Investigating the prevalence of antimicrobial resistance and virulence factors of *Escherichia coli* isolated from caecum in broiler chickens

Submitted in partial fulfilment of the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and that the use of all material from other sources has been properly and fully acknowledged.

Ahran Lee

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Abstract

The growing prevalence of antimicrobial resistance (AMR) by bacteria is a concern for both human and animal health. *Escherichia coli* (*E. coli*), found in the intestinal tracts of all animals and birds, has been widely used as a sentinel species for the determination of AMR. Antioxidants such as selenium and beneficial microbes such as yeasts and lactic acid bacteria may help prevent the proliferation of pathogenic bacteria and potentially AMR in the gut through mechanisms such as competitive exclusion, neutralization of toxins, bactericidal activity or enhanced immune competence. The aim of this study was to determine the evolution of AMR and virulence associated gene (VAG) of *E. coli* isolated from the caecum taken throughout the life of broiler chickens, and how this is influenced by administration of selenium, lactic acid bacteria and yeast.

No antibiotics were administered to the birds at any time. There was no significant effect of either selenium or yeasts or lactic acid bacteria on the prevalence of AMR and VAG in these studies. The birds were most susceptible to antibiotic resistant infection (78.57% and 100% of *E. coli* colony forming units showing resistance to TET and AMP) at the end of the starter phase (around 8 days old). The carriage of AMR and VAG by *E. coli* then declined during the grower and finisher phases. However, in the final bird study, resistance by *E. coli* to ampicillin (ranging from 60.4% to 71.8%) and tetracycline (ranging from 89.0% to 99.4%) was maintained throughout the birds' life, but the prevalence of VAG did decrease (being between 11.1% and 44.4% when birds were 28 days of age). The evolution of *E. coli* as the birds got older tended toward more commensal *E. coli* that were susceptible to antibiotics. The ability of *E. coli* to utilise sucrose, sorbose and dulcitol was associated with the carriage of VAG. Furthermore, AMR was associated with iron-uptake related genes. This suggests that further work investigating the manipulation of nutrient and iron availability in the chicken gut might provide a means of controlling the proliferation of putative pathogenic and AMR coliforms in young broiler chickens.

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List of abbreviations

^{1}H NMR	Proton nuclear magnetic resonance
AFEC	Avian faecal *Escherichia coli*
AMP	Ampicillin
AMPR	Ampicillin resistance
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
APEC	Avian pathogenic *Escherichia coli*
API	Analytical profile index
ARG	Antimicrobial resistance gene
*ast*A	Arginine succinyl transferase A
AU	Arbitrary unit
bp	Base pair
CDC	Centers for Disease Control and Prevention
C. famata	*Candida famata*
CFU	Colony forming unit
CHL	Chloramphenicol
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CP	Crude protein
Cu	Copper
D_2O	Deuterium oxide
DAEC	Diffuse-adhering *Escherichia coli*
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double-distilled water
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Thymidine triphosphate
E. coli	*Escherichia coli*

EAEC	Enteroaggregative *Escherichia coli*
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FAO	Food and Agriculture Organisation of the United Nations
FCR	Feed conversion ratio
FDA	Food and Drug Administration
Fe	Iron
FID	Free induction decay
GARDP	Global Antibiotic Research and Development Partnership
GSH-PX	Glutathione peroxidase
HGT	Horizontal gene transfer
*hly*A	Hemolysin A
HMDB	Human Metabolome Database
I	Iodine
*irp*2	Iron-repressible protein 2
iss	Increased serum survival
IU	International unit
*iuc*D	Iron uptake D
Kbp	Kilo base pair
KH_2PO_4	Potassium phosphate monobasic
L. fermentum	*Lactobacillus fermentum*
LAB	Lactic acid bacteria
LB	Luria-Bertani
LPS	Lipopolysaccharide

M	Mutability
M	Molar
M9	Minimal salts medium
ME	Metabolisable energy
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MHz	Megahertz
MLST	Multilocus sequence typing
Mn	Manganese
Mo	Molybdenum
MRS	De Man, Rogosa and Sharpe
NA	Nalidixic acid
Na_2HPO_4	Disodium hydrogen orthophosphate
$Na_2HPO_4 \cdot 7H_2O$	Sodium phosphate dibasic heptahydrate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen orthophosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
NMEC	Neonatal meningitis *Escherichia coli*
O_2	Oxygen
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
OIE	World Organisation for Animal Health
omp	Outer membrane protease
P	Probability
*pap*C	Pyelonephritis associated with pili C
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
Pmol	Picomole

RNA	Ribonucleic acid
rpm	Revolutions per minute
S. boulardii	*Saccharomyces boulardii*
SDA	Sabouraud dextrose agar
Se	Selenium
SEM	Standard error of the mean
SEPEC	Sepsis-associated *Escherichia coli*
sp.	Species
ST	Serotype
TBE	Tris-borate-EDTA
TET	Tetracycline
tsh	Temperature sensitive haemagglutinin
TSP	Tetradeuteropropionic acid
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
VAG	Virulence associated gene
WGS	Whole genome sequencing
WHO	World Health Organisation
x^2	Chi-square
YPD	Yeast extract peptone dextrose
Zn	Zinc

Chapter 1 Introduction

Antibiotics are antimicrobial substances produced naturally from living microorganisms which either kill or inhibit the growth of bacteria (Aminov, 2010; Hosain *et al.*, 2021). Antibiotics have been extensively used for a long time and these have played an important role in the prevention and treatment of bacterial infections. The discovery of antibiotics was a great achievement in medicine as antibiotics and chemotherapy have increased life expectancy and saved numerous lives in both humans and animals (Aminov, 2010; Blair *et al.*, 2015). Antibiotics have also been used agricultural livestock production and have contributed to improvements in productivity and disease prevention as well as for the treatment of infections (Singer and Hofacre, 2006; Brown *et al.*, 2017). Klein *et al.* (2018) investigated antibiotic consumption between 2000 and 2015 in 76 countries. Antibiotic consumption increased 65% (from 21.1 to 34.8 billion defined daily doses (DDDs)). Antibiotic use in animals is greater than antibiotic use in humans. For example, 72.5% (8,893,103 kg) of antibiotics considered medically important for human health by the FDA were sold in the USA for use in animals compared with 27.5% (2,279,226 kg) used in humans (O'Neill, 2015).

The dramatic increase in the use of antibiotics is a great global concern because this may increase the resistance to antibiotics rendering them ineffective. A growing prevalence of antimicrobial resistance (AMR) by bacteria is therefore a threat to public health (Marshall and Levy, 2011; Ma *et al.*, 2021). *Escherichia coli* (*E. coli*) is a gram-negative bacteria and consists of commensal and pathogenic strains. *E. coli* is associated with AMR and deemed a potential reservoir of AMR genes (Ewers *et al.*, 2009; Xiong *et al.*, 2018). AMR *E. coli* can quickly spread between animals, into

the food supply, the environment (soil and water), and into humans. All sectors are connected, and AMR *E. coli* are a One Health challenge (Pokharel *et al.*, 2020). The misuse or overuse of antibiotics without prescription or through inappropriate action can cause the development of AMR and spread between people through direct or indirect contact both in healthcare facilities and in the community (Larson, 2007). In addition, the wastes or contaminants produced from patients or healthcare facilities can contribute to antimicrobial resistance in the environment (for example the soil or watercourses). AMR *E. coli* in the environment can then spread to humans and animals (Durso and Cook, 2014).

Antibiotic use in intensive livestock production systems, particularly at sub-therapeutic doses for growth promotion purposes, have led to the emergence of multi-drug resistant bacteria. These bacteria can then transfer to the environment and people (Wall *et al.*, 2016). The AMR increase can make treatments on animals and people ineffective and lead to serious infections such as food poisoning, blood poisoning and urinary infections (CIWF, 2011). Thus, AMR can affect chicken productivity and health in poultry industry (Vidovic and Vidovic, 2020; Kasimanickam *et al.*, 2021). Furthermore, in U.S, AMR causes more than 2.8 million infections and more than 35,000 deaths every year (CDC, 2019). By 2050, it is estimated that the proliferation in AMR will result in 10 million deaths globally and an economic loss of $100 trillion annually (O'Neill, 2016).

The study of AMR has increased and many researchers have studied AMR mechanisms, and the emergence and transmission of AMR in animals and humans (Blair *et al.*, 2015; Holmes *et al.*, 2016; Vidovic and Vidovic, 2020). There is increasing awareness of the AMR issue and many countries and organisations such as World Health Organisation (WHO), Food and Agriculture Organisation of the

United Nations (FAO), World Organisation for Animal Health (OIE) and Global Antibiotic Research and Development Partnership (GARDP) have regulated antibiotic use (type, dosage and duration) and banned particular antimicrobials (for example fluoroquinolones) to try and reduce AMR problems (Landers *et al.*, 2012; O'Neill, 2016; More, 2020). Many experts have also investigated the effect of useful microorganisms, phage therapy, bioactive compounds, CRISPR-Cas9 gene editing technology and detecting efflux inhibitors as means of controlling the spread of AMR bacteria (Piddock *et al.*, 2010; Gholizadeh *et al.*, 2020; El-Tarabily *et al.*, 2021; Li *et al.*, 2021).

However, Miles *et al.* (2006) showed that there was still a high prevalence of AMR *E. coli* from excreta samples in older broiler chickens even though no antibiotic had been administered. There is still a considerable gap in the understanding of the complex mechanisms that lead to the emergence and prevalence of AMR *E. coli* in chickens that have not been exposed to antibiotics. In addition, understanding of any changes in the prevalence of AMR *E. coli* based on bird age is lacking.

This study seeks to obtain data which would help to address these research gaps and this thesis investigates the evolution of AMR genes in *E. coli* isolated from the broiler caecum as broiler chickens aged. Furthermore, AMR *E. coli* might be considered whether there might be some selective advantage through the association between antimicrobial resistant genes and virulence genes of *E. coli*. Feed additives can modulate the gut microbiome and they might decrease the population of AMR and pathogenic bacteria from the chicken gut. This study therefore set out to assess the effect of dietary interventions on the prevalence of AMR and putative pathogenic *E. coli*. It is anticipated that knowledge of the characteristics of AMR bacteria, and evolution of AMR with increasing bird age and

association between AMR and virulence genes might help future work to better understand means of combatting AMR bacteria.

Chapter 2 Literature review

2.1 Antimicrobials use in livestock production

With the intensification of the livestock industry, a number of antimicrobial agents were identified and added to feed for non-therapeutic purposes (Singer and Hofacre, 2006; CIWF, 2011). This was often for prophylactic use, but also for growth promoting purposes and this has now been practiced for several decades (Gustafson and Bowen, 1997; Van Boeckel *et al.*, 2015; Brown *et al.*, 2017). Antimicrobials, when used as growth promoters, were administered in concentrations between 2.5 mg/kg and 125 mg/kg, depending on the type of antibiotic and animal species (FAO *et al.*, 2004; Brown *et al.*, 2017). A wide range of antimicrobials have been researched and developed to improve daily weight gain and treat disease (Phillips *et al.*, 2004). The US Food and Drug Administration (FDA) first approved the use of antibiotics as feed additives in the animal industry without prescription in 1951 (Jones and Ricke, 2003; Brown *et al.*, 2017). Consequently, antimicrobial growth promoters in livestock production have been commonly applied worldwide with an increase of 10-to 20 fold since the 1950s (Brown *et al.*, 2017).

2.1.1 Current practices in use of antibiotics

Antibiotic usage considerably varies depending on countries. Currently, the largest consumers of antibiotics in food animals are China, US and Brazil (Tiseo *et al.*, 2020; Hosain *et al.*, 2021). Antibiotic usage is also practised in developing countries such as Bangladesh, Bhutan, India, Indonesia, Myanmar, Nepal, Sri Lanka and Thailand, and usage in developing countries has rapidly increased (Hosain *et al.*, 2021). Antibiotic usage in low-income countries may be poorly managed or involve the usage of drugs that are not approved for veterinary use, or which may be critically important, but may still be readily sold in an unregulated market (Magnusson *et al.*, 2019). In all countries, antibiotics have been administered via feed or drinking water with no veterinary supervision or prescription (Wall *et al.*, 2016; Van *et al.*, 2020; Hosain *et al.*, 2021). According to Van Boeckel *et al.* (2015), the increase of antimicrobial consumption results from a high demand for animal products in low and middle-income countries and conversion to large scale and intensive farming systems.

In the past, developed countries were responsible for a substantial use of antibiotics for animal production and health. Many researchers in Europe between 1980s and 1990s became concerned that the use of antimicrobial growth promoters in animals involved the same antibiotics that were consumed by humans (CIWF, 2011; More, 2020). Recently, some high-income countries realised the seriousness of AMR issues and have regulated the antibiotic use in livestock to try and prevent the proliferation of AMR (Magnusson *et al.*, 2019).

2.1.2 Guidelines in usage of antibiotics

The European Union banned in 2006 the use of antimicrobials as growth promoters including virginiamycin, tylosin phosphate, bacitracin zinc, spiramycin, avilamycin, flavophospholipol, monensin, salinomycin, carbadox and olaquindox (CIWF, 2011; Van *et al.*, 2020). Northern Europe limits antibiotic use for therapeutic and prophylactic purposes and only with veterinary prescription. The Netherlands have monitored and surveyed antibiotic usage in animal production, and they prohibited the mixing of antibiotics in feed. In addition, antibiotic use is strictly managed by veterinarians, and awareness of antibiotic regulation and AMR issues have been increased through education (Dewulf *et al.*, 2020).

However, in some countries, guidelines for the use of antibiotics are not strictly defined and antibiotics which are banned as growth promoters are still used as therapeutics and prophylactics resulting in an increasing AMR issue (Wall *et al.*, 2016; Van *et al.*, 2020; Hosain *et al.*, 2021). These countries unnecessarily used antibiotics for disease treatment and prevention. Tylosin is a banned growth promoter, but is available to use in feed for infection control in pigs and chickens (EFSA, 2010; CIWF, 2011). Some antibiotics are still used as coccidiostats such as lasalocid, monensin and salinomycin. Coccidiostats are added in feed to inhibit coccidioides, but the prolonged use of coccidiostats can result in antibiotic resistance. The EU previously banned monensin and salinomycin as growth promoters (More, 2020; Ma *et al.*, 2021), but these are still authorized as coccidiostats (CIWF, 2011). Therefore, the antibiotics should be prudently used and administered only by prescription. Many countries have continuously established the policies and guidelines of antibiotic use and are making considerable efforts to reduce the AMR issues. This will be discussed further in section 2.7.

2.1.3 Global trend of antibiotic use

Worldwide antibiotic usage is still increasing, and several countries still approve antibiotic use for animal production. Global antibiotic consumption for animal production is projected to increase by 67% from 63,151 (± 1,560) t in 2010 to 105,596 (± 3,605) t in 2030. The five countries projected to show the greatest proportional increase in antibiotic consumption are Myanmar (205%), Indonesia (202%), Nigeria (163%), Peru (160%), and Vietnam (157%) (Van Boeckel *et al.*, 2015). In addition, the consumption of antibiotics for animals in Brazil, Russia, India, China and South Africa is projected to rise by 99% from 2010 to 2030 (Van Boeckel *et al.*, 2015).

2.1.4 Types of antimicrobials used

Prolonged antibiotic use in livestock and humans is associated with the development and proliferation of antimicrobial resistant bacteria (Xiong *et al.*, 2018; Kasimanickam *et al.*, 2021). β-lactams, tetracyclines, aminoglycosides, lincosamides, quinolones, polypeptides, amphenicols, macrolides, and sulfonamides are commonly used antibiotics for animals (Chowdhury *et al.*, 2009; Marshall and Levy, 2011; Hosain *et al.*, 2021). Resistance to β-lactams, penicillin, tetracyclines and sulfonamides have been frequently observed in animal food, digesta and humans (Koga *et al.*, 2015; Hesp *et al.*, 2019; Majewski *et al.*, 2020; Aworh *et al.*, 2021; Ma *et al.*, 2021). Since both humans and animals can be infected by the same or similar pathogenic bacteria, they may be treated with similar antibiotics to treat infectious diseases. Aminoglycosides, cephalosporins (3rd, 4th and 5th generation), macrolides, ketolides, penicillin and polymyxins are used in

animals even though WHO (2019) classified these antibiotics as critically important in human medicine. Therefore, antibiotics used in livestock can pose the huge threat to human and public health (CIWF, 2011; Aidara-Kane *et al.*, 2018; CDC, 2019).

2.2 Emergence and mechanism of antimicrobial resistance (AMR)

The awareness about the potential issue of antibiotic resistance has increased over the past several decades (Guerra *et al.*, 2003; Persoons *et al.*, 2010; Grace, 2015; Marquardt and Li, 2018). The emergence and high prevalence of AMR is dependent on the quantity, duration and frequency of antibiotic use (McEwen, 2006). The overuse and improper management of antibiotics in both animals and humans increases the emergence and spread of AMR bacteria (Aminov and Mackie, 2007; Wall *et al.*, 2016).

