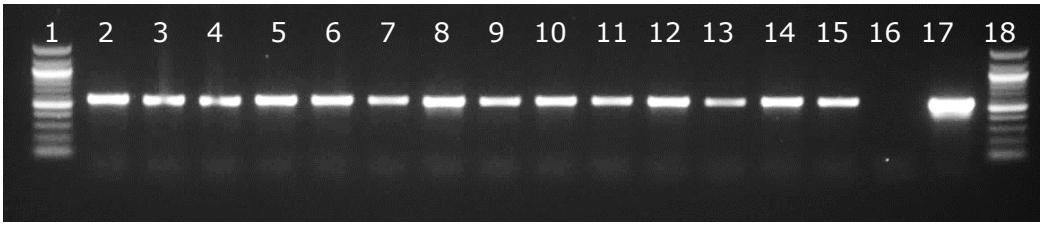


Figure 2.2.5 Agarose gels amplification products of PCRs by using Ravi primers exhibiting target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of control group challenged with *Salmonella* (G3) electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 3.22.1, Lane 3 *IntI1* Ravi sample 3.22.2, Lane 4 *IntI1* Ravi sample 3.22.3, Lane 5 *IntI1* Ravi sample 3.22.4, Lane 6 *IntI1* Ravi sample 3.22.5, Lane 7 *IntI1* Ravi sample 3.22.6, Lane 8 *IntI1* Ravi sample 3.22.7, Lane 9 *IntI1* Ravi sample 3.24.1, Lane 10 *IntI1* Ravi sample 3.24.2, Lane 11 *IntI1* Ravi sample 3.24.3, Lane 12 *IntI1* Ravi sample 3.24.4, Lane 13 *IntI1* Ravi sample 3.24.5, Lane 14 *IntI1* Ravi sample 3.24.6, Lane 15 *IntI1* Ravi sample 3.24.7, Lane 16 *IntI1* Ravi -ve control, Lane 17 *IntI1* Ravi +ve control, Lane 18 100 bp ladder (BioLab).

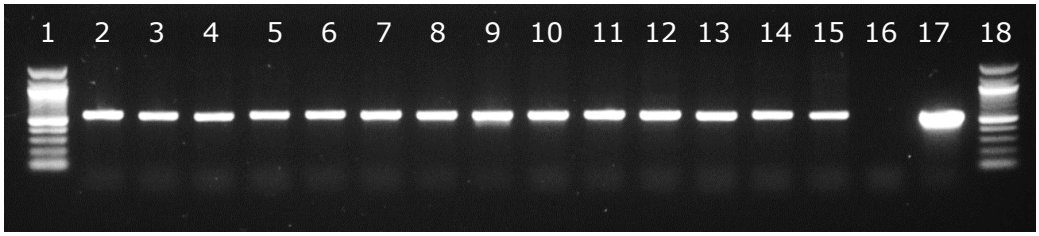


Figure 2.2.6 Agarose gels amplification products of PCRs by using Ravi primers showing target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of G3 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 IntI1 Ravi sample 3.28.1, Lane 3 IntI1 Ravi sample 3.28.2, Lane 4 IntI1 Ravi sample 3.28.3, Lane 5 IntI1 Ravi sample 3.28.4, Lane 6 IntI1 Ravi sample 3.28.5, Lane 7 IntI1 Ravi sample 3.28.6, Lane 8 IntI1 Ravi sample 3.28.7, Lane 9 IntI1 Ravi sample 3.35.1, Lane 10 IntI1 Ravi sample 3.35.2, Lane 11 IntI1 Ravi sample 3.35.3, Lane 12 IntI1 Ravi sample 3.35.4, Lane 13 IntI1 Ravi sample 3.35.5, Lane 14 IntI1 Ravi sample 3.35.6, Lane 15 IntI1 Ravi sample 3.35.7, Lane 17 IntI1 Ravi -ve control, Lane 17 IntI1 Ravi +ve control.

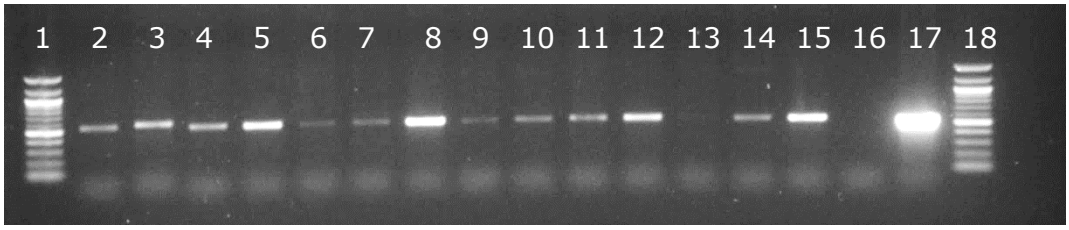


Figure 2.2.7 Agarose gels amplification products of PCRs by using Ravi primers showing target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of GOS supplementary dietary challenged by *Salmonella* (G4) electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 4.22.1, Lane 3 *IntI1* Ravi sample 4.22.2, Lane 4 *IntI1* Ravi sample 4.22.3, Lane 5 *IntI1* Ravi sample 4.22.4, Lane 6 I*ntI1* Ravi sample 4.22.5, Lane 7 *IntI1* Ravi sample 4.22.6, Lane 8 *IntI1* Ravi sample 4.22.7, Lane 9 *IntI1* Ravi sample 4.24.1, Lane 10 *IntI1* Ravi sample 4.24.2, Lane 11 *IntI1* Ravi sample 4.24.3, Lane 12 *IntI1* Ravi sample 4.24.4, Lane 13 *IntI1* Ravi sample 4.24.5, Lane 14 *IntI1* Ravi sample 4.24.6, Lane 15 *IntI1* Ravi sample 4.24.7, Lane 16 *IntI1* Ravi -ve control, Lane 17 *IntI1* Ravi +ve control, Lane 18 100 bp ladder (BioLab).

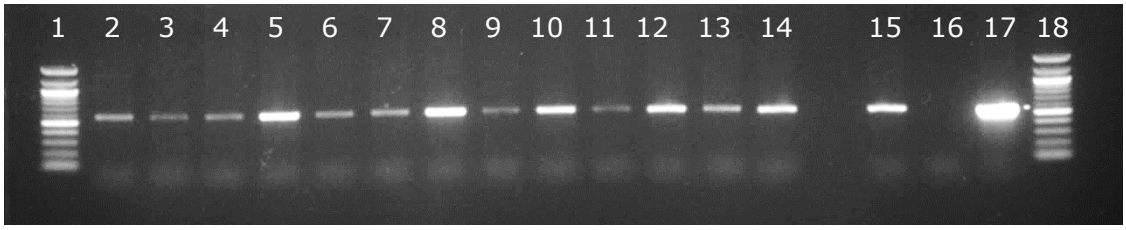


Figure 2.2.8 Agarose gels amplification products of PCRs by using Ravi primers showing target genes the 546 bp PCRs products of class 1 integrase gene (*intI1* Ravi, Chapter 2 Table 2.4) obtained from biosecure condition chicken caecal DNA samples of G4 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 4.28.1, Lane 3 *IntI1* Ravi sample 4.28.2, Lane 4 *IntI1* Ravi sample 4.28.3, Lane 5 *IntI1* Ravi sample 4.28.4, Lane 6 *IntI1* Ravi sample 4.28.5, Lane 7 *IntI1* Ravi sample 4.28.6, Lane 8 *IntI1* Ravi sample 4.28.7 , Lane 9 *IntI1* Ravi sample 4.35.1, Lane 10 *IntI1* Ravi sample 4.35.2, Lane 11 *IntI1* Ravi sample 4.35.3, Lane 12 *IntI1* Ravi sample 4.35.4, Lane 13 *IntI1* Ravi sample 4.35.5, Lane 14 *IntI1* Ravi sample 4.35.6, Lane 15 *IntI1* Ravi sample 4.35.7, Lane 16 *IntI1* Ravi -ve control, Lane 17 *IntI1* Ravi +ve control, Lane 18 100 bp ladder (BioLab).

Appendix 2.3 The PCR ampilicon product of class 1 integron (*intI1* H primer) of biosecure birds

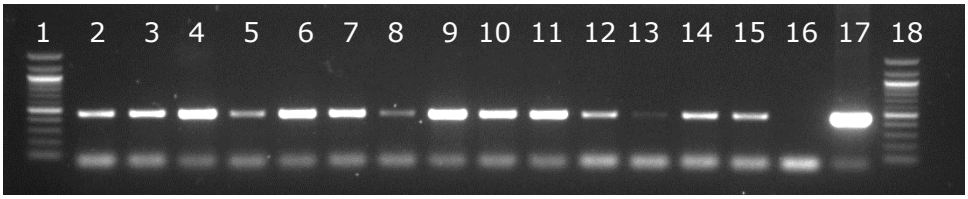


Figure 2.3.1 Agarose gels amplification products of PCRs by using *intI1* HS464/HS463a primers (Chapter 2 Table 2.4) showing target genes the 471 bp PCRs products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples of G1 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* H sample 1.22.1, Lane 3 *IntI*1 H sample 1.22.2, Lane 4 *IntI1* H sample 1.22.3, Lane 5 *IntI1* H sample 1.22.4, Lane 6 *IntI1* H sample 1.22.5, Lane 7 *IntI1* H sample 1.22.6, Lane 8 *IntI1* H sample 1.22.7, Lane 9 *IntI1* H sample 1.24.1, Lane 10 *IntI1* H sample 1.24.2, Lane 11 *IntI1* H sample 1.24.3, Lane 12 *IntI1* H sample 1.24.4, Lane 13 *IntI1* H sample 1.24.5, Lane 14 *IntI1* H sample 1.24.6, Lane 15 *IntI1* H sample 1.24.7, Lane 16 *IntI1* H -ve control, Lane 17 *IntI1* H +ve control, Lane 18 100 bp ladder (BioLab).

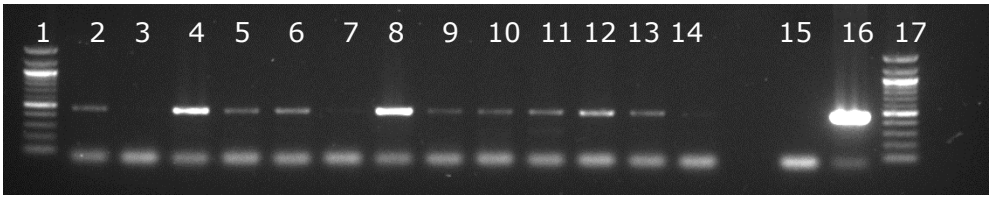


Figure 2.3.2 Agarose gels amplification products of PCRs by using *intI1* HS464/HS463a primers showing target genes the 471 bp PCRs products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples of G1 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* H sample 1.28.1, Lane 3 *IntI*1 H sample 1.28.2, Lane 4 *IntI1* H sample 1.28.3, Lane 5 *IntI1* H sample 1.28.4, Lane 6 *IntI1* H sample 1.28.5, Lane 7 *IntI1* H sample 1.28.6, Lane 8 *IntI1* H sample 1.28.7, Lane 9 *IntI1* H sample 1.35.1, Lane 10 *IntI1* H sample 1.35.2, Lane 11 *IntI1* H sample 1.35.4, Lane 12, *IntI1* H sample 1.35.5, Lane 13 *IntI1* H sample 1.35.6, Lane 14 *IntI1* H sample 1.35.7, Lane 15 *IntI1* H -ve control, Lane 16 *IntI1* H +ve control, Lane 17100 bp ladder (BioLab).

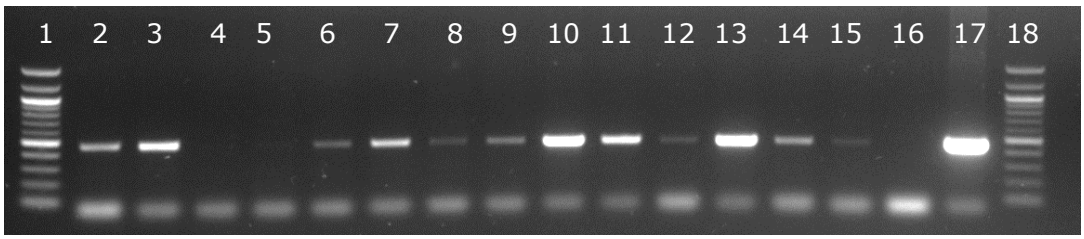


Figure 2.3.3 Agarose gels amplification products of PCRs by using *intI1* HS464/HS463a primers showing target genes the 471 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples of G2 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* H sample 2.22.1, Lane 3 *IntI*1 H sample 2.22.2, Lane 4 *IntI1* H sample 2.22.3, Lane 5 *IntI1* H sample 2.22.4, Lane 6 *IntI1* HS464 sample 2.22.5, Lane 7 *IntI1* H sample 2.22.6, Lane 8 *IntI1* H sample 2.22.7, Lane 9 *IntI1* H sample 2.24.1, Lane 10 *IntI1* H sample 2.24.2, Lane 11 *IntI1* H sample 2.24.3, Lane 12 *IntI1* H sample 2.24.4, Lane 13 *IntI1* H sample 2.24.5, Lane 14 *IntI1* H sample 2.24.6, Lane 15 *IntI1* H sample 2.24.7, Lane 16 *IntI1* H -ve control, Lane 17 *IntI1* H +ve control, Lane 18 100 bp ladder (BioLab).

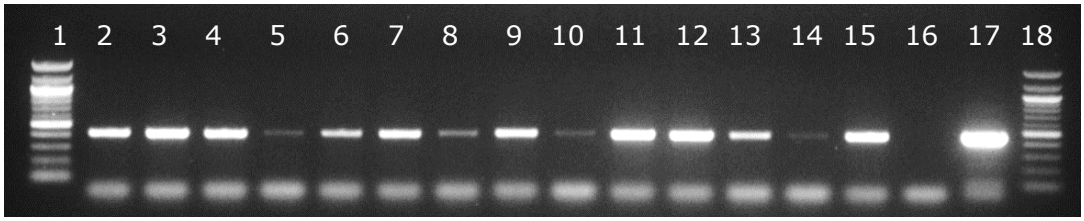


Figure 2.3.4 Agarose gels amplification products of PCRs by using *intI1* HS464/HS463a primers showing target genes the 471 bp PCRs products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples of G2 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* H sample 2.28.1, Lane 3 *IntI1* H sample 2.28.2, Lane 4 *IntI1* H sample 2.28.3, Lane 5 *IntI1* H sample 2.28.4, Lane 6 *IntI1* H sample 2.28.5, Lane 7 *IntI1* H sample 2.28.6, Lane 8 *IntI1* H sample 2.28.7, Lane 9 *IntI1* H sample 2.35.1, Lane 10 *IntI1* H sample 2.35.2, Lane 11 *IntI1* H sample 2.35.3, Lane 12 *IntI1* H sample 2.35.4, Lane 13 *IntI1* H sample 2.35.5, Lane 14 *IntI1* H sample 2.35.6, Lane 15 *IntI1* H sample 2.35.7, Lane 16 *IntI1* H -ve control, Lane 17 *IntI1* H +ve control, Lane 18 100 bp ladder (BioLab).

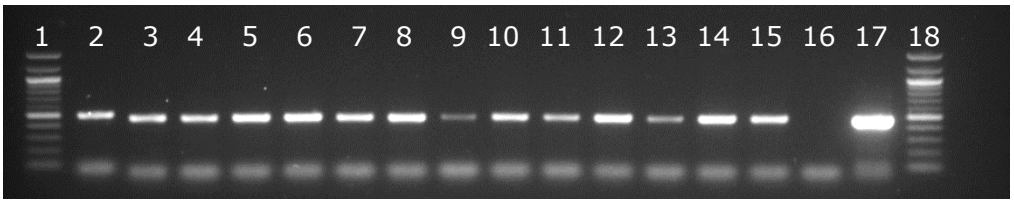


Figure 2.3.5 Agarose gels amplification products of PCRs by using *intl1* HS464/HS463a primers showing target genes the 471 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples G3 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* H sample 3.22.1, Lane 3 *IntI*1 H sample 3.22.2, Lane 4 *IntI1* H sample 3.22.3, Lane 5 *IntI1* H sample 3.22.4, Lane 6 *IntI1* H sample 3.22.5, Lane 7 *IntI1* H sample 3.22.6, Lane 8 *IntI1* H sample 3.22.7, Lane 9 *IntI1* H sample 3.24.1 Lane 10 *IntI1* H sample 3.24.2, Lane 11 *IntI1* H sample 3.24.3, Lane 12 *IntI1* H sample 3.24.4, Lane 13 *IntI1* H sample 3.24.5, Lane 14 *IntI1* H sample 3.24.6, Lane 15 *IntI1* H sample 3.24.7, Lane 16 *IntI1* H -ve control, Lane 17 *IntI1* H +ve control, Lane 18 100 bp ladder (BioLab).

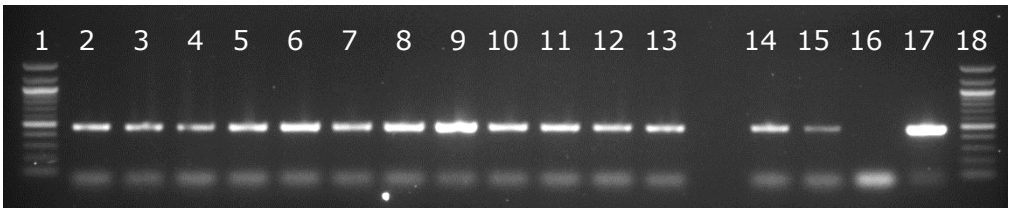


Figure 2.3.6 Agarose gels amplification products of PCRs by using *intl1* HS464/HS463a primers showing target genes the 471 bp PCRs products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples of G3 electrophoresed on a 1 % TAE gel

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* H sample 3.28.1, Lane 3 *IntI1* H sample 3.28.2, Lane 4 *IntI1* H sample 3.28.3, Lane 5 *IntI1* H sample 3.28.4, Lane 6 *IntI1* H sample 3.28.5, Lane 7 *IntI1* H sample 3.28.6, Lane 8 *IntI1* H sample 3.28.7, Lane 9 *IntI1* H sample 3.35.1 Lane 10 *IntI1* H sample 3.35.2, Lane 11 *IntI1* H sample 3.35.3, Lane 12 *IntI1* H sample 3.35.4, Lane 13 *IntI1* H sample 3.35.5, Lane 14 *IntI1* H sample 3.35.6, Lane 15 *IntI1* H sample 3.35.7, Lane 16 *IntI1* H -ve control, Lane 17 *IntI1* H +ve control, Lane 18 100 bp ladder (BioLab).

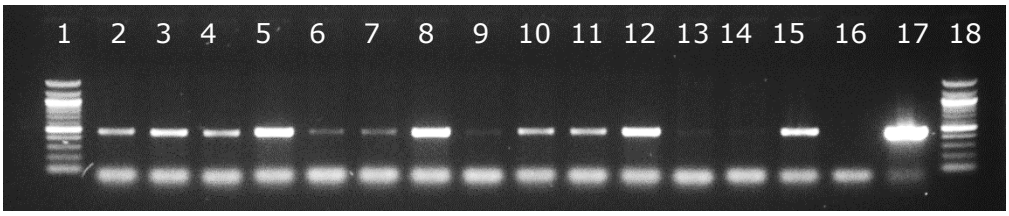


Figure 2.3.7 Agarose gels amplification products of PCRs by using *intI1* HS464/HS463a primers showing target genes the 471 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples G4 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* HS464 sample 4.22.1, Lane 3 *IntI*1 H sample 4.22.2, Lane 4 *IntI1* H sample 4.22.3, Lane 5 *IntI1* H sample 4.22.4, Lane 6 *IntI1* H sample 4.22.5, Lane 7 *IntI1* H sample 4.22.6, Lane 8 *IntI1* H sample 4.22.7, Lane 9 *IntI1* H sample 4.24.1, Lane 10 *IntI1* H sample 4.24.2, Lane 11 *IntI1* H sample 4.24.3, Lane 12 IntI1 H sample 4.24.4, Lane 13 *IntI1* H sample 4.24.5, Lane 14 *IntI1* H sample 4.24.6, Lane 15 *IntI1* H sample 4.24.7, Lane 16 *IntI1* H -ve control, Lane 17 *IntI1* H +ve control, Lane 18 100 bp ladder (BioLab).

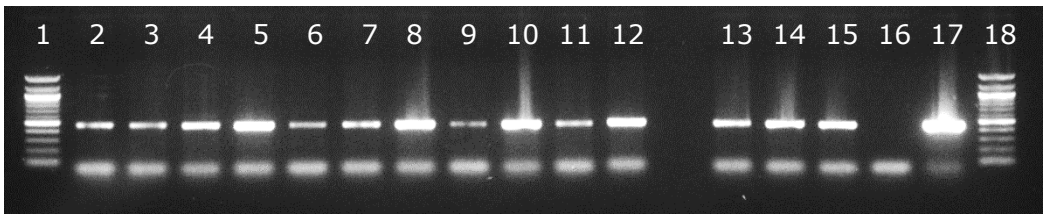


Figure 2.3.8 Agarose gels amplification products of PCRs by using *intI1* HS464/HS463a primers showing target genes the 471 bp PCRs products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples of G4 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* H sample 4.28.1, Lane 3 *IntI1* H sample 4.28.2, Lane 4 *IntI1* H sample 4.28.3, Lane 5 *IntI1* H sample 4.28.4, Lane 6 *IntI1* H sample 4.28.5, Lane 7 *IntI1* H sample 4.28.6, Lane 8 *IntI1* H sample 4.28.7, Lane 9 *IntI1* H sample 4.35.1 Lane 10 *IntI1* H sample 4.35.2, Lane 11 *IntI1* H sample 4.35.3, Lane 12 *IntI1* H sample 4.35.4, Lane 13 *IntI1* H sample 4.35.5, Lane 14 *IntI1* H sample 4.35.6, Lane 15 *IntI1* H sample 1.35.7, Lane 16 *IntI1* H -ve control, Lane 17 *IntI1* H +ve control, Lane 18 100 bp ladder (BioLab).

Appendix 2.4 The PCR ampilicon product of class 1 integron (Clinical integrons gene, *intI1* C) of biosecure birds

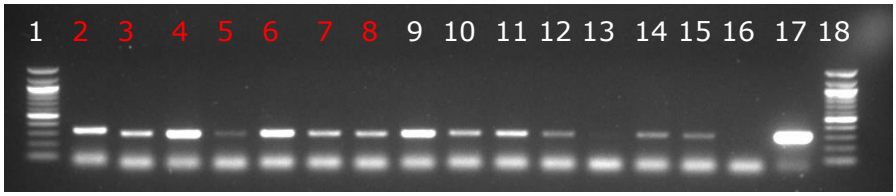


Figure 2.4.1 Agarose gels amplification products of PCRs by using *intI1* clinical primers (F165/R476, Chapter 2, Table 2.4) showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples G1 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 1.22.1, Lane 3 *IntI1 C* sample 1.22.2, Lane 4 *IntI1 C* sample 1.22.3, Lane 5 *IntI1 C* sample 1.22.4, Lane 6 *IntI1 C* sample 1.22.5, Lane 7 *IntI1 C* sample 1.22.6, Lane 8 *IntI1 C* sample 1.22.7, Lane 9 *IntI1 C* sample 1.24.1, Lane 10 *IntI1 C* sample 1.24.2, Lane 11 *IntI1 C* sample 1.24.3, Lane 12 *IntI1 C* sample 1.24.4, Lane 13 *IntI1 C* sample 1.24.5, Lane 14 *IntI1 C* sample 1.24.6, Lane 15 *IntI1 C* sample 1.24.7, Lane 16 *IntI1 C*-ve control, Lane 17 *IntI1 C* +ve control, Lane 18 100 bp ladder (BioLab).

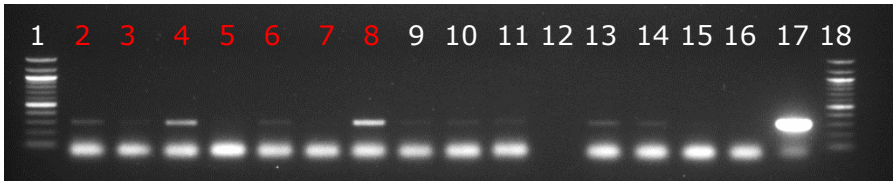


Figure 2.4.2 Agarose gels amplification products of PCRs by using *intI1* clinical primers (F165/R476, Chapter 2, Table 2.4) showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples of G1 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 1.28.1, Lane 3 *IntI1 C* sample 1.28.2, Lane 4 *IntI1 C* sample 1.28.3, Lane 5 *IntI1 C* sample 1.28.4, Lane 6 *IntI1 C* sample 1.28.5, Lane 7 *IntI1 C* sample 1.28.6, Lane 8 *IntI1 C* sample 1.28.7, Lane 9 *IntI1 C* sample 1.35.1, Lane 10 *IntI1 C* sample 1.35.2, Lane 11 *IntI1 C* sample 1.35.4, Lane 12 *IntI1 C* sample 1.35.5, Lane 13 *IntI1 C* sample 1.35.6, Lane 14 *IntI1 C* sample 1.35.7, Lane 15 *IntI1 C*+ve control, Lane 16 *IntI1 C*-ve control, Lane 17 100 bp ladder (BioLab).

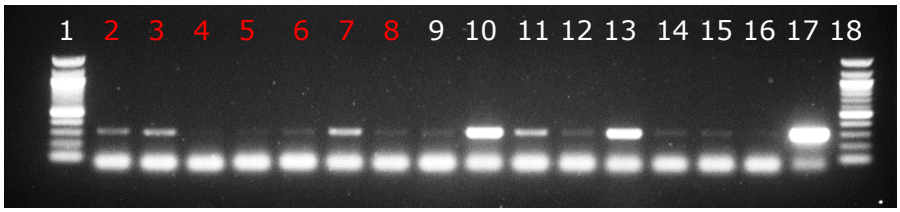


Figure 2.4.3 Agarose gels amplification products of PCRs by using *intI1* clinical primers (F165/R476, Chapter 2, Table 2.4) showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples of G2 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 2.22.1, Lane 3 *IntI1 C* sample 2.22.2, Lane 4 *IntI1 C* sample 2.22.3, Lane 5 *IntI1 C* sample 2.22.4, Lane 6 *IntI1 C* sample 2.22.5, Lane 7 *IntI1 C* sample 2.22.6, Lane 8 *IntI1 C* sample 2.22.7, Lane 9 *IntI1 C* sample 2.24.1, Lane 10 *IntI1 C* sample 2.24.2, Lane 11 *IntI1 C* sample 2.24.3, Lane 12 *IntI1 C* sample 2.24.4, Lane 13 *IntI1 C* sample 2.24.5, Lane 14 *IntI1 C* sample 2.24.6, Lane 15 *IntI1 C* sample 2.24.7, Lane 16 *IntI1 C*-ve control, Lane 17 *IntI1 C* +ve control, Lane 18 100 bp ladder (BioLab).

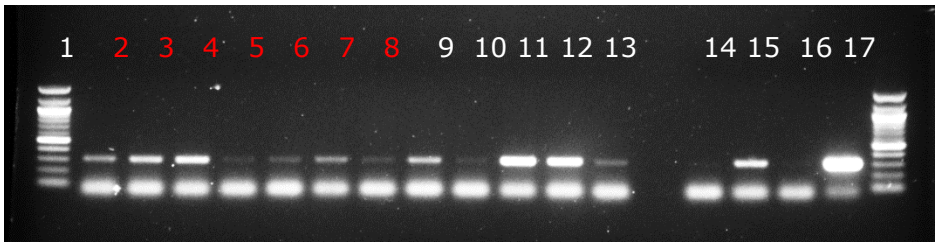


Figure 2.4.4 Agarose gels amplification products of PCRs by using *intI1* clinical primers (F165/R476, Chapter 2, Table 2.4) showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples of G2 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 2.28.1, Lane 3 *IntI1 C* sample 2 .28.2, Lane 4 *IntI1 C* sample 2.28.3, Lane 5 *IntI1 C* sample 2.28.4, Lane 6 *IntI1 C* sample 2.28.5, Lane 7 *IntI1 C* sample 2.28.6, Lane 8 *IntI1 C* sample 2.28.7, Lane 9 *IntI1 C* sample 2.35.1, Lane 10 *IntI1 C* sample 2.35.2, Lane 11 *IntI1 C* sample 2.35.4, Lane 12 *IntI1 C* sample 2.35.5, Lane 13 *IntI1 C* sample 2.35.6, Lane 14 *IntI1 C* sample 2.35.7, Lane 15 *IntI1 C*+ve control, Lane 16 *IntI1 C*-ve control, Lane 17 100 bp ladder (BioLab).