A high prevalence of antimicrobial resistant bacteria has been detected in the gut digesta and meat of healthy animals, including those which have never been exposed to antibiotics (Miles *et al.*, 2006; Roth *et al.*, 2017). The emergence and proliferation of antimicrobial resistant bacteria results from selective pressure, intrinsic resistance and horizontal transmission (Persoons *et al.*, 2010). Selective pressures are external factors such as antibiotic exposure that encourage the proliferation of bacteria with intrinsic resistance or carriage of antimicrobial resistance genes through genetic mutation or via mobile genetic elements (Aminov, 2009). Intrinsic resistance is the innate traits of a bacterial species which confer resistance to particular antibiotics through the inherent structural or functional characteristics of the bacteria, which allow it to be insensitive to an antimicrobial

class such as aminoglycosides, flouroquinolones or β-lactams (Blair *et al.*, 2015; Wall *et al.*, 2016).

Horizontal gene transfer (HGT) is the movement of genetic material between bacteria (Holmes *et al.*, 2016). HGT has three main mechanisms (Figure 2.1): (1) transformation, in which free DNA is transferred to a bacterial cell; (2) transduction, which involves the mobilization of bacterial DNA from one bacterium to another by a bacteriophage; and (3) conjugation, which is the transfer of DNA from a donor bacterium to a recipient bacterium during physical contact (Holmes *et al.*, 2016; Wall *et al.*, 2016). HGT is a major driver of antimicrobial resistance, resulting in the multiplication of antimicrobial resistant bacteria via transposable elements to the same or different species (McEwen, 2006).

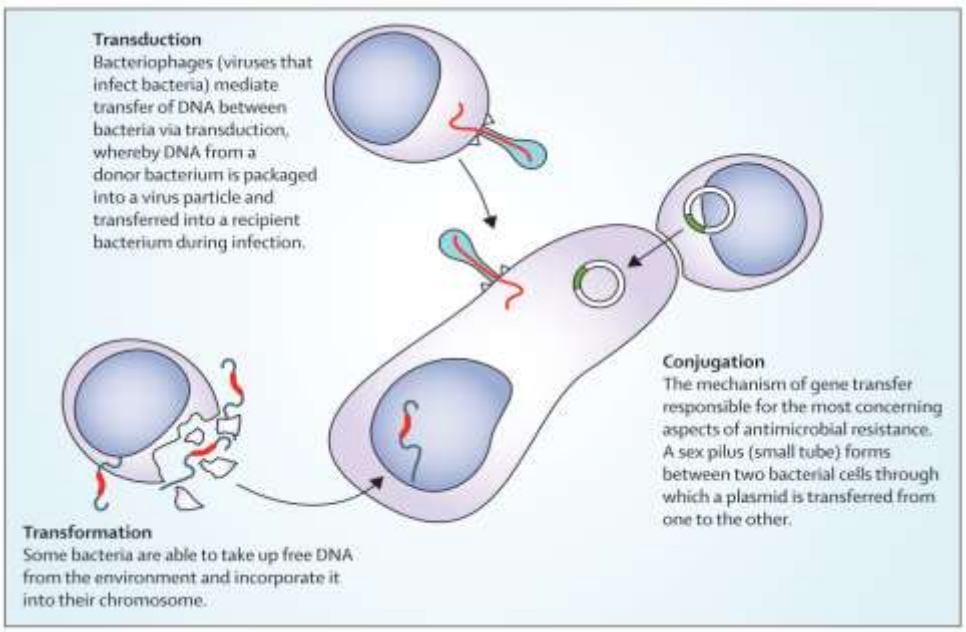


Figure 2.1 The transfer of genetical materials between bacteria (Holmes et al., 2016).

Both pathogenic and commensal bacteria may carry one or more resistance genes (Persoons *et al.*, 2010; Thorsteinsdottir *et al.*, 2010; Wasyl *et al.*, 2013). The mechanisms of antimicrobial resistance have four main mechanisms: (1) limiting uptake of antimicrobial agents; (2) modification of a drug target; (3) inactivation of an antibiotic; (4) active drug efflux. Figure 2.2 illustrates these generalised antimicrobial resistance mechanisms (Reygaert, 2018).

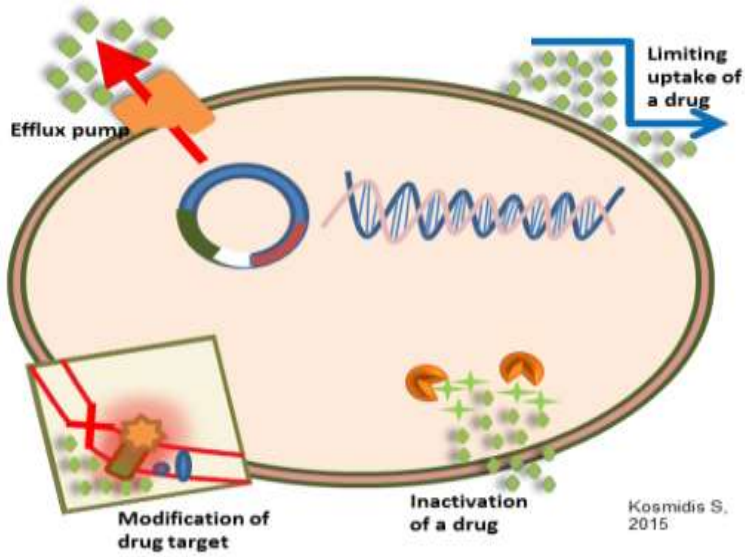


Figure 2.2 General antimicrobial resistance mechanisms (Reygaert, 2018).

2.3 Impact of AMR on public health

AMR bacteria can spread through the environment and between animals and humans, thus becoming a One Health challenge (Collignon and McEwen, 2019; Magnusson *et al.*, 2019). This section discusses how AMR is transmitted from livestock to the environment and thence to humans, and the impact of AMR on human health.

2.3.1 AMR transmission from livestock to human

AMR bacteria can transfer resistance to susceptible bacteria via mobile genetic elements and these AMR bacteria may then proliferate and spread rapidly to all sectors (Xiong *et al.*, 2018; Vidovic and Vidovic, 2020). The transmission pathways of antimicrobial resistance from livestock to humans are illustrated in Figure 2.3 (CFIA, 2017). Antimicrobial resistant bacteria can transfer to humans through direct contact with infected animals on the farm or through the consumption of undercooked or contaminated animal products, or via animal manure, water and contaminated crops (Dolejska *et al.*, 2012; O'Neill, 2015). In addition, the AMR in animal foods may spread nationally and internationally through the global trade of animal food products (Aarestrup, 2004; McEwen, 2006) and these can rapidly cause antibiotic-resistant infection throughout the world (Aarestrup, 2004; Landers *et al.*, 2012).

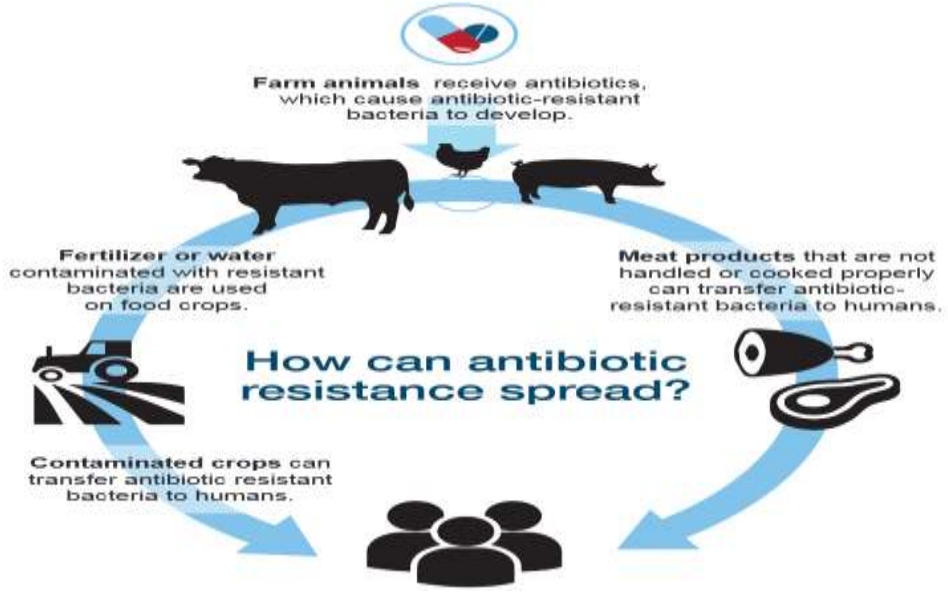


Figure 2.3 Transmission pathways of antimicrobial resistance from livestock to human (CFIA, 2017).

Thorsteinsdottir *et al.* (2010) reported on the genetic relatedness of AMR *E. coli* isolates from animals, animal foods, slaughterhouse personnel and outpatients in Iceland. The rate of antimicrobial resistant *E. coli* in samples taken from slaughterhouse personnel was 39.1%, while that of hospital outpatients (with diarrhoea) was 23.1%. The *E. coli* isolated from broiler meat and a slaughterhouse worker was observed to have the same resistance pattern and pulsotype (Thorsteinsdottir *et al.*, 2010). These findings suggest a high prevalence of antimicrobial resistance in *E. coli* isolates taken from slaughtered animals, and more worryingly the potential transfer of resistance genes by *E. coli* from animals or environmental factors such as contaminated feed to people working in close association with these animals (slaughterhouse personnel compared with hospital outpatients). The direct association of AMR transfer and public health is still controversial, but clearly there is potential for AMR *E. coli* to transfer to humans from faecal contamination during carcass processing, via direct or indirect pathways, and poses a potential risk of AMR infection to humans.

2.3.2 Threat of AMR to human health

Antimicrobial resistance is a significant threat to global public health (Ma *et al.*, 2021). Accumulation of antimicrobial resistance may cause serious results such as an ineffectiveness of initial antibiotic treatment, severe illnesses and increase of death rate (CIWF, 2011). In the US, more than 2.8 million people are infected with bacteria diseases annually, and deaths of more than 35,000 people arise from antimicrobial resistance (CDC, 2019). In addition, about 223,900 people in the US needed treatment for *Clostridioides difficile* and at least 12,800 people were killed in 2017 (CDC, 2019). Chen *et al.* (2019) reported that 195,763 cases of

pneumococcal disease have been unresolved due to antimicrobial resistance and 2,925 children died every year in Ethiopia. It also caused a first treatment failure rate of 29.4%. Murray *et al.* (2022) estimated the number of deaths resulting from AMR bacteria in 204 countries in 2019. As a consequence of AMR, around 27.3 people per 100,000 have died, and this was highest in western sub-Saharan Africa and lowest in Australasia at 6.5 per 100,000. O'Neill (2014) reported that around 700,000 people worldwide die each year from drug resistant strains of common bacterial infections such as tuberculosis and malaria. It is estimated that 10 million deaths globally and a cumulative cost of $100 trillion will result from antimicrobial resistance by 2050 (O'Neill, 2016; Kasimanickam *et al.*, 2021).

A number of AMR bacteria inhabit the intestinal tract of chickens and the chicken gut functions as a significant reservoir of antimicrobial resistant genes (Diaz Carrasco *et al.*, 2019; Sood *et al.*, 2019). To explore the evolution of AMR bacteria and prevalence within the intestinal tract throughout broiler chicken's life, it is imperative to understand the gut physiology and development of the gut microbiome over time, and the interaction between host and bacteria.

2.4 Gastrointestinal tract and gut microbiome of the chicken

The chicken has a digestive system different from the intestinal tract of mammals. The chicken does not have teeth but has a crop, proventriculus, gizzard, intestines and cloaca (Jacob *et al.*, 2011; Svihus, 2014). The main function of the chicken gut is the digestion of food and absorption of nutritional constituents (Scanes and Pierzchala-Koziec, 2014). Table 2.1 shows the average retention time and pH in the digestive tract of broiler chickens (Ravindran, 2013). The average gut transit time

from feed intake to excreta is approximately 3 to 4 hours (Ravindran, 2013). The bacterial community in the gut plays a crucial role in nutrient absorption, improvement of immunity and defending against pathogens (Rehman *et al.*, 2007; Grond *et al.*, 2018; Diaz Carrasco *et al.*, 2019). This section will explain the processes occurring in different sections of the gastrointestinal tract, the establishment and composition of the gut microbiome, and the interaction between microbiome and host.

Table 2.1 Average retention time and pH in digestive tract of broiler chickens (Ravindran, 2013).

Segment	pH	Transit time, min
Crop	5.5	10-50
Proventriculus/gizzard	2.5-3.5	30-90
Duodenum	5-6	5-10
Jejunum	6.5-7.0	20-30
Ileum	7.0-7.5	50-70
Caecum/colon	8.0	20-30

2.4.1 Gastrointestinal tract of chicken

Oesophagus and crop

The bird gathers feed into its mouth via its beak, and the feed is quickly swallowed and enters the oesophagus. The oesophagus is a long and flexible tube that connects the mouth to the rest of digestive tract. This transfers food and water to the crop and proventriculus (Svihus, 2014). The crop is a large pocket of the oesophagus and much food can be stored in here awaiting digestion (Jacob *et al.*, 2011).

Proventriculus

The wall of the proventriculus is thickened and the proventriculus has multiple secretory tubules and deep gastric glands with lobules (Scanes and Pierzchala-Koziec, 2014; Svihus, 2014). The proventriculus is the glandular stomach in poultry and acts as the 'true stomach' (Svihus, 2014). The process of digestion begins in the proventriculus and gastrin hormone stimulates the oxynticopeptic cells to secrete the proenzyme known as pepsinogen, as well as hydrochloric acid (Borda-Molina *et al.*, 2018). The acid converts the inactive pepsinogen into active pepsin. Pepsin is proteolytic enzyme and breaks down proteins into smaller peptides and amino acids. The pH of the proventriculus is very low (2.5) and this inhibits bacterial growth (Scanes and Pierzchala-Koziec, 2014).

Gizzard

The gizzard is the muscular ‘stomach’ and acts as the bird’s teeth. Consumed foods are mixed and ground by small stones or grit that accumulate in the gizzard (Jacob *et al.*, 2011). The gizzard works between 2 and 5 contractions per minute depending on the consistency of the feed particle. The size of the gizzard grows considerably during pre- and post- hatching and the numbers of muscle cells increase greatly (Gabella, 1989; Scanes and Pierzchala-Koziec, 2014). The gizzard is a relatively large organ especially when filled with ingesta. The gizzard becomes bigger and the pH of the digesta is lower when chickens are fed coarse, insoluble fibres compared to fine particles (Sacranie *et al.*, 2012; Scanes and Pierzchala-Koziec, 2014).

Small intestine

The small intestine consists of the duodenum, jejunum and ileum. Relative growth of the small intestine is greater compared to the body weight of the bird post hatch and peaks from 6 to 10 days (Sklan, 2001; Lilburn and Loeffler, 2015). The villi and crypt system of the jejunum rapidly develops in the chicks after hatching and the epithelial cells also proliferate (Uni *et al.*, 2000; Scanes and Pierzchala-Koziec, 2014).

Their main functions are food digestion by the action of digestive enzymes and nutrient absorption across the villi and into the blood stream (Jacob *et al.*, 2011). The enzymes in the digestive juices produced by the pancreas digest carbohydrates, protein and fat in the duodenum. Glucose is absorbed in the duodenum by active transport. The pancreas, which is located in the duodenal loop also secretes

bicarbonate that protects the duodenum by neutralizing the digesta and acid coming from the gizzard (Borda-Molina *et al.*, 2018).

Meckel's diverticulum is a small pouch located at the junction between the jejunum and ileum (Scanes and Pierzchala-Koziec, 2014). The Meckel's diverticulum is formed during the chicken's embryonic development and is derived from the yolk sac. The yolk sac in the egg provides nutrients for the embryo's growth, and in early life post hatch. The yolk sac gets to the navel cavity of the embryo right before hatch and then the remaining tiny sac is the Meckel's diverticulum (Jacob *et al.*, 2011).

Cholecystokinin hormone produced from the duodenum stimulates the gall bladder to secrete bile when fat is in the duodenum. Bile plays an important role in digestion and absorption of lipids. Bile emulsifies lipids to enable them to be more easily digested (to free fatty acids and glycerol) and helps in the absorption of fat-soluble vitamins (A, D, E, and K) in the jejunum. Digesta then pass into the ileum, which is the site of amino acid, fatty acid and glycerol absorption into the lymphatic vessels (Svihus, 2014).

Caeca

The caeca are two blind sacs that are located at the junction of the ileum and colon. Bacteria in the caeca digest and ferment undigested material. Caecal digesta are emptied about every 24 hours and appear as the light brown, frothy excreta (Svihus, 2014). According to Karasawa and Maeda (1995), caecal proteins and amino acids can also be moved to the small intestine by reverse peristalsis from where they may be digested and absorbed.

The caeca have a crucial role in the absorption of water and electrolytes such as sodium and chloride. The anaerobic fermentation of carbohydrates in the caeca results in the production (and absorption) of volatile fatty acids, which contributes to the energy balance of the bird, and B group vitamins are also produced by the bacteria in the caeca (Son, 2012; Scanes and Pierzchala-Koziec, 2014).

Large intestine and cloaca

The large intestine is a very short organ in the bird, connecting the ileum to the cloaca. The large intestine chiefly absorbs water and dries out undigested food (Scanes and Pierzchala-Koziec, 2014). The cloaca is the single external opening in the bird, and undigested food, waste, urine and, eggs from the oviduct passes through the cloaca or vent (Jacob *et al.*, 2011). Chickens don't urinate because there is no bladder. Urine produced by the kidney are semi-solid, white crystals of uric acid which cover the excreta (Borda-Molina *et al.*, 2018).

2.4.2 Importance and role of the gut microbiome

The gut microbiome is a complex and diverse community of microorganisms including bacteria, fungi, archaea and viruses and they play a significant role in maintaining a conducive milieu for the chickens (Diaz Carrasco *et al.*, 2019; Mahmood and Guo, 2020). A microbiome community may be found throughout the gut, but it increases in size and diversity in the more distal sections of the gut. There is only a limited microbial population in the gut of day-old chicks. However, the number of gut microorganism rapidly increases in the first days post hatch. The composition of the microbiome is affected by bird age, and by environmental factors

such as exposure to excreta and reused litter (Wang *et al.*, 2016; Rychlik, 2020). The diversity and abundance of the microbiome initially increases with bird age (Shang *et al.*, 2018; Diaz Carrasco *et al.*, 2019; Rinninella *et al.*, 2019; Rychlik, 2020). Kumar *et al.* (2018) reported that Firmicutes were the most abundant phylum in both the ileum and the caecum throughout birds' life (from day 0 to day 42), except day 42 in the caecum where Bacteroidetes were dominant. Awad *et al.* (2016) and Tonks (2018) observed that Proteobacteria was the most abundant phylum in the caecum of the very young bird (<9 d of age) but agree that Firmicutes then becomes the dominant phylum.

The gut microbiome and its metabolites improve nutrient digestion, absorption metabolism and stimulate the development of immune system (Shang *et al.*, 2018; Yadav and Jha, 2019; Rychlik, 2020). It can also reduce the risk of disease through the competitive exclusion of pathogens (Yadav and Jha, 2019; Smits *et al.*, 2021). Therefore, the gut microbiome is associated with the development and health of the chicken. The bacterial composition, the status of AMR or pathogenic bacteria in the gut will be changed depending on the diet and the development of the bird. Dietary interventions, such as the inclusion of feed additives may contribute to an improvement in the bacterial composition.

2.5 Modulating gut microbiome

The composition and diversity of microbiome can be changed by factors such as dietary intervention (Pan and Yu, 2014; Wang *et al.*, 2017). Supplements and feed additives such as probiotics, prebiotics, synbiotics, organic acids, phytogenic compounds, exogenous digestive enzymes and coccidiostats have been used as

antibiotic alternatives (Yadav and Jha, 2019). They can modulate the gut microbiome and their effects have been shown the improvement of growth performance, nutrient utilisation, immunity and health of chickens (Mahmood and Guo, 2020).

Probiotics have been commonly used in broiler chickens and they are well known to have beneficial effects on improvement of productivity, the balance of gut microbiota and prevention of the pathogens proliferation in broiler chickens (Edens, 2003; Jha *et al.*, 2020). The genera of probiotics commonly used includes *Bacillus*, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Enterococcus*, and yeasts such as *Candida* and *Saccharomyces* (Kabir, 2009; Alagawany *et al.*, 2018; Jha *et al.*, 2020). There are several mechanisms of action of probiotics. The first is antibacterial activity. Probiotics produce secondary metabolites (such as hydrogen peroxide, alcohols and organic acids including volatile fatty acids). The organic acids reduce the pH of the gut environment and this, together with the activity of the other metabolites, inhibits the growth of pathogenic bacteria (Edens, 2003; Khan and Naz, 2013; Alagawany *et al.*, 2018). The second mode of action is competitive exclusion, whereby the probiotics prevent the growth of pathogenic bacteria by outcompeting them and adhering to the intestinal epithelium so that the pathogenic bacteria are unable to establish and proliferate (Edens, 2003; Khan and Naz, 2013; Alagawany *et al.*, 2018). The third mode of action is the stimulation of the immune system of the host (Khan and Naz, 2013; Alagawany *et al.*, 2018). The fourth is a decrease in bacterial enzyme activity, whereby probiotics prevent pathogenic bacteria producing toxin activating enzymes such as β-glucosidase and β-glucuronidase (Khan and Naz, 2013; Alagawany *et al.*, 2018). Finally, by competition for nutrients, probiotics suppress pathogenic bacteria by outcompeting them for the acquisition of the

nutrients required for survival and growth (Edens, 2003; Khan and Naz, 2013; Alagawany *et al.*, 2018).

Prebiotics are composed of non-digestible carbohydrates such as fructo-oligosaccharides, galacto-oligosaccharides (GOS), and mannan-oligosaccharides (MOS) that stimulate the growth or activity of healthy bacteria and fungi in the gut (Dhama *et al.*, 2008; Dankowiakowska *et al.*, 2013; Pourabedin and Zhao, 2015). Synbiotics are mixtures of probiotics and prebiotics, and this can help the survival and growth of beneficial microorganisms which can positively influence the balance of gut bacteria, metabolism and immunity (Dankowiakowska *et al.*, 2013). Therefore, probiotic, prebiotics and synbiotics can alter the composition of gut microbiome and they are able to increase the population of lactic acid bacteria, Bifidobacteria and other species which affect improvement of productivity and health in broiler chickens.

Other feed additives such as organic acids, exogenous digestive enzymes, phytogenic compounds and coccidiostats also have useful substances and metabolites which can modulate the gut microbiome and improve chicken health (Pan and Yu, 2014; Gadde *et al.*, 2017; Wang *et al.*, 2017). Organic acids include lactate, acetate, propionate, butyrate, tannic and fumaric. Supplementing the diet with organic acids inhibits the growth of bacteria, including pathogens, by producing a low pH (Gadde *et al.*, 2017). Exogenous digestive enzymes commonly used in poultry feed are amylase, xylanase, β-glucanase , α-galactosidase, protease, lipase and phytase (Yadav and Jha, 2019). These enzymes hydrolyse compounds that cannot be attacked by endogenous enzymes, and they can counteract anti-nutritional factors in the poultry diet (Gadde *et al.*, 2017). In doing this, the profile of nutrients in the gut is altered, which may help to promote a more beneficial microbiome by producing nutrients for them (Bedford and Cowieson, 2012; Gadde

et al., 2017; Yadav and Jha, 2019). When exogenous enzymes were added to break down non-starch polysaccharides (NSP) in a barley-based diet, the gut microbiome was altered significantly in the ileum and caecum (Torok *et al.*, 2008; Singh and Kim, 2021).

Phytogenic compounds have also been used as feed additives and antibiotic alternatives (Clavijo and Flórez, 2017; Gadde *et al.*, 2017). Phytogenic compounds are essential oils, botanicals and herbal extracts derived from plants. Phytogenic feed additives have antimicrobial activity which can inhibit pathogenic bacteria and alter the balance of the bacterial community (Gadde *et al.*, 2017). Coccidiostats incldue the polyether ionophore antibiotics such as monensin, salinomycin, lasalocid, maduramycin, semduramycin and narasin. The coccidiostasts prevent the growth of coccidia, but also inhibit Gram positive bacteria, particularly *Clostridium perfringens* (Engberg *et al.*, 2000; Bjerrum *et al.*, 2006). Trela *et al.* (2020) reported that a combination of salinomycin and *Bacillus licheniformis* decreased the alpha diversity and *Enterobacteriaceae* and encouraged *Bacillaceae* communities in the jejunum. The combination also decreased *Clostridiaceae* in the caeca. This study showed the effect of the synchronous use of coccidiostats and probiotics, however the improper use of coccidiostats might lead to antibiotic resistance and serious health risks.

Trace minerals are important nutrients in feed for poultry and selenium is one such element that has a wide range of activities in the animal. Selenium has important roles in antioxidant activity and is a component of the glutathione peroxidase (GSH-PX) enzyme which helps prevent oxidative damage of cell membranes (Choct *et al.*, 2004; Heindl *et al.*, 2010; Suchý *et al.*, 2014). The effect of selenium on the improvement of the bird's antioxidant status and its immune system has been

demonstrated, and it was reported by Yang *et al.* (2021) that supplementation of the diet with inorganic selenium increased the species richness and diversity in the ileal mucous membrane.

This overview of studies investigating the effects of different feed additives on the composition of the gut microbiome demonstrate that it is possible to alter the composition of the microbiome. What is unknown is what impact this might have on bacteria that carry antibiotic resistant genes. The focus of this thesis will explore the impact of lactic acid bacteria and yeast on the population of coliforms, and the prevalence of AMR and putative pathogenic *E. coli* in the chicken caecum throughout the birds' life.

2.6 Current broiler production system

With high demand for chicken meat, many countries have converted to intensive systems of broiler production (Constance *et al.*, 2010; Augère-Granier, 2019). Both chicken welfare and high profitability in broiler production systems are importantly considered (Constance *et al.*, 2010; Mottet and Tempio, 2017). Large flocks of birds kept indoors and selected for rapid growth rates require high levels of biosecurity, and are more susceptible to challenges from pathogens because of the ease with which disease can spread in such conditions (Mottet and Tempio, 2017). Intensive broiler production systems may facilitate the spread and proliferation of AMR and pathogenic bacteria (Omeira *et al.*, 2006; Saraiva *et al.*, 2020; Ngogang *et al.*, 2021). To explore the challenges of current broiler production systems regarding AMR bacteria, this section will discuss the trend of global chicken production, current broiler farming systems and the practice of reused poultry litter management.

2.6.1 Broiler production and consumption

Poultry meat has many benefits, being a high protein, nutritious and versatile food at an affordable price (Grashorn, 2007). The trend of world poultry meat production from 2017 to 2029 is shown in Figure 2.4, showing that production of poultry meats is projected to increase by approximately 145.7 million metric tonnes until 2029 (OECD and FAO, 2020). The poultry industry has been growing rapidly over several decades, driving the increased trade of the global poultry market and contributing positively to the economy (Windhorst, 2006). In 2017, the largest supplier of chickens was the USA (around 19 million t, 18% of global production), followed by Brazil (around 13 million t, 12.5%) and China (around 12 million t, 11.8%, (CIWF, 2019b)). The European Union 28 (EU) produced around 12.8 million t of poultry meat in 2017, with the UK contributing 1.6 million t and this is increasing annually (AVEC, 2018; CIWF, 2019b). Chicken is widely accepted, with few cultural taboos associated with it (Augère-Granier, 2019; OECD and FAO, 2020).

The average per capita global consumption of poultry meat in 2017 was 14 kg. The largest consumer in 2017 was the USA (48 kg) followed by Brazil (44.2 kg) (OECD and FAO, 2018; CIWF, 2019b). In the case of the EU 28, the average per capita consumption in 2018 was 21.4 kg, while in the UK per capita consumption increased from 22.9 kg in 2015 to 23.2 kg in 2016 (AVEC, 2018; CIWF, 2019b). These statistics illustrate the global reliance on poultry meat. Poultry meat provides people with valuable nutrients such as high quality protein, iron, zinc, selenium, vitamin B_2, vitamin B_6 and niacin (Soriano-Santos, 2010; Pereira and Vicente, 2013). Chicken meat is low in fat and high in protein with a high concentration of essential amino acids (Soriano-Santos, 2010). Additionally, poultry meat contains more essential unsaturated fatty acids than less healthy fatty acids (Farrell, 2013). Chicken breast

also provides beneficial vitamins and 100 g of chicken breast meat supplies 56% of the daily requirement for niacin and 27% of the daily requirement for vitamin B_6 (Pereira and Vicente, 2013).

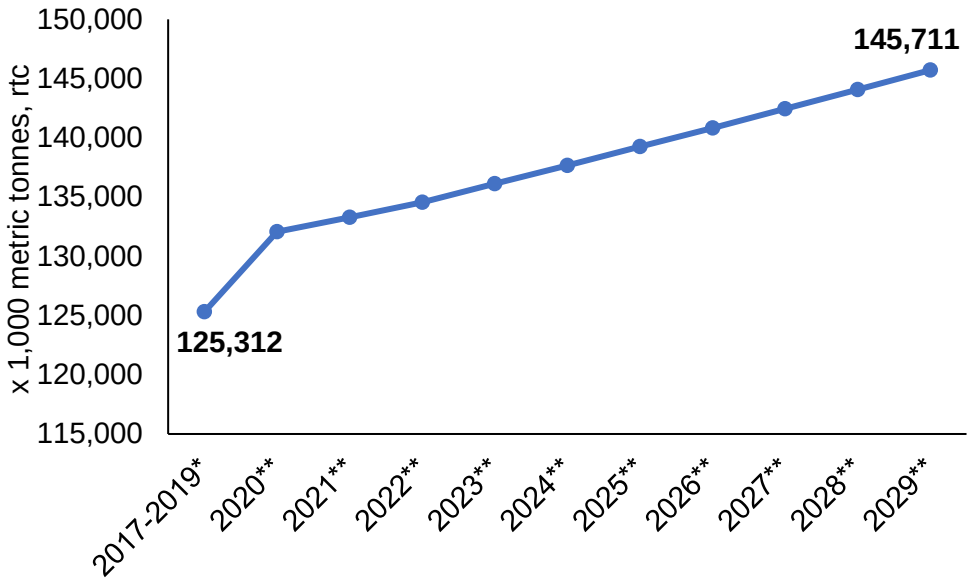


Figure 2.4 The trend of global poultry meat production (2017-2029).

*average 2017-19est; **projection, rtc: ready to cook equivalent.

Source: OECD and FAO (2020), OECD-FAO Agricultural Outlook.

2.6.2 Intensive poultry production

To meet the increasing demand for poultry meat, poultry production has changed from being a minor and subsidiary operation to a highly specialized and vertically integrated industry (Constance *et al.*, 2010). The process of broiler production begins with broiler breeder farm. Fertilised eggs collected from breeder birds are delivered to hatcheries. The eggs are stored under optimum conditions and eggs hatch around 21 days. The chicks are moved to broiler farms, and they are grown for around five to seven weeks before they are slaughtered (Mottet and Tempio,

2017; CIWF, 2019a). Clean water should be provided for birds' growth performance and heath when birds are reared in farms. Water sources used in the poultry industry are mainly farm boreholes, streams, rivers, and rainwater (Sorvala *et al.*, 2008; Etuk *et al.*, 2014).

Many poultry are reared in a confined space with high stocking densities, with little room to move (Wall *et al.*, 2016). There are a number of challenges associated with intensive poultry production. These include compromised bird welfare, increased susceptibility to infection and disease and the potential spread of antimicrobial resistance (AMR) within poultry flocks and into humans (CIWF, 2011).

2.6.3 Poultry litter

Bedding materials that are used in poultry production include wood shavings, sawdust, straw, peanut shells and rice hulls. Poultry litter consists of bedding material and bird excreta, spilt feed, water and feathers (Pan and Yu, 2014). In UK and northern Europe, all litter is removed when the shed is emptied and fresh material is put in before the next batch of chicks. However, in other systems (such as in Brazil, US and Australia), poultry litter may be reused for several consecutive growth cycles to reduce production costs and the challenges of litter disposal (Pan and Yu, 2014; Diaz Carrasco *et al.*, 2019). Reused litter amasses faecal matter and other wastes, and is the medium for a complex and diverse microbiota (Wang *et al.*, 2016). Day old chicks begin pecking and ingesting bedding material as soon as they are placed. If chicks are placed in a reused litter system, they will be exposed to a very different microbial load than if they are placed on fresh bedding material. The bacteria ingested can move to the chicken gut, thus whether chicks are brooded on

fresh or reused litter may affect the chicken gut microbiome (Borda-Molina *et al.*, 2018).

Some research demonstrates a correlation between the microbial community of the litter and the gut microbiome of chickens (Cressman *et al.*, 2010; Wang *et al.*, 2016). Reused litter can act as a reservoir of diverse microbial populations and enable the exchange of microorganisms with the host (Diaz Carrasco *et al.*, 2019). Cressman *et al.* (2010) reported that there were more environmental bacteria in fresh litter but that reused litter had more intestinal bacteria. In addition, *Lactobacillus* spp. was predominant in the ileum of chicks reared on fresh litter while *Clostridiales* was predominant in the ileum of chicks reared on reused litter. Rearing chicks on reused litter changed the gut microbiome of chickens and increased the population of commensal bacteria such as *Faecalibacterium prausnitzii* and commensal butyrate-producing species in the caecum of young chickens (Wang *et al.*, 2016).