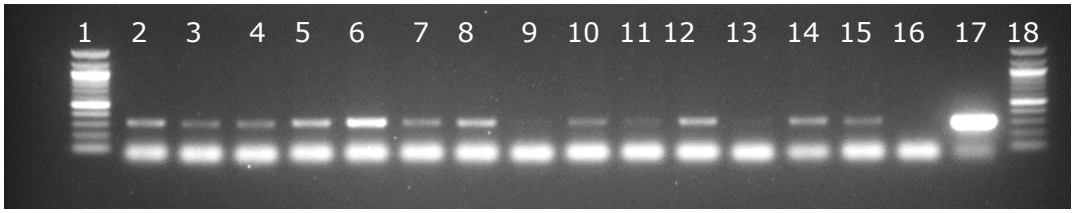


Figure 2.4.5 Agarose gels amplification products of PCRs by using *intI1* clinical primers (F165/R476, Chapter 2, Table 2.4) showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples G3 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 3.22.1, Lane 3 *IntI1 C* sample 3.22.2, Lane 4 *IntI1 C* sample 3.22.3, Lane 5 *IntI1 C* sample 3.22.4, Lane 6 *IntI1 C* sample 3.22.5, Lane 7 *IntI1 C* sample 3.22.6, Lane 8 *IntI1 C* sample 3.22.7, Lane 9 *IntI1 C* sample 3.24.1, Lane 10 *IntI1 C* sample 3.24.2, Lane 11 *IntI1 C* sample 3.24.3, Lane 12 *IntI1 C* sample 3.24.4, Lane 13 *IntI1 C* sample 3.24.5, Lane 14 *IntI1 C* sample 3.24.6, Lane 15 *IntI1 C* sample 3.24.7, Lane 16 *IntI1 C*-ve control, Lane 17 *IntI1 C* +ve control, Lane 18 100 bp ladder (BioLab).

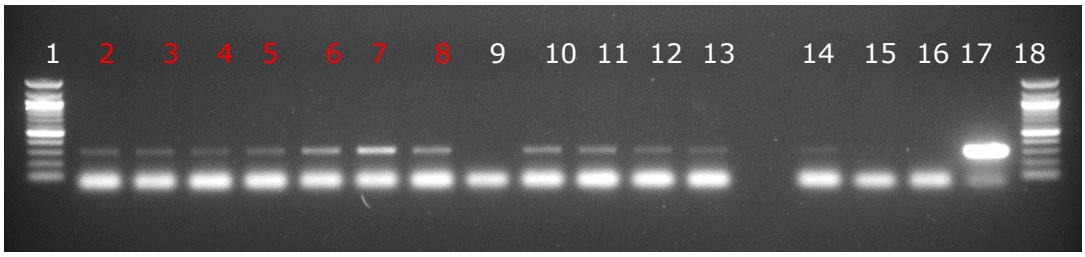


Figure 2.4.6 Agarose gels amplification products of PCRs by using *intI1* clinical primers showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples G3 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 3.28.1, Lane 3 *IntI1 C* sample 3.28.2, Lane 4 *IntI1 C* sample 3.28.3, Lane 5 *IntI1 C* sample 3.28.4, Lane 6 *IntI1 C* sample 3.28.5, Lane 7 *IntI1 C* sample 3.28.6, Lane 8 *IntI1 C* sample 3.28.7, Lane 9 *IntI1 C* sample 3.35.1, Lane 10 *IntI1 C* sample 3.35.2, Lane 11 *IntI1 C* sample 3.35.4, Lane 12 *IntI1 C* sample 3.35.5, Lane 13 *IntI1 C* sample 3.35.6, Lane 14 *IntI1 C* sample 3.35.7, Lane 15 *IntI1 C*+ve control, Lane 16 *IntI1 C*-ve control, Lane 17 100 bp ladder (BioLab).

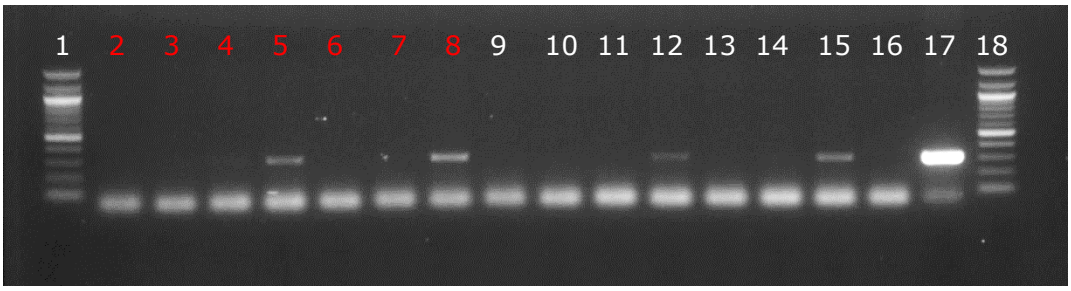


Figure 2.4.7 Agarose gels amplification products of PCRs by using *intI1* clinical primers showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from biosecure condition chicken caecal DNA samples G4 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 4.22.1, Lane 3 *IntI1 C* sample 4.22.2, Lane 4 *IntI1 C* sample 4.22.3, Lane 5 *IntI1 C* sample 4.22.4, Lane 6 *IntI1 C* sample 4.22.5, Lane 7 *IntI1 C* sample 4.22.6, Lane 8 *IntI1 C* sample 4.22.7, Lane 9 *IntI1 C* sample 4.24.1, Lane 10 *IntI1 C* sample 4.24.2, Lane 11 *IntI1 C* sample 4.24.3, Lane 12 *IntI1 C* sample 4.24.4, Lane 13 *IntI1 C* sample 4.24.5, Lane 14 *IntI1 C* sample 4.24.6, Lane 15 *IntI1 C* sample 4.24.7, Lane 16 *IntI1 C*-ve control, Lane 17 *IntI1 C* +ve control, Lane 18 100 bp ladder (BioLab).

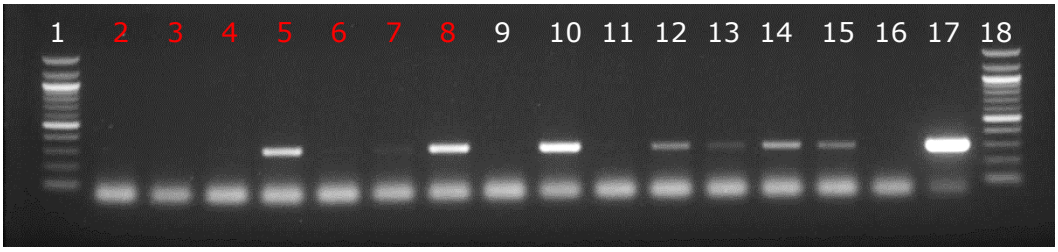


Figure 2.4.8 Agarose gels amplification products of PCRs by using *intI1* clinical primers showing target genes the 311 bp PCRs products of class 1 integrase gene (obtained from Biosecure condition chicken caecal DNA samples G4 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1 C* sample 4.28.1, Lane 3 *IntI1 C* sample 4.28.2, Lane 4 *IntI1 C* sample 4.28.3, Lane 5 *IntI1 C* sample 4.28.4, Lane 6 *IntI1 C* sample 4.28.5, Lane 7 *IntI1 C* sample 4.28.6, Lane 8 *IntI1 C* sample 4.28.7, Lane 9 *IntI1 C* sample 4.35.1, Lane 10 *IntI1 C* sample 4.35.2, Lane 11 *IntI1 C* sample 4.35.4, Lane 12 *IntI1 C* sample 4.35.5, Lane 13 *IntI1 C* sample 4.35.6, Lane 14 *IntI1 C* sample 4.35.7, Lane 15 *IntI1 C*+ve control, Lane 16 *IntI1 C*-ve control, Lane 17 100 bp ladder (BioLab).

Appendix 2.5 Sulfonamides resistance gene (*sul1*) of biosecure birds

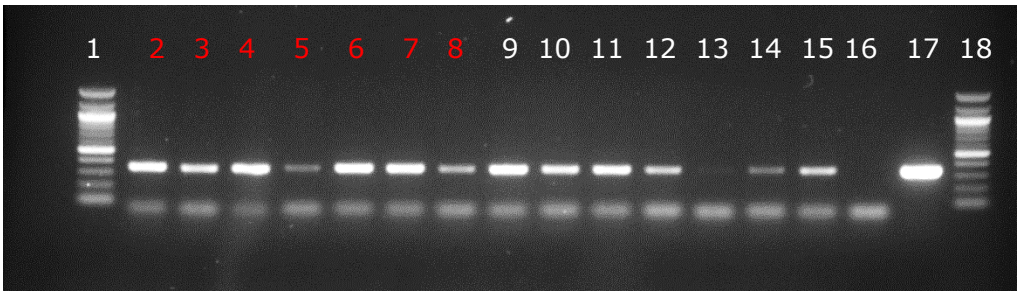


Figure 2.5.1 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene was 364 bp PCR products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G1 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 1.22.1, Lane 3 *sul1* sample 1.22.2, Lane 4 *sul1* sample 1.22.3, Lane 5 *sul1* sample 1.22.4, Lane 6 *sul1* sample 1.22.5, Lane 7 *sul1* sample 1.22.6, Lane 8 *sul1* sample 1.22.7, Lane 9 *sul1* sample 1.24.1, Lane 10 *sul1* sample 1.24.2, Lane 11 *sul1* sample 1.24.3, Lane 12 *sul1* sample 1.24.4, Lane 13 *sul1* sample 1.24.5, Lane 14 *sul1* sample 1.24.6, Lane 15 *sul1* sample 1.24.7, Lane 16 *sul1*-ve control, Lane 17 *sul1*+ve control, Lane 18 100 bp ladder (BioLab).

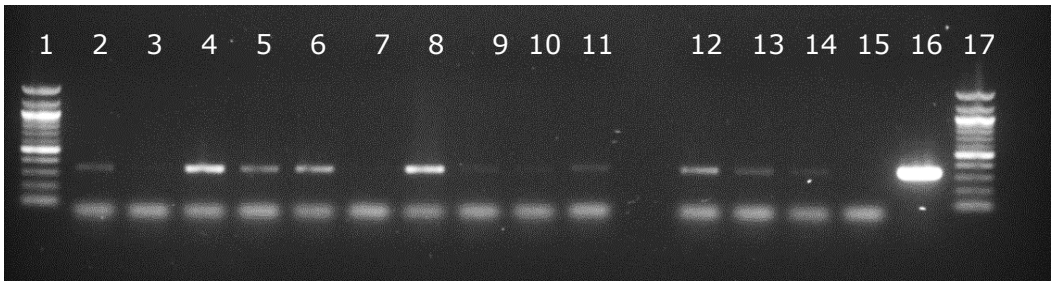


Figure 2.5.2 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene was 364 bp PCR products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G1 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 1.28.1, Lane 3 *sul1* sample 1.28.2, Lane 4 *sul1* sample 1.28.3, Lane 5 *sul1* sample 1.28.4, Lane 6 *sul1* sample 1.28.5, Lane 7 *sul1* sample 1.28.6, Lane 8 *sul1* sample 1.28.7, Lane 9 *sul1* sample 1.35.1, Lane 10 *sul1* sample 1.35.2, Lane 11 *sul1* sample 1.35.4, Lane 12 *sul1* sample 1.35.5, Lane 13 *sul1* sample 1.35.6, Lane 14 *sul1* sample 1.35.7, Lane 15 *sul1*+ve control, Lane 16 *sul1*-ve control, Lane 17 100 bp ladder (BioLab).

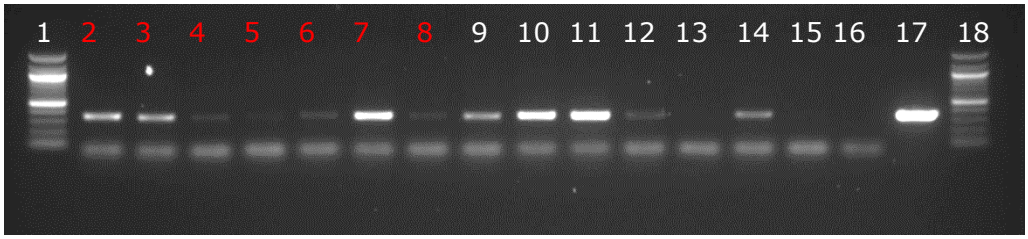


Figure 2.5.3 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene was 364 bp PCR products of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G2 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 2.22.1, Lane 3 *sul1* sample 2.22.2, Lane 4 *sul1* sample 2.22.3, Lane 5 *sul1* sample 2.22.4, Lane 6 *sul1* sample 2.22.5, Lane 7 *sul1* sample 2.22.6, Lane 8 *sul1* sample 2.22.7, Lane 9 *sul1* sample 2.24.1, Lane 10 *sul1* sample 2.24.2, Lane 11 *sul1* sample 2.24.3, Lane 12 *sul1* sample 2.24.4, Lane 13 *sul1* sample 2.24.5, Lane 14 *sul1* sample 2.24.6, Lane 15 *sul1* sample 2.24.7, Lane 16 *sul1*-ve control, Lane 17 *sul1*+ve control, Lane 18 100 bp ladder (BioLab).

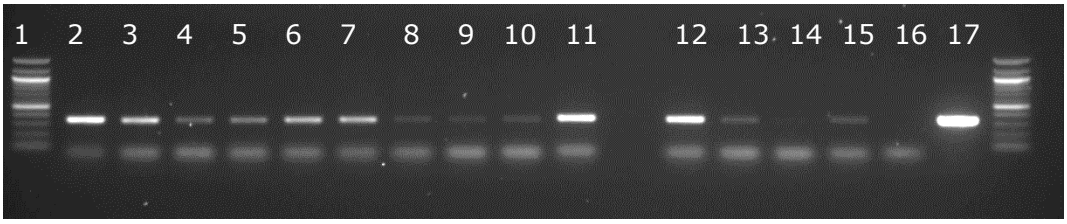


Figure 2.5.4 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene size was 364 bp of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G2 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 2.28.1, Lane 3 *sul1* sample 2.28.2, Lane 4 *sul1* sample 2.28.3, Lane 5 *sul1* sample 2.28.4, Lane 6 *sul1*, sample 2.28.5, Lane 7 *sul1* sample 2.28.6, Lane 8 *sul1* sample 2.28.7, Lane 9 *sul1* sample 2.35.1, Lane 10 *sul1* sample 2.35.2, Lane 11 *sul1* sample 2.35.3, Lane 12 *sul1* sample 2.35.4, Lane 13 *sul1* sample 2.35.5, Lane 14 *sul1* sample 2.35.6, Lane 15 *sul1* sample 1.35.7, Lane 16 *sul1*-ve control, Lane 17 *sul1*+ve control, Lane 18 100 bp ladder (BioLab).

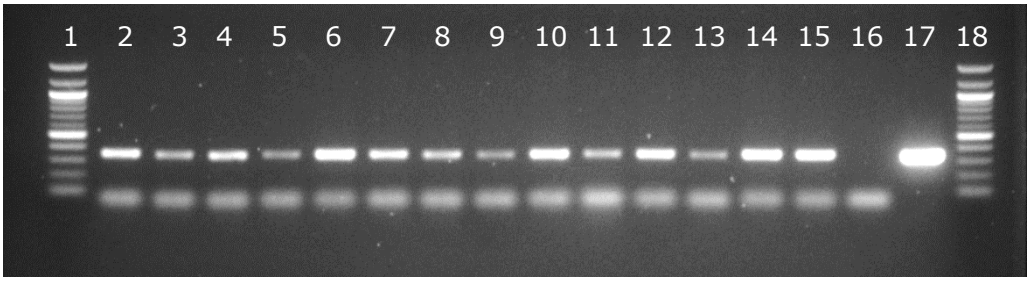


Figure 2.5.5 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene size was 364 bp of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G3 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 3.22.1, Lane 3 *sul1* sample 3.22.2, Lane 4 *sul1* sample 3.22.3, Lane 5 *sul1* sample 3.22.4, Lane 6 *sul1*, sample 3.22.5, Lane 7 *sul1* sample 3.22.6, Lane 8 *sul1* sample 3.22.7, Lane 9 *sul1* sample 3.24.1, Lane 10 *sul1* sample 3.24.2, Lane 11 *sul1* sample 3.24.3, Lane 12 *sul1* sample 3.24.4. Lane 13 *sul1* sample 3.24.5, Lane 14 *sul1* sample 3.24.6, Lane 15 *sul1* sample 3.24.7, Lane 16 *sul1*-ve control, Lane 17 sul1+ve control, Lane 18 100 bp ladder (BioLab).

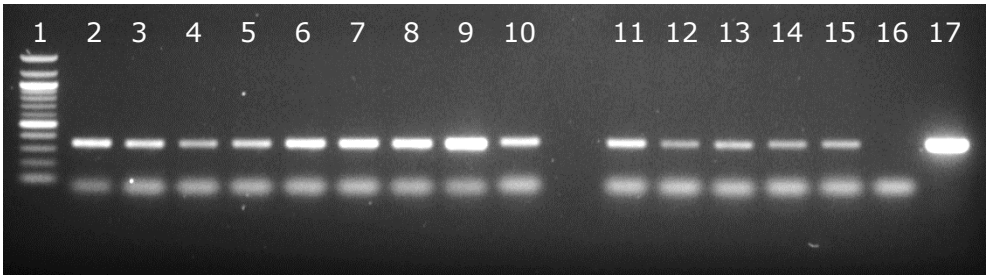


Figure 2.5.6 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene size was 364 bp of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G3 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 3.28.1, Lane 3 *sul1* sample 3.28.2, Lane 4 *sul1* sample 3.28.3, Lane 5 *sul1* sample 3.28.4, Lane 6 *sul1* sample 3.28.5, Lane 7 *sul1* sample 3.28.6, Lane 8 *sul1* sample 3.28.7, Lane 9 *sul1* sample 3.35.1, Lane 10 *sul1* sample 3.35.2, Lane 11 *sul1* sample 3.35.3, Lane 12 *sul1* sample 3.35.4 Lane 13 *sul1* sample 3.35.5, Lane 14 *sul1* sample 3.35.6, Lane 15 *sul1* sample 3.35.7, Lane 16 *sul1*-ve control, Lane 17 sul1+ve control.

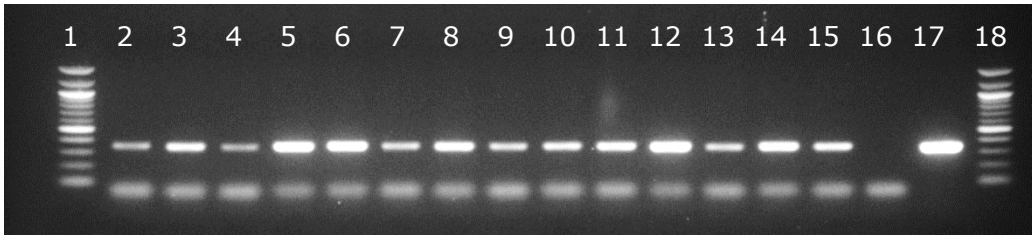


Figure 2.5.7 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene size was 364 bp of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G4 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 4.22.1, Lane 3 *sul1* sample 4.22.2, Lane 4 *sul1* sample 4.22.3, Lane 5 *sul1* sample 4.22.4, Lane 6 *sul1* sample 4.22.5, Lane 7 *sul1* sample 4.22.6, Lane 8 *sul1* sample 4.22.7, Lane 9 *sul1* sample 4.24.1, Lane 10 *sul1* sample 4.24.2, Lane 11 *sul1* sample 4.24.3, Lane 12 *sul1* sample 4.24.4, Lane 13 *sul1* sample 4.24.5, Lane 14 *sul1* sample 4.24.6, Lane 15 *sul1* sample 4.24.7, Lane 16 *sul1*-ve control, Lane 17 *sul1*+ve control, Lane 18 100 bp ladder (BioLab).

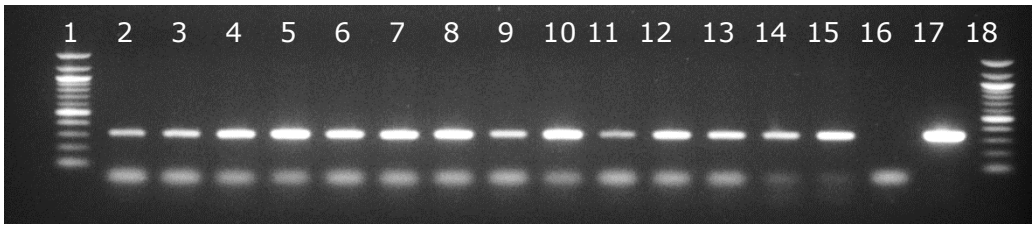


Figure 2.5.8 Agarose gel shows PCRs amplification products of sulphonamides resistance gene at 3 conserved regions. The target gene size was 364 bp of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G4 electrophoresed on a 1 % TAE gel.

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 4.28.1, Lane 3 *sul1* sample 4.28.2, Lane 4 *sul1* sample 4.28.3, Lane 5 *sul1* sample 4.28.4, Lane 6 *sul1* sample 4.28.5, Lane 7 *sul1* sample 4.28.6, Lane 8 *sul1* sample 4.28.7, Lane 9 *sul1* sample 4.35.1, Lane 10 *sul1* sample 4.35.2, Lane 11 *sul1* sample 4.35.3, Lane 12 *sul1* sample 4.35.4, Lane 13 *sul1* sample 4.35.5, Lane 14 *sul1* sample 4.35.6, Lane 15 *sul1* sample 4.35.7, Lane 16 *sul1*-ve control, Lane 17 *sul1*+ve control, Lane 18 100 bp ladder (BioLab).

Appendix 2.6 Quaternary ammonium compounds resistance (*qacE*Δ1) of biosecure birds

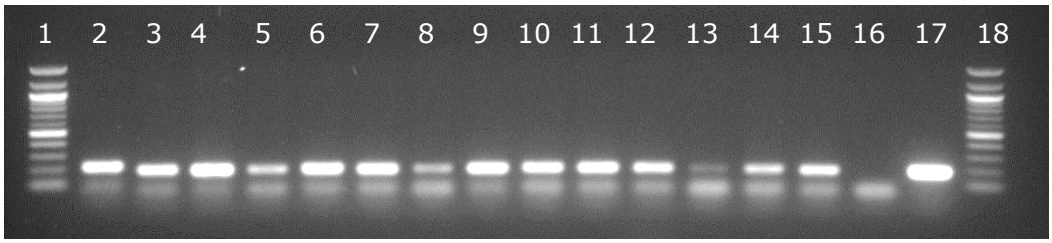


Figure 2.6.1 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G1 electrophoresed on a 1 % TAE gel. The target gene size is 200 bp.

Lane 1 100 bp ladder (BioLab), Lane 2 q*ac*EΔ1 sample 1.22.1, Lane 3 *qacE*Δ1 sample 1.22.2, Lane 4 *qacE*Δ1 sample 1.22.3, Lane 5 *qacE*Δ1 sample 1.22.4, Lane 6 *qacE*Δ1 sample 1.22.5, Lane 7 *qacE*Δ1 sample 1.22.6, Lane 8 *qacE*Δ1, sample 1.22.7, Lane 9 *qacE*Δ1 sample 1.24.1, Lane 10 *qacE*Δ1 sample 1.24.2, Lane 11 *qacE*Δ1 sample 1.24.3, Lane 12 *qacE*Δ1 sample 1.24.4, Lane 13 *qacE*Δ1 sample 1.24.5, Lane 14 *qacE*Δ1 sample 1.24.6, Lane 15 *qacE*Δ1 sample 1.24.7 Lane 16 *qacE*Δ1 -ve control, Lane 17 *qacE*Δ1 +ve control, Lane 18 100 bp ladder (BioLab).

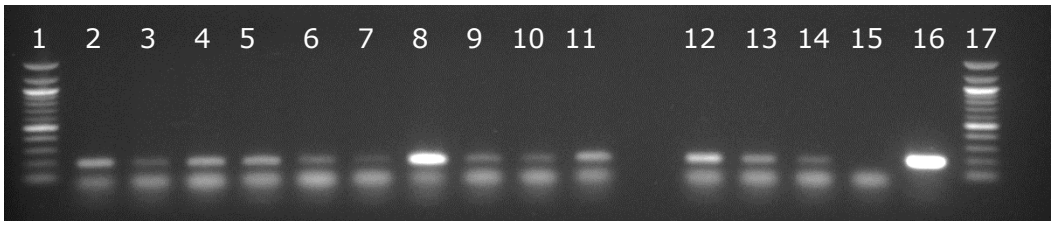


Figure 2.6.2 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G1electrophoresed on a 1 % TAE gel. The target gene size is 200 bp.

Lane 1 100 bp ladder (BioLab), Lane 2 *qacE*Δ1 sample 1.28.1, Lane 3 *qacE*Δ1 sample 1.28.2, Lane 4 *qacE*Δ1 sample 1.28.3, Lane 5 *qacE*Δ1 sample 1.28.4 , Lane 6 qacEΔ1 sample 1.28.5, Lane 7 *qacE*Δ1 sample 1.28.6, Lane 8 *qacE*Δ1 sample 1.28.7, Lane 9 *qacE*Δ1 sample 1.35.1, Lane 10 *qacE*Δ1 sample 1.35.2, Lane 11 *qacE*Δ1 sample 1.35.4, Lane 12 *qacE*Δ1 sample 1.35.5, Lane 13 *qacE*Δ1 sample 1.35.6, Lane 14 *qacE*Δ1 sample 1.35.7, Lane 15 *qacE*Δ1 -ve control, Lane 16 *qacE*Δ1 -ve control, Lane 17 100 bp ladder (BioLab).

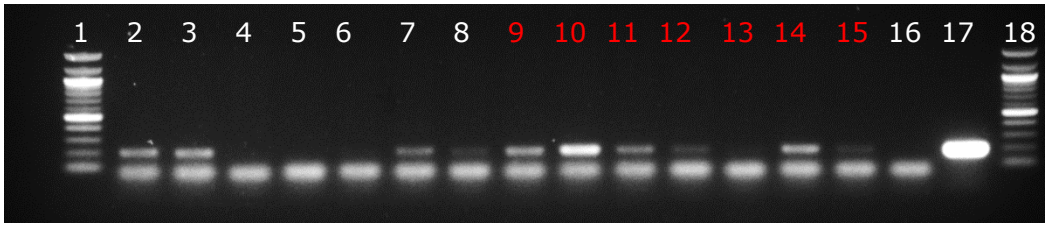


Figure 2.6.3 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G2 electrophoresed on a 1 % TAE gel. The target gene size is 200 bp.

Lane 1 100 bp ladder (BioLab), Lane 2 *qacE*Δ1 sample 2.22.1, Lane 3 *qacE*Δ1 sample 2.22.2, Lane 4 *qacE*Δ1 sample 2.22.3, Lane 5 *qacE*Δ1 sample 2.22.4 Lane 6 *qacE*Δ1 sample 2.22.5, Lane 7 *qacE*Δ1 sample 2.22.6, Lane 8 *qacE*Δ1 sample 2.22.7, Lane 9 *qacE*Δ1 sample 2.24.1, Lane 10 *qacE*Δ1 sample 2.24.2, Lane 11 *qacE*Δ1 sample 2.24.3, Lane 12 *qacE*Δ1 sample 2.24.4, Lane 13 *qacE*Δ1 sample 2.24.5, Lane 14 *qacE*Δ1 sample 2.24.6, Lane 15 *qacE*Δ1 sample 2.24.7, Lane 16 *qacE*Δ1 -ve control, Lane 17 *qacE*Δ1 +ve control, Lane 18 100 bp ladder (BioLab).

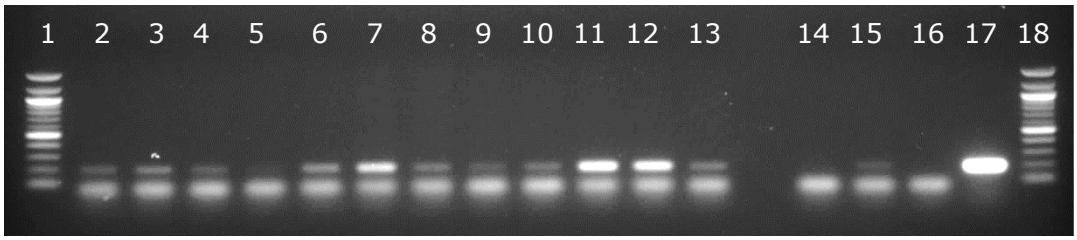


Figure 2.6.4 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G2 electrophoresed on a 1 % TAE gel. The target gene size is 200 bp

Lane 1 100 bp ladder (BioLab), Lane 2 *qacE*Δ1 sample 2.28.1, Lane 3 *qacE*Δ1 sample 2.28.2, Lane 4 *qacE*Δ1 sample 2.28.3, Lane 5 *qacE*Δ1 sample 2.28.4, Lane 6 *qacE*Δ1 sample 2.28.5, Lane 7 *qacE*Δ1 sample 2.28.6, Lane 8 *qacE*Δ1, sample 2.28.7, Lane 9 *qacE*Δ1 sample 2.35.1, Lane 10 *qacE*Δ1 sample 2.35.2, Lane 11 *qacE*Δ1 sample 2.35.3, Lane 12 *qacE*Δ1 sample 2.35.4, Lane 13 *qacE*Δ1 sample 2.35.5, Lane 14 *qacE*Δ1 sample 2.35.6, Lane 15 *qacE*Δ1 sample 2.35.7, Lane 16 *qacE*Δ1 -ve control, Lane 18 100 bp ladder (BioLab).

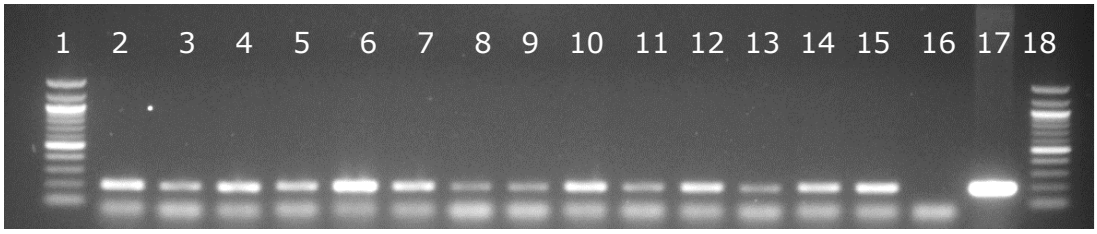


Figure 2.6.5 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G3 electrophoresed on a 1 % TAE gel. The target gene size is 200 bp.

Lane 1 100 bp ladder (BioLab), Lane 2 *qacEΔ1* sample 3.22.1, Lane 3 *qacE*Δ1 sample 3.22.2, Lane 4 *qacEΔ1* sample 3.22.3, Lane 5 *qacE*Δ1 sample 3.22.4, Lane 6 *qacE*Δ1 sample 3.22.5, Lane 7 *qacE*Δ1 sample 3.22.6, Lane 8 *qacE*Δ1 sample 3.22.7, Lane 9 *qacE*Δ1 sample 3.24.1, Lane 10 *qacE*Δ1 sample 3.24.2, Lane 11 *qacE*Δ1 sample 3.24.3, Lane 12 *qacE*Δ1 sample 3.24.4, Lane 13 *qacE*Δ1 sample 3.24.5, Lane 14 *qacE*Δ1 sample 3.24.6, Lane 15 *qacE*Δ1 sample 3.24.7, Lane 16 *qacE*Δ1-ve control, Lane 17 *qacE*Δ1+ve control, Lane 18 100 bp ladder (BioLab).

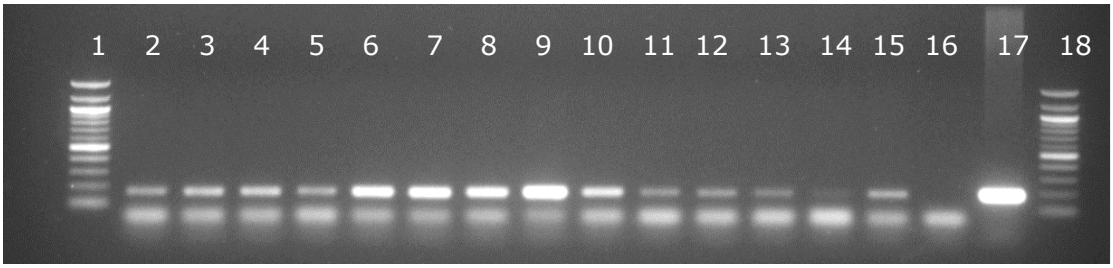


Figure 2.6.6 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G3 electrophoresed on a 1 % TAE gel. The target gene size is 200 bp.

Lane 1 100 bp ladder (BioLab), Lane 2 *qacE*Δ1 sample 3.28.1, Lane 3 *qacEΔ1* sample 3.28.2, Lane 4 *qacE*Δ1 sample 3.28.3, Lane 5 q*acE*Δ1 sample 3.28.4, Lane 6 *qacE*Δ1 sample 3.28.5, Lane 7 *qacE*Δ1 sample 3.28.6, Lane 8 *qacE*Δ1 sample 3.28.7, Lane 9 *qacE*Δ1 sample 3.35.1, Lane 10 *qacE*Δ1 sample 3.35.2, Lane 11 *qacE*Δ1 sample 3.35.3, Lane 12 *qacE*Δ1 sample 3.35.4, Lane 13 *qacE*Δ1 sample 3.35.5, Lane 14 *qacE*Δ1 sample 3.35.6, Lane 15 *qacE*Δ1 sample 3.35.7, Lane 16 *qacE*Δ1 -ve control, Lane 18 100 bp ladder (BioLab).

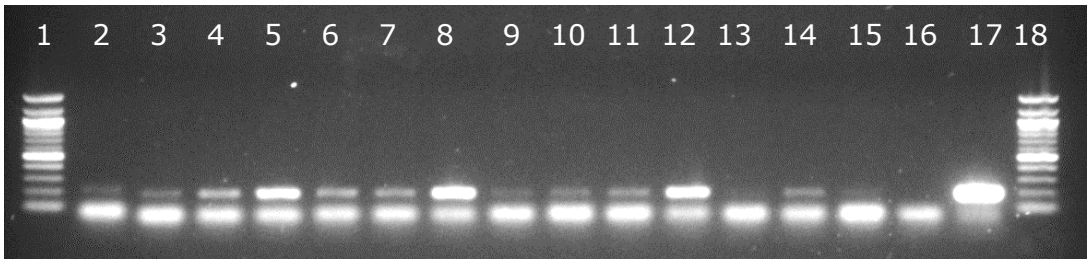


Figure 2.6.7 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G4 electrophoresed on a 1 % TAE gel. The target gene size is 200 bp.

Lane 1 100 bp ladder (BioLab), Lane 2 *qacE*Δ1 sample 4.22.1, Lane 3 *qacEΔ1* sample 4.22.2, Lane 4 *qacE*Δ1 sample 4.22.3, Lane 5 *qacE*Δ1 sample 4.22.4, Lane 6 *qacE*Δ1 sample 4.22.5, Lane 7 *qacE*Δ1 sample 4.22.6, Lane 8 *qacE*Δ1 sample 4.22.7, Lane 9 *qacE*Δ1 sample 4.24.1, Lane 10 *qacE*Δ1 sample 4.24.2, Lane 11 *qacE*Δ1 sample 4.24.3, Lane 12 *qacE*Δ1 sample 4.24.4, Lane 13 *qacE*Δ1 sample 4.24.5, Lane 14 *qacE*Δ1 sample 4.24.6, Lane 15 *qacE*Δ1 sample 4.24.7 Lane 16 *qacE*Δ1 -ve control, Lane 17 *qacE*Δ1 +ve control, Lane 18 100 bp ladder (BioLab).

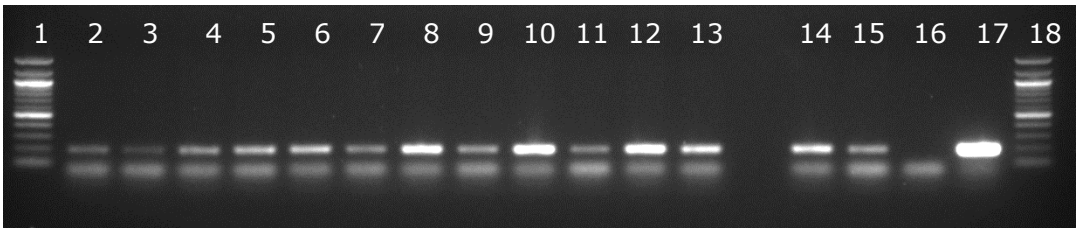


Figure 2.6.8 Agarose gel shows PCRs amplification products of quaternary ammonium compound resistance gene (*qacE*Δ1) at 3 conserved region of class 1 integrase gene obtained from biosecure condition chicken caecal DNA samples G4 electrophoresed on a 1 % TAE gel. The target gene size is 200 bp.

Lane 1 100 bp ladder (BioLab), Lane 2 *qacEΔ1* sample 4.28.1, Lane 3 *qacEΔ1* sample 4.28.2, Lane 4 *qacEΔ1* sample 4.28.3, Lane 5 *qacEΔ1* sample 4.28.4, Lane 6 *qacEΔ1* sample 4.28.5, Lane 7 *qacEΔ1* sample 4.28.6, Lane 8 *qacEΔ1* sample 4.28.7, Lane 9 *qacEΔ1* sample 4.35.1, Lane 10 *qacEΔ1* sample 4.35.2, Lane 11 *qacE*Δ1 sample 4.35.3, Lane 12 *qacEΔ1* sample 4.35.4, Lane 13 *qacE*Δ1 sample 4.35.5, Lane 14 *qacEΔ1* sample 4.35.6, Lane 15 *qacE*Δ1 sample 4.35.7, Lane 16 *qacEΔ1* -ve control, Lane 17 *qacE*Δ1 +ve control, Lane 18 100 bp ladder (BioLab).

Appendix 2.7 Table 2.7.1 The prevalence of conserved genes of class 1 integrons of caecal contents of commercial birds

Sample	feed type	age	*intI1* Ravi	*intI1* EB	*intI1* H	*intl1* C	*sul1*	*qacEΔ1*
1	ctl	30	–	–	–	–	–	+
2	ctl	30	+	–	+	+	+	+
3	ctl	30	+	–	+	+	+	+
4	ctl	30	–	–	+	–	+	+
5	ctl	30	+	+	+	+	+	+
6	ctl	30	+	–	+	–	+	+
7	ctl	30	+	–	+	–	+	+
8	ctl	30	+	+	+	+	+	+
9	ctl	30	+	–	+	+	+	+
10	ctl	30	+	–	+	+	+	+
11	GOS	30	+	–	+	+	+	+
12	GOS	30	+	–	+	+	+	+
13	GOS	30	+	–	–	–	+	+
14	GOS	30	+	+	–	+	+	+
15	GOS	30	+	–	+	+	+	+
16	GOS	30	+	+	+	+	+	+
17	GOS	30	+	–	+	–	+	+
18	GOS	30	+	+	+	+	+	+
19	GOS	30	+	–	+	+	+	+
20	GOS	30	+	+	+	+	+	+
21	GOS	37	+	+	+	+	+	+
22	GOS	37	+	–	+	–	+	+
23	GOS	37	+	+	+	+	+	+
24	GOS	37	+	+	+	+	+	+
25	GOS	37	+	+	+	+	+	+
26	GOS	37	–	–	+	+	+	+
27	GOS	37	+	+	+	+	+	+
28	GOS	37	+	+	+	+	+	+
29	GOS	37	+	+	+	+	+	+
30	GOS	37	+	+	+	+	+	+
31	ctl	37	+	–	+	+	+	+
32	ctl	37	+	–	+	+	+	+
33	ctl	37	+	–	+	+	+	+
34	ctl	37	+	–	+	+	+	+
35	ctl	37	+	–	+	+	+	+
36	ctl	37	+	–	+	–	+	+
37	ctl	37	+	–	+	+	+	+
38	ctl	37	+	–	+	+	+	+
39	ctl	37	+	–	+	–	+	+
40	ctl	37	+	+	+	–	+	+

Appendix 2.8 The PCR ampilicon product of class 1 integron (*intl1* Ravi, *intl1* EB, *sul1* and *qacEΔ1*) of commerical birds (Samples 1-7)

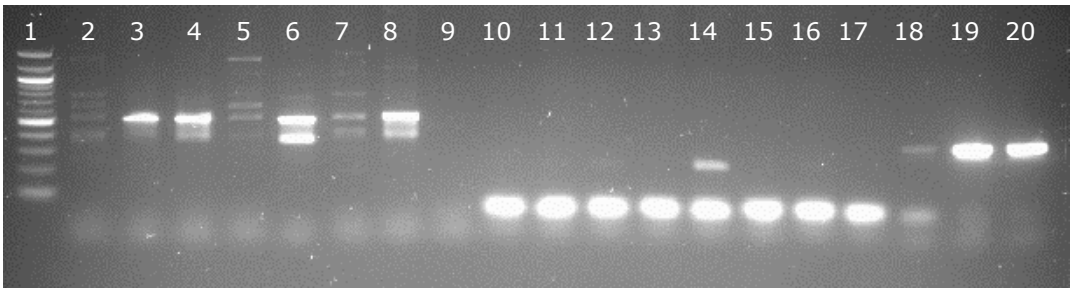


Figure 2.8.1 The PCR amplification using the *IntI1* Ravi (546 bp), *IntI1* EB (254 bp) and *sul1* (346 bp) primers to examine the presence of resistance genes in commercial chicken's caecal DNA (sample 1-7).

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 1, Lane 3 *IntI1* Ravi sample 2, Lane 4 IntI1 Ravi sample 3, Lane 5 *IntI1* Ravi sample 4, Lane 6 *IntI1* Ravi sample 5, Lane 7 *IntI1* Ravi sample 6, Lane 8 *IntI1* Ravi sample 7, Lane 9 *IntI1* Ravi –ve control, Lane 10 *IntI1* EB sample 1, Lane 11 *IntI1* EB sample 2, Lane 12 *IntI1* EB sample 3, Lane 13 *IntI1* EB sample 4, Lane 14 *IntI1* EB sample 5, Lane 15 *IntI1* EB sample 6, Lane 16 *IntI1* EB sample 7, Lane 17 *IntI1* EB +ve control, Lane 18 *sul1* sample 1, Lane 19 *sul1* sample 2, Lane 20 *sul1* sample 3.

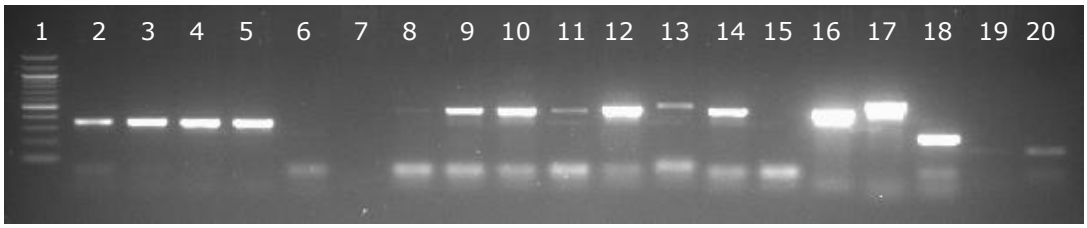


Figure 2.8.2 PCR amplification using the *sul1* (346 bp) and *intl1* H primer (471 bp) to examine the presence of sulphonamides and integrase resistance genes in commercial chicken's caecal DNA (sample 1-7).

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 4, Lane 3 *sul1* sample 5, Lane 4 *sul1* sample 6, Lane 5 *sul1* sample 7, Lane 6 *sul1 S*.T.U288, Lane 7 *sul1* –ve control, Lane 8 *intl1* H sample 1, Lane 9 *intl1* H sample 2, Lane 10 *intl1* H sample 3, Lane 11 *intl1* H sample 4, Lane 12 *intl1* H sample 5, Lane 13 *intl1* H sample 6, Lane 14 *intl1* H sample 7, Lane 15 *intl1* H –ve control, Lane 16 *intl1* H *S*.T.U288, Lane 17 *intl1* Ravi +ve, Lane 18 +ve EB, Lane 19 –ve *qacEΔ1*, Lane 20 +ve control

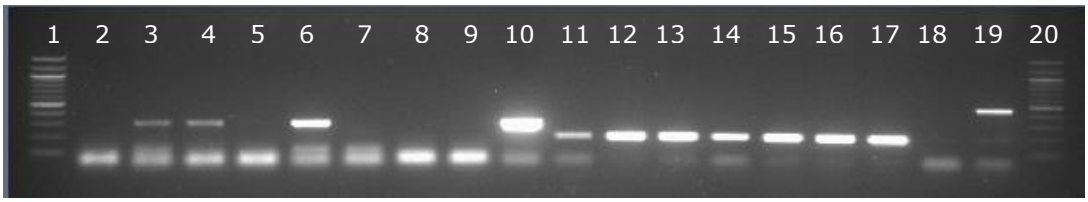


Figure 2.8.3 The PCR amplification using the *intl1 C* (311 bp) and *qacEΔ1* (200 bp) to examine the presence of integrase resistance genes in commercial chicken's caecal DNA (sample 1-7).

Lane 1 100 bp ladder (BioLab*)*, Lane 2 *intl1* C sample 1, Lane 3 *intl* C sample 2, Lane 4 *intl1* C sample 3, Lane 5 *intl1* C sample 4, Lane 6 *intl1* C sample 5*,* Lane 7 *intl* C sample 6, Lane 8 *intl1* C sample 7 Lane 9 *intl1* C –ve, Lane 10 *intl1* C +ve, Lane 11 *qac*EΔ sample 1, Lane 12 *qacEΔ1* sample 2, Lane 13 *qacEΔ1* sample 3, Lane 14 *qacEΔ1* sample 4, Lane 15 *qacEΔ1* sample 5, Lane 16 *qacEΔ1* sample 6, Lane 17 *qacEΔ1* sample 7, Lane 18 *qacEΔ1 S*.T U288, Lane 19 *qacEΔ1* -ve control, Lane 20 100 bp ladder DNA.

Appendix 2.9 The PCR ampilicon product of class 1 integron (*intl1* Ravi, *intl1* EB, *sul1* and *qacEΔ1*) of commerical birds (Samples 8-16)

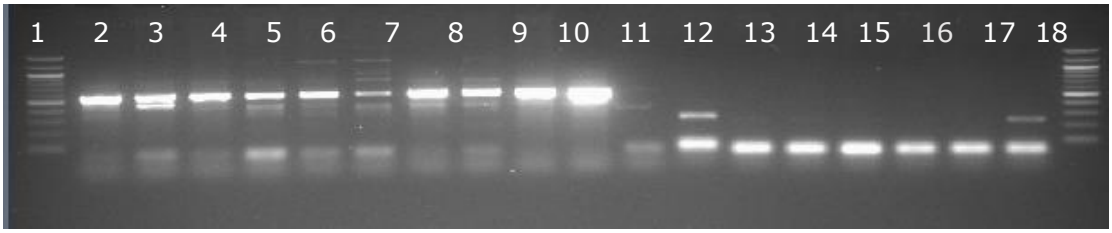


Figure 2.9.1 The PCR amplification using the *IntI1* Ravi (546 bp) and *IntI1* EB (254 bp) primers to examine the presence of resistance genes in commercial chicken's caecal DNA (sample 8-16).

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 8, Lane 3 *IntI1* Ravi sample 9, Lane 4 *IntI1* Ravi sample 10, Lane 5 *IntI1* Ravi sample 11, Lane 6 *IntI1* Ravi sample 12, Lane 7 *IntI1* Ravi sample 13, Lane 8 *IntI1* Ravi sample 14, Lane 9 *IntI1* Ravi sample 15, Lane 10 *IntI1* Ravi sample 16, Lane 11 *IntI1* Ravi +ve control, Lane 12 *IntI1* –ve control, Lane 13 *IntI1* EB sample 8, Lane 14 *IntI1* EB sample 9, Lane 15 *IntI1* EB sample 10, Lane 16 *IntI1* EB sample 11, Lane 17 *IntI1* EB sample 12, Lane 18 *IntI1* EB sample 13, Lane 19 *IntI1* EB sample 14, Lane 20 100 bp ladder (BioLab).

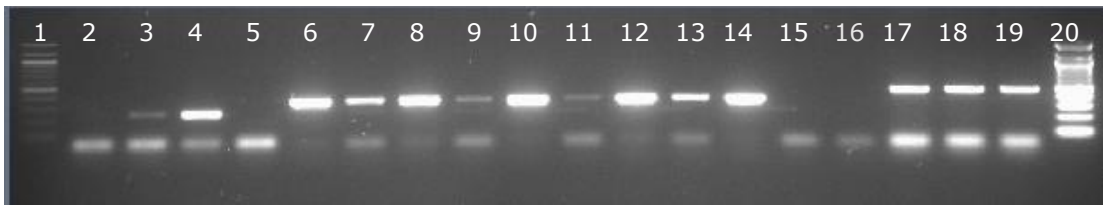


Figure 2.9.2 The PCR amplification using the *IntI1* EB (254 bp), *sul1* (346 bp) and *intl1* H (471 bp) primers to examine the presence resistance genes in commercial chicken's caecal DNA (sample 8-16).

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* EB sample 15, Lane 3 IntI1 EB sample 16, Lane 4 IntI1 EB +ve control, Lane 5 IntI1 EB -ve control, Lane 6 *sul1* sample 8, Lane 7 *sul1* sample 9, Lane 8 *sul1* sample 10, Lane 9 *sul1* sample 11 Lane 10 *sul1* sample 12, Lane 11 *sul1* sample 13, Lane 12 *sul1* sample 14, Lane 13 *sul1* sample 15, Lane 14 *sul1* sample 16, Lane 15 *sul1 S*.T.U 288, Lane 16 *sul1* –ve, Lane 17 *intl1* H sample 8, Lane 18 *intl1* H sample 9, Lane 19 *intl1* H sample 10, Lane 20 100 bp ladder (BioLab).

Figure 2.9.3 The PCR amplification using the *intl1* H (471 bp) and *intl1 C* (311 bp) to examine the presence of integrase resistance genes in commercial chicken's caecal DNA (sample 8-16).

Lane 1 100 bp ladder (BioLab), Lane 2 *intl1* H sample 11, Lane 3 *intl1* H sample 12, Lane 4 *intl1* H sample 13, Lane 5 *intl1* H sample 14, Lane 6 *intl1* H sample 15, Lane 7 *intl1* H sample 16, Lane 8 *intl1* H *S*.T.U288 (+ve control), Lane 9 *intl1* H -ve control, 10 empty, Lane 11 *intl* C sample 8, Lane 12 *intl* C sample 9, Lane 13 *intl* C sample 10, Lane 14 *int1l* C sample 11, Lane 15 *intl* C sample 12, Lane 16 *intl* C sample 13, Lane 17 *int1l* C sample 14, Lane 18 *intl* C sample 15, Lane 19 *intl* C sample 16, Lane 20 100 bp ladder (BioLab).

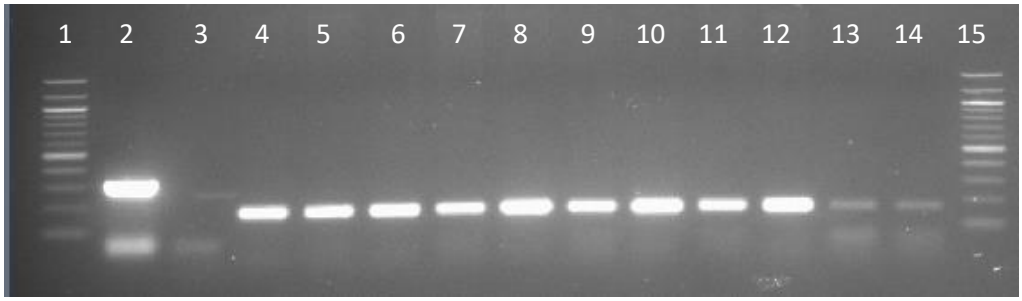


Figure 2.9.4 The PCR amplification using the *intI1 C* (311 bp) and *qacEΔ1* (200 bp) to examine the presence of integrase resistance genes in commercial chicken's caecal DNA (sample 8-16).

Lane 1 100 bp ladder (BioLab), Lane 2 *intI* C +ve , Lane 3 *intI* C –ve, Lane 4 *qac*EΔ sample 8, Lane 5 *qacEΔ1*sample 9, Lane 6 *qacEΔ1 sample* 10, Lane 7 *qacEΔ1* sample 11, Lane 8 *qacEΔ1* sample 12, Lane 9 *qacEΔ1* sample 13, Lane 10 *qacEΔ1* sample 14, Lane 11 *qacEΔ1* sample 15, Lane 12 *qacEΔ1* sample 16, Lane 13 *qacEΔ1 S*.T U288, Lane 14 *qacEΔ1* -ve control, Lane 15 100 bp Ladder DNA.

Appendix 2.10 The PCR ampilicon product of class 1 integron (*intI1* ravi, *intI1* EB, *sul1* and *qacEΔ1*) of commerical birds (Samples 17-26)

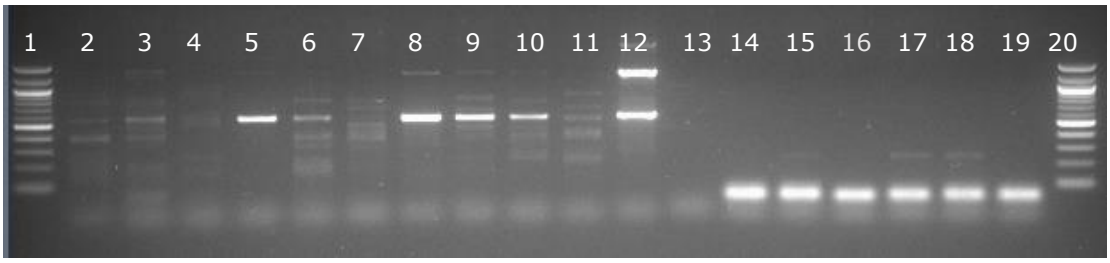


Figure 2.10.1 The PCR amplification using the *IntI1* Ravi (546 bp) and *IntI1* EB (254 bp) primers to examine the presence of resistance genes in commercial chicken's caecal DNA (sample 17-26).

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 17, Lane 3 *IntI1* Ravi sample 18, Lane 4 *IntI1* Ravi sample 19, Lane 5 *IntI1* Ravi sample 20, Lane 6 *IntI1* Ravi sample 21, Lane 7 *IntI1* Ravi sample 22, Lane 8 *IntI1* Ravi sample 23, Lane 9 *IntI1* Ravi sample 24, Lane 10 *IntI1* Ravi sample 25, Lane 11 *IntI1* Ravi sample 26, Lane 12 *IntI1* +ve control, Lane 13 *IntI1* Ravi -ve control, Lane 14 IntI1 EB sample 17, Lane 15 *IntI1* EB sample 18, Lane 16 *IntI1* EB sample 19, Lane 17 *IntI1* EB sample 20, Lane 18 *IntI1* EB sample 21, Lane 19 *IntI1* EB sample 22, Lane 20 100 bp ladder (BioLab).

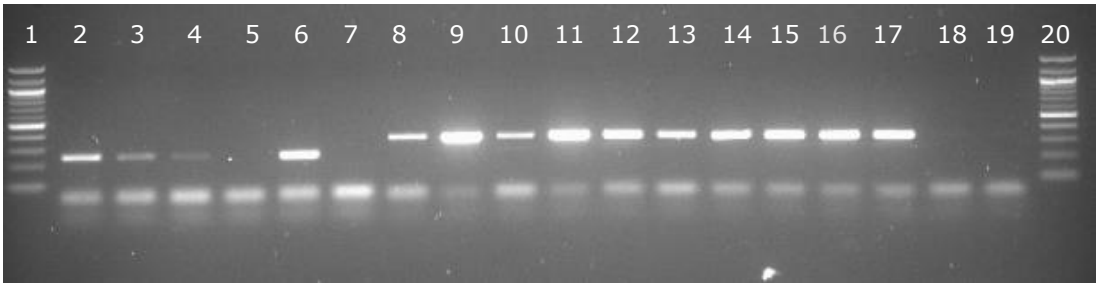


Figure 2.10.2 The PCR amplification using the *IntI1* EB (254 bp) and *sul1* (346 bp) to examine the presence resistance genes in commercial chicken's caecal DNA (sample 17-26).

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* EB sample 23, Lane 3 *IntI1* EB sample 24, Lane 4 *IntI1* EB sample 25, Lane 5 *IntI1* EB sample 26, Lane 6 *IntI1* EB +ve control, Lane 7 *IntI1* EB –ve control, Lane 8 *sul1* sample 17, Lane 9 *sul1* sample 18, Lane 10 *sul1* sample 19, Lane 11 *sul1* sample 20, Lane 12 *sul1* sample 21, Lane 13 *sul1* sample 22, Lane 14 *sul1* sample 23, Lane 15 *sul1* sample 24, Lane 16 *sul1* sample 25, Lane 17 *sul1* sample 26, Lane 18 *sul1 S*.T.U 288, Lane 19 *sul1* –ve, Lane 20 100 bp ladder (BioLab).