Repeatedly used litter in poor management systems can lead to higher litter moisture contents and an increase of ammonia, pH and pathogens (Omeira *et al.*, 2006; Wang *et al.*, 2016). If pathogenic or AMR bacteria are observed in litter from the previous flocks, it could pose a potential health risk by transferring the pathogens to subsequent flocks if litter is reused. However, if well managed, reused litter may have a role as a probiotic to improve the health of chickens. Correct litter management in the poultry industry is important to decrease the pathogen load in the chicken and improve the gut environment. Many more studies are needed to better understand the correlation between the litter and gut microbiome and to balance the bacterial community in litter and improve chicken gut health.

2.7 Action to combat AMR

There have been many efforts to control AMR issues (Magnusson *et al.*, 2019; More, 2020). The use of antibiotics as a growth promoter have been banned in many areas of the world and the unnecessary prophylactic use of antibiotics has also been regulated to mitigate the emergence and transmission of AMR (FAO, 2016; Sugden *et al.*, 2016; Smits *et al.*, 2021). Several European countries including Belgium, Denmark, Sweden and the Netherlands have closely surveyed antibiotic use in livestock and the prevalence of AMR. They have strictly regulated and prohibited much antibiotic use in the livestock industry. Consequently, consumption of antibiotics in these countries has reduced considerably without a negative effect on animal production (Dewulf *et al.*, 2020). In many developed countries, antibiotics are only used for therapeutic purposes and only following a veterinarian's diagnosis and prescription (Magnusson *et al.*, 2019). Vaccination and good husbandry, management, welfare and hygiene on the farm can prevent bacterial infections without using antibiotics (Magnusson *et al.*, 2019).

From 28 January 2022, the EU implemented a new regulation to control AMR by banning the preventive use of antibiotics (More, 2020). Many countries and organisations have surveyed and collected data on antibiotic consumption and AMR prevalence over several decades. FAO, OIE, WHO and CDC are continuously cooperating, planning and establishing the regulation of antibiotic use to combat the AMR problem (FAO *et al.*, 2004; FAO, 2016; O'Neill, 2016; WHO, 2018; CDC, 2019).

Many countries and organisations have continuously made efforts to survey and regulate antibiotic use but the prevalence of antimicrobial resistance bacteria is still high and continuing to develop and spread. Strategies to reduce the prevalence of

AMR bacteria in the poultry gut are therefore needed. There are few studies that investigate the effect of dietary interventions on the control of AMR bacteria in the digesta of broiler chickens. Therefore, more research is needed to characterise AMR bacteria and seek to reduce the prevalence of AMR bacteria in livestock, the environment and in humans. *E. coli* is commonly used as a representative indicator of gut bacteria and this thesis explores changes in the AMR and pathogenic characteristics of *E. coli* as birds age.

2.8 *Escherichia coli* (*E. coli*):

E. coli is used as a sentinel microorganism in the characterisation of microbial communities and in the determination of antimicrobial resistance and virulence factors (Phillips *et al.*, 2004). *E. coli* is a rod-shaped, ubiquitous gram negative microorganism found in the intestinal tracts of all birds and mammals, and it has been extensively studied in both microbiology and biotechnology (Jang *et al.*, 2017). There is considerable diversity in the *E. coli* population and most of them are harmless and act as commensal bacteria being beneficial to the host's health by providing vitamin K_2 and preventing the proliferation of pathogenic bacteria (Tenaillon *et al.*, 2010).

Serotypes of *E. coli* can be identified on the basis of their somatic (O), capsular (K), fimbrial (F) and flagellar (H) antigens and 150 to 200 serotypes have been identified (Figure 2.5) (Stenutz *et al.*, 2006). The somatic (O) component of *E. coli* contains genes coding for lipopolysaccharide (LPS) molecules, which consists of three structures. The first is lipid A, which is a hydrophobic glycolipid; the second is a core oligosaccharide, which is a nonrepeating oligosaccharide with sugars (heptose and

keto-deoxyoctulosonate); the third is the O antigen, which comprises multiple oligosaccharide repeating units (O units) (Liu *et al.*, 2020). The serotyping of gram-negative bacteria can be classified through the variation of their O antigen (Liu *et al.*, 2020).

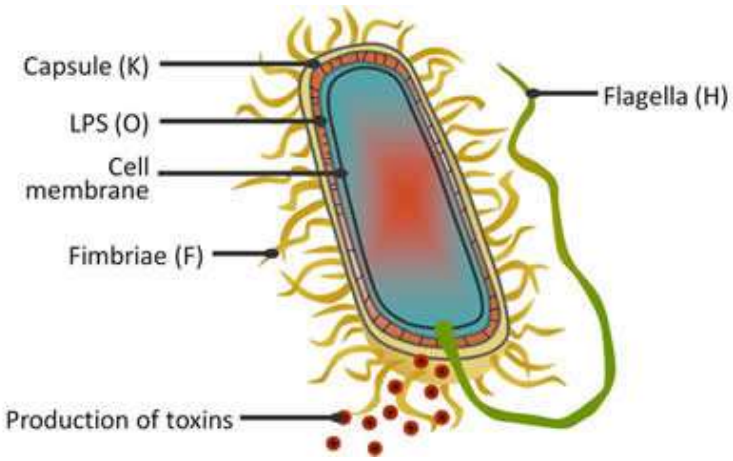


Figure 2.5 Diagram illustrating the serotyping of *E. coli* (Stenutz et al., 2006).
Source: Reference Laboratory for *Escherichia coli* (EcL).
LPS: Lipopolysaccharides.

2.8.1 The prevalence of AMR *E. coli* in animals

E. coli may also carry resistance to important antibiotics, and a high prevalence of antimicrobial resistant *E. coli* have been observed even in day-old chicks in Austria (Roth et al., 2017). The EFSA and ECDC (2016) reported that samples of *E. coli* isolated from broilers had resistance to ciprofloxacin (66%), ampicillin (59%), sulfamethoxazole (53%) and tetracycline (53%) in all 27 countries of the EU. A study by Smith *et al.* (2007a) reported that *E. coli* isolated from caecal droppings on commercial chicken farms in northeast Georgia had a high resistance to tetracycline (36 to 97%), sulfathiazole (50% to 100%), and streptomycin (53% to 100%) from 3

week old to 5 week old birds. Similarly, isolates of *E. coli* taken from cloacal swabs of broilers in Poland between 2009 and 2012 were observed to be resistant to ampicillin, nalidixic acid, ciprofloxacin, streptomycin, sulfametoxazole, trimethoprim and tetracycline, while rectal swabs from cattle were generally not resistant to these antibiotics (Wasyl *et al.*, 2013). The high prevalence of AMR to more than one antibiotic might be a result of linkage of resistance genes on multi-resistance encoding plasmids and the horizontal transfer of resistant genes (Zankari *et al.*, 2012; Rozwandowicz *et al.*, 2018).

2.8.2 Pathogenic *E. coli*

Specific strains of *E. coli* carry virulence factors and may lead to a variety of infections in humans and in animals. Pathogenic *E. coli* produce toxins and have virulence properties which may cause disease or death in the host (Kaper *et al.*, 2004; Pitout, 2012). Depending on the *E. coli* strain, it can infect either intestinal or extraintestinal sites. The six intestinal pathogenic *E. coli* strains are categorised as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and diffuse-adhering *E. coli* (DAEC) (Schroeder *et al.*, 2004; Jang *et al.*, 2017). These enteric pathogenic *E. coli* mainly cause diarrhoea (Schroeder *et al.*, 2004; Jang *et al.*, 2017). The extraintestinal pathogenic *E. coli* (ExPEC) strains are associated with infection outside the intestine (Smith *et al.*, 2007b). ExPEC are also potential zoonotic pathogenic bacteria and lead to a variety of diseases in humans such as neonatal meningitis *E. coli* (NMEC), sepsis-associated *E. coli* (SEPEC, causing sepsis) and uropathogenic *E. coli* (UPEC, causing urinary tract infections) (Sarowska *et al.*, 2019). Avian pathogenic *E. coli* (APEC) is the main causal

organism of colibacillosis in poultry and APEC infection gives rise to significant mortality by colonising the respiratory and gastrointestinal tracts of poultry (Pitout, 2012; Stromberg *et al.*, 2017).

As noted above, most *E. coli* strains are commensal and are adapted to survive in the intestine, but some *E. coli* strains are pathogenic and lead to various infectious diseases (Kaper *et al.*, 2004; Dobrindt, 2005). Colibacillosis is one of the most common bacterial infections in cattle, swine and poultry (Mayrhofer *et al.*, 2004; Lee *et al.*, 2009). Avian pathogenic *E. coli* (APEC) causes severe diseases such as septicaemia, air sacculitis, and cellulitis in broiler chickens, and peritonitis and salpingitis in broiler breeders (Pitout, 2012). A variety of virulence associated genes have been implicated in causing these extraintestinal diseases in birds (Paixao *et al.*, 2016).

2.8.3 Virulence-associated factors of *E. coli*

The virulence factor genes consist of adhesins (these include firstly pyelonephritis associated with pili C (*pap*C), and secondly temperature sensitive haemagglutinin (*tsh*)); invasion (invasion of brain endothelium (*ibe*A, B, C)); resistance to the immune system of the host (this would include the iron-repressible high-molecular-weight proteins 2 (*irp2*), iron-uptake systems of *E. coli* D (*iuc*D), aerobactin receptor (iutA), catecholate siderophore (salmochelin) receptor (iroN), and increased serum survival (*iss*), outer membrane protease T (*omp*T)); and also toxins (such as arginine succinyl transferase A (*ast*A) and haemolysin (*hly*A, hlyF)) (Delicato *et al.*, 2003; Paixao *et al.*, 2016; Sarowska *et al.*, 2019). The virulence gene of pyelonephritis associated with pili C (*pap*C) is closely related to the genes

associated with urinary tract infections in humans and the virulence gene of *pap*C attaches to the P blood group antigen that includes D-galactose-D-galactose (Karami *et al.*, 2017). The temperature-sensitive hemagglutinin (*tsh*) is an autotransporter group of proteins whose domain is the outer membrane (Dozois *et al.*, 2000). Iron is an important and essential element for growth of host and bacteria (Litwin and Calderwood, 1993; Sarowska *et al.*, 2019). Iron-uptake related genes of *E. coli* produce siderophores which utilise haem or haem-containing proteins to supply the *E. coli* with the iron that it needs (Thariath *et al.*, 1993). Increased serum survival (*Iss*) protein confers resistance to serum proteins by the *E. coli*, protects against phagocytosis and confers virulence (Dziva and Stevens, 2008; Sarowska *et al.*, 2019). Outer membrane protein (*omp*) enables intracellular survival and evasion from the body's defence mechanisms (Sarowska *et al.*, 2019). The arginine succinyl transferase A (*ast*A) gene encodes an enterotoxin (de Sousa and Dubreuil, 2001). Haemolysins (*hly*) damage cell membranes which leads to lysis of red blood cells (Ewers *et al.*, 2004), and thereby a supply of haem iron to the *E. coli*. The acquisition of these virulence genes by *E. coli* may give them a selective advantage over more benign serotypes, enabling them to better establish in the host but potentially causing serious diseases in the host animal (Delicato *et al.*, 2003; Paixao *et al.*, 2016).

The many virulence associated genes were associated with infection and colibacillosis in poultry (Mellata *et al.*, 2003; McPeake *et al.*, 2005; Nakazato *et al.*, 2009). Virulence genes are generally located in mobile factors such as plasmids, allowing them to transfer to other (formerly) commensal *E. coli* (Johnson *et al.*, 2012). Reducing the selective advantage conferred by these virulence factors by enabling the establishment of more benign serotypes (perhaps through dietary

intervention to alter the gut environment) may be a means of reducing the spread of pathogenic *E. coli* and, potentially, AMR if these are transferred with the virulence genes. One such way of addressing this selective advantage of potentially pathogenic *E. coli* may be through the use of probiotics that may alter the supply of nutrients in the gut, the gut environment or the composition of the gut microbiome. The impact of probiotics (specifically lactic acid bacteria and yeast probiotics) on improvement of bird performance and inhibition of pathogen is discussed in the next section.

2.9 Probiotics and the chicken microbiome

There is considerable evidence demonstrating the positive effect of administering probiotics such as *Bacillus* sp., lactic acid bacteria or yeast on bird performance, feed efficiency, inhibition of pathogenic bacteria (such as *Salmonella* spp., *E. coli, Staphylococcus aureus*, *Clostridium perfringens* and *Campylobacter*) and improvement of the immune system (Khaksefidi and Rahimi, 2005; Murry *et al.*, 2006; Khan and Naz, 2013; Olnood *et al.*, 2015). Lactic acid bacteria and yeast produce many beneficial metabolites and they can manipulate the intestinal microbiome and improve the gut environment. Many studies have reported that lactic acid bacteria or yeast intervention improved production efficiency, nutrient utilisation and health of chickens (Khan and Naz, 2013; Gadde *et al.*, 2017; Jha *et al.*, 2020). This review focuses on exploring lactic acid bacteria and yeast interventions as means of manipulating the intestinal microbiome to improve the growth performance and health of the host chicken.

2.9.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of gram positive facultative anaerobic and rod-shaped or spherical bacteria that produce lactic acid as a product of the fermentation of substrates for energy. LAB include various genera such as *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus* and *Leuconostoc* (Kabir, 2009; Ohashi and Ushida, 2009; Khan and Naz, 2013). LAB have numerous beneficial effects including antimicrobial activity and improvement of the productivity, and immune system of poultry (Murry *et al.*, 2006; Fijan, 2014; Olnood *et al.*, 2015).

Kalavathy *et al.* (2003) reported that a mixture of 12 *Lactobacillus* stains (LC) fed to broilers improved their body weight gain and feed conversion ratio (FCR) from 1 to 42 days of age and decreased their serum total cholesterol, low density lipoprotein cholesterol and triglycerides from the ages of 21 to 42 days. There was no significant effect on serum high density lipoprotein cholesterol and the weight of organs between control and LC treatments. Saint-Cyr *et al.* (2017) observed that administering *Lactobacillus salivarius* SMXD51 reduced the population of *Campylobacter* loads in broiler chicken caecal contents compared to control on days 14 and 35 of age. Forte *et al.* (2018) demonstrated that 2.0 g/100 kg of *Lactobacillus acidophilus* supplemented feed improved the body weight and FCR on days 21 and 42 of age in chickens. The population size of *Lactobacillus* sp. in intestinal contents increased in the treated *Lactobacillus acidophilus* group. On the other hand, there was no significant difference between the control and *Lactobacillus acidophilus* treated group in the population of *Enterococci*, *Staphylococci*, and *E. coli*. Murry *et al.* (2006) observed that administering a botanical probiotic containing *Lactobacillus* sp. in the diet improved feed intake and

FCR and increased the population of *Lactobacillus* sp. while reducing the population of *Clostridium perfringens* in the cloaca of broilers. However, Olnood *et al.* (2015) reported that four strains of *Lactobacillus* (*Lactobacillus johnsonii*, *Lactobacillus crispatus*, *Lactobacillus salivarius* and an unidentified *Lactobacillus* sp.) had no effect on the weight gain, feed intake and FCR of broilers, although it did increase the weight of the jejunum and ileum and decreased the population of *Enterobacteria* in the caecum on day 35 of bird age.

2.9.2 Yeasts

Yeast have been extensively used in animal feeds for many years (Vohra *et al.*, 2016; Ogbuewu *et al.*, 2019). Yeast genera include *Saccharomyces*, *Pichia*, *Metschnikowia*, *Yarrowia*, *Candida*, *Debaryomyces*, *Isaatchenkia* and *Kluyveromyces* (Vohra *et al.*, 2016). They have desirable amino acids in their proteins, as well as supplying zinc, choline, magnesium, and B group vitamins (Ogbuewu *et al.*, 2019). In addition, they secrete valuable extracellular enzymes such as amylase, β-galactosidase, and phytases (Rima *et al.*, 2012; Vohra *et al.*, 2016).

Supplementing birds with yeast has been reported to improve nutrient utilisation, FCR, and immunity in broilers. Chen *et al.* (2017) reported that an inclusion rate of 2.5 g/kg of *Saccharomyces cerevisiae* in the diet of broilers had a positive effect on their feed intake and FCR. Gao *et al.* (2008) also observed that a 2.5 g/kg inclusion of a commercial yeast product in the diet improved the average daily weight gain and FCR in broiler chickens. In addition, there was a significant effect of yeast administration on the availability of calcium and phosphorus, gut mucosal

morphology and the immune functions of broilers. Aluwong *et al.* (2013) observed that administering *Saccharomyces cerevisiae* via the drinking water improved body weight and antioxidant (glutathione peroxidase) activity in broilers. He *et al.* (2021) demonstrated that the administration of live yeast (*Saccharomyces cerevisiae*) increased the body weight and crude protein retention of broilers. After birds were vaccinated for Newcastle disease and infectious bursal disease, the antibody titre was higher in birds treated with live yeast. The administration of yeast increased catalase activity (another antioxidant) and the height of villi in the jejunum and ileum. Live yeast added to the diet was observed to reduce serum cholesterol in broilers (He *et al.*, 2021).