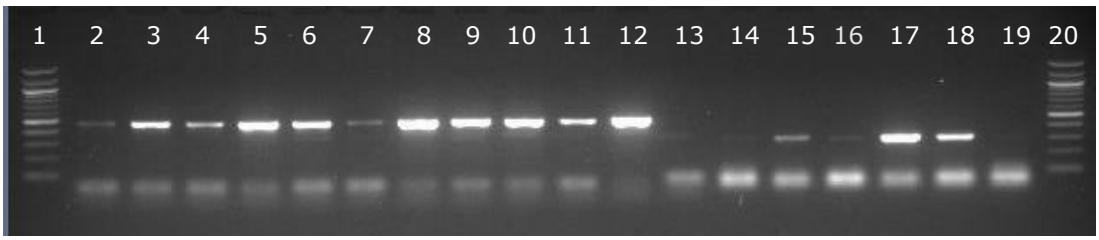


Figure 2.10.3 The PCR amplification using the *intl1* H (471 bp) and *intl1 C* (311 bp) to examine the presence of integrase resistance genes in commercial chicken's caecal DNA (sample 17-26).

Lane 1 100 bp ladder, Lane 2 *intl1* H sample 17, Lane 3 *intl1* H sample 18, Lane 4 *intl1* H sample 19, Lane 5 *intl1* H sample 20, Lane 6 *intl1* H sample 21, Lane 7 *intl1* H sample 22, Lane 8 *intl1* H sample 23, Lane 9 *intl1* H sample 24 Lane 10 *intl1* H sample 25, Lane 11 *intl1* H sample 26, Lane 12 *intl1* H *S*.T.U288 (+ve control)Lane 13 *int1l* H –ve, Lane 14 *intl1* C sample 17, Lane 15 *intl1* C, sample 18, Lane 16 *intl1* C sample 19, Lane 17 *intl1* C sample 20, Lane 18 *intl1* C sample 21, Lane 19 *intl1* C sample 22, Lane 20 100 bp ladder DNA.

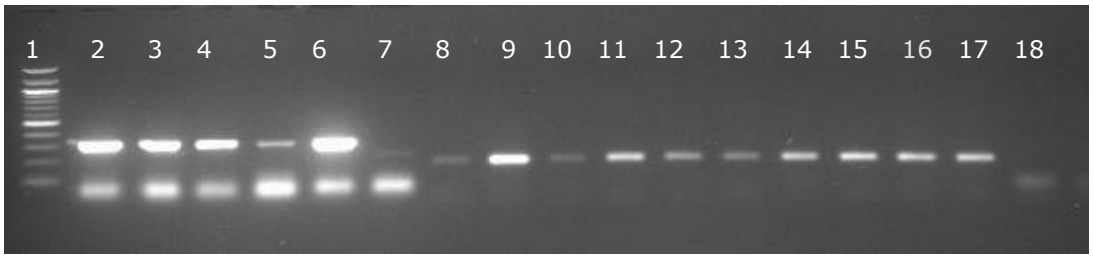


Figure 2.10.4 The PCR amplification using the *intI1 C* (311 bp) and *qacEΔ1* (200 bp) to examine the presence of integrase resistance genes in commercial chickens caecal DNA (sample 17-26).

Lane 1 100 bp ladder, Lane 2 *intI1* C sample 23, Lane 3 *intI1* C sample 24, Lane 4 *intI1* C sample 25, Lane 5 *intI1* C sample 26, Lane 6 *intI1* C +ve, Lane 7 *intI1* C –ve, Lane 8 *qac*EΔ1 sample17, Lane 9 *qacEΔ1* sample 18, Lane 10 *qacEΔ1* sample 19, Lane 11 *qacEΔ1* sample 20 , Lane 12 *qacEΔ1* sample 21, Lane 13 *qacEΔ1* sample 22, Lane 14 *qacEΔ1* sample 23, Lane 15 *qacEΔ1* sample 24, Lane 16 *qacEΔ1* sample 25, Lane 17 *qacEΔ1* sample 26, Lane 18 *qacEΔ1* EΔ1-ve.

Appendix 2.11 The PCR ampilicon product of class 1 integron (*intI1* ravi, *intI1* EB, *sul1* and *qacEΔ1*) of commerical birds (Samples 27-40)

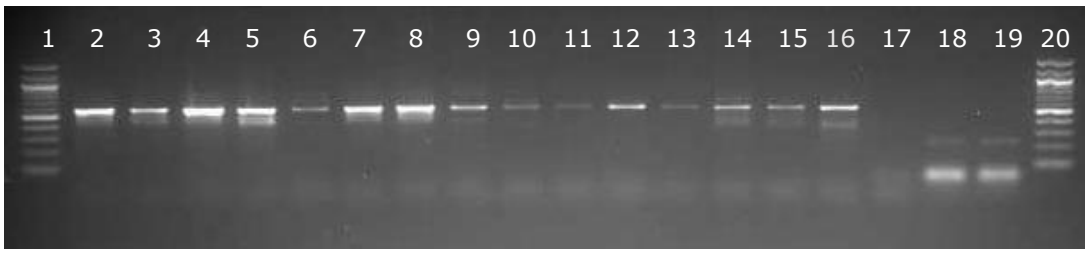


Figure 2.11.1 The PCR amplification using the *IntI1* Ravi (546 bp) and *IntI1* EB (254 bp) primers to examine the presence of resistance genes in commercial chickens caecal DNA (sample 27-40).

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* Ravi sample 27, Lane 3 *IntI1* Ravi sample 28, Lane 4 IntI1 Ravi sample 29, Lane 5 *IntI1* Ravi sample 30, Lane 6 *IntI1* Ravi sample 31, Lane 7 *IntI1* Ravi sample 32, Lane 8 *IntI1* Ravi sample 33, Lane 9 *IntI1* Ravi sample 34, Lane 10 *IntI1* Ravi sample 35, Lane 11 *IntI1* Ravi sample 36, Lane 12 *IntI1* Ravi sample 37, Lane 13 *IntI1* Ravi sample 38, Lane 14 *IntI1* Ravi sample 39, Lane 15 *IntI1* Ravi sample 40 Lane 16 *IntI1* +ve control, Lane 17 *IntI1* Ravi -ve control, Lane 18 *IntI1* EB sample 27, Lane 19 *IntI1* EB sample 28, Lane 20 100 bp ladder (BioLab).

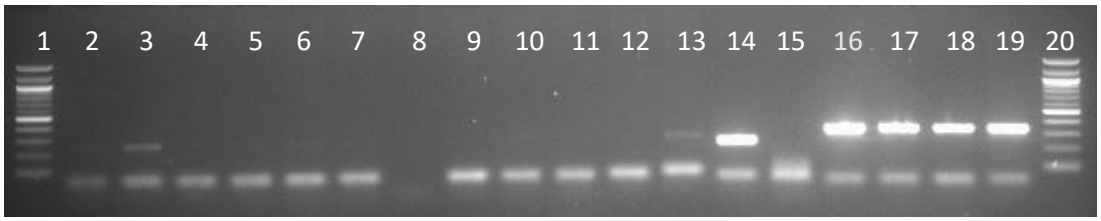


Figure 2.11.2 The PCR amplification using the *IntI1* EB (254 bp) and *sul1* (346 bp) to examine the presence resistance genes in commercial chickens caecal DNA (sample 27-40).

Lane 1 100 bp ladder (BioLab), Lane 2 *IntI1* EB sample 29, Lane 3 *IntI1* EB sample 30, Lane 4 *IntI1* EB sample 31, Lane 5 *IntI1* EB sample 32, Lane 6 *IntI1* EB sample 33, Lane 7 *IntI1* EB sample 34, Lane 8 *IntI1* EB sample 35, Lane 9 *IntI1* EB sample 36, Lane 10 *IntI1* EB sample 37, Lane 11 *IntI1* EB sample 38, Lane 12 *IntI1* EB sample 39, Lane 13 *IntI1* EB sample 40, Lane 14 *IntI1* EB +ve control, Lane 15 IntI1 EB–ve control, Lane 16 *sul1* sample 27, Lane 17 *sul1* sample 28, Lane 18 *sul1* sample 29, Lane 19 *sul1* sample 30, Lane 20 100 bp ladder (BioLab).

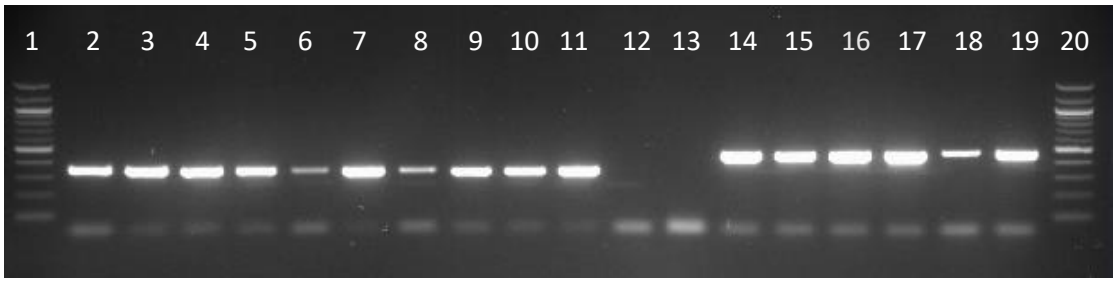


Figure 2.11.3 The PCR amplification using the *sul1* (346 bp) and *intl1* H (471 bp) to examine the presence of integrase resistance genes in commercial chickens caecal DNA (sample 27-40).

Lane 1 100 bp ladder (BioLab), Lane 2 *sul1* sample 31, Lane 3 *sul1* sample 32 Lane 4 *sul1* sample 33, Lane 5 *sul1* sample 34, Lane 6 *sul1* sample 35, Lane 7 *sul1* sample 36, Lane 8 *sul1* sample 37, Lane 9 *sul1* sample 38,Lane 10 *sul1* sample 39, Lane 11 *sul1* sample 40, Lane 12 *sul1 S*.T.U 288, Lane 13 *sul1*–ve, Lane 14 *intl1* H sample 27, Lane 15 *intl1* H sample 28, Lane 16 *intl1* H sample 29, Lane 17 *intl1* H sample 30, Lane 18 *intl1* H sample 31, Lane 19 *intl1* H sample 32, Lane 20 100 bp ladder (BioLab).

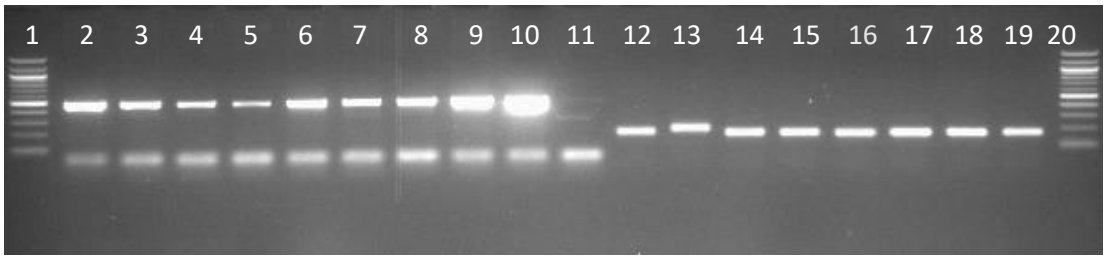


Figure 2.11.4 The PCR amplification using the *intl1 C* (311 bp) and *qacEΔ1* (200 bp) to examine the presence of integrase resistance genes in commercial chickens caecal DNA (sample 27-40).

Lane 1 100 bp ladder (BioLab), Lane 2 *intl1* H sample 33, Lane 3 *intl1* H sample 34, Lane 4 *intl1* H sample 35, Lane 5 *intl1* H sample 36, Lane 6 *intl1* H sample 37, Lane 7 *intl1* H sample 38, Lane 8 *intl1* H sample 39, Lane 9 *intl1* H sample 40, Lane 10 *intl1* H *S*.T.U288, Lane 11 *intl1* H –ve control, Lane 12 *qacEΔ1* sample 27, Lane 13 *qacEΔ1* sample 28, Lane 14 *qacEΔ1* sample 29, Lane 15 *qacEΔ1* sample 30, Lane 16 *qacEΔ1* sample 31, Lane 17 *qacEΔ1* sample 32, Lane 18 *qacEΔ1* sample 33,Lane 19 *qacEΔ1* sample 34,Lane 20 100 bp ladder DNA.

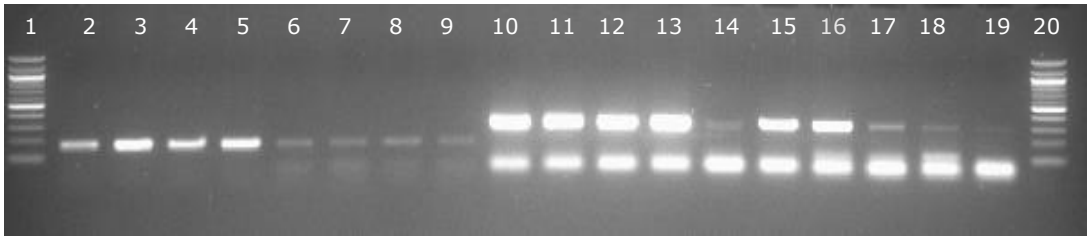


Figure 2.11.5 The PCR amplification using the *intl1 C* (311 bp) and *qacEΔ1* (200 bp) to examine the presence of integrase resistance genes in commercial chickens caecal DNA (sample 27-40).

Lane 1 100 bp ladder (BioLab), Lane 2 *qacEΔ1* sample 35, Lane 3 *qacEΔ1* sample 36, Lane 4 *qacEΔ1* sample 37, Lane 5 *qacEΔ1* sample 38, Lane 6 *qacEΔ1* sample 39, Lane 7 *qacEΔ1* sample 40, Lane 8 *qacEΔ1 S*.T U288, Lane 9 *qacEΔ1* -ve control, Lane 10 *intl1* C sample 27, Lane 11 *intl1* C sample 28, Lane 12 *intl1* C sample 29, Lane 13 *intl1* C sample 30, Lane 14 *intl1* C sample 31,Lane 15 *intl1* C sample 32, Lane 16 *intl1* C sample 33, Lane 17 *intl1* C sample 34 , Lane 18 *int1l* C sample 35, Lane 19 *intl1* C sample 36, Lane 20 100 bp ladder

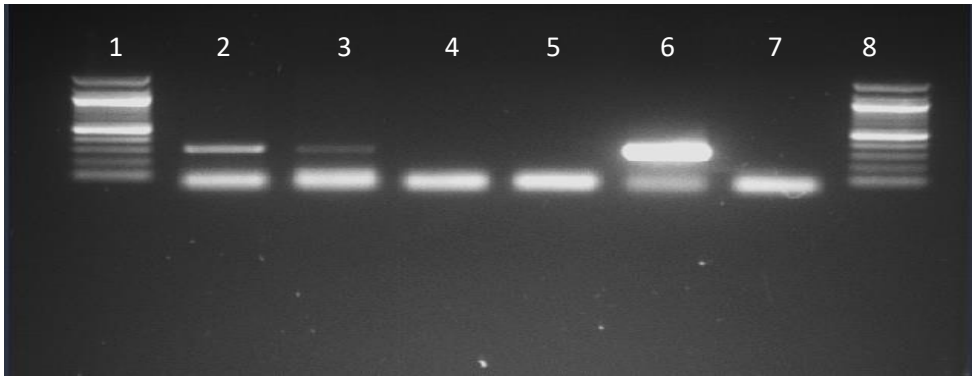


Figure 2.11.6 The PCR amplification using the *intl1 C* (311 bp) to examine the presence of integrase resistance genes in commercial chickens caecal DNA (sample 27-40).

Lane 1 100 bp ladder (BioLab), Lane 2 *intl1* C sample 37, Lane 3 *intl1* C sample 38 Lane 4 *intl1* C sample 39, Lane 5 *intl1* C sample 40, Lane 6 *int1l* C +ve, Lane 7 *intl1* C -ve

Appendix 2.12 Initial test for selecting GCs primers

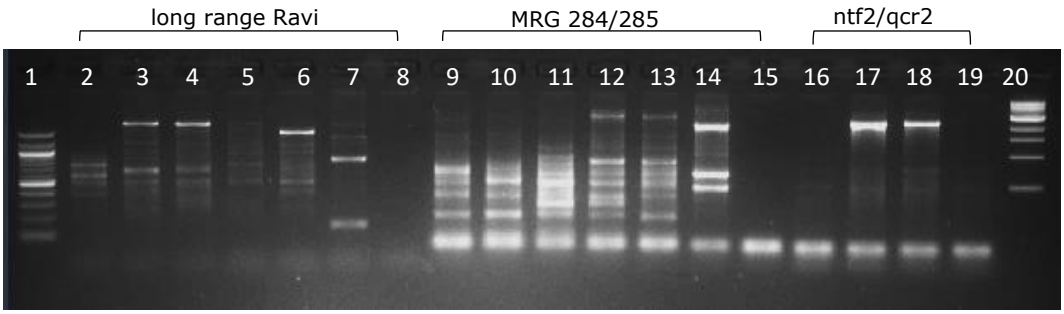


Figure 2.12. 1A The PCR amplification of GCs of broiler caecal DNA by using four different primers (Table 2.5, Chapter 2) for selecting optimum primers (1 min extension time) electrophoresed on 0.8% TAE agarose gel.

Lane 1 100 bp ladder (BioLab), Lane 2 long range Ravi sample 1, Lane 3 long range Ravi sample 2, Lane 4 long range Ravi sample 3, Lane 5 long range Ravi sample 4, Lane 6 long range Ravi sample 5, Lane 7 long range Ravi +ve control, Lane 8 long range Ravi -ve control. Lane 9 MRG 284/285 sample 1, Lane 10 MRG 284/285 sample 2, Lane 11 MRG 284/285 sample 3, Lane 12 MRG 284/285 sample 4, Lane 13 MRG 284/285 sample 5, Lane 14 MRG 284/285 +ve, Lane 15 MRG 284/285 –ve, Lane 16 ntf2/qcr2 sample 1, Lane 17 ntf2/qcr2 sample 2, Lane 18 ntf2/qcr2 sample 3, Lane 19 ntf2/qcr2 sample 4, Lane 20 1 kb ladder (BioLab).

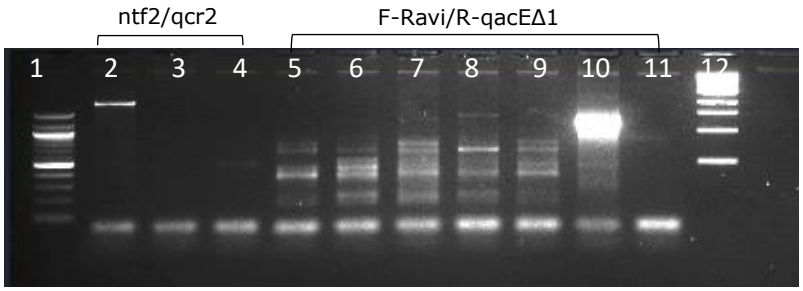


Figure 2.12.2A The PCR amplification of GCs of broiler caecal DNA by using four different primers (Table 2.5, Chapter 2) for selecting optimum primers (1 min extension time) electrophoresed on 0.8% TAE agarose gel.

Lane 1 100 bp ladder (BioLab), Lane 2 ntf2/qcr2 sample 5, Lane 3 ntf2/qcr2 -ve, Lane 4 ntf2/qcr2 +ve control, Lane 5 F-Ravi/R-*qacEΔ1* sample 1, Lane 6 F-Ravi/R- *qacEΔ1* sample 2, Lane 7 F-Ravi/R-*qacEΔ1* sample 3, Lane 8 F-Ravi/R-*qacEΔ1* sample 4, Lane 9 F-Ravi/R-*qacEΔ1*, sample 5, Lane 10 F-Ravi/R-*qacEΔ1* +ve control, Lane 11 F-Ravi/R-*qacEΔ1* -ve control, Lane 12 1 kb ladder (BioLab).

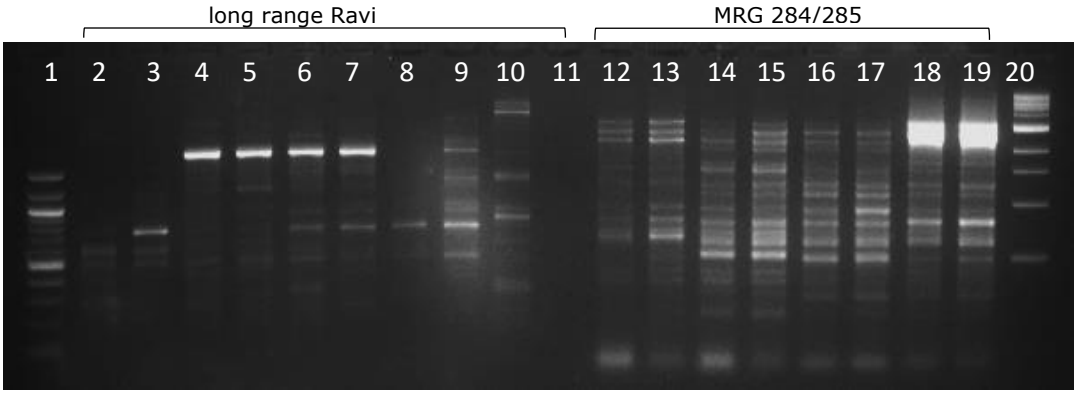


Figure 2.12.1B The PCR amplification of GCs of broiler caecal DNA by using four different primers (Table 2.5, Chapter 2) for selecting optimum primers (3 min extension time) electrophoresed on 0.8% TAE agarose gel.

Lane 1 100 bp ladder (BioLab), Lane 2 long range Ravi sample 1, Lane 3 long range Ravi sample 1 (Replicate), Lane 4 long range Ravi sample 2, Lane 5 long range Ravi sample 2, Lane 6 long range Ravi sample 3, Lane 7 long range Ravi sample 3, Lane 8 long range Ravi sample 4, Lane 9 long range Ravi sample 4, Lane 10 long range Ravi +ve control, Lane 11 long range Ravi -ve control, Lane 12 MRG 284/285 sample 1, Lane 13 MRG 284/285 sample 1, Lane 14 MRG 284/285 sample 2, Lane 15 MRG 284/285 sample 2, Lane 16 MRG 284/285 sample 3, Lane 17 MRG 284/285 sample 3, Lane 18 MRG 284/285 sample 4, Lane 19 MRG 284/285 sample 4, Lane 20 1 kb ladder DNA.

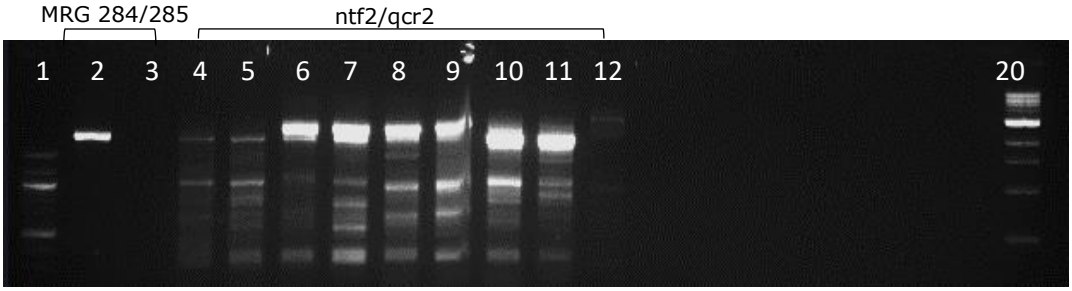


Figure 2.12.2B The PCR amplification of GCs of broiler caecal DNA by using four different primers (Table 2.5, Chapter 2) for selecting optimum primers (3 min extension time) electrophoresed on 0.8% TAE agarose gel.

Lane 1 100 bp ladder (BioLab), Lane 2 MRG 284/285 +ve, Lane 3 MRG 284/285 –ve, Lane 4 ntf2/qcr2 sample 1, Lane 5 ntf2/qcr2 sample 2, Lane 6 ntf2/qcr2 sample 3, Lane 7 ntf2/qcr2 sample 4, Lane 8 ntf2/qcr2 sample 5, Lane 9 ntf2/qcr2 sample 6, Lane 10 ntf2/qcr2 sample 7, Lane 11 ntf2/qcr2 sample 8, Lane 12 ntf2/qcr2 sample 9, Lane 20 1 kb ladder (BioLab).

Appendix 2.13 The gene cassettes sequence data

Figure 2.13.1 class_1_integron_group_A (*aad*A1, *lin*F).

Figure 2.13.2 Class_ 1 _integron group_B-1 (dfrA1), hypothetical protein, and aminoglycoside-3'-adenyltransferase (aadA24) genes, and QacEdelta1 gene.

AAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCC
AGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTA
TGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCG
TGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCT
AAAACAAAGTTAACCTCTGAGGAAGAATTGTGAAACTATCACTAATGGTAGCTATATCG
AAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGCT
CCTGTTTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAAT F2-GCs-B
CAATGGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCT
GACAATGAGAACGTATTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAA
AATAACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGATC
AAGTAGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTT
CCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCCTCTAACATAAA
TTATAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGGCAGCAACGGATTCGCAAACCT
GTCACGCCTTTTGTACCAAAAGCCGCGCCAGGTTTGCGATCCGCTGTGCCAGGCGTTAG F2-GCs—B-1
GTCGCACAGGTACCACATGGAAAGAAATACTGCCGAAGATATATTTGCTGCCTGCGAGC
GTGCATTAGCGAATCTTATGGAGGCAGAGATTGCAATTGCTCAAATCTCCGATCCAGTA
GAGCGCGCCGAGCTCATGAAGGCACTTTTGAGTTCACTCGCCGAAATAACTGCAGGTGT
TAGGGCCTCGGCTTTGCGCCAGTATCCTGACATTCATCCGCCGGAGCCACATGGAGCGC
CTGATACAACAATCGTTGAAGAAGATGTCGCTATAGTCTCCCAACTGACCACTATTGAC
ATTACCGCCATAGACAAAGCCCTCCTAGCAGAGTGCGCATCTTCTTGGCAAAAGGTTGC
CCGTGTTGTGGGGGATGCATTGCACTCTTCCTCCCCAAACCTCAAAAAAGTTCCAGTTG
GCTATTACGCCCAAAGGATTATTGCGCTGGTCGAGCTCGGAAAGCTTGAATCTCAAGGA
AATCTTCATTACATCCGAAGCAGTGAGGTCAGGCTTCCAAATGACAGTAAAAGTGCAGC
CTAACCCTTCAATCAACAGGGACAGTCCAAAGCTAGCGCTTTGTCCTGCCCCTTATTTC
AAACGTTAGACATCATGAGGGACGCAGTGATCGCCGAAATTTCGACACAACTGTTAGAG
GTGCTTAGTGTCATTGAGCGCCATCTGGAGCCGACGTTGCTGGCCGTGCATTTGTACGG
CTCCGCAGTGAATGGCGGCCTGAAGCCATACAGCGATATTGATTTGCTGGTTACTGTGA
CTGTAAGGCTTAATGAAACAACGCGGCGAGCTTTGCTCAACGACCTTCTGGAGGTTTCG
ACTTTCCCCGGCGAGAGTGAGGCTCTCCGCGCTATAGAAGTCACCATTGTCGTGCACGA
CGACATCATTCCGTGGCGTTATCCAGCTAAGCGAGAACTGCAATTTGGAGAATGGCAGC R2-GCs—B-1
GCAATGACATTCTTGCGGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATC
TTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACT
CTTTGATCCGGTCCCTGAACAGGATCTATTCGAGGCACTAAATGAAACCTTGAAGCTAT
GGAACTCGCAGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCTCGT
ATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGC
AATGGAGCGCCTACCGGCCCAGTATCAGCCCGTCTTGCTTGAAGCTAGACAGGCTTATC
TTGGACAAGAAGAAGATCGCTTGGCCTTGCACGCTGATCAGTTGGAAGAATTTGTTCAC
TACGTGAAAGGCGAGAGCACCAAGGTAGTCGGCAAATGATGTCTAACAATTCGTTCAAG
CCGACGCCGCTTCGCGGCGCGGCTTAACTCGAGCGTTAGATGCACTAAGCACATAATTG
CTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTATTATTTTTAAGCGTGCATAATAAG
CCCTACACAAATTGGGAGATATATCATGAAAGGC

Figure 2.13.3 class_1_integron_group_B-2 (*dfr*A1, *aad*A1)

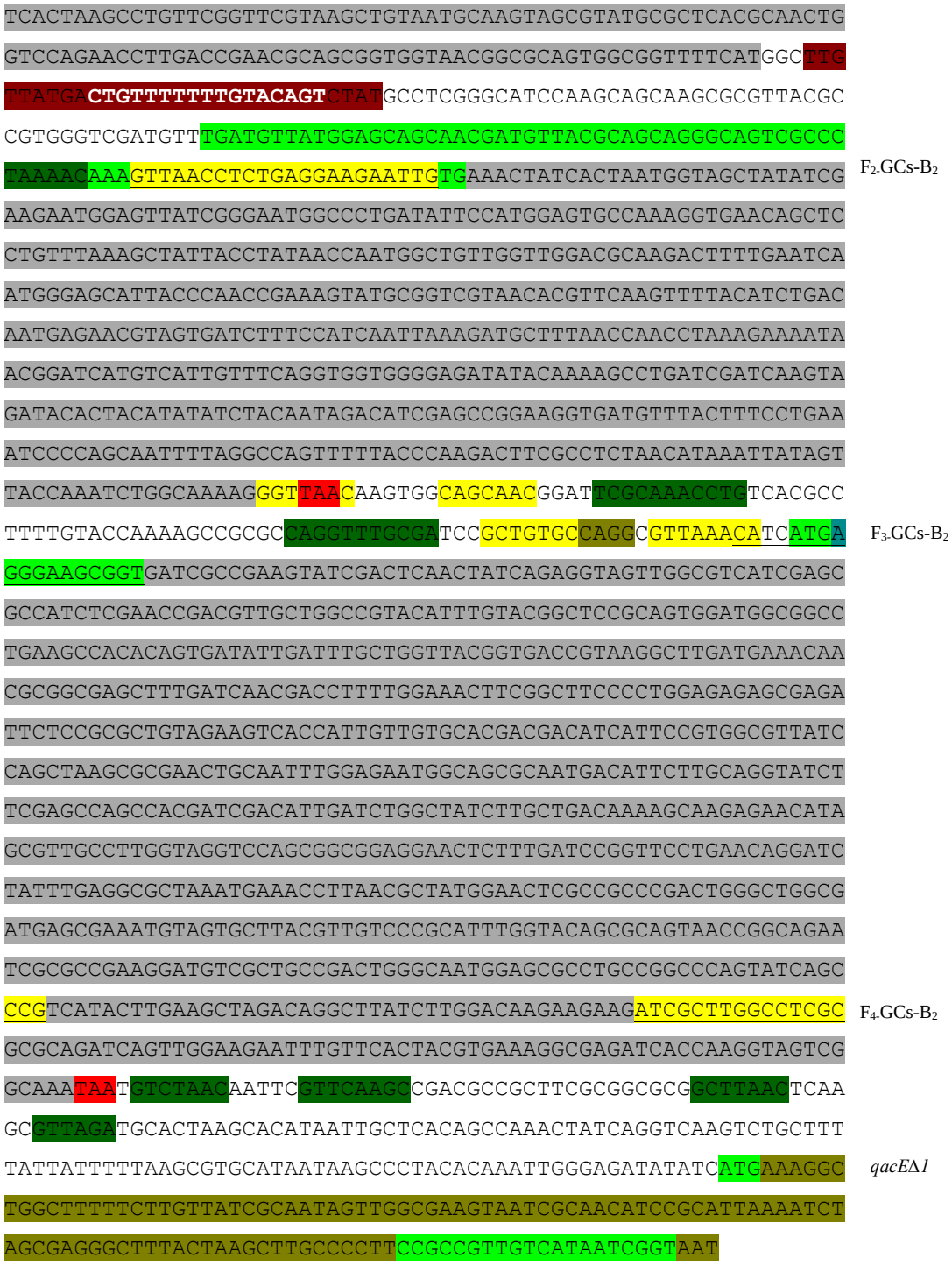


Figure 2.13.4 class_1_integron_group_C-2 (*aadA1*)

Figure 2.13.5 class_1_integron_group_ (*aadA9*)

GACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGC

AACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTT

TCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCA

GCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTT

ACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGACATGATGAGCAACTCTATAC

ACACCGGAATCTCAAGGCAGCTTCAGGCACGCGATGTAATTAAACGCCATTTGGC

ATCAACGCTGAAAGCCATACACTTGTATGGTTCTGCAATTGATGGTGGCCTCAAA

CCATATAGCGACATTGATCTGCTGGTTACCGTGGATGCACGCTTGGATGAAGCTA

CCAGACGCTCCCTGATGCTCGATTTCTTGAATATCTCGGCACCACCATGCGAAAG F_2.GCs-F

CTCAATACTCCGGCCGCTAGAGGTAACTGTTGTTGCATGCAACGAAGTAGTGCCT

TGGCGTTATCCGGCACGACGAGAACTGCAGTTCGGGGAGTGGCTGCGGGAGGATA

TTCTTGAAGGTGTCTTCGAGCCAGCCGCCTTGGACGCCGACCTTGCAATTCTAAT

AACGAAAGCTAGGCAACACAGCATCGCTTTAGTAGGTCCAGTGGCTCAAAAAGTC

TTCATGCCGGTGCCAGAGCATGACTTTCTCCAGGTGCTTTCCGATACCCTTAAGC

TGTGGAATACTCATGAGGATTGGGAAAATGAGGAGCGGAACATCGTACTCACGTT

AGCTCGGATCTGGTATAGCACTGAAACTGGAGGAATCGTCCCCAAGGATGTGGCC

GCCGAATGGGTTTTAGAGCGCTTGCCAGCTGAGCATAAGCCAATACTGGTTGAGG

CGCGGCAAGCCTATCTTGGGCTTTGCAAGGATAGTCTTGCTTTGCGTGCAGATGA

GACTTCGGCGTTCATTGGCTATGCAAAGTCTGCGGTCGCTGATTTGCTCGAAAAG

CGAAAATCTCAAACTTCGCATATTTGCGATGGCGCCAAGAACGTCTAACGTCTAA

CTATTCATTTAAGCCGAAGCCGCTTCGCGGCTCGGCTTAATTCAGGCGTTAGATG

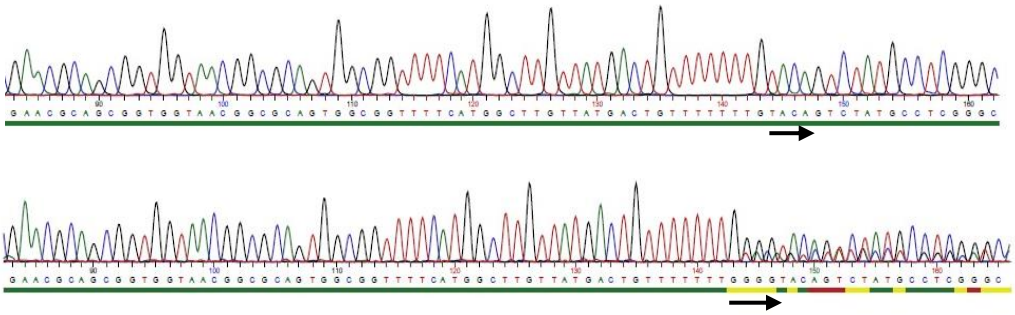
CACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTATTA

TTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGAAAGGC

TGGCTTTTTCTTGTTATCGCAATAGTTGGCGAAGTAATCGCAACATCCGCATTAA *qacEΔ1*

AATCTAGCGAGGGCTTTACTAAGCTTGCCCCTTCC

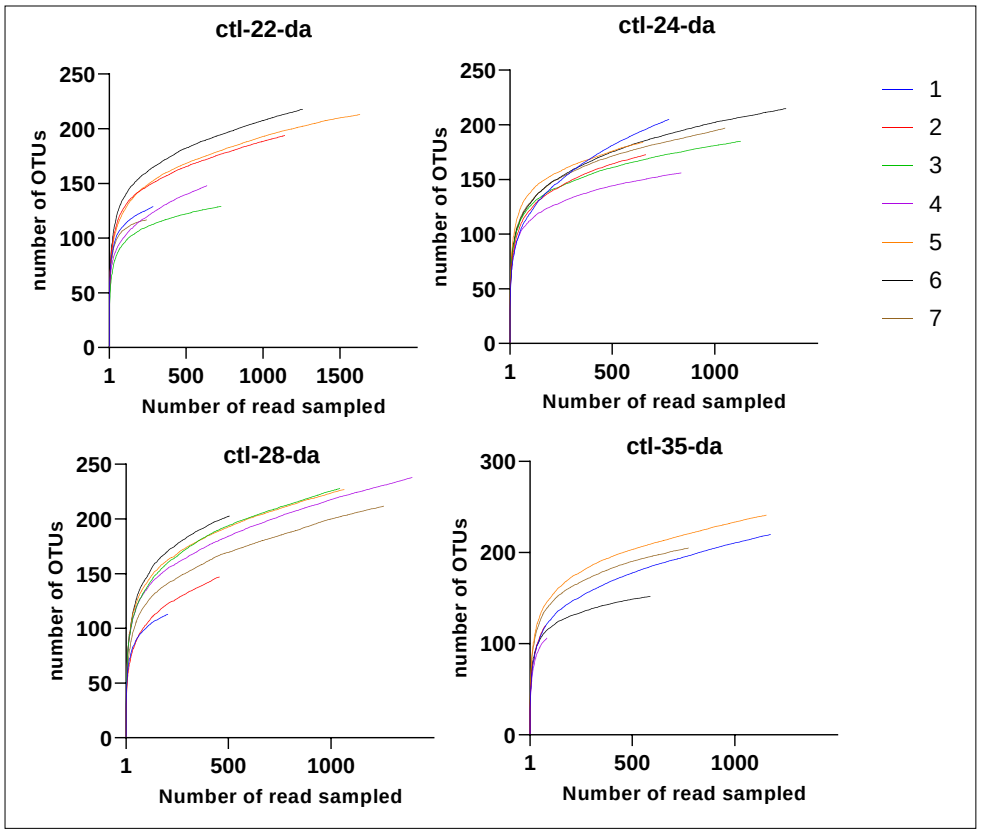
Appendix 2.14 The insertion of three Gs in integrase gene sequence found in commercial flock samples compared with the same GC components in controlled housing chicken Forward reads for 2.35.4 and sample 40.



Appendix 3

Figure 3.1 Rarefactions curves of observed OTUs of caecal samples microbiota collected from A biosecure birds and B commercial birds.

A-biosecured birds



B-commercial birds

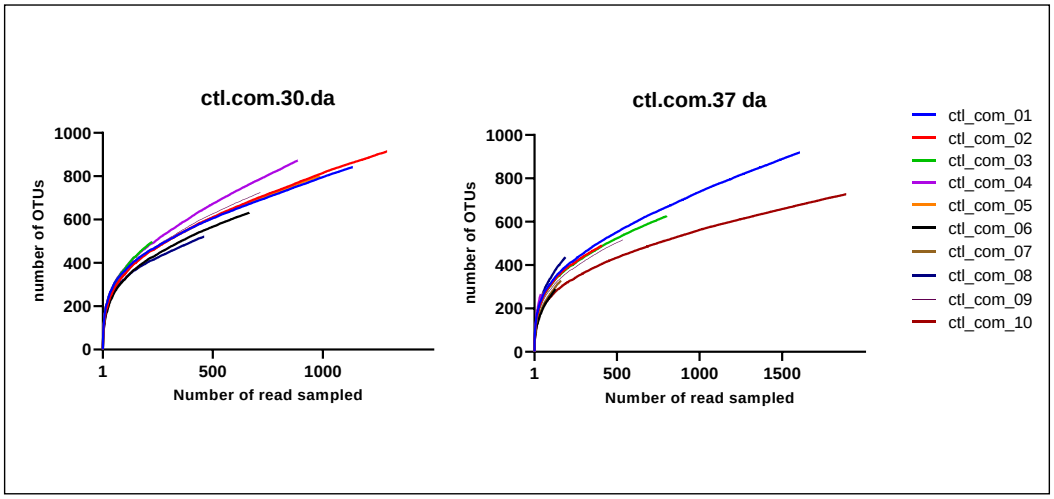
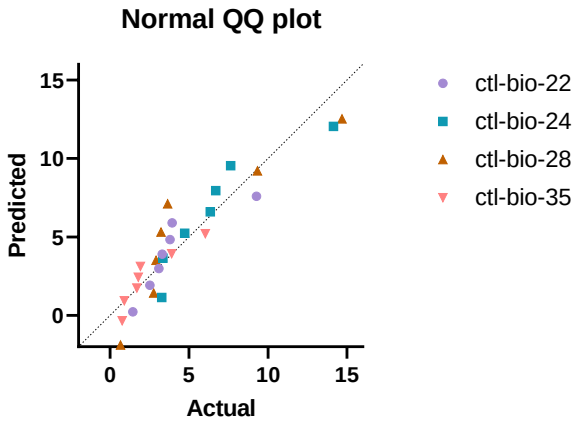


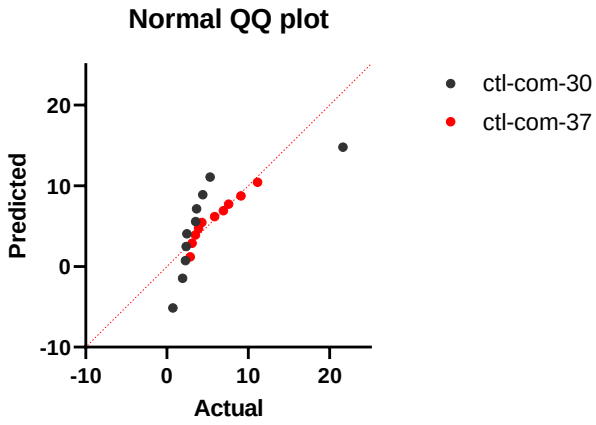
Figure 3.2 The relative abundance of Proteobacteria phylum in control diet groups.

A-biosecure birds



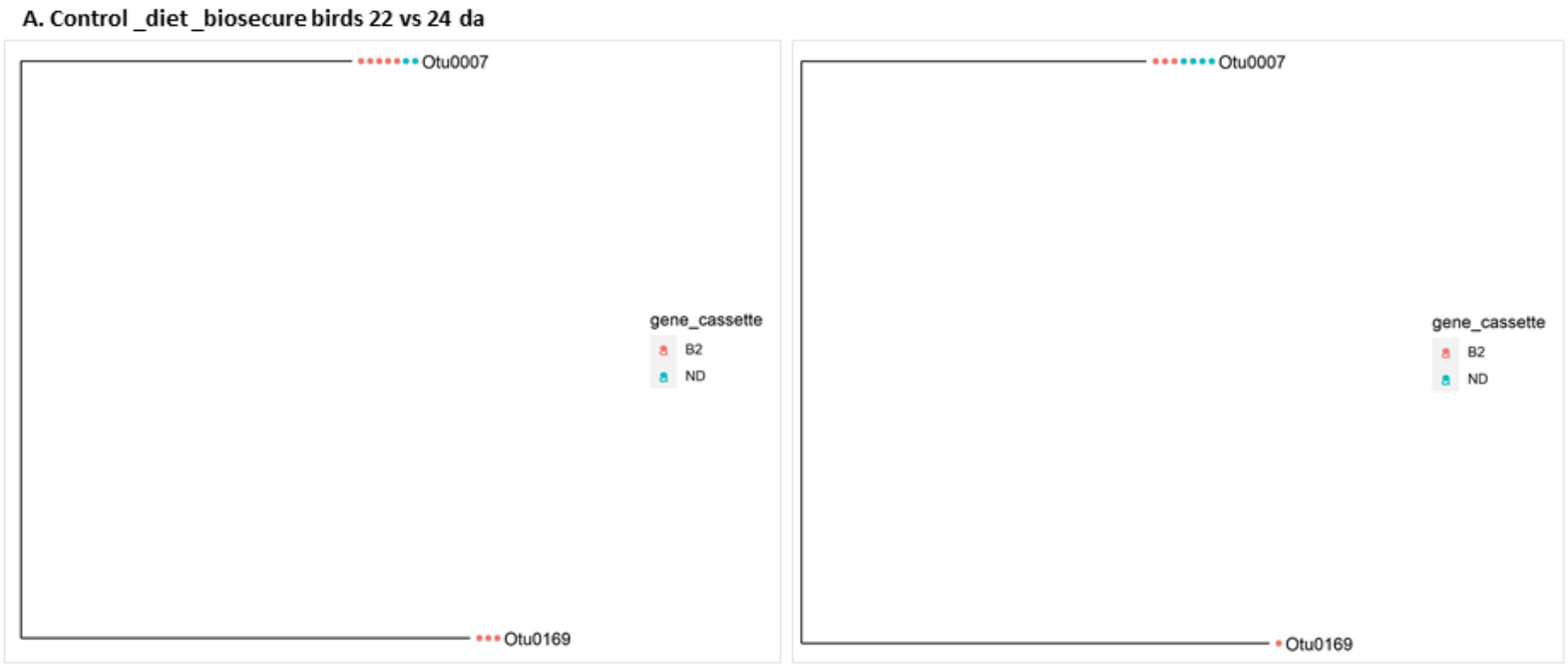
Data not normally distributed at 22 da (Shapiro-Wilk normality test= 0.0215), however other sampling points show data normally distributed across cohorts (p-value 0.0957, 0.05, 0.0807 respectively).

B-commercial birds

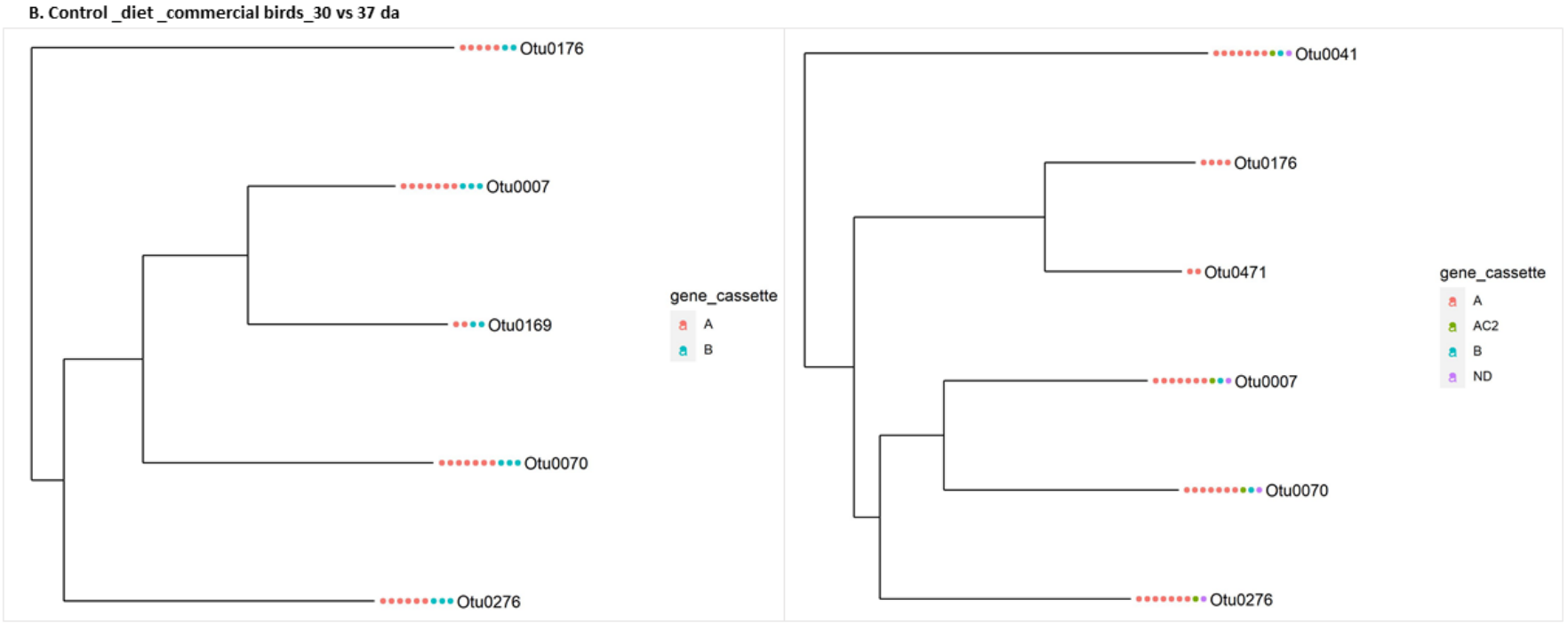


The abundance of Proteobacteria was not normally distributed at 30 da (Shapiro-Wilk normality test= 0.0001), while this abundance of Proteobacteria was normally distributed at 37 da (p-value=0.2762)

Figure 3.3 Phylogenetic tree of GCs distribution among sampling points in A biosecure birds, and B commercial birds.



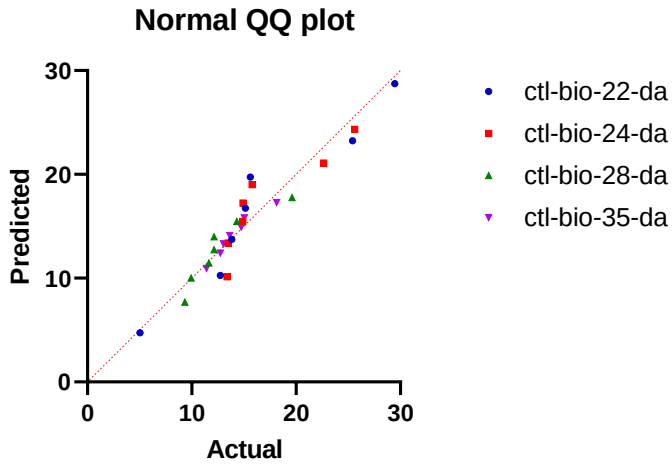
The phylogenetic tree of Proteobacteria phylum shows the OTUs associated with distribution of GCs groups in broiler chicken microbiota reared in biosecure conditions. No Proteobacteria detected at 28 and 35 da with bootstrap value ≥20.



The phylogenetic tree of Proteobacteria phylum shows the OTUs associated with distribution of GCs groups in broiler chicken microbiota reared under commercial conditions. It is constructed by a neighbour-joining method with Bootstrap value ≥ 20.

Figure 3.4 QQ plot of control fed birds (all sampling days) reared in controlled housed conditions

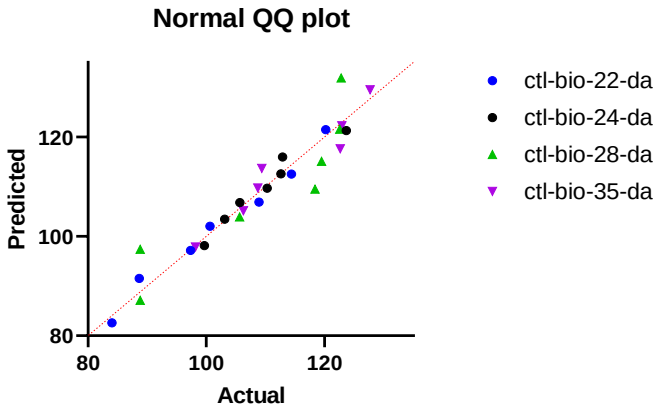
A-inverse-Simpson index



Normality test for invsimpson index CH birds

Data not normally distributed (Shapiro-Wilk normality test) mainly at 24 da (p-value 0.0279) however other sampling days are normally distributed p-value >0.05 as it passes normality tests.

B-Chao index

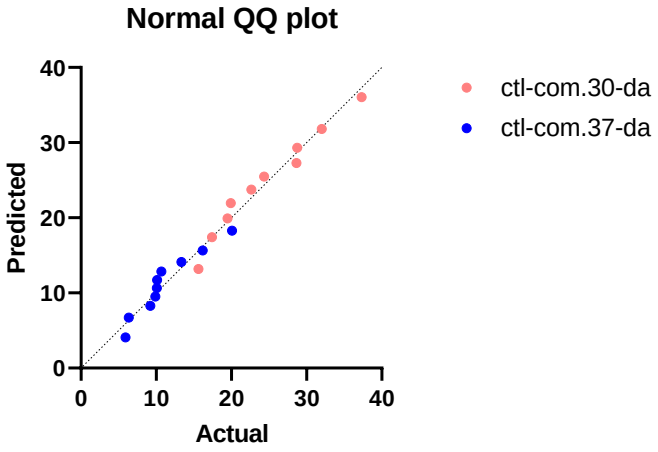


Normality test for Chao index CH birds

Data not normally distributed based Shapiro-Wilk normality test indicated that at day 28 data not normally distributed but the other sampling days are normally distributed as it passes both normality tests (p-value respectively of all Sampling da are: 0.8737, 0.7649, 0.0378 and 0.4811.

Figure 3.5 QQ plot of control fed birds (all sampling days) reared in commercial conditions

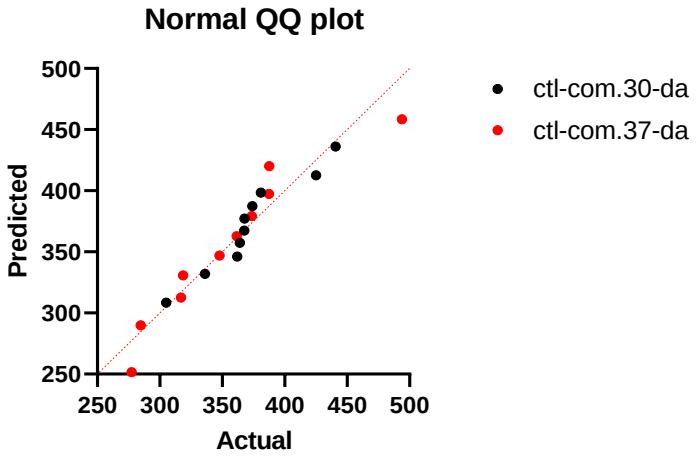
A-inverse-Simpson index



Normality test for invsimpson index commberical birds

Data normally distributed at 30 and 37 da (Shapiro-Wilk normality test= 0.7348, 0.2637 respectively) therefore it passes normality tests.

B-Chao index



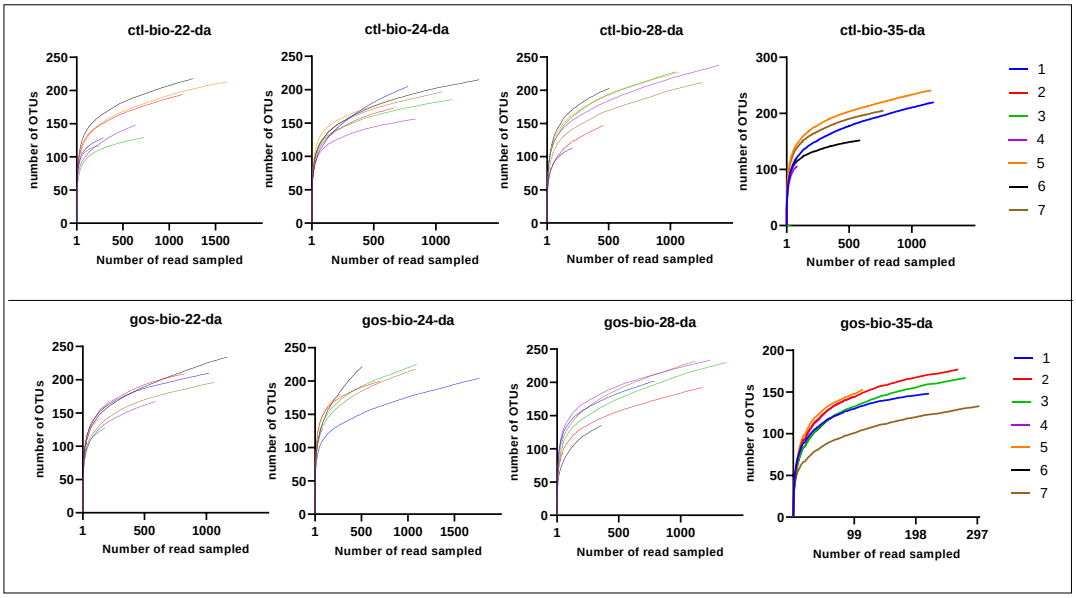
Normality test for Chao index commberical birds

Data normally distributed at 30 and 37 da (Shapiro-Wilk normality test= 0.4431, 0.3210 respectively) therefore it passes normality tests.

Appendix 4

Figure 4.1 Rarefactions curves of observed OTUs of caecal samples microbiota collected from A biosecure birds and B commercial birds.