These observations demonstrate the beneficial effect of LAB and yeast on bird performance and health. As LAB and yeast establish and proliferate in the birds' gut, they may have an effect on the composition of the gut microbiome. This in turn may affect the evolution of *E. coli* in the gut of birds; potentially encouraging the proliferation of more commensal (and antibiotic sensitive) strains at the expense of AMR pathogenic strains. It is hypothesised that LAB and yeast interventions may reduce the prevalence of pathogenic and AMR *E. coli* isolated from chicken caecum, and this will be investigated in this thesis.

2.10 Aims and objectives

Thus far, many studies have focused on the prevalence of antimicrobial resistance in animals. Little is known about the effect of dietary interventions on this prevalence, nor on the relationship between antimicrobial resistance and bacterial virulence, and whether this changes as the bird ages. There has been no detailed investigation on how AMR and virulence factors of *E. coli* from broiler caecum change and evolve throughout broiler's life. The status of AMR and virulence in *E. coli* may evolve as the bird ages and develops. If the relation between bird age and prevalence of AMR *E. coli* can be identified, the evolution and development of *E. coli* may be better understood, enabling the development of dietary or management interventions to manipulate the prevalence of AMR *E. coli*.

Bird age and dietary interventions may influence the balance between commensal (susceptible to antibiotic) *E. coli* and AMR pathogenic *E. coli*. It was noted in the review of probiotics that these had a variety of biological activities and positive effects on the increase of beneficial gut bacteria and prevention of pathogen growth, and that this intervention influenced overall performance and health of broilers.

This thesis will explore the association between bird age and prevalence of AMR and putative pathogenic *E. coli*. It will also investigate whether there is a correlation between AMR and virulence in *E. coli* and whether dietary interventions affect the prevalence of AMR and putative pathogenic *E. coli*. These findings will contribute to a deeper understanding of AMR and virulent *E. coli* evolution during the birds' life and suggest the development of potential crude dietary interventions to reduce the prevalence of AMR and putative pathogenic *E. coli* in the gut of growing broiler chickens.

It is hypothesised that:

- Changes in the gut microbiome with increasing bird age might change the prevalence of AMR and putative pathogenic *E. coli*.
- Manipulating the broiler gut environment with crude interventions might alter the prevalence of *E. coli* with antimicrobial resistance and pathogenicity traits.

The objectives of this thesis were therefore:

- To determine the evolution of AMR and virulence of *E. coli* isolated from the gut of chickens during the broiler chickens' life, and how this is affected by crude interventions such as the administration of selenium, LAB and yeast.
- To isolate, identify and characterise lactic acid bacteria (LAB) from the broiler caecum, and investigate their association with the prevalence of virulent and AMR *E. coli* in the broiler caecum.

Chapter 3. Effect of administering selenium and yeast on broiler performance, prevalence of antimicrobial resistance, virulence genes and nutrient utilisation by *E. coli* isolated from the broiler caecum

3.1 Introduction

There are a number of dietary and management interventions that might be used in poultry husbandry to enhance bird health, performance, and improve the AMR status of the bird (Patterson and Burkholder, 2003; Shakeri *et al.*, 2014; Chen *et al.*, 2017). Selenium administration is essential to poultry and contributes to their antioxidant status, helping to prevent oxidative stress which damages the cells of both host and microorganisms (Salami *et al.*, 2015). Administering poultry diets with selenium has resulted in improved productivity and FCR (Choct *et al.*, 2004; Ševčíková *et al.*, 2006). However, little is known about the impact of selenium administration on the prevalence of AMR in the birds. The investigation of selenium administration and its effect on AMR prevalence that is reported in this chapter was an opportunistic study. In this study, therefore, selenium was added to broiler diets to determine whether selenium has the potential to prevent the growth of AMR and pathogenic bacteria.

Beneficial bacteria have also been used both as feed additives and as alternatives to antibiotics (Edens, 2003; Gadde *et al.*, 2017). Interest in yeasts is increasing, and there are a number of studies on the effect of yeast on performance and health in broilers (Gao *et al.*, 2008; Kabir, 2009; Rima *et al.*, 2012; Ezema, 2013). In the study reported in this chapter, *Saccharomyces boulardii* and *Candida famata* were

administered to young broiler chicks to determine their effect on the evolution of the *E. coli* in the caecum, as an indicator of the AMR and pathogenic challenge faced by the bird. Again, this was an opportunistic study and as the yeast isolates that were used were untested and unlicensed a cautious approach was adopted using a low dose for just three days (days 7, 8 and 9 of age).

Saccharomyces boulardii is well known to have beneficial probiotic properties and effects of administering *S. boulardii* on improvement of growth performance and growth inhibition of pathogenic bacteria in the broiler gut have been reported (Line *et al.*, 1998; Gil De Los Santos *et al.*, 2005; Czerucka *et al.*, 2007; Abudabos *et al.*, 2019). *Candida famata* is naturally isolated from the caecum of the chicken and its potential value is being determined as part of a separate study. *Candida famata* Y5 isolated from the chicken caecum showed an absence of haemolytic activity and cytotoxicity, and the isolate was able to adhere to the intestinal epithelial cell (Al-Seraih *et al.*, 2015).

Thus far, most research on AMR status has focused on the prevalence of antimicrobial resistance in broiler chickens. Little is known about the relationship between antimicrobial resistance and bacterial characteristics such as virulence and their ability to utilise different carbon sources. It is also not known how these evolve as the bird ages, but the competition between different *E. coli* strains determines the pathogenicity of this ubiquitous microorganism (Fabich *et al.*, 2008). Pathogenic strains compete with the resident, commensal microbiota for nutrients and space in the intestinal tract (Fabich *et al.*, 2008). It is assumed that pathogenic and AMR *E. coli* may have a different profile of nutrients they can utilise as carbon sources compared with commensal (and perhaps antimicrobial sensitive) strains. Understanding these differences may allow a means of encouraging the rapid

development of a healthy gut in the growing chick, which would reduce the prevalence of *E. coli* with multiple antimicrobial resistance and pathogenic genetic constructs.

It is hypothesised that the administration of *Candida famata* and *Saccharomyces boulardii* might positively influence the proliferation of commensal bacteria and thus decrease the coliform population and the prevalence of antibiotic resistance and presumptive pathogenic bacteria. The objectives of this study were therefore to determine the effect of administration of selenium and yeast (*Candida famata* compared with *Saccharomyces boulardii*) on the prevalence of AMR, virulence associated genes and the utilisation of different carbon sources by *E. coli* isolated from the caecum of growing broiler chickens.

3.2 Material and Methods

3.2.1 Experimental design, birds and diets

All birds were housed at the Centre for Dairy Research (CEDAR), University of Reading. All animal care procedures were approved by the Institutional Animal Care and Use Committee of the University of Reading. Chickens were kept according to the Recommendations for the Welfare of Meat Chickens and Breeding Chickens (DEFRA, 2018) and the Ross 308 broiler management handbook (Aviagen, 2018).

A total of 280 chicks (day-old, male Ross 308, purchased from PD Hook, Cote, Oxfordshire) were procured for this experiment. 42 chicks were used in an independent, undergraduate study and the remaining chicks were used in this experiment, or were sacrificed on day 1. All chicks were weighed on arrival, wing

tagged, blocked by liveweight, and then randomly allocated to one of six brooder rings (37 chicks per ring; two replicate rings for each Control, *Candida famata*, and *Saccharomyces boulardii* treatment) bedded on white wood shavings, and fed a common starter diet until the birds were 14 days of age. *Candida famata* (isolated from a chicken) and *Saccharomyces boulardii* (isolated from a chicken) was administered via the drinking water (taken from a borehole) as the yeast cultures could be more evenly mixed with water rather than broiler feed. Water from the borehole was not treated prior to supplying the birds. Stock cultures of each *C. famata* and S*. boulardii* isolate were incubated overnight in Yeast Extract Peptone Dextrose (YPD) broth. Population density was then estimated by serial dilution and measurement of the optical density of the incubation medium at 600 nm. 30 ml of yeast suspension (10^6 CFU/ml) was prepared to add to 2 L drinking water in the hopper on day 9 and 20 ml of yeast suspension (10^6 CFU/ml) was added to 1980 ml water on days 10 and 11 to attain the desired final concentration of yeast.

In a preliminary test, *Candida* sp. was isolated from the small intestine and caecum on days 7 and 22 of bird age. This indicated that *Candida* sp. colonisation was more optimised in growing chicks rather than day-old chicks. The yeast administering period was only three days (9, 10 and 11 day) as the birds' response to this yeast was uncertain and so a very cautious yeast dose was selected. Water (from a borehole) was therefore provided either untreated (Control) or with a preparation of *C. famata (*10^5 CFU/ml water offered) or *S. boulardii* (10^5 CFU/ml water offered) in water hoppers on days 9, 10 and 11. Water hoppers were replenished with fresh (untreated) water when required.

On day 14, 168 birds were weighed and allocated to one of 24 pens with respect to yeast (eight replicate pens), selenium administration (12 replicate pens) and

stocking density (either nine or five birds/pen; 12 replicates). A litter of fresh shavings was added to each pen and a solid plygene wall (1 m diameter) surrounded each pen to exclude draughts and prevent cross-contamination. There were two replicate pens for each yeast x selenium x stocking density interaction. The variation in prevalence of AMR was unknown, but the amount of replication required to detect (with 80% power at $P<0.05$) a 40% difference assuming a coefficient of variation of 20% was used. This would require a minimum of n=2. The experimental design was also constrained as there was only room for 24 pens, and it is acknowledged that smaller differences between treatments may not be detected because there was insufficient replication. Subsequent analysis showed no significant effect of stocking density, and so this factor was then excluded from the model. There were therefore four replicate pens for each yeast x selenium interaction (which would allow a 28% difference to be detected). All birds were then fed a common grower/finisher diet that was either not supplemented with additional selenium (control) or supplemented with 16 g/kg of a hydroxy analogue of selenomethionine, (Selisseo, Adisseo, France, 0.3 mg Se/kg diet). The selenium contents of the diets were not determined, but were formulated (based on database selenium contents of the ingredients used) to supply 0.2 and 0.5 mg Se/kg for the control and Se diets respectively, which would be sufficient to meet the birds' requirement of 0.2-0.3 mg/kg (Surai, 2018).

The ingredient and calculated nutrient composition of the diets is shown in Table 3.1. The diets were fed in the form of a mash, and were manufactured by Target Feeds (Whitchurch, UK). Samples of each diet were analysed for crude protein, starch, oil, sugar, methionine, and lysine (Sciantec, York, UK). The experimental design and treatments are presented in Table 3.2. Lighting was via incandescent

lights with 23 h continuous light per 24 h period for the first seven days, followed by 18 h continuous light (6 h darkness) in each 24 h period. The birds were brooded according to the breeder's recommendations using infrared lights to provide supplementary heat when necessary.

Table 3.1 Ingredient and nutrient composition (as-fed basis) of chickens' diets.

	Starter (0-14 d)	Grower/ Finisher (15-36 d)	
		Control	+Se
Ingredient composition (g/kg)			
Barley	40	40	39
Wheat (12.5% CP)	500	550	541
Soya bean meal (48% CP)	320	265	261
Rapeseed meal	42	42	41
Soya bean oil	50	65	64
L-lysine HCl	4	1	0.9
DL-methionine	3.45	2.42	2.38
L-threonine	2.05	2.02	1.99
Sodium bicarbonate	2.5	2.5	2.5
Salt	2	2.5	2.5
Limestone	11	8.56	8.42
Poultry vitamins/minerals (Se free)[1]	2	2	2
Hydroxy analogue of selenomethionine[2]	0	0	16
Dicalcium phosphate (QPRDC)	20	16	15.7
Titanium dioxide	1	1	0.9
Nutrient composition (g/kg as fed)			
ME (MJ/kg)	12.4	12.5	12.5
Crude Protein	227	192	205
Starch	345	371	335
Total Oil (Oil B)	71.3	79.5	85.8
Sugar	48.9	43.8	56.8
Methionine	6.3	5.3	5.2
Lysine	12.8	10.2	11.2

ME: Metabolisable Energy

[1] The vitamin/mineral premix supplied per kg of starter diets: vitamin A 6750 IU, vitamin D_3 2500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Mo 0.25 mg.
The vitamin/mineral premix supplied per kg of grower/finisher diets: vitamin A 5000 IU, vitamin D_3 2500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Mo 0.25 mg.

[2] supplemented with 16 g/kg of a hydroxy analogue of selenomethionine, (Selisseo, Adisseo, France, 0.3 mg Se/kg diet).

Table 3.2 The experimental design and treatment.

Added Se (mg/kg)	Yeast
0.2	None
0.5	None
0.2	*C. famata*
0.5	*C. famata*
0.2	*S. boulardii*
0.5	*S. boulardii*

3.2.2 Sample collection

Birds were vaccinated prior to arrival, and they had no coccidiostats, medication or vaccinations during the study. On day 1, a total of 16 birds were randomly selected and humanely slaughtered by cervical dislocation for sampling. A swab was taken from the lumen of the yolk sac, duodenum and caecum of each chick. On day 14, four birds from each brooder ring were sacrificed and a swab was taken from the lumen of the caecum. After the birds were allocated to their pens, one bird from each pen was randomly selected and sacrificed on days 22 and 36. All samples of caecal lumen digesta taken by swab were placed on ice for transportation and analysed for the determination of the population size (CFU/ml) of coliforms, and for the determination of the prevalence of antimicrobial resistant *E. coli* on the same day. All remaining birds were euthanased by cervical dislocation on day 36.

3.2.3 Bird performance

Birds were weighed individually on days 1, 14, 22 and 36. Mean body weight increase was then calculated on a pen basis during both the starter period and grower/finisher period. Feed intake was recorded on a pen basis and FCR was then calculated on a pen basis (feed intake/body weight gain) during the grower/finisher period.

3.2.4 Determination of viable *E. coli* population

The swab taken from the yolk sac, duodenal, and caecal lumen on day-old chicks was placed in 4 ml of sterile nutrient broth and the suspension was then serially diluted with nutrient broth. The swab taken from the caecum on days 14, 22 and 36 was diluted in 10 ml of sterile nutrient broth and the suspension was then serially diluted with nutrient broth. 100 µl of the diluted suspension was spread onto a MacConkey agar plate containing either 0 or 50 µg/ml ampicillin (AMP). Plates were incubated at 37°C overnight. This was to determine the population of both total and ampicillin resistant *E. coli*.

3.2.5 Antimicrobial susceptibility

Antimicrobial susceptibility was evaluated using the disc diffusion method. Ampicillin resistance was first screened to evaluate whether isolates were resistant to both ampicillin and another antibiotic. One distinct colony from each plate containing either 0 or 50 µg/ml ampicillin was picked using a sterile plastic inoculating loop and transferred to a microcentrifuge tube containing 500 µl nutrient broth. The tube was

vortexed, and then 100 µl suspension was spread evenly onto a nutrient agar plate. The suspension was allowed to air dry, and then well-spaced antibiotic discs were placed on the plate. The antibiotic discs used were chloramphenicol (10 µg), streptomycin (10 µg); tetracycline (10 µg) and ciprofloxacin (5 µg). The plates were incubated at 37°C overnight. The radius of the inhibition zone was measured and antimicrobial susceptibility was determined according to the Clinical and Laboratory Standards Institute guidelines (Table 3.3) although the concentrations of chloramphenicol and tetracycline in the discs used were lower than those required by CLSI. These lower concentrations of chloramphenicol and tetracycline were selected based on the availability of discs that could be acquired at the time and to enable the detection of resistance to low concentrations of the antibiotic.

Table 3.3 Antimicrobials and resistance breakpoints (radius of inhibition zone, mm).

Antimicrobial agent	Disc content (µg)	Resistant	Intermediate	Susceptible
Chloramphenicol	10	≤12	13-17	≥18
Streptomycin	10	≤11	12-14	≥15
Tetracycline	10	≤11	12-14	≥15
Ciprofloxacin	5	≤15	16-20	≥21

CLSI guidelines (Clinical and Laboratory Standards Institute, 2017).

3.2.6 Isolation of *E. coli*

All plates with *E. coli* colonies were then refrigerated (<5°C) for 3 months. A single pink colony was then taken from each plate and transferred to a microcentrifuge tube containing 1 ml of 15% v/v glycerol (45 ml of glycerol plus 255 ml of nutrient broth). The single colony of *E. coli* picked from the MacConkey agar plates containing 50 µg/ml ampicillin were put in microcentrifuge tubes containing 1 ml of 15% v/v glycerol and 0.5 µl of 50 µg/ml ampicillin. This culture was incubated at 37°C for 18-24 hours. 500 µl of the resulting *E. coli* suspension was then transferred to a cryovial (Mast group, Mastdisks, UK) and stored at -80°C pending further analysis of carbon source utilisation and presence of virulence genes.