A-biosecure birds



B-commercial birds

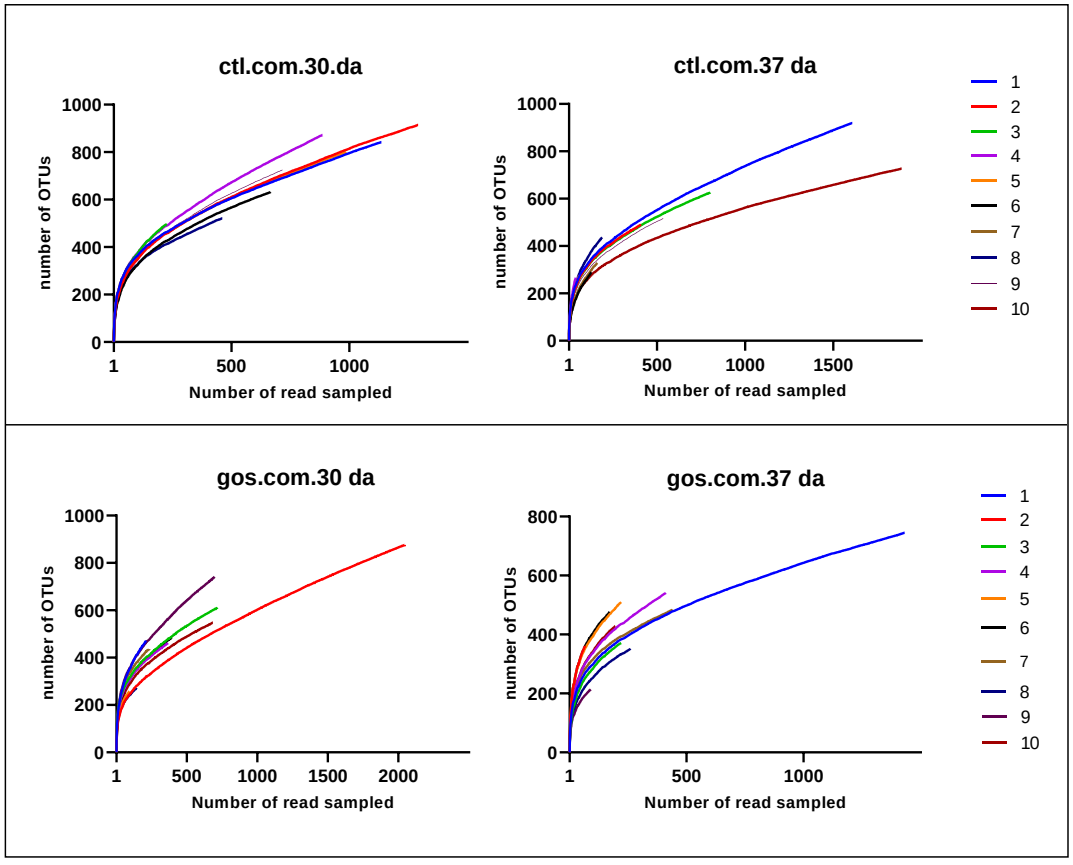
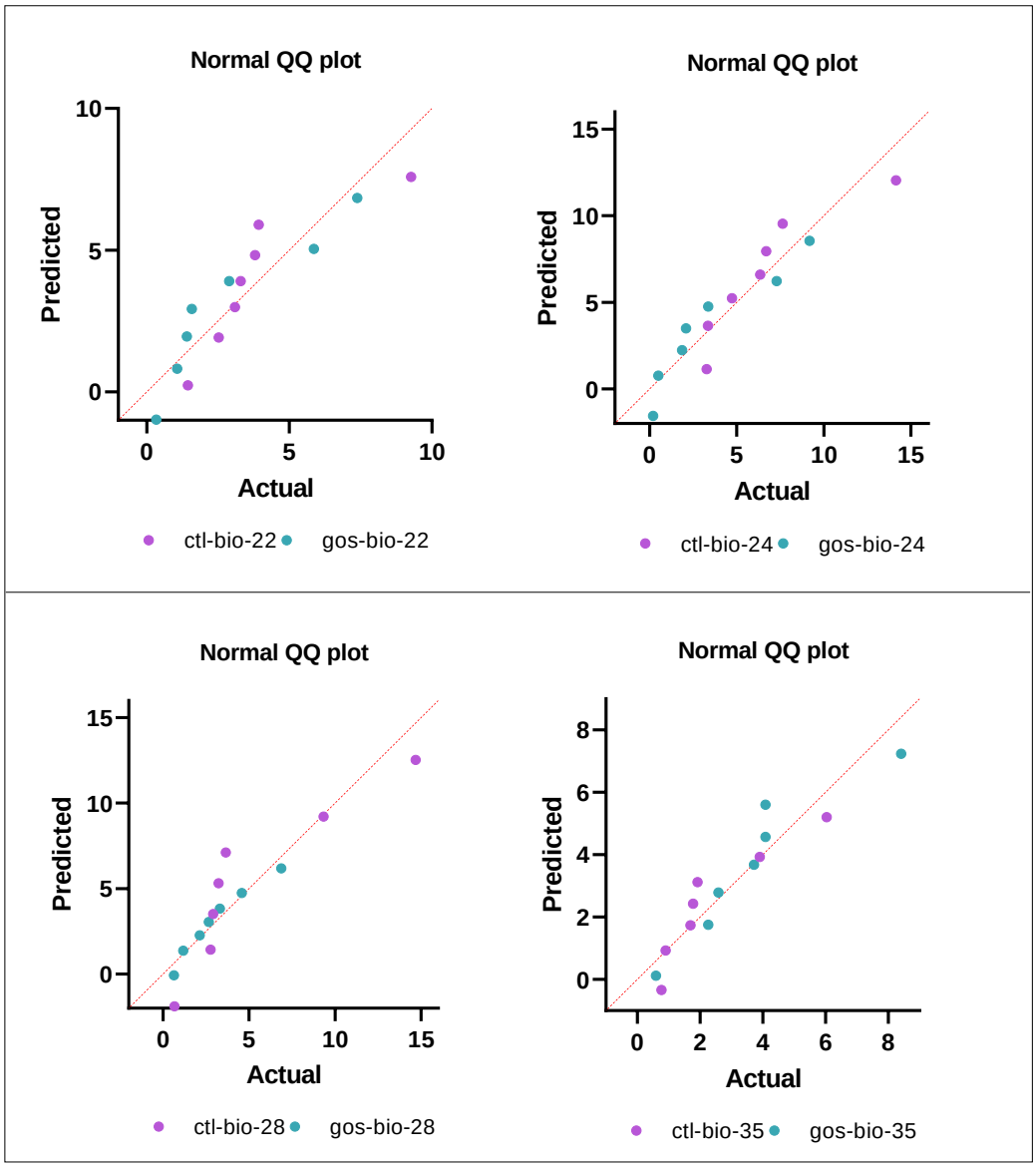


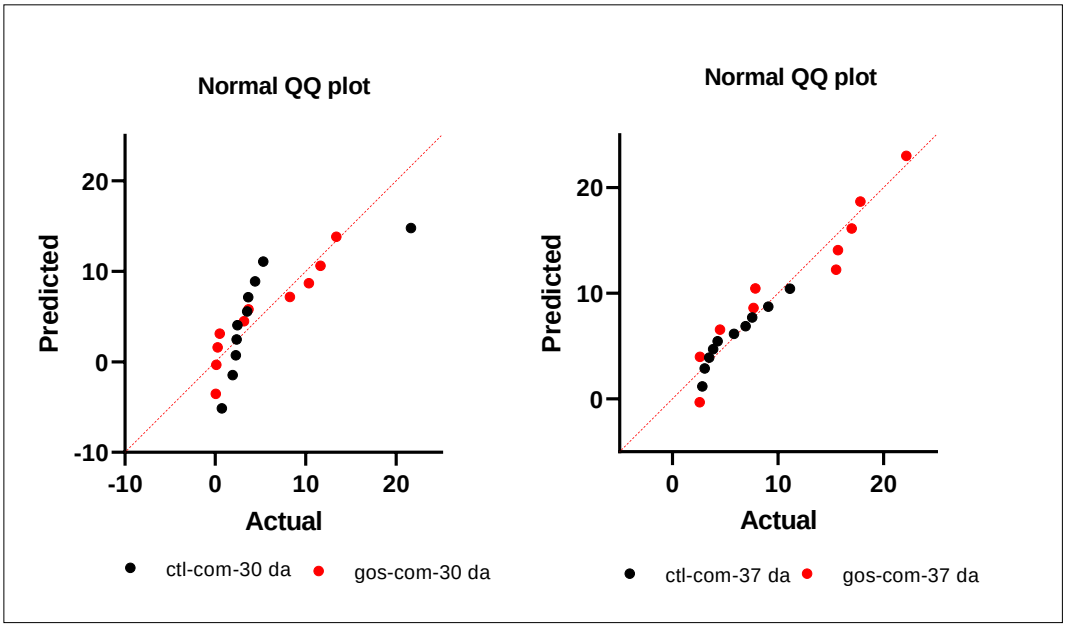
Figure 4.2 The relative abundance of Proteobacteria phylum in control and GOS diets of commercial and biosecure birds.

A-Biosecure birds



The data were not normally distributed in control diet at 22 da (Shapiro-Wilk normality test= 0.0215), however other sampling points show the data are normally distributed across cohorts (p-value > 0.05).

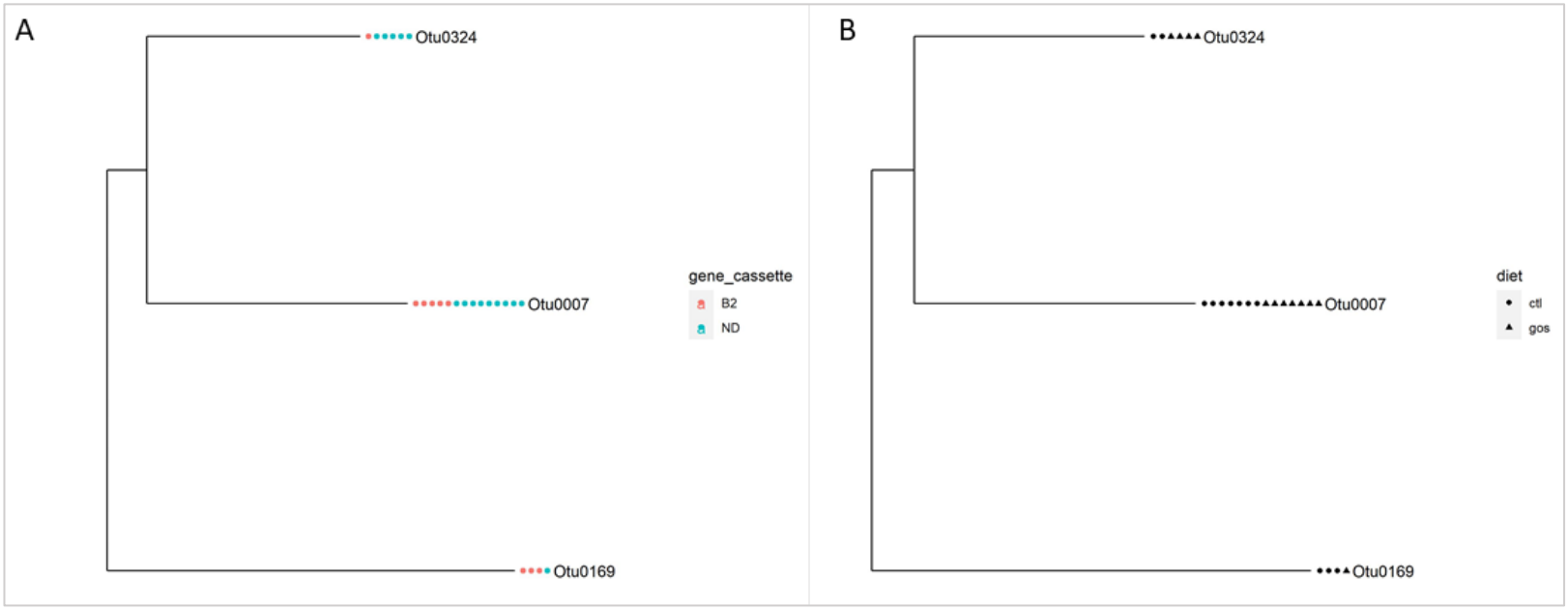
B-Commercial birds



The abundance of Proteobacteria was not normally distributed at control-30 da (Shapiro-Wilk normality test= 0.0001), while all other sampling points for both diets showing the Proteobacteria abundance was normally distributed (p-value>0.05).

Appendix 4.3.1 The coincidence of resistance GCs arrays in Proteobacteria phylum of biosecure birds. Figure A shows distribution of GCs and Figure B demonstrates feed type

1-Biosecured birds-22 da

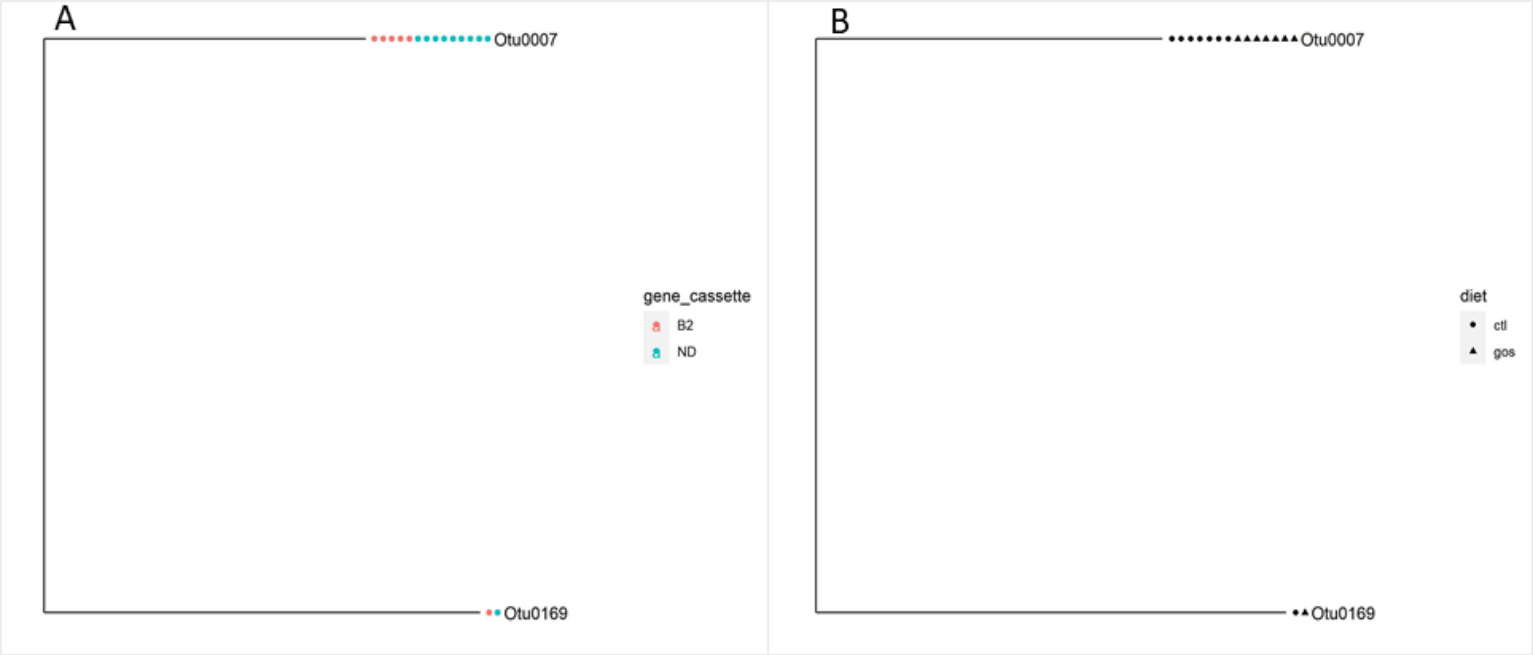


The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*).

*** ND not detected.**

Appendix 4.3.2 The abundance of resistance GCs arrays in Proteobacteria phylum in biosecure housing birds. Figure A shows distribution of GCs and Figure B demonstrates feed type

2-Biosecure birds-24 da

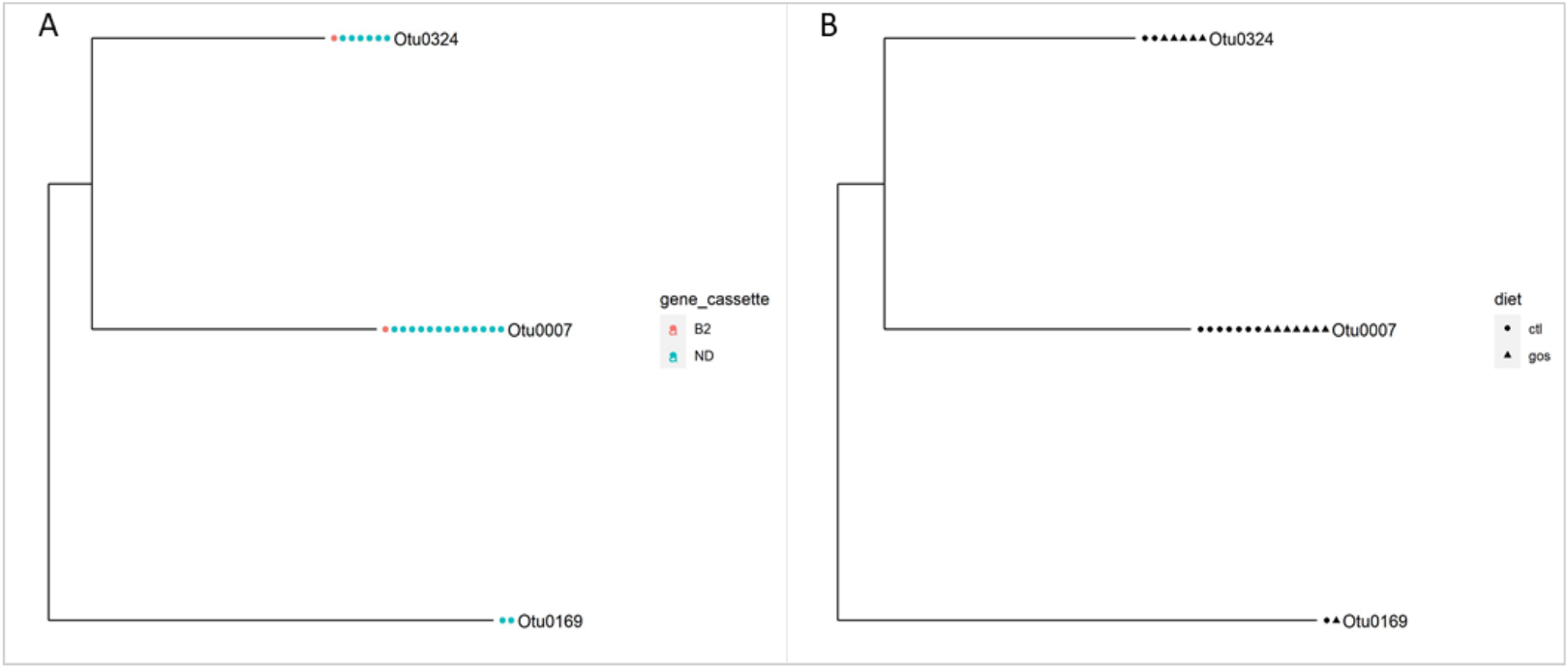


The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*).

*** ND not detected.**

Appendix 4.3.3 The abundance of resistance GCs arrays in *Proteobacteria* phylum in biosecure housing birds. Figure A shows distribution of GCs and Figure B demonstrates feed type

3-Biosecure birds-28 da

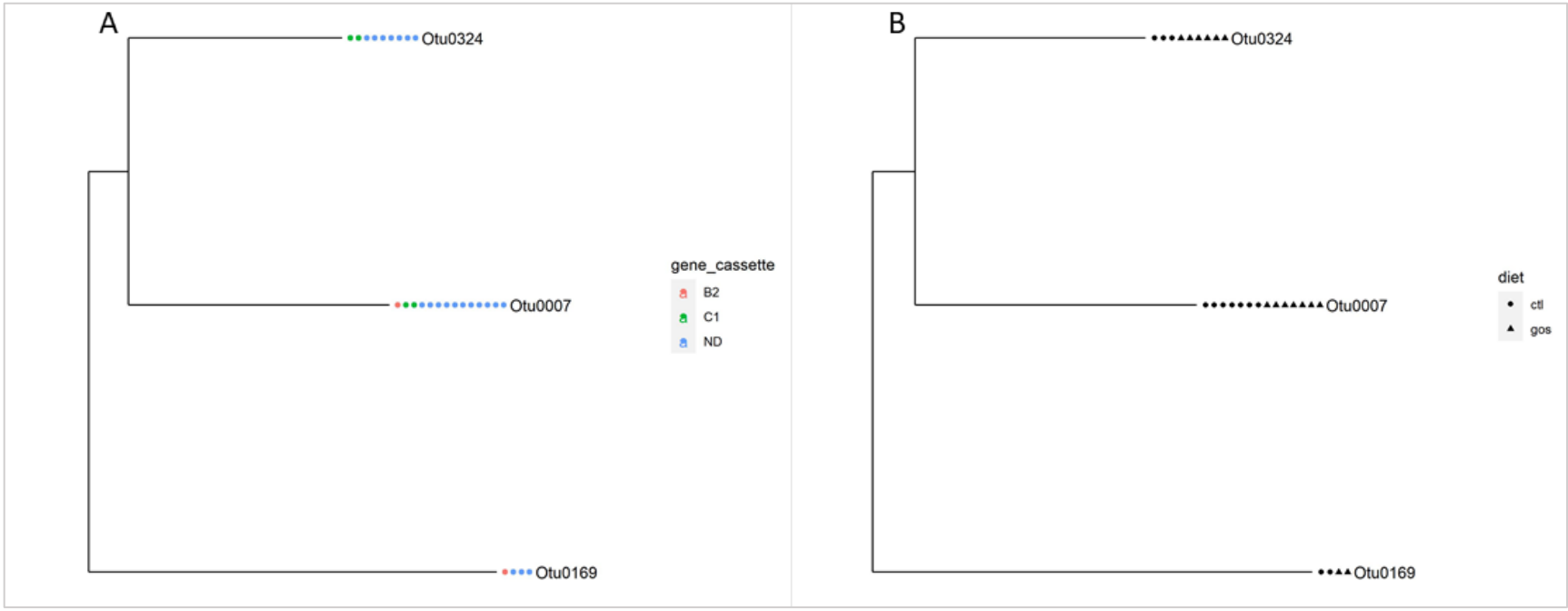


The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*).

*** ND not detected.**

Appendix 4.3.4 The abundance of resistance GCs arrays in *Proteobacteria* phylum in biosecure housing birds. Figure A shows distribution of GCs and Figure B demonstrates feed type

4-Biosecure birds-35 da

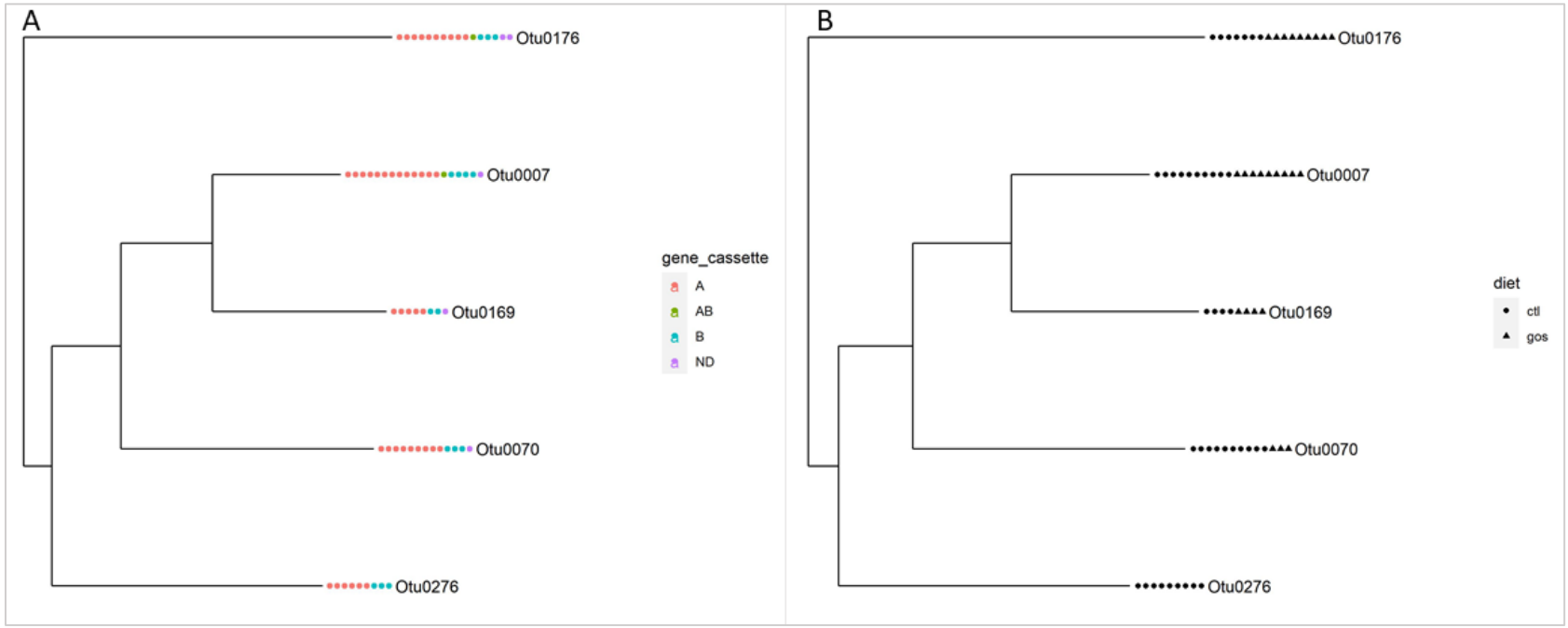


The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU 0324 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*).