3.2.7 Samples of *E. coli* isolates selected for analysis of virulence and carbon source utilisation

A single colony from each isolate of *E. coli* stored in cryovials was taken and streaked on to MacConkey agar plates (with and without AMP, 50 µg/ml) in order to evaluate the growth and status of ampicillin resistance. Of the original isolates that were prepared, only 36 were found to be viable. These were selected to determine the presence of virulence genes and the isolate's ability to utilise different carbon sources. The provenance of the viable isolates is summarised in Table 3.4.

Table 3.4 The provenance of the viable *E. coli* isolates taken from the caecum in broiler chickens.

ID of isolate	Response to ampicillin (50 µg/ml)	Age (d) of bird from which isolate was taken	Exposure of bird to yeast treatment	Exposure of bird to selenium administration
CR2	Sensitive	1	-	-
CR3	Sensitive	1	-	-
CR4	Sensitive	1	-	-
CR6	Resistant	1	-	-
CR7	Resistant	1	-	-
CR8	Sensitive	1	-	-
CR11	Sensitive	1	-	-
CR12	Sensitive	1	-	-
CR134	Resistant	14	Control	-
CR138	Resistant	14	*S. boulardii*	-
CR146	Resistant	14	Control	-
CR153	Resistant	14	Control	-
CR160	Resistant	22	Control	No
CR57	Resistant	22	Control	No
CR64	Resistant	22	Control	No
CR68	Sensitive	22	*C. Famata*	No
CR86	Sensitive	36	Control	No
CR91	Resistant	36	Control	Yes
CR93	Sensitive	36	*S. boulardii*	No
CR97	Resistant	36	*C. Famata*	Yes
CR98	Sensitive	36	*C. Famata*	Yes
CR99	Resistant	36	Control	No
CR103	Resistant	36	Control	Yes
CR104	Resistant	36	*C. Famata*	Yes
CR107	Sensitive	36	*S. boulardii*	No
CR109	Sensitive	36	*C. Famata*	Yes
CR110	Sensitive	36	*C. Famata*	No
CR111	Sensitive	36	*S. boulardii*	Yes
CR185	Resistant	36	Control	Yes
CR188	Resistant	36	Control	No
CR192	Resistant	36	Control	Yes
CR196	Resistant	36	*C. Famata*	Yes
CR197	Sensitive	36	*C. Famata*	Yes
CR200	Resistant	36	*C. Famata*	No
CR204	Resistant	36	*C. Famata*	Yes
CR212	Resistant	36	*S. boulardii*	Yes

3.2.8 Carbon source utilisation

E. coli colonies were characterised on the basis of their ability to utilise five sources of carbon. These sources were sucrose, adonitol (ribitol), arabitol, dulcitol (galacitol), sorbose and the method was described by Alkandari (2017). A single bead from each stored *E. coli* cryovial was inoculated in nutrient broth and incubated at 37°C overnight. The suspension of *E. coli* was then streaked onto nutrient agar plates and incubated at 37°C overnight. Then, a single colony was streaked on minimal salts medium (M9) agar. Minimal medium was prepared in 1,000 ml volumes for each component. The salt solution was prepared as a 5X concentrated stock solution with 1.5% potassium dihydrogen phosphate (KH_2PO_4), 3.39% disodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), 0.25% sodium chloride (NaCl), 0.5% ammonium chloride (NH_4Cl) dissolved in 1 l water and autoclaved. To prepare the 500 ml of minimal medium, 50 ml of autoclaved salt solution and 15 g of purified agar was autoclaved with 450 ml double-distilled water (ddH_2O). After cooling the autoclaved minimal medium to 50°C, 1M magnesium sulphate ($MgSO_4$, 1 M, 1 ml), calcium chloride hexahydrate ($CaCl_2 \cdot 6H_2O$, 1 M, 0.1 ml) and 10 ml of the carbon source (20% w/v prepared by dissolving in sterile distilled water and filter sterilizing, Sartorius sterilin, PES 0.22) were added separately in multiple preparations. 20 ml of media were poured per sterile petri dish and allowed to solidify at room temperature. A total of 36 individual isolated *E. coli* colonies, described above, were streaked (one on each plate). The plates were incubated at 37°C for 18-24 hours, then incubated at 25°C for up to a further seven days. Results were scored after 7 days' incubation. Scoring was as follows: clear growth was scored positive (+), absence of discernible growth was scored negative (-). Mutability (M) that is defined here as rare colonies on a background of very weak or no growth was also scored.

3.2.9 Extraction of genomic DNA

The genomic DNA from the 36 *E. coli* isolates was extracted to determine the presence of *E. coli* virulence factors. Genomic DNA was extracted using Pure-gene yeast/bact Kit B (Qiagen, Venlo, Netherlands) and standard protocols for fresh samples of Gram-negative bacterial cultures were followed. Isolated cultures of *E. coli* were streaked on nutrient agar plates and incubated at 37°C overnight. Colonies of *E. coli* were then taken from these plates and transferred to a 1.5 ml sterile microcentrifuge tube. These were then incubated at 80°C for 5 min after adding 300 µl of cell lysis solution to the pellet. The tubes were put on ice for 20 minutes. 100 µl of protein precipitation solution was added and vortexed vigorously for 20 s at high speed. The mixture was then centrifuged at 13,400 rpm for 3 min (Eppendorf minispin, Germany). The supernatant was transferred to a clean 1.5 ml microcentrifuge containing about 700 µl of 99.5% ethanol and mixed by gently inverting 50 times. The mixture was centrifuged at 13,400 rpm for 1 min, and the supernatant was carefully discarded. A total of 300 µl of 70% ethanol was added to the DNA pellet and inverted several times. The mixture was then centrifuged at 13,400 rpm for 1 min, and again the supernatant was discarded and the pellet was allowed to air dry for 5 min. 100 µl of DNA hydration solution was added and the mixture was vortexed for 5 s, incubated at 65°C for 1 h, followed by incubation overnight at room temperature with gentle shaking. The purification of DNA was determined with a Nanodrop spectrophotometer (ND 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The Nanodrop tube was cleaned by pipetting 1.5 µl of distilled water onto it and wiping with medical wipe. The Nanodrop was blanked with 1.5 µl of distilled water. 1.5 µl DNA solution was added to the measuring stage and the DNA concentration was recorded in ng/µl, and the 260:280 nm ratio was

also recorded (1.92 ± 0.08 was required). The DNA size was assessed by electrophoresis (75 V for 1 h) with a 1% agarose gel stained with ethidium bromide in 0.5X TBE buffer. A 100bp and 1kbp DNA ladder was used as a reference. Gel images were captured using G-BOX Chemi-XR5, (Syngene), connected to a computer.

3.2.10 Virulence genotyping

The DNA extracts were then analysed for seven avian pathogenic *E. coli* (APEC) virulence factors using PCR assays. The method used was described by Alkandari (2017). The virulence genes investigated (*pap*C, *iuc*D, *tsh*, *irp*2, *iss*, *ast*A and *hly*A) are described in Table 3.5, with their primer sequences. PCR was performed using GE healthcare illustra™ PuReTaq Ready-To-Go™ PCR Beads (Thermo Fisher Scientific, UK) containing 2.5 units of recombinant PuReTaq DNA Polymerase, dATP, dCTP, dGTP, dTTP, stabilizers, bovine serum albumen and reaction buffer. PCR assays of *pap*C and *iuc*D were performed with 25 µl of PCR mixtures containing 5 µl of DNA extraction, each 1 µl (25 pmol) of forward and reverse primer pairs, and 18 µl of sterile double-distilled water. The other virulence genes (*tsh*, *irp*2, *iss*, *ast*A and *hly*A) were determined using 25 µl of PCR mixtures containing 5 µl of DNA, 1 µl (25 pmol) each of forward and reverse primer pairs, magnesium chloride ($MgCl_2$) (1.25 µl, 10 mM) and 16.75 µl of sterile double-distilled water. The PCR amplification was carried out using a 96-well MJ Thermal Cycler (Bio-Rad, UK) with the following protocol: initial denaturation at 94˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 58˚C for 30 s, extension at 68˚C for 3 min, and the final extension step at 72˚C for 5 min. Analysis of the amplified products

was performed by electrophoresis (75 V for 1 h) with a 1.5% agarose gel stained with ethidium bromide in 0.5 X Tris-borate-EDTA (TBE) buffer. 100 bp and 1 Kbp DNA ladders were used. 2 µl of loading dye and 2 µl of PCR products was mixed and put into wells. Gel images were captured using G-BOX Chemi-XR5, (Syngene, UK), connected to a computer. The analysis of specific band sizes for different virulence genes tested was scored as present (+) or absent (-).

Table 3.5 The primers used for detection of the various genes by PCR, amplicon size, encoded virulence factors and primer references were used.

Gene	Description	Primer sequence	Amplicon size (bp)	References
Adhesins				
papC	P-fimbriae, pyelonephritis associated pili	TGATATCACGCAGTCAGTAG CCGGCCATATTCACATA	501	(Sanger *et al.*, 1977; Janßen *et al.*, 2001)
tsh	Temperature-sensitive haemagglutinin	ACTATTCTCTGCAGGAAGT CTTCCGATGTTCTGAACG	824	(Dozois *et al.*, 1992; Ewers *et al.*, 2007)
Iron acquisition				
iucD	Aerobactin synthesis, iron uptake chelate	ACAAAAAGTTCTATCGCTTC CCTGATCCAGATGATGCT	714	(Sanger *et al.*, 1977; Janßen *et al.*, 2001)
irp2	Iron-repressible protein associated with yersinabactin synthesis	AAGGATTCGCTGTTACCGGA AACTCCTGATACAGGTGG	413	(Dozois *et al.*, 1992)
Serum survival				
iss	Increase serum survival	ATCACATAGGATTCTGCC CAGCGGAGTATAGATGCC	309	(Ewers *et al.*, 2007)
Toxins				
astA	Arginine succinyl transferase A (enteroaggregative heat-stable toxin)	TGCCATCAACACAGTATATC TCAGGTCGCGAGTGACGG	116	(Franck *et al.*, 1998)
hlyA	Haemolysin A	GTCCATTGCCGATAAGTTT AAGTAATTTTTGCCGTGTTTT	1177	(Janßen *et al.*, 2001; Ewers *et al.*, 2004)

3.2.11 Data analysis

The effect of treatment on bird performance (body weight gain, feed intake, feed conversion ratio) and population of ampicillin resistant *E. coli* was determined by analysis of variance using the general linear model of Minitab (Minitab 17, Minitab Inc., PA, USA). Tukey's post-hoc test was used to compare means to observe significance at the level of $P < 0.05$. Chi-square analysis was used to determine the association between bird age, yeast and selenium administration and the measured characteristics of *E. coli* (resistance to antibiotics, carbon utilisation and carriage of virulence genes). Associations between the phenotypic expression of antimicrobial resistance, carbon utilisation and the carriage of virulence genes were also determined by Chi-square analysis.

3.3 Results

3.3.1 Growth performance

On arrival, the average weight of all 238 chicks was 40.0 ± 2.62 g. There was no significant effect of yeast administration on body weight gain during the starter period (see Appendix 1). There was no significant effect of either yeast or selenium on FCR, and no significant interaction between these two main effects on FCR (Table 3.6).

Table 3.6 Effect of administering the drinking water with yeast, or the diet with selenium, on the feed conversion ratio.

	Yeast treatment (Y)[1]						SEM	*P*-value		
	Control		*C. famata*		*S. boulardii*			Y	Se	YxSe
	-Se[2]	+Se	-Se	+Se	-Se	+Se				
Grower/finisher period (g/bird/d, day 14-36)										
Weight gain	82.26	82.55	80.19	82.11	80.82	83.19	6.81	0.982	0.785	0.987
Feed intake	127.8	127.8	125.9	127.5	126.1	129.2	13.8	0.996	0.892	0.994
FCR	1.53	1.52	1.55	1.53	1.54	1.53	0.04	0.957	0.775	0.972

[1]Birds were administered with either no yeast (Control) or with *C. famata* (10^5 CFU/ml) or *S. boulardii* (10^5 CFU/ml) in their drinking water between 9 and 11 d of age.
[2]From 14-36 d of age, birds' diets were administered with either 0.2 mg selenium/kg diet (-Se) or 0.5 mg selenium/kg diet (+Se).

3.3.2 Counting of viable cells

The number of *E. coli* from the duodenum, yolk sac and caecum in day-old birds is shown in Figure 3.1. The population density of the duodenum and yolk sac was similar (3.83 and 2.79 $\log_{10}$ CFU/ml respectively), and lower than that observed in the caecum (6.65 $\log_{10}$ CFU/ml). Ampicillin resistant *E. coli* from the duodenum and yolk sac was found in only two out of 16 samples, and from the caecum only one out of 16 samples on day 1.

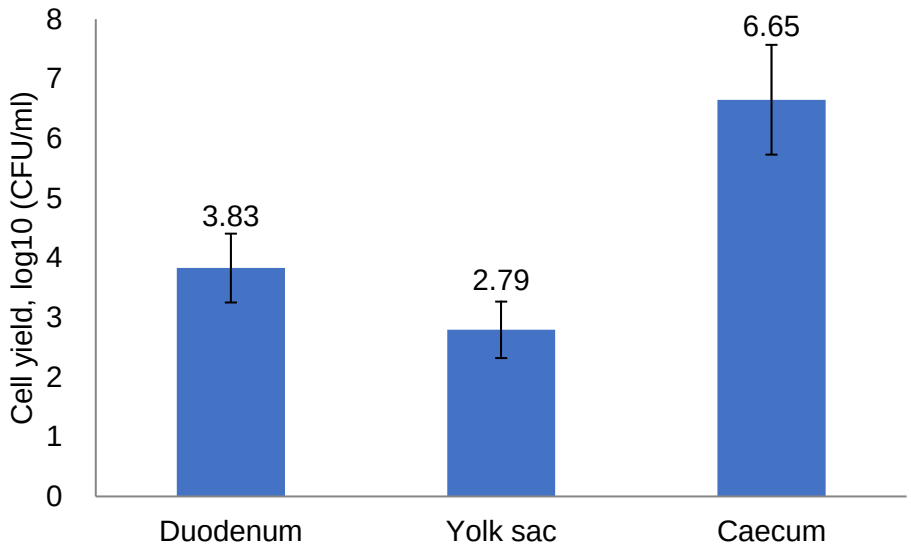


Figure 3.1 Growth of total *E. coli* from duodenum, yolk sac and caecum of chick day 1.

There was no significant effect of yeast on the population of *E. coli* (both total and ampicillin resistant), but it was noticeable that nearly all the birds sampled on day 14, 22 and 36 had caecal *E. coli* that were ampicillin resistant, and the population size of *E. coli* grown in the presence or absence of ampicillin was virtually the same (98.93 ± 2.03%) during the grower/finisher phase. This was in the absence of any

challenge with ampicillin. *E. coli* from the duodenum was found in only 5 samples of 24 birds (day 14), suggesting *E. coli* might have difficulty colonising the duodenum in older birds. Thus, only caecal samples were taken on days 22 and 36.

There was no significant interaction between yeast and selenium administration on the *E. coli* population (both total and ampicillin resistant, AmpR) when birds were 22 days of age. However, the administration of *S. boulardii* (5.29 $\log_{10}$ CFU/ml) decreased the total and AmpR *E. coli* count compared with the control and *C. famata* treatment (6.05 and 5.74 $\log_{10}$ CFU/ml respectively) ($P < 0.001$) (Figure 3.2).

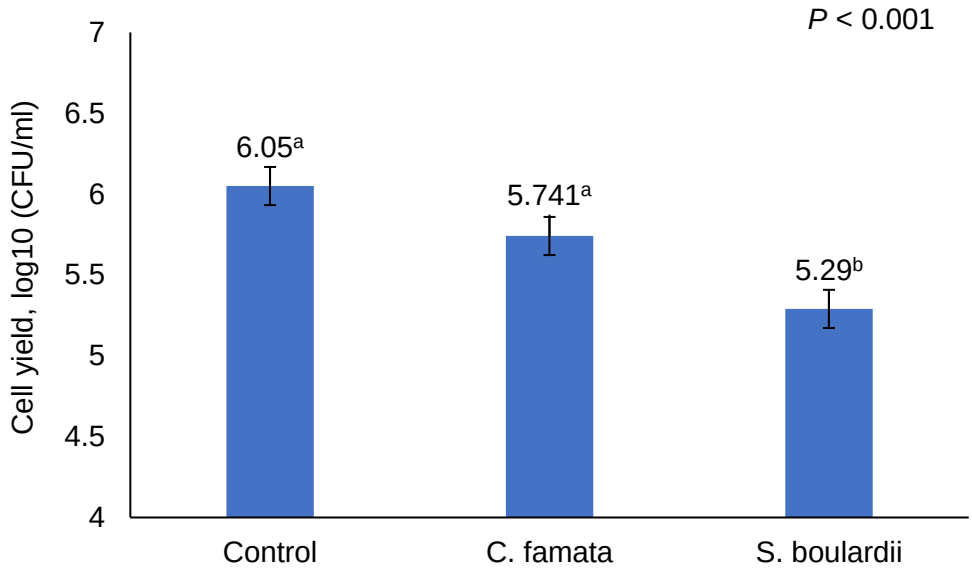


Figure 3.2 The effect of yeast on total and ampicillin resistant *E. coli* counts from caecum of bird day 22.

Values with different superscripts (a-b) are significantly different ($P < 0.05$).

There was no significant interaction between main effects on *E. coli* population when birds were 36 days of age. However, the total and ampicillin resistant *E. coli* count of birds supplemented with selenium (5.86 log_{10} CFU/ml) tended (*P*=0.064) to increase compared with the untreated selenium group (5.43 log_{10} CFU/ml) (Figure 3.3).

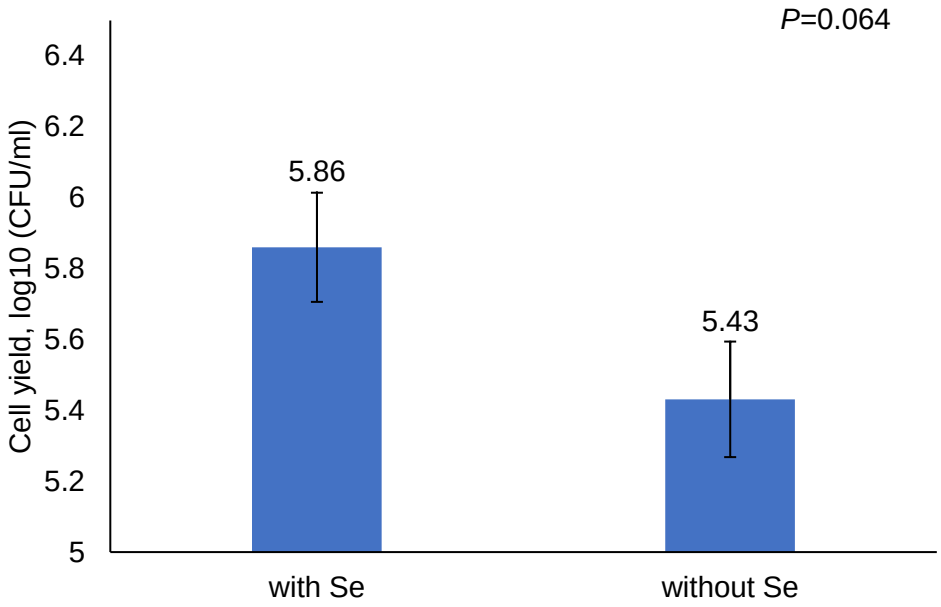


Figure 3.3 The effect of selenium on total and ampicillin resistant *E. coli* counts from caecum of bird day 36.

3.3.3 Antimicrobial resistance of *E. coli*

No significant effect of administering selenium or yeast was detected by Chi-square analysis (Figure 3.4). Total and ampicillin resistant *E. coli* had a high incidence of cross resistance to tetracycline, but not to other classes of antibiotics throughout the study. On day 1, no colonies of ampicillin resistant *E. coli* from the caecum were detected. The highest rate of resistance to tetracycline in both total *E. coli* (26.09%) and ampicillin resistant *E. coli* (34.78%) was observed on day 14. However, there was no significant effect of bird age on total and ampicillin resistant *E. coli* or on resistance to tetracycline.

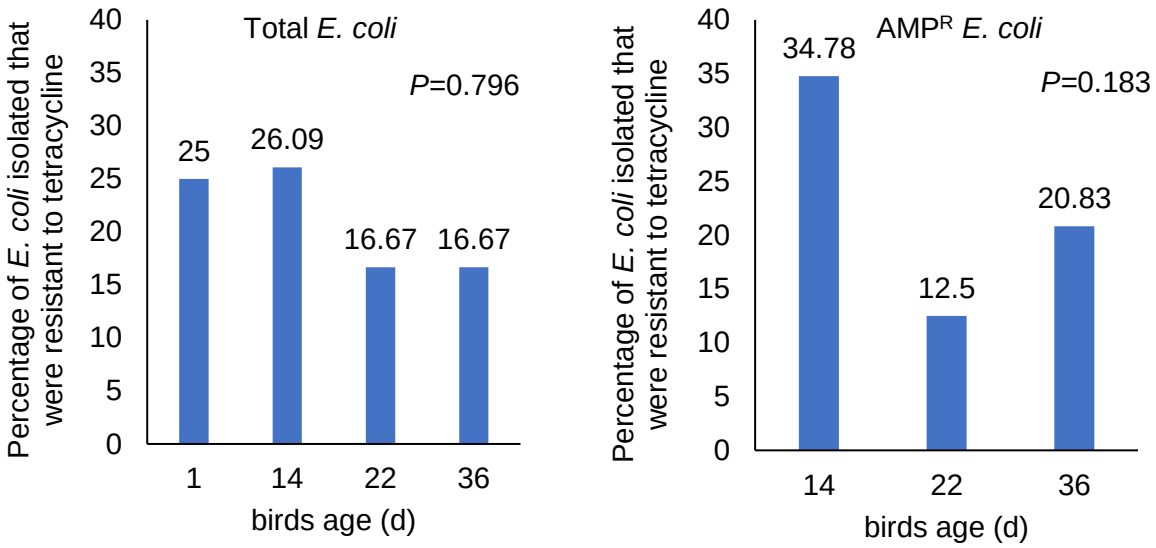


Figure 3.4 The percentage of tetracycline resistance of total *E. coli* and ampicillin resistant *E. coli* from caecum (from day 1 to day 36).

3.3.4 Carbon source utilisation

The percentage of *E*. *coli* that were able to utilise the different carbon sources is shown in Figure 3.5. Of a total of 36 *E. coli* samples isolated from the caecum, 80.56% could utilise sucrose and dulcitol, 41.67% sorbose, 2.78% arabitol and none could utilise adonitol. There was no significant association between the administration of yeast or selenium and the AMP resistant status on carbon utilisation of *E. coli,* as determined by Chi-square analysis. The weakness in the methodology was only 36 *E. coli* isolates were used for evaluation of carbon source utilisation and virulence genes as isolates were not prepared and frozen immediately. *E. coli* isolates in subsequent studies were prepared and frozen promptly.

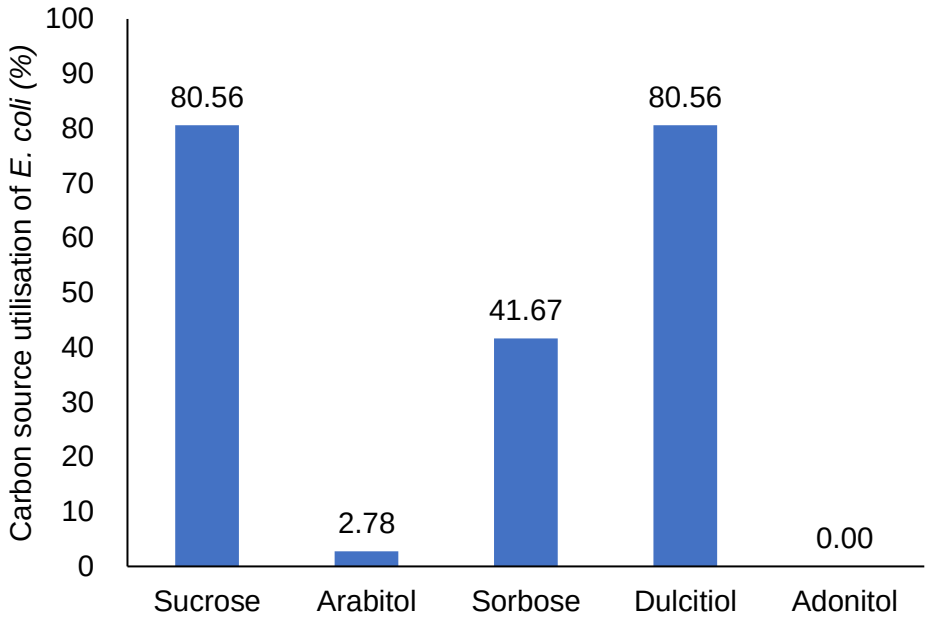


Figure 3.5 The percentage of carbon source utilisation in *E. coli*.

3.3.5 *E. coli* virulence and its association with AMR and carbon source utilisation

The percentage of virulence-associated genes in *E. coli* is shown in Figure 3.6. The most frequently detected gene was *iss* (Increased serum survival) observed in 77.78% of cases, followed by *iuc*D (iron-uptake systems of *E. coli* D) in 52.78% of cases, while 44.44% of cases had *ast*A (arginine succinyl transferase A) and *tsh* (temperature-sensitive haemagglutinin), present in 38.89% of cases. In addition, 36.11% gave positive results for iron-repressible protein *irp*2 gene and 19.44% possessed the *hly*A (haemolysin) gene. The *pap*C gene (pyelonephritis associated with pili C) was not detected in any isolate. Coliforms isolated on day 14 had a much higher prevalence of *iuc*D in 75% of cases (X^2=7.211, *P*=0.065), *tsh* in 100% of cases (X^2=11.49, *P*=0.009) and *irp*2 in 100% of cases (X^2=11.944, *P*=0.008), which then reduced after day 14 (Figure 3.7). However, there was no association between the administration of yeast or selenium and the carriage of virulence genes in *E. coli* isolates.

The carriage of the *irp*2 gene was more associated with ampicillin resistant *E. coli* (52.38%) rather than ampicillin sensitive *E. coli* (13.33%) (X^2=5.78, *P*=0.016 Figure 3.8). Utilisation of sucrose and dulcitol by *E. coli* was associated with the carriage of *iuc*D in 62% (X^2=5.17, *P*=0.023) and *tsh* genes in 48% (X^2=5.53, *P*=0.019), Figure 3.9. In addition, utilisation of sorbose was associated with the carriage of the *tsh* gene in 67% (X^2=8.35, *P*=0.04). There was a tendency (although not significant) for sorbose utilisation to be associated with the carriage of the *irp*2 gene in 53% (X^2=3.31, *P*=0.069).

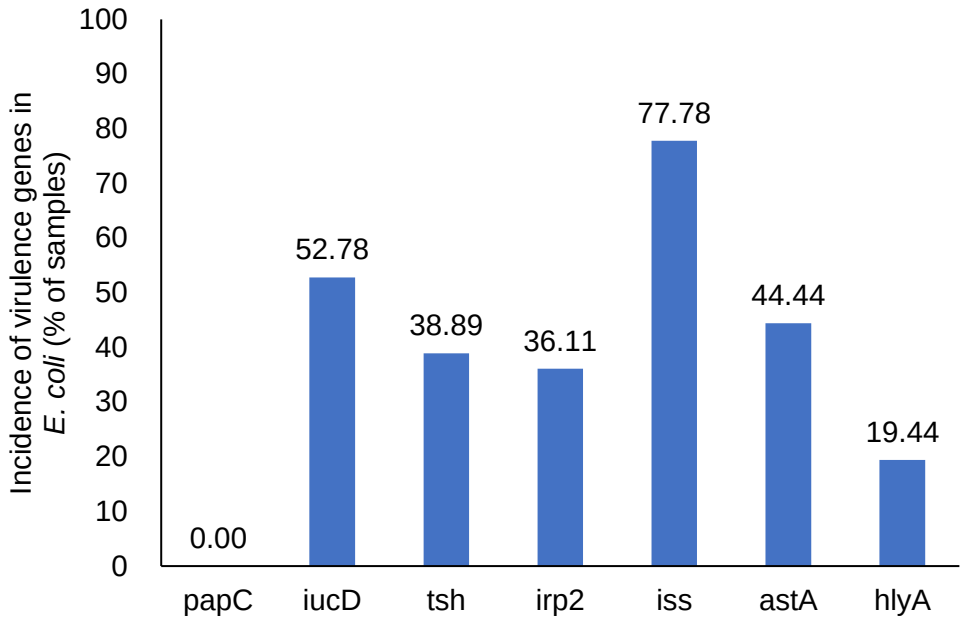


Figure 3.6 The percentage of *E. coli* isolates carrying different virulence-associated genes.

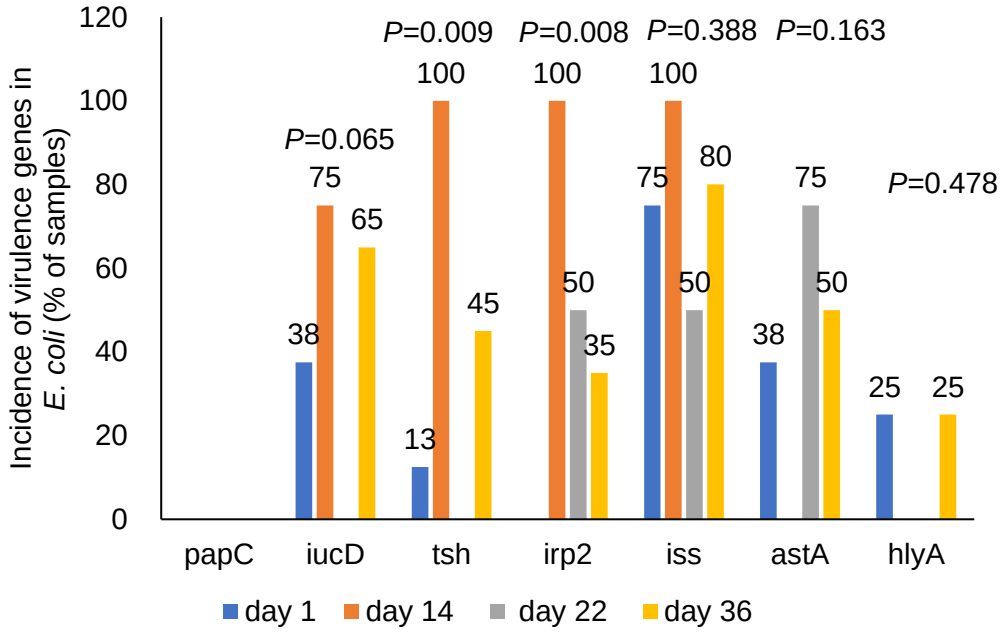


Figure 3.7 The effect of bird age on the carriage of virulence-associated genes in isolates of *E. coli* from the caecum of broiler chickens.

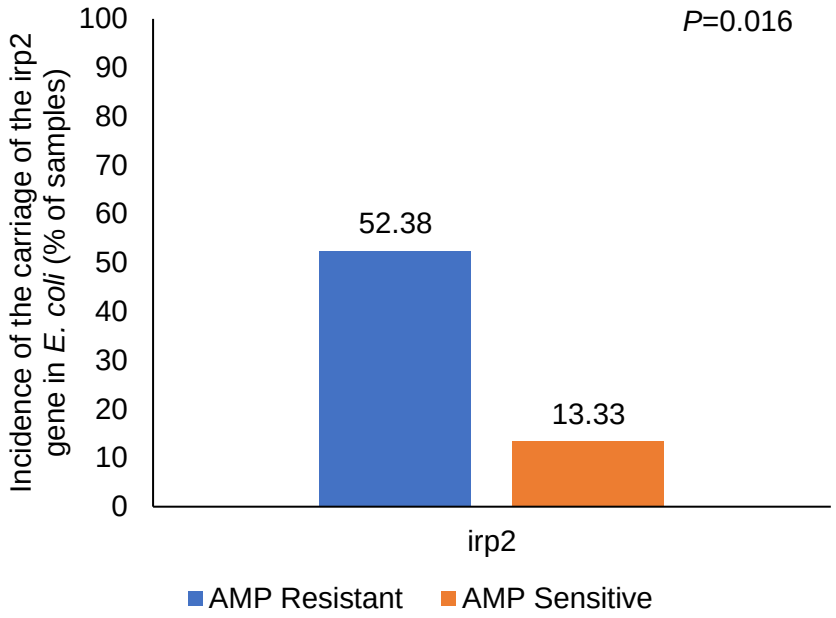
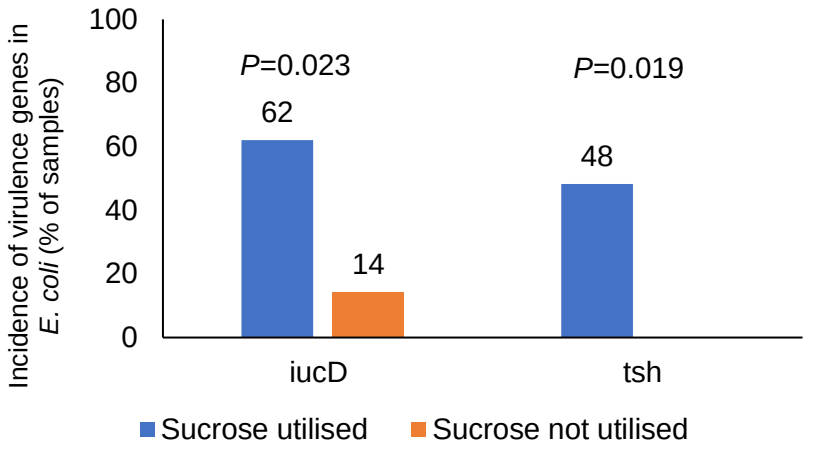
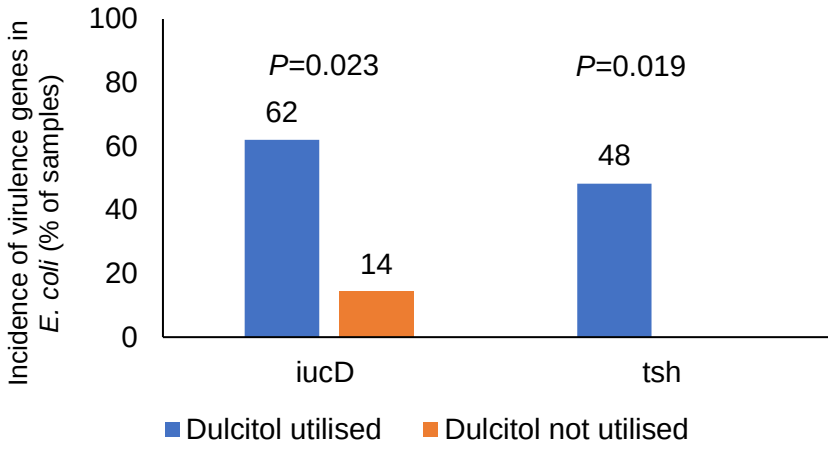


Figure 3.8 The association between the carriage of the *irp*2 and the phenotypic sensitivity or resistance by isolates of *E. coli* to ampicillin (50 µg/ml).





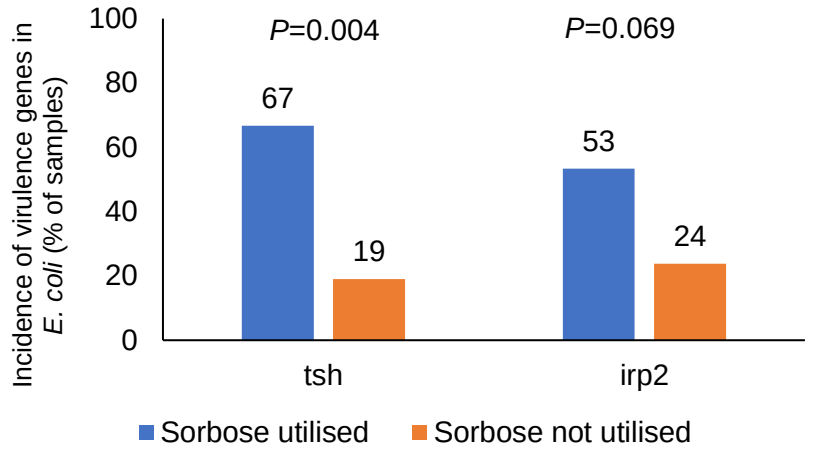


Figure 3.9 The association between the carriage of virulence genes and the sucrose, sorbose and dulcitol source utilisation of *E. coli*.

3.4 Discussion

3.4.1 Growth performance

Bird performance was recorded as an indicator of bird health. Birds consumed slightly less feed than the 142.3 g/d expected (Aviagen, 2019) and so grew slightly less than the expected 89.0 g/d. FCR was also higher than would be expected. However, birds were fed a mash diet rather than a pellet, which reduces feed intake and results in greater wastage, and so their performance does not suggest they were suffering any subclinical infection or other stress. The interaction between selenium and yeast administration had no significant effect on growth performance. The benefit of selenium administration on performance, antioxidant activity, meat quality and animal health has been extensively demonstrated. Choct *et al.* (2004) observed that diets with a high selenium content reduced the feed intakes. Ševčíková *et al.* (2006) reported that Se and Se-enriched yeast supplements improved the liveweight of broilers. Zhou and Wang (2011) reported that adding 0.30 mg/kg of Nano-Se to diets improved the daily weight gain, FCR, breast drip loss and glutathione peroxidase (GSH-Px) activities in the serum and liver. However, some studies reported the lack of effect of selenium on the growth performance in broiler chickens (Perić *et al.*, 2009; Chen *et al.*, 2013; Chen *et al.*, 2014). These previous findings were consistent with this study.

S. boulardii is known as a non-pathogenic yeast which has a beneficial effect on intestinal health and the immune system (Buts and De Keyser, 2006; Nari *et al.*, 2020). *S. boulardii* has been widely used as a probiotic and can improve performance and bird health. Some studies have reported that administering *S. boulardii* increased bird weight gain (Bradley *et al.*, 1994; Gil De Los Santos *et al.*,

2005). Few beneficial effects were observed in this study, and it was included as a positive control to compare with *C. famata.* On the basis of bird performance, there was no evidence, from this study, of any real benefit in supplementing birds with *C. famata.* However, the administration period (days 9-11 of age) was extremely short thus it might be insufficient to have beneficial effects on growth performance of broiler chickens.

3.4.2 *E.* coli counts from caecum and cross-resistance to antibiotics

Yeast culture was added to drinking water from borehole water. The borehole water is generally clean and includes no or low levels of pathogenic bacteria. However, borehole water could be contaminated by poor hygienic protection at the top of the bore hole or by leaking septic and fuel tanks (Aviagen, 2018). There was, however, no evidence of any issues with water quality throughout this study.

Yeast colonies isolated from the caecum (as part of a separate study) were rarely observed. This might result from the low dosage or short administration period, or be an indication that or yeast does not colonise the broiler caecum. Administration with *S. boulardii* was associated with a significant decrease in the population density of both total and ampicillin resistant *E. coli* from the caecum of birds on day 22. This is a promising finding, particularly if this effect could persist throughout the bird's life. A similar finding was reported by Line *et al.* (1998), who discovered that administering dried *S. boulardii* in the feed for 23 days decreased the population of *Salmonella* from the caecum of broiler chickens. Many researchers have reported that administering bacteria and yeast to broiler chickens decreased pathogenic *E. coli*, *Salmonella* and *Campylobacter* populations in broilers (Trela *et al.*, 2020; Li *et*

al., 2021; Śmiałek *et al.*, 2021). The absence of any observable effect in this study may well be a consequence of the small amount of yeast that was administered and for such a short duration. Higher doses of bacteria and yeast were therefore administered for longer periods in subsequent studies.

The population size of total *E. coli* and ampicillin resistant *E. coli* was determined over the experimental period. However, from these plates, a single *E. coli* isolate was used to evaluate resistance to other antibiotics by disc diffusion. A weakness in this methodology is that the isolate selected may not be representative of the coliform population. In the present study, *E. coli* from the caecum was mostly sensitive to ampicillin on day 1. However, nearly all birds sampled on days 14, 22 and 36 had caecal *E. coli* that were ampicillin resistant, and the population size of *E. coli* grown in the presence or absence of ampicillin was virtually the same. This would suggest that ampicillin resistant *E. coli* might have some selective advantage over ampicillin sensitive *E. coli,* even when there is no exposure to ampicillin, such that it is easier to colonize the caecum if the *E. coli* carries the ampicillin resistant plasmid. Wasyl *et al.* (2013) demonstrated a high prevalence of resistance to ampicillin (75.5%), nalidixic acid (74%) and ciprofloxacin (79.8%) in broilers and AMR resistance was much higher in poultry compared with swine and cattle. Similarly, a high rate (50% or more) of resistance to ampicillin, nalidixic acid, tetracycline, streptomycin and the combination trimethoprim-sulfonamide was observed by Persoons *et al.* (2010). This study also reported *E. coli* from the broiler caecum had a high prevalence of resistance to ampicillin and tetracycline. It had been assumed that yeast, which produces many useful bioactive compounds, might manipulate the gut microbial community and thereby decrease the prevalence of AMR and presumptive pathogenic *E. coli*. However, there was no effect of *C. famata*

and *S. boulardii* intervention on the prevalence of AMR and presumptive pathogenic *E. coli* and so there was no evidence to support this hypothesis.

In the present study, ampicillin resistant *E. coli* had high cross resistance to tetracycline compared to other types of antibiotics. Many other researchers have observed a high prevalence of resistance to one or more antibiotics by *E. coli* in poultry (Yang *et al.*, 2004; Johnson *et al.*, 2010; Persoons *et al.*, 2010; Wasyl *et al.*, 2013). The high prevalence of multi-drug antimicrobial resistance can cause severe issues of public health (Wall *et al.*, 2016). High incidence of multi drug resistance may be associated with mobile elements such as plasmids and integrons carrying genes coding for resistance to more than one antibiotic (Diarra *et al.*, 2007; Thorsteinsdottir *et al.*, 2010). What was promising in this study was the reduction in AMP resistance, and of cross resistance to AMP and TET, after 14 d. These results reflect those of Diarra *et al.* (2007) who also found that antimicrobial resistance decreased as birds aged. In addition, the management of diets and environments can influence the microbial status of the gut (Apajalahti *et al.*, 2004). This study suggests that while chicks may have been infected with highly resistant *E. coli* (often APEC types) from the hatchery or early in life, over the lifespan of the birds these types were replaced by more robust non-antibiotic-resistant commensal types.

Altering the composition of the gut microbiome in young birds might decrease the potential risk of antibiotic resistant infections. If the development of the microbiome and the gut environment found in the older bird could be advanced so that it was established earlier in life, then birds may be protected from the very vulnerable ‘window’ that was observed during the starter phase (when the prevalence of both AMR and virulence genes was very high).

3.4.3 Carbon source utilisation

Most of the *E. coli* populations isolated from the caecum were able to utilise sucrose (83.78%), dulcitol (81.58%), and sorbose (44.74%). Ratnam *et al.* (1988) also reported that pathogenic *E. coli* O157:H7 taken from humans could utilise dulcitol and raffinose. In addition, *E. coli* O157:H7 was better able to utilise sucrose compared with other *E. coli* strains. Durso *et al.* (2004) investigated pathogenic *E. coli* isolated from cattle and observed they were better able to utilise dulcitol, sucrose and L-galactonic acid δ -lactone compared with commensal *E. coli* strains. Thus, commensal and pathogenic *E. coli* could be partly differentiated through the test of carbon source utilisation.

Fabich *et al.* (2008) observed that pathogenic *E. coli* EDL933 and commensal *E. coli* MG1655 co-metabolized 13 sugars in a similar order *in vitro*. However, a systematic mutation analysis showed that the two strains used different carbon sources for colonization of the streptomycin-treated mouse. Both pathogenic *E. coli* EDL933 and commensal *E. coli* MG1655 utilised arabinose, fucose, and N-acetylglucosamine in the intestine. In addition, *E. coli* EDL933 used galactose, hexuronates, mannose, and ribose, while *E. coli* MG1655 utilised gluconate and N-acetylneuraminic acid. These results indicate that the pathogen strains may be able to proliferate and successfully invade because of their competitive advantage in being able to utilise nutrients found in the mouse intestine that are not used by commensal *E. coli* and the normal microbiota. These previous observations support the findings of this study and it can thus be suggested that the carriage of various virulent genes by *E. coli* might be associated with the ability to better utilise carbon sources such as dulcitol and sucrose compared with commensal *E. coli*. There is a

need to determine and better understand the relationship between utilisation of carbon source and pathogenicity of *E. coli*, as it may offer another strategy, through the manipulation of the host animal diet, to control the growth of APEC.

3.4.4 *E. coli* virulence and its association with AMR

In the current study, the increased serum survival (*iss*) gene was the most prevalent virulence gene, being carried by 78.95% of the *E. coli* isolates. Adhesins (*tsh*), toxins (*ast*A) and iron-uptake system (*iucD*, *irp2*) genes showed similar prevalence of virulence genes around 38.47% - 55.26%. However, *hly*A was less prevalent at 18.42% and there was no evidence of the carriage of *pap*C. The *irp*2 and *tsh* genes were very prevalent when birds were 14 d old, but these genes then declined as the birds aged. Some caution should be applied to this interpretation as the numbers of isolates were not balanced across the bird ages (n=8 for day 1, n=20 for day 36) with a smaller number being successfully preserved from samples taken when birds were 14 (n=4) and 22 d (n=4) of age. In addition, the number of *E. coli* isolates across control (n=10), *C. famata* (n=13) and *S. boulardii* (n=5) treatment was different. There was no difference between control and *C. famata* group on the prevalence of virulence genes in *E. coli* isolates. The *S. boulardii* treatment was excluded for this analysis because of the small number of samples. This finding refutes the hypothesis that the administration of yeast might reduce the prevalence of virulence genes in *E. coli.* However, this may in part be a consequence of the low dosage and short administration period of yeast that was used in this experiment.

Paixao *et al.* (2016) demonstrated that *fim*C adhesins were most prevalent in APEC and Avian Faecal *Escherichia coli* (AFEC). The *ast*A gene had a high prevalence in

AFEC while *pap*C, *fim*C, *Vat*, *Cva*A/B, *iuc*D, *irp2*, *fyu*A and *iss* had a high prevalence in APEC. According to Paixao *et al.* (2016), APEC isolates had a higher prevalence of the iron uptake-related genes and the serum survival genes compared with commensal strains of *E. coli.* This is because iron is an essential nutrient for *E. coli* and is of more limited availability in extraintestinal tissues compared with digesta. There is therefore a selective advantage for APEC to carry genes such as *iuc*D and *irp*2. What was interesting in this study was the association between *irp*2 and ampicillin resistance. As observed before, it is unclear what selective advantage there is to the carriage of genes coding for ampicillin (or any other antibiotic) resistance when no antibiotics have been administered. However, it is possible that these virulence genes are carried on the same plasmid (or other mobile genetic element) as the genes coding for ampicillin resistance and the advantage (to APEC) is in the greater ability to scavenge and accumulate iron (Janßen *et al.*, 2001). Other researchers have also demonstrated that virulence factors were associated with antimicrobial resistance by *E. coli* isolates in animal digesta. de Verdier *et al.* (2012) reported that multi antimicrobial resistant *E. coli* was more common with the virulence genes *esp*P, *irp*, and *fyu*A in Swedish dairy calves. Likewise, Johnson *et al.* (2012) indicated avian extraintestinal pathogenic *E. coli* possessed more multidrug resistance compared to commensal *E. coli*. Virulence genes of *E. coli* are often placed on plasmids or transposons thus antimicrobial resistance by *E. coli* can be correlated with virulence genes as these factors might be linked to AMR genes (Rosengren *et al.*, 2009).

3.5 Conclusions

Administering selenium, *C. famata* and/or *S. boulardi* had no effect on bird performance and did not affect the prevalence of AMR and carriage of virulence genes by *E. coli*. There was also no adverse impact of these treatments on broiler chickens. There was a high prevalence, in young birds, of ampicillin resistant *E. coli*, and there was also a high prevalence of cross resistance to tetracycline (although not significant). The *tsh* and *irp2* virulence genes were highly prevalent in *E. coli* isolates taken from birds 14 d of age, but then they declined as the birds got older. The carriage of *irp*2 was associated with ampicillin resistant *E. coli*. In addition, *E. coli* utilisation of sucrose and dulcitol was correlated with the carriage of the *iucD* and *tsh* genes, while sorbose utilisation was associated with the carriage of the *irp*2 and *tsh*. Iron acquisition is an important trait for *E. coli* and may be the key to understanding the prevalence of AMR *E. coli* in situations where no antibiotics are administered. The relationship between iron acquisition systems, antimicrobial resistance and utilisation of different nutrients needs clarification.

Advancing the development of a more mature microbiome (with a lower prevalence of virulent and AMR *E. coli*, as was observed in the older birds in this study) may reduce the vulnerability of the young bird. One means of doing this may be through the use of yeast which are administered throughout the bird's life (36 days) rather than the relatively low dose over a three-day period that was done in this study. This might positively influence bird performance and prevent the proliferation of AMR and pathogenic *E. coli*. In addition, whole genome sequencing (WGS) of the *E. coli* may shed more light on the identification and characterisation of *E. coli* found in the chicken gut as it ages. This is to be addressed in the next chapter.

Chapter 4. The effect of *Candida famata* and *Lactobacillus fermentum* on the microbial population in the chicken gut and the prevalence of antimicrobial resistance and virulence of *E. coli* isolated from the broiler caecum

This study has been reported in part in the paper:

Lee, A., Aldeieg, M., Woodward, M., Juniper, D.T., Rymer, C., 2021. The effect of *Candida famata* and *Lactobacillus plantarum* on the number of coliforms and the antibiotic resistance and virulence of *Escherichia coli* in the gut of broilers. *Animal* (Accepted 8 June 2021).

4.1 Introduction

Dietary interventions may alter the composition of the gut microbiome and develop the gut (Olnood *et al.*, 2015). In doing this, the AMR status of the bird (and its products) may be altered. *Lactobacillus* sp. and yeasts