*** ND not detected.**

Appendix 4.4.1 The abundance of resistance GCs arrays in Proteobacteria phylum in commercial birds. Figure A shows distribution of GCs and Figure B demonstrates feed type

1-Commerical birds-30 da

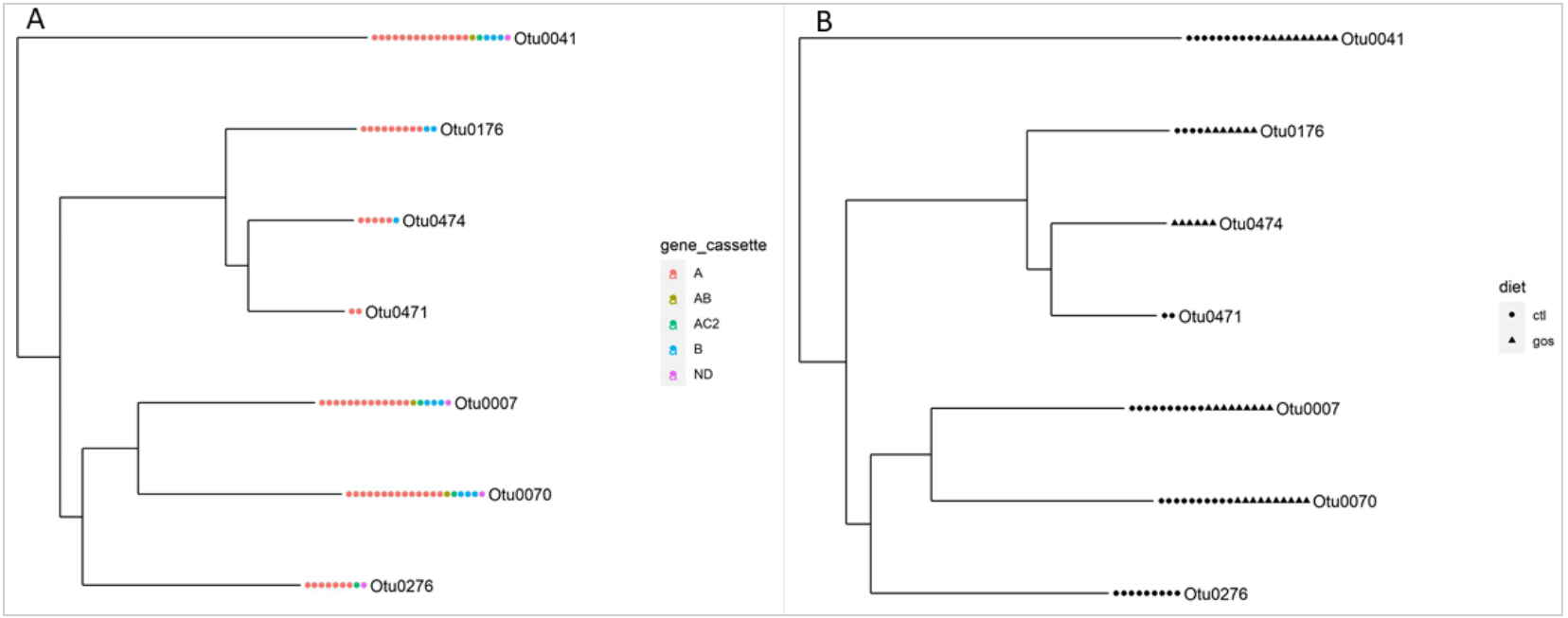


The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU 0169 is Gamma-proteobacteria (*Proteus*), OTU0176 is Alpha-proteobacteria (*Aestuariispira*), OTU0070 is Beta-proteobacteria (*Parasutterella*), OTU0276 is *Proteobacteria_unclassified.*

*** ND not detected.**

Appendix 4.4.2 The abundance of resistance GCs arrays in Proteobacteria phylum in commercial birds. Figure A shows distribution of GCs and Figure B demonstrates feed type

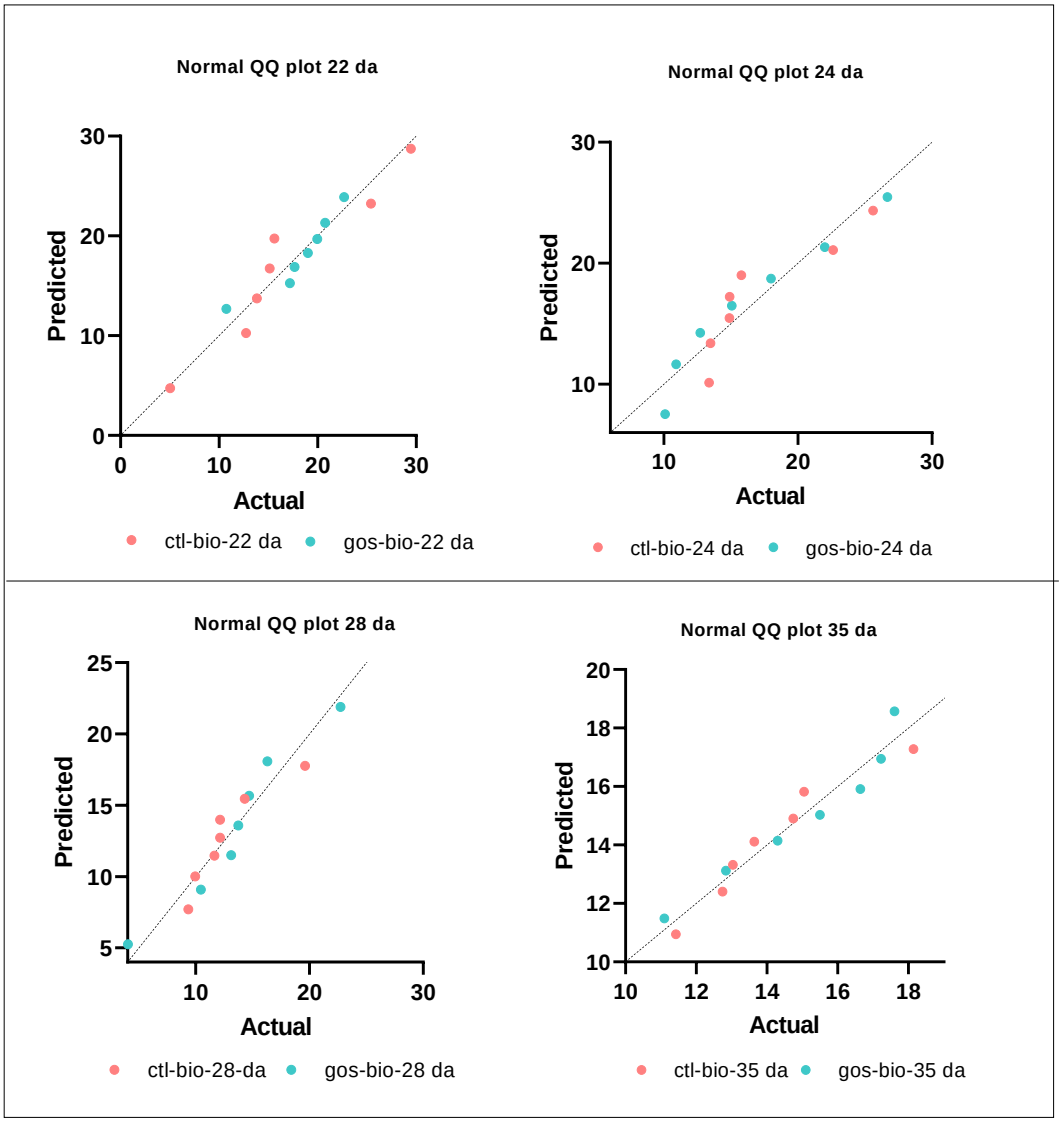
2-Commerical birds-37 da



The scale bar represents a sequence divergence of 3%. The bacterial OTUs are: OTU0041 is Epsilonproteobacteria (*Campylobacter*), OTU0176 is Alpha-proteobacteria (*Aestuariispira*), OTU0474 is *Alpha-proteobacteria_unclassified*, OTU0471 is Alpha-proteobacteria (*Alphaproteobacteria_unclassified* OTU0007 is Gamma-proteobacteria (*Enterobacteriaceae_unclassified*), OTU0070 is Beta-proteobacteria (*Parasutterella*), OTU0276 is *Proteobacteria_unclassified.* *** ND not detected.**

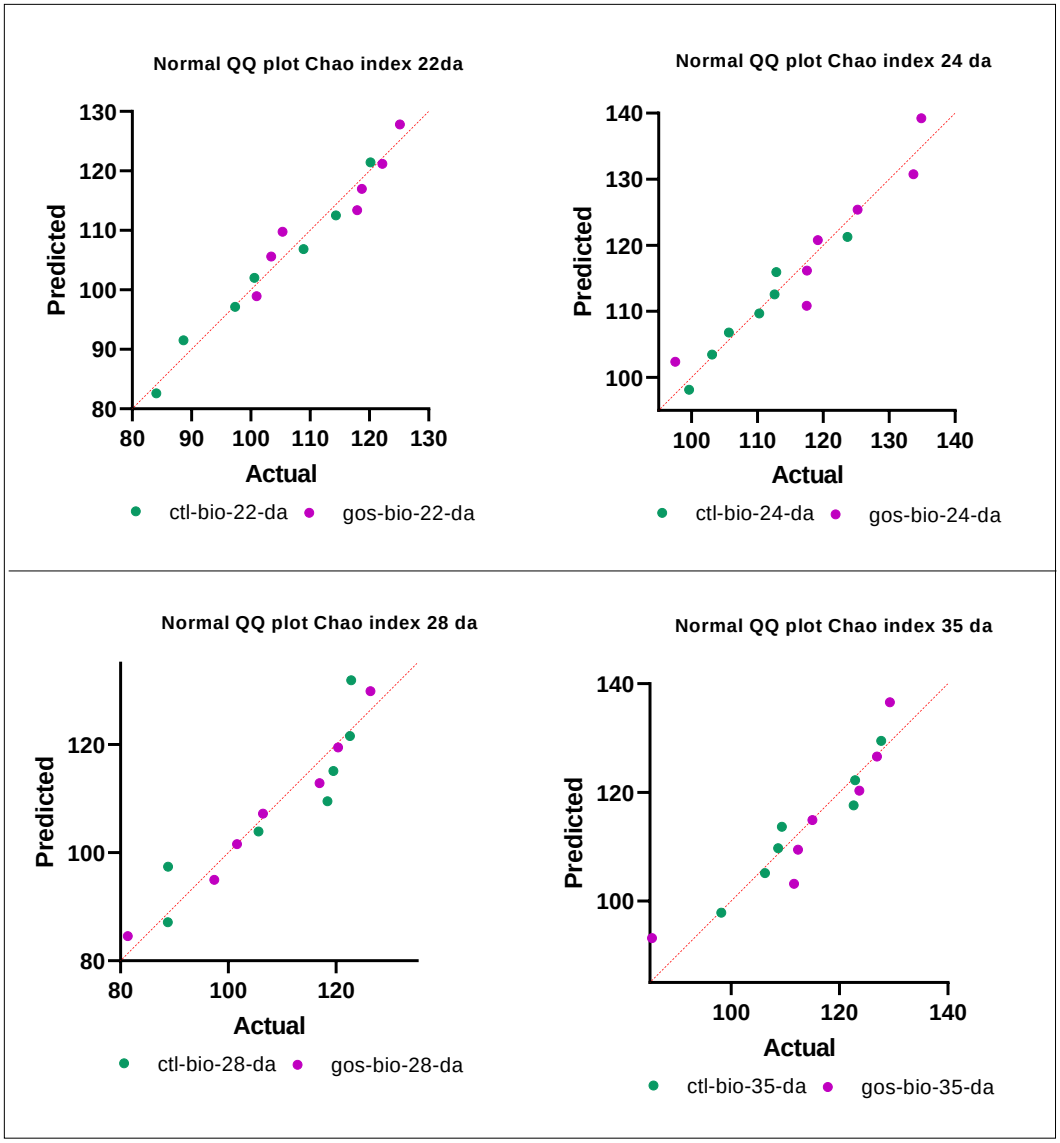
Appendix 4.5 QQ plot of normality test of control and GOS diets (all sampling days) for controlled housed birds or biosecure reading birds

A-inverse-Simpson index



The Shapiro-Wilk normality test shows that inverse Simpson data were not not normally distributed at control-24 da (p-value 0.0279) however other sampling days are of both diets were normally distributed p-value (>0.05).

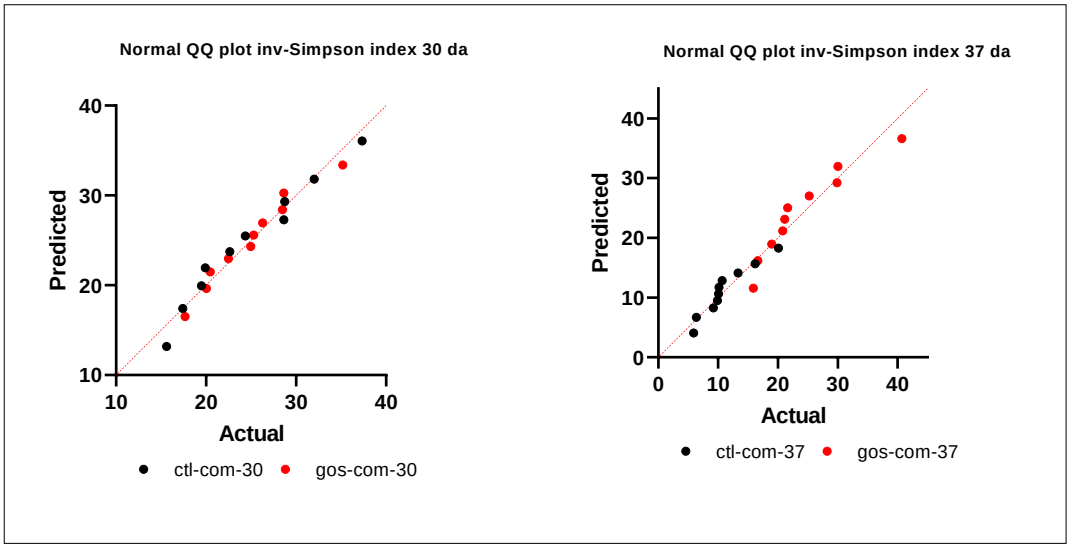
B-Chao index



The Shapiro-Wilk normality test shows that Chao index data were not normally distributed at control-28 da (p-value 0.0378) however other sampling days are of both diets were normally distributed p-value (>0.05).

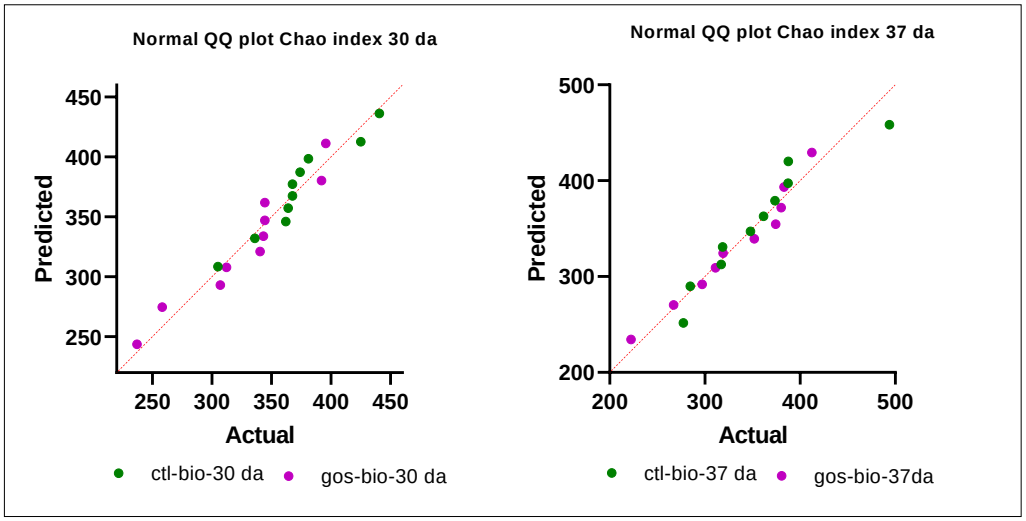
Appendix 4.6 QQ plot of normality test of control and GOS diets (all sampling days) for commercial birds

A-inverse-Simpson index



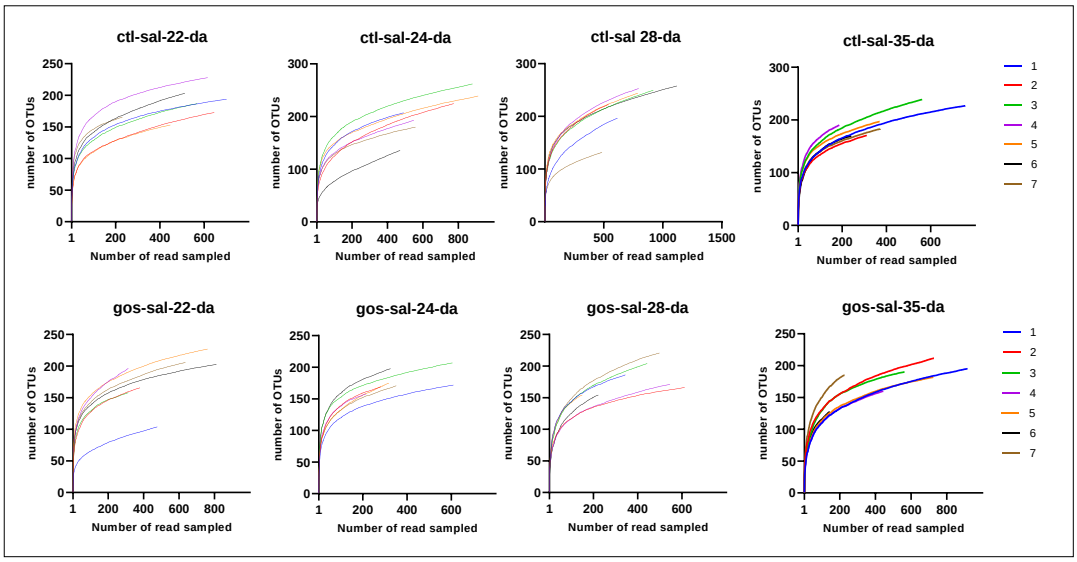
The Shapiro-Wilk normality test shows that data normally distributed at 30 and 37 da (p-value = 0.7348, 0.8189 for control-com vs GOS-com 30da; p-value 0.2637, 0.1736 among control-com vs GOS -com at 37 da).

B-Chao index

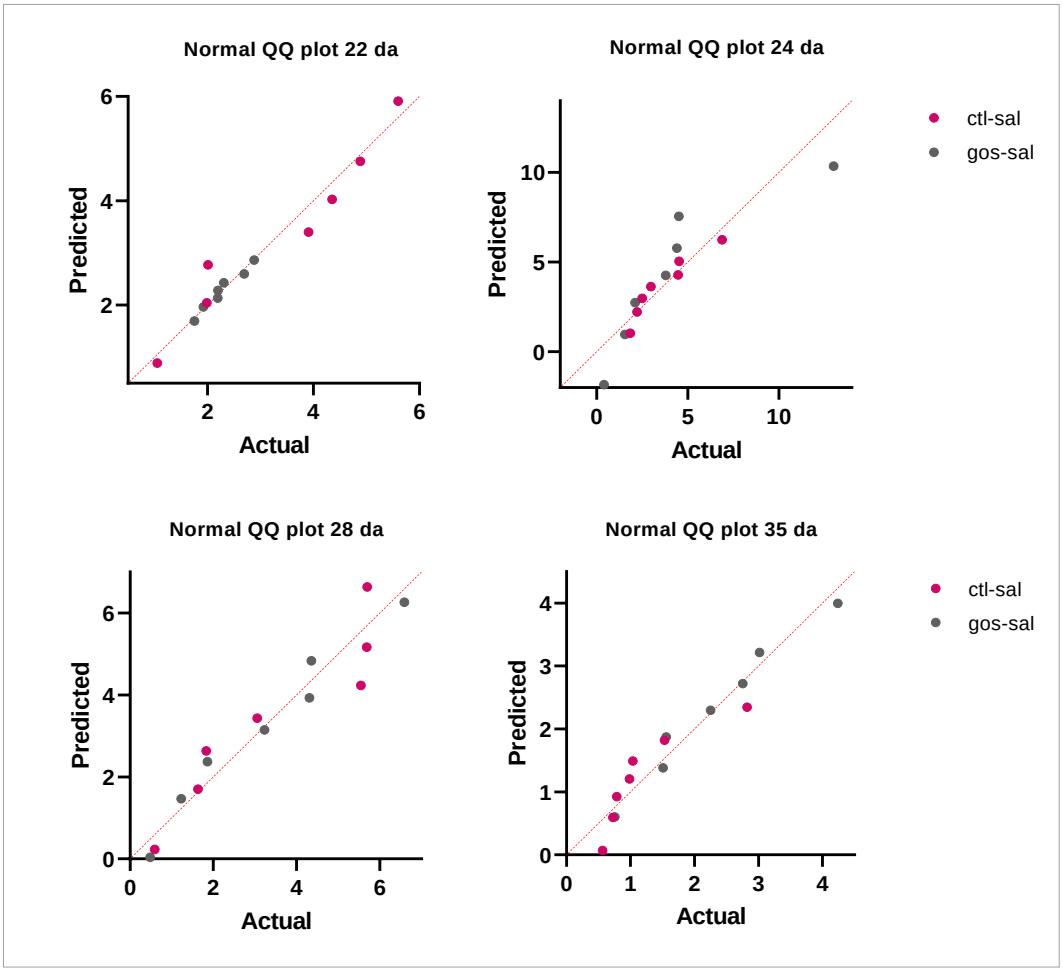


The Shapiro-Wilk normality test shows that data normally distributed across cohorts (p-value >0.05).

Appendix 5.1 Rarefactions curves of observed OTUs of caecal samples microbiota collected from challenged groups fed both control and GOS diets reared in controlled housing condition

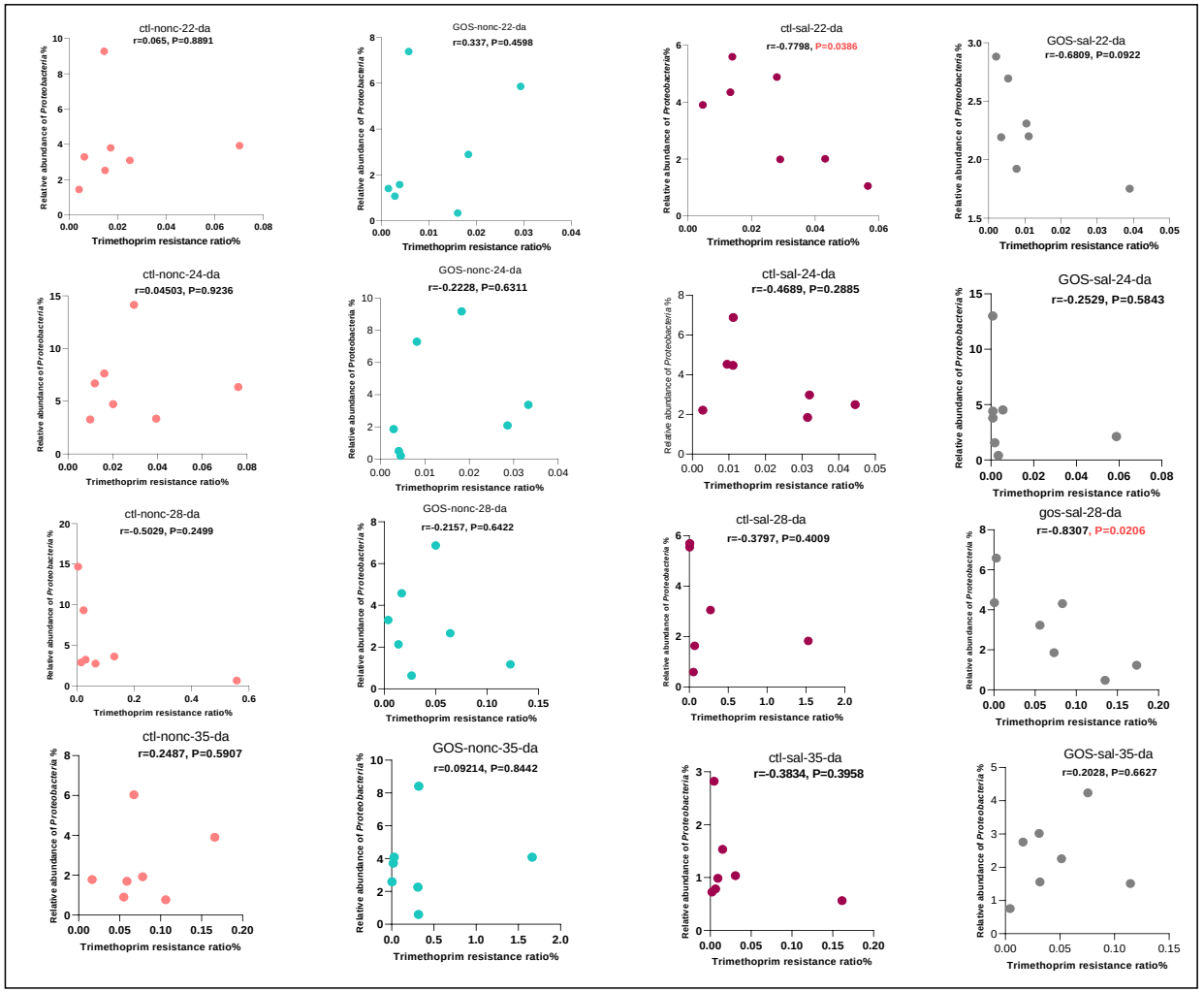


Appendix 5.2 The relative abundance of Proteobacteria phylum in control and GOS diets of controlled housing birds.



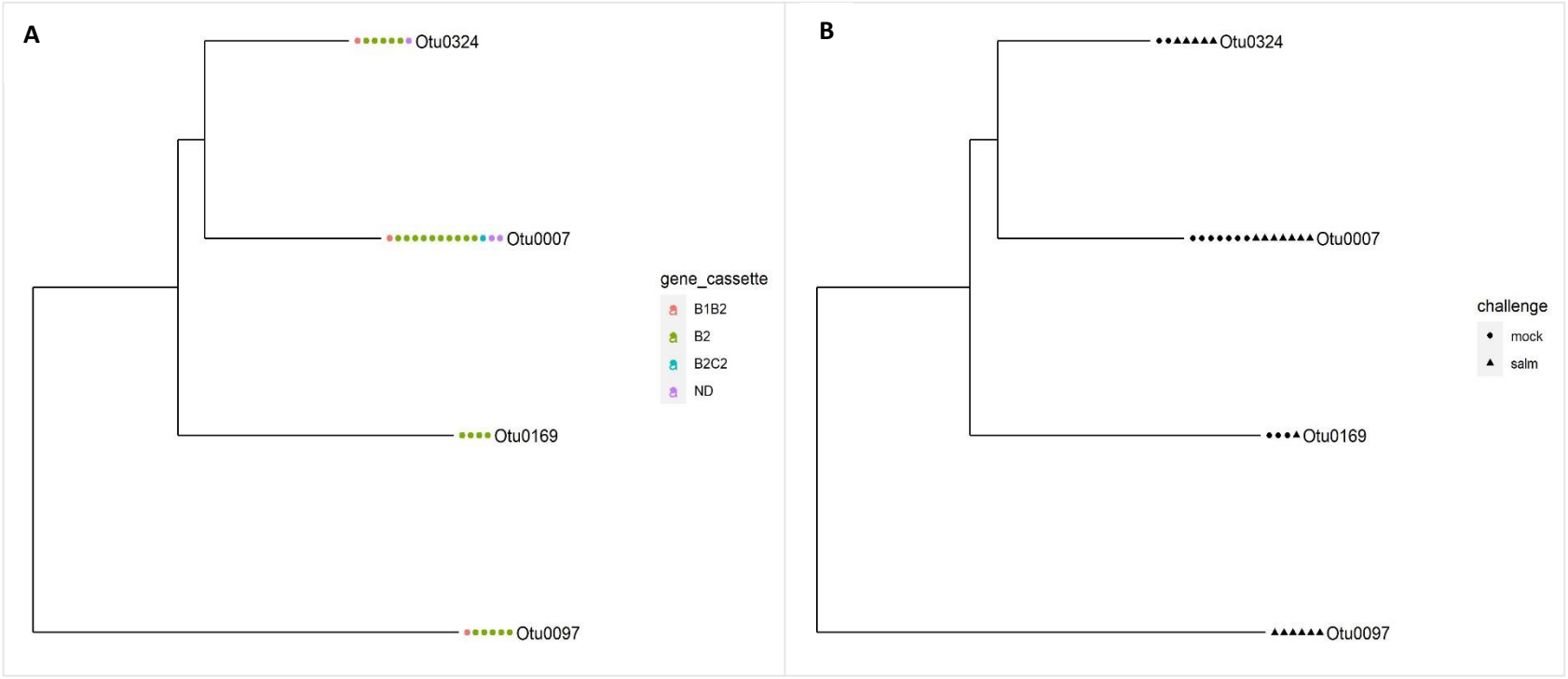
The Shapiro–Wilk test of normality displayed that the data of abundance Proteobacteria were not normally distributed at 24 da in gos-sal barns(p-value=0.0358) as well as in ctl-sal barns at 35 da old birds (p-value=0.0304) otherwise all data showed normal distribution (p- value >0.05).

Appendix 5.3 Correlation coefficient between the relative abundance of Proteobacteria and proportional of trimethoprim resistance population (Ratio). Significance calculated by using Pearson's correlation coefficient method.



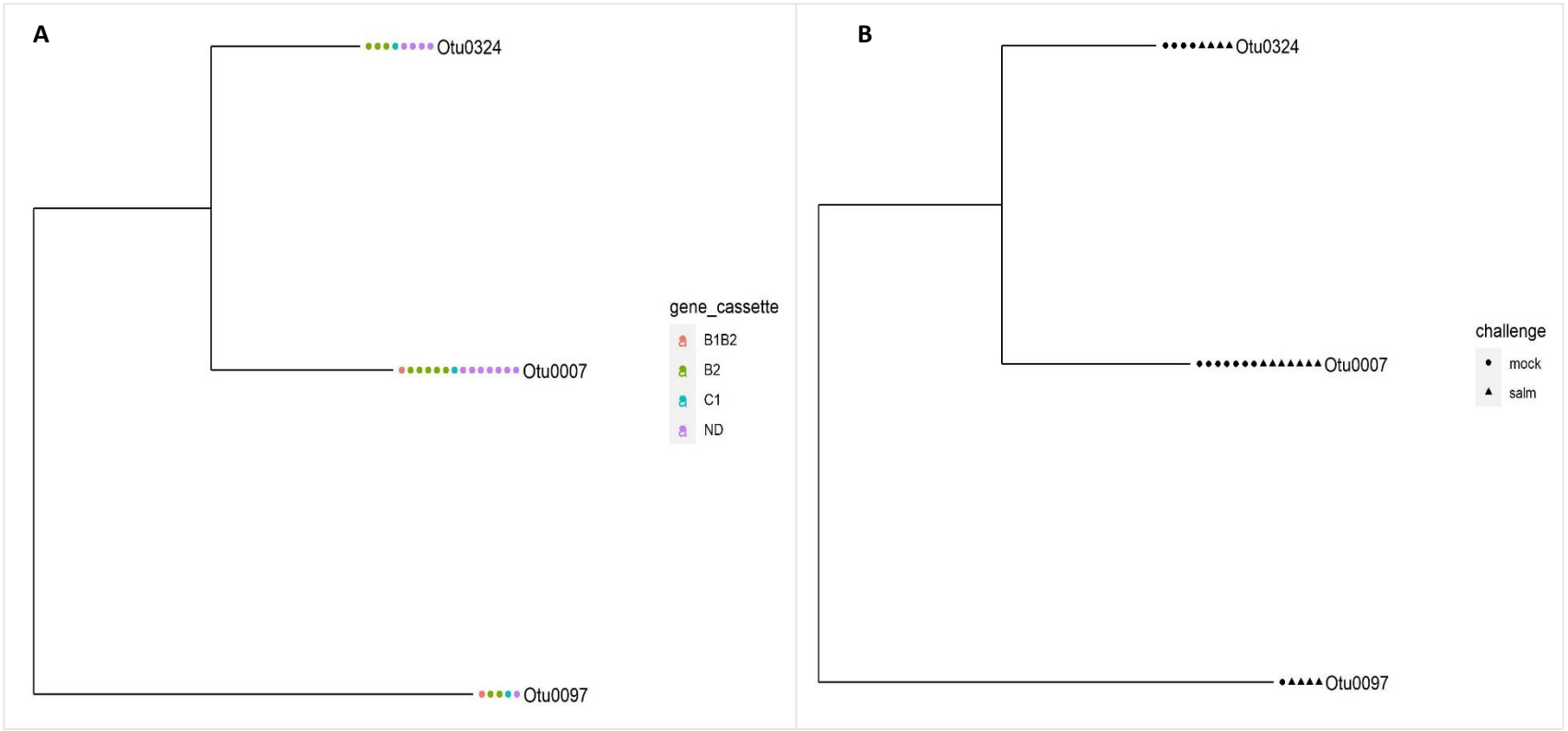
Appendix 5.4 A The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

1. ctl_nonc and ctl_sal groups_ at 22 da



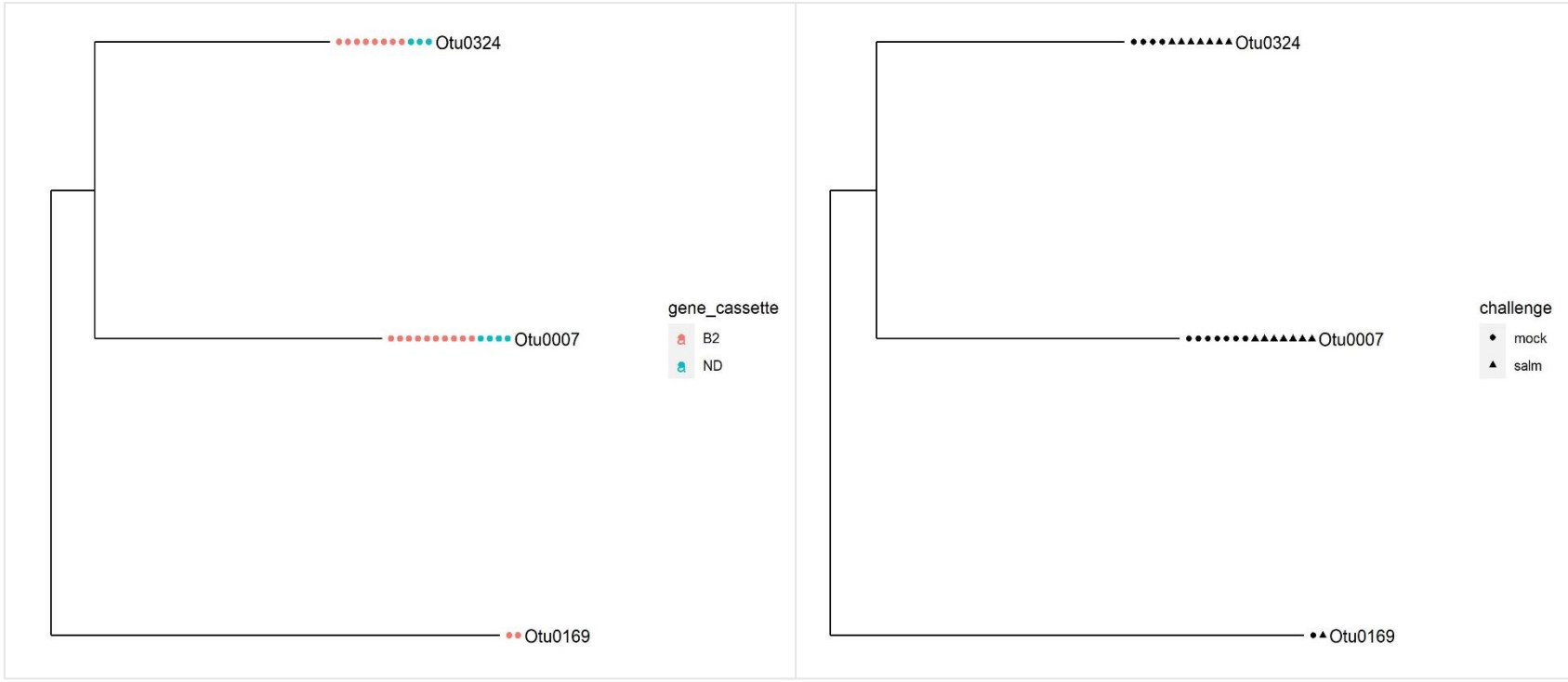
Appendix 5.4 B The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

2. gos_nonc and gos_sal groups_ at 22 da



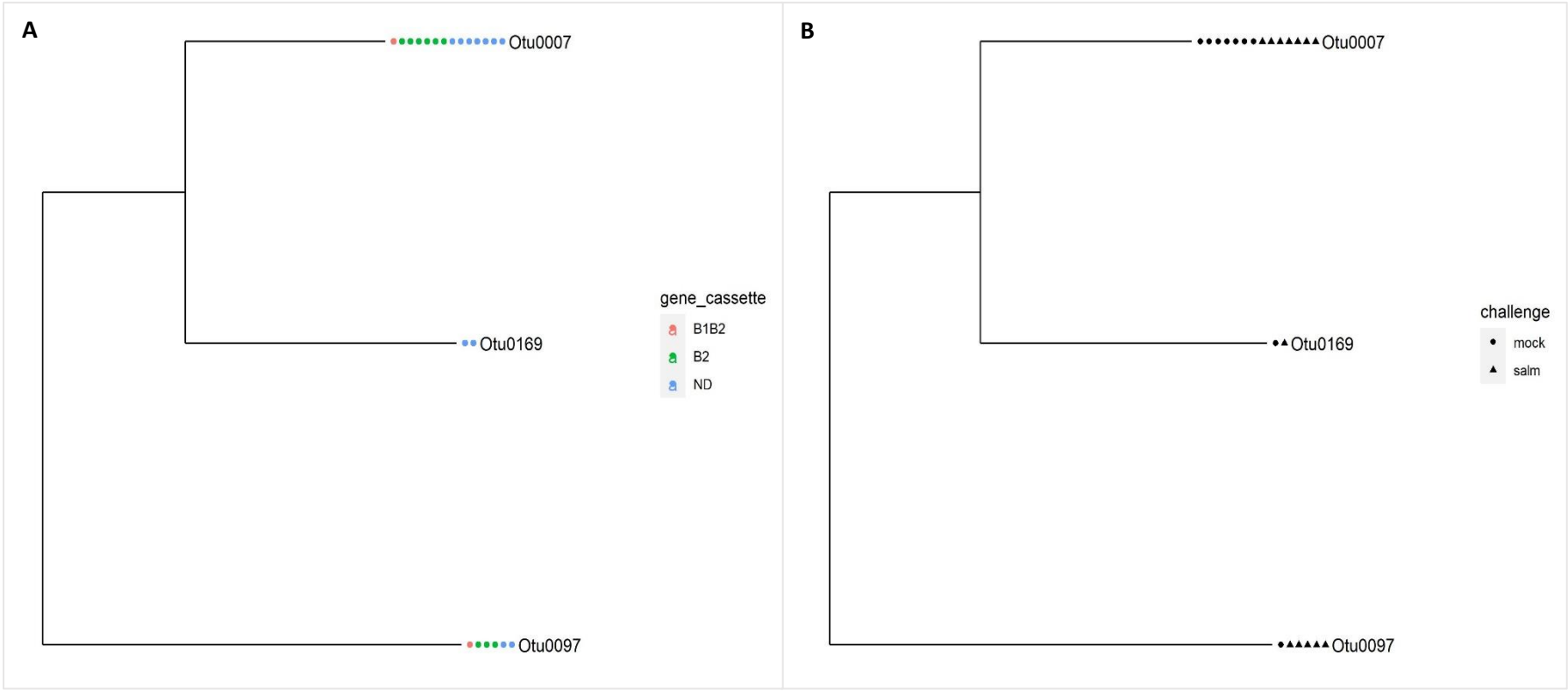
Appendix 5.4 C The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

3. ctl_nonc and ctl_sal groups_ at 24 da



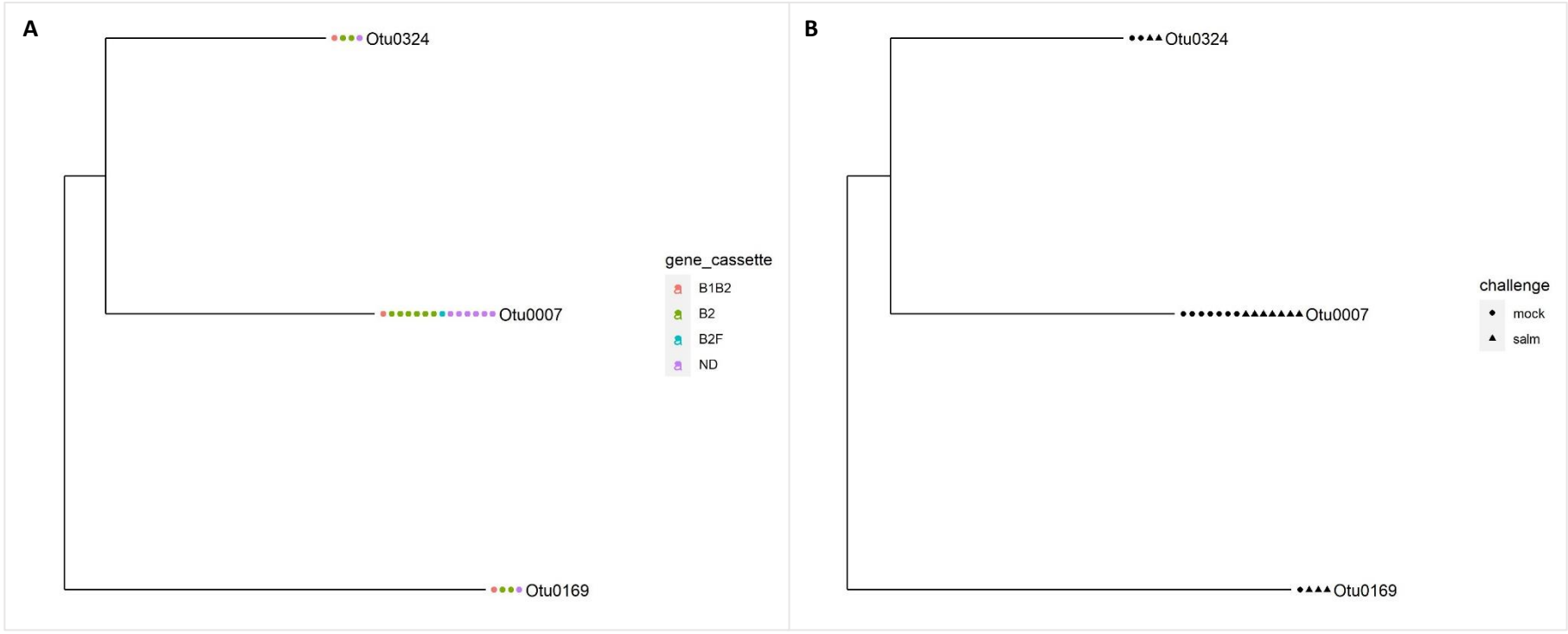
Appendix 5.4 D The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

4. gos_nonc and gos_sal groups_ at 24 da



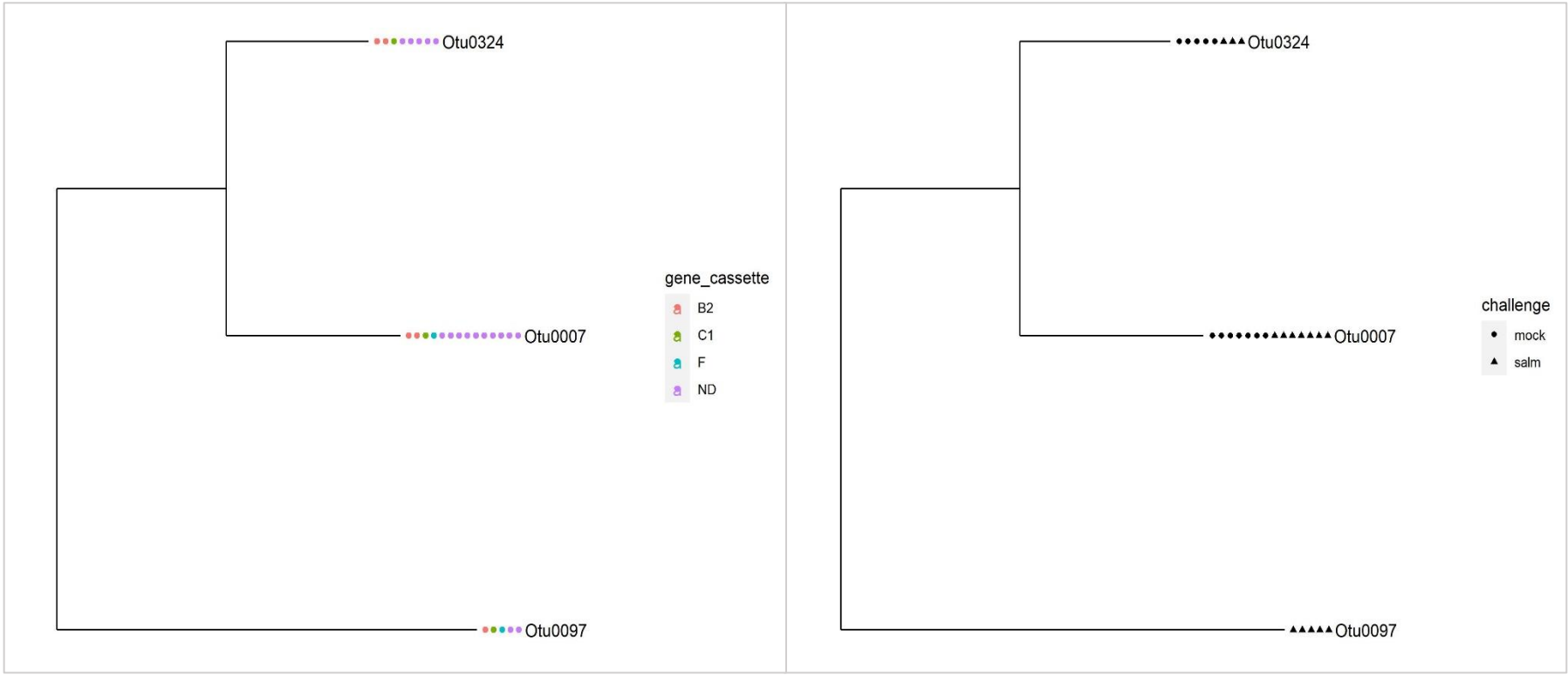
Appendix 5.4 E The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

5. ctl_nonc and ctl_sal groups_ at 28 da



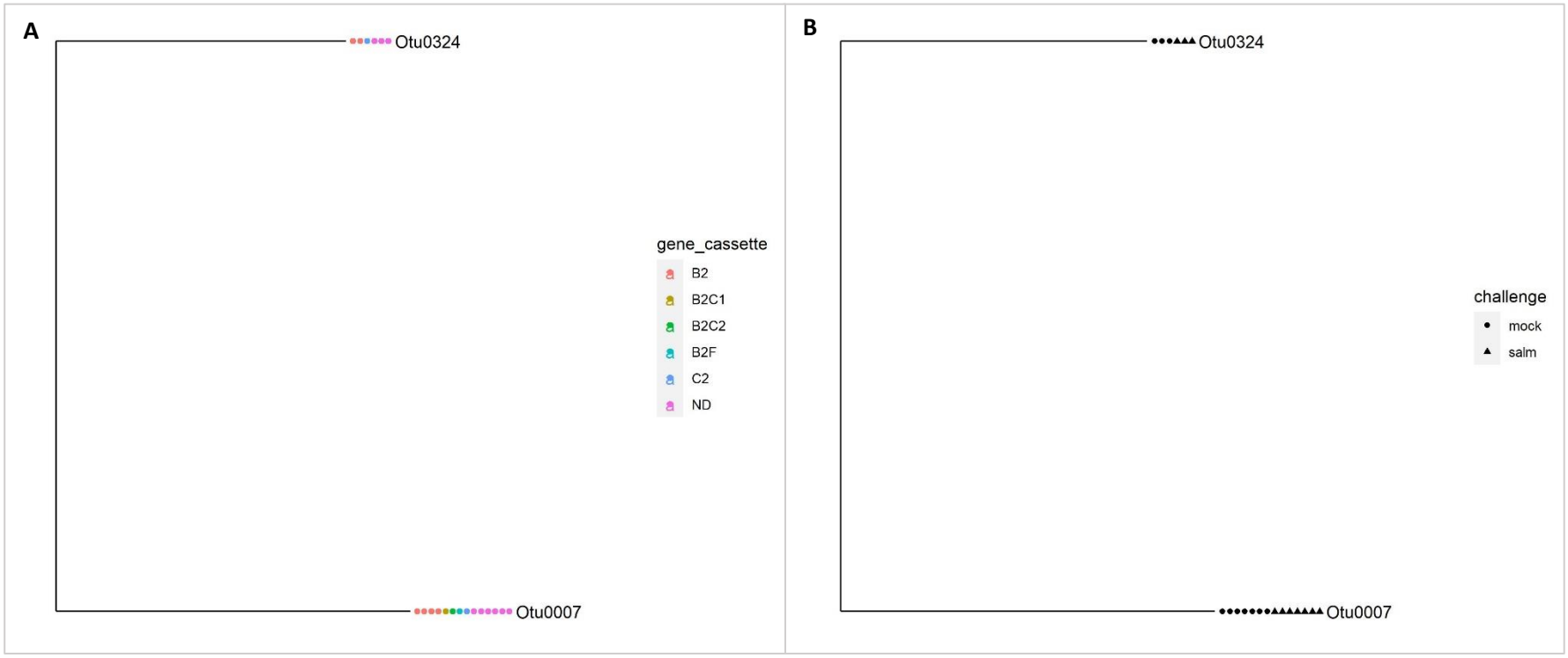
Appendix 5.4 F The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

6. gos_nonc and gos_sal groups_ at 28 da



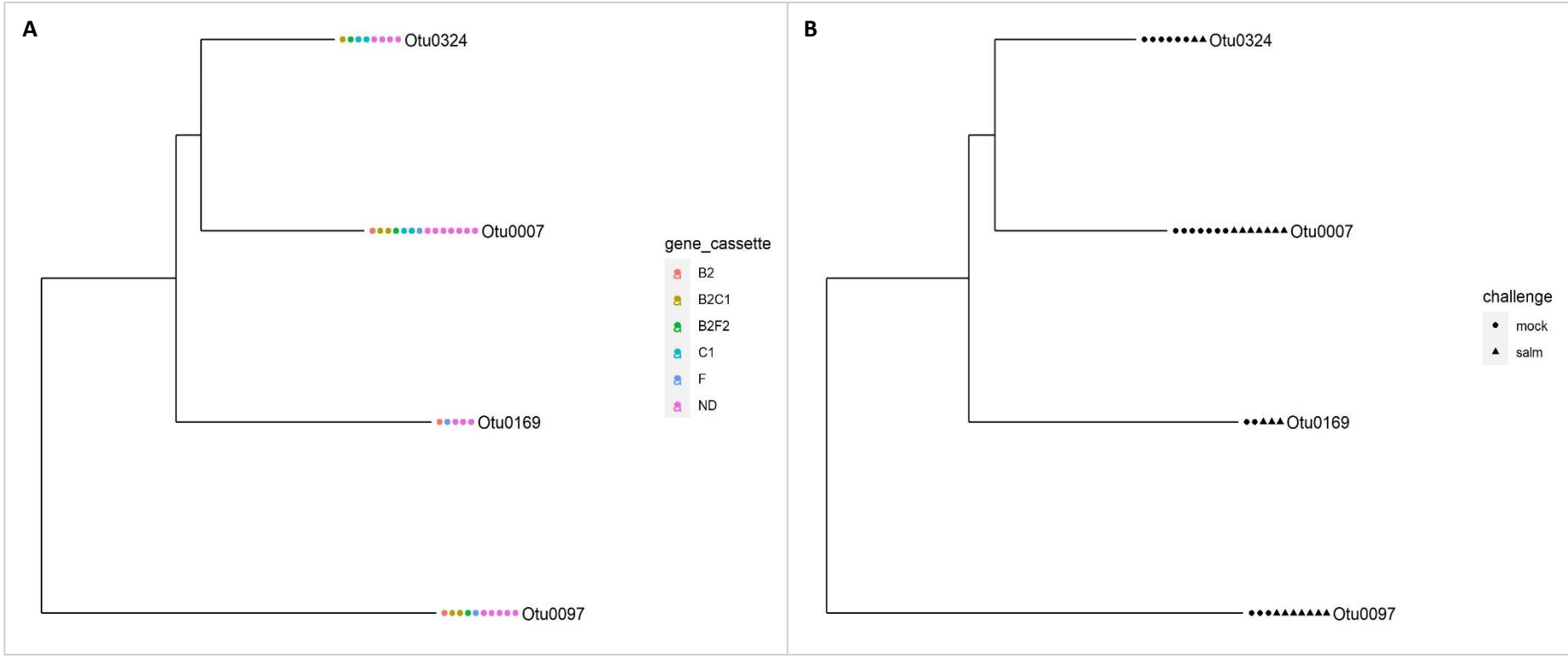
Appendix 5.4 G The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

7. ctl_nonc and ctl_sal groups_ at 35 da



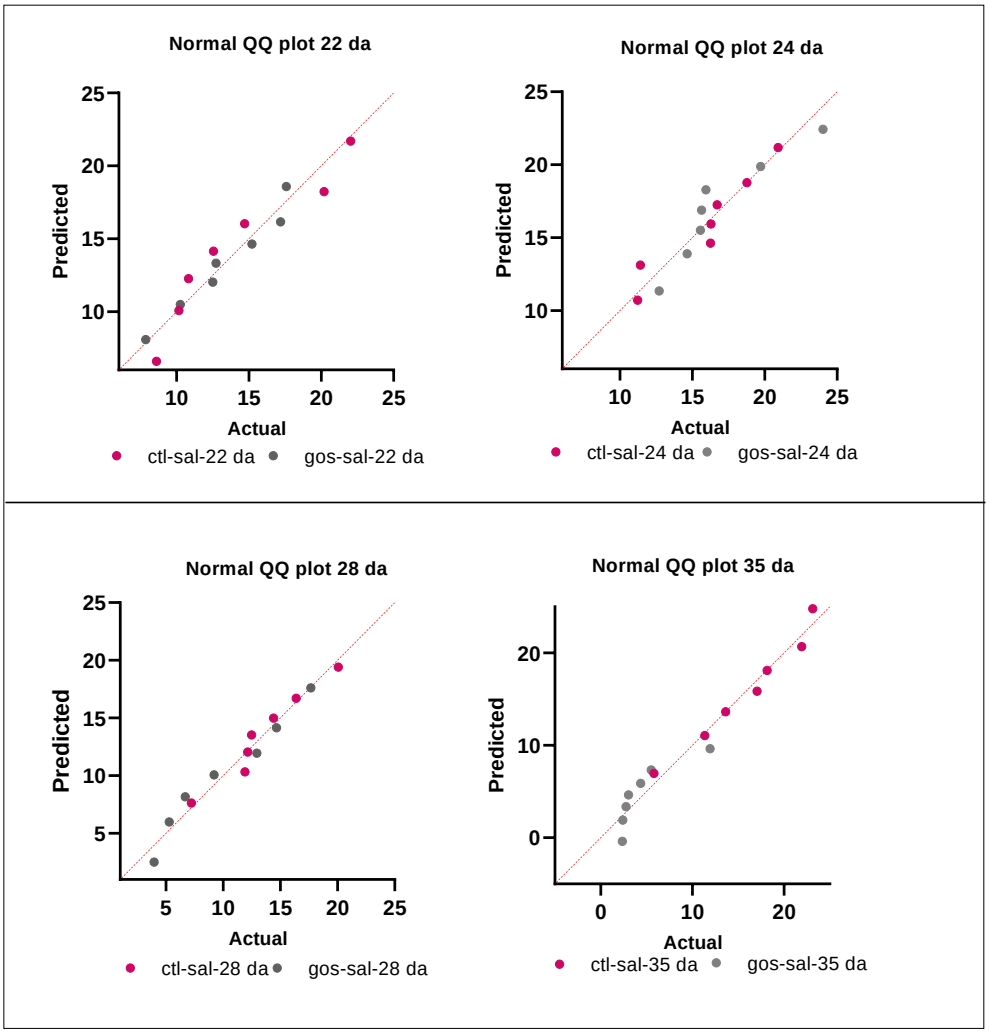
Appendix 5.4 H The abundance of resistance GCs arrays in Proteobacteria phylum in control fed birds colonised and non-colonised birds housing in controlled condition. Figure A shows distribution of GCs and Figure B demonstrates feed type

8. gos_nonc and gos_sal groups_ at 35 da



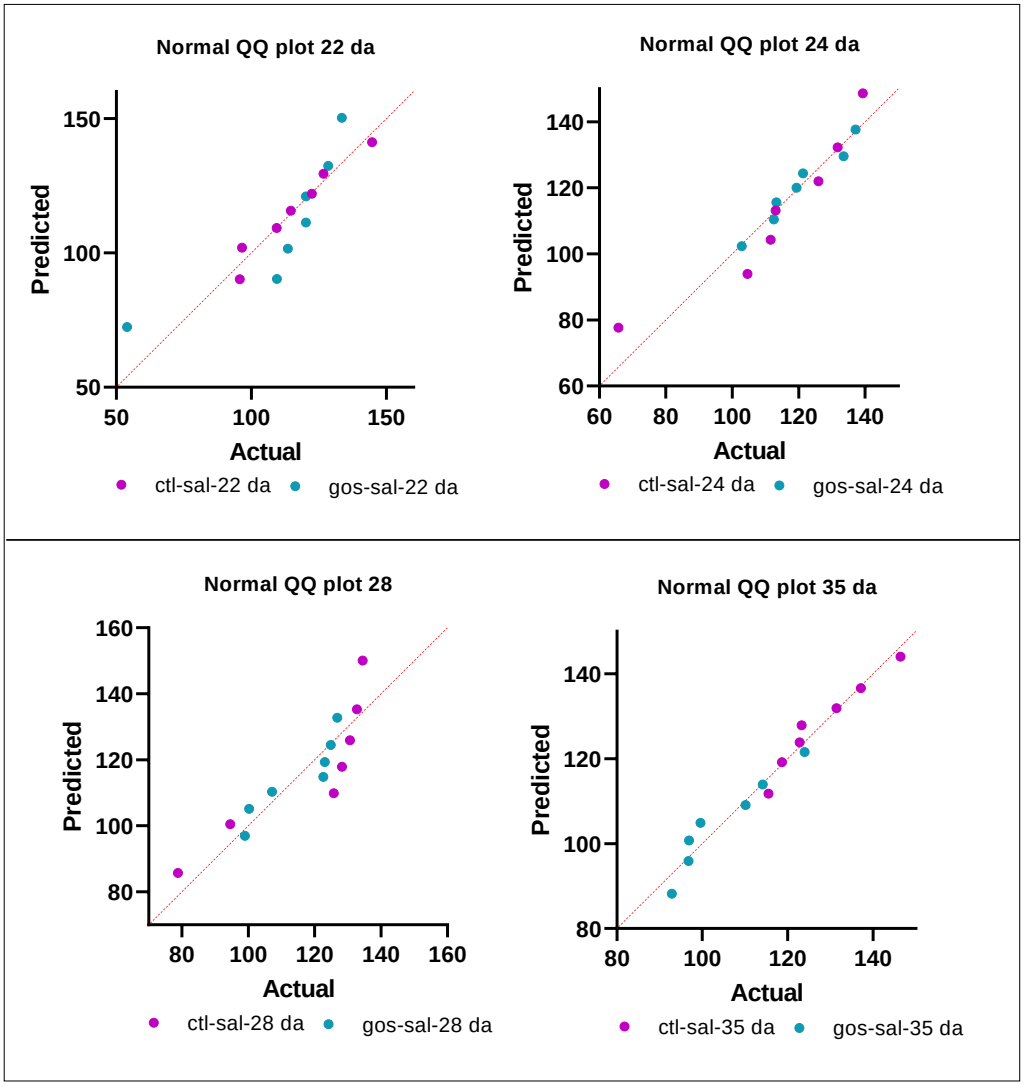
Appendix 5.5 QQ plot of normality test of control and GOS diets (all sampling days) for controlled housed birds or biosecure reading birds

A-inverse Simpson



The Shapiro–Wilk test of normality displayed that the data of inverse Simpson indices were not normally distributed at 35 da in gos-sal barns (p-value= 0.0064) otherwise all data showed normal distribution (p-value >0.05).

B-Chao index



The Shapiro–Wilk test of normality displayed that the data of Chao indices were not normally distributed at 22 da in gos-sal barns (p-value= 0.0116) as well as at 28 da in ctl-sal groups (p-value=0.0163) otherwise all data showed normal distribution (p-value >0.05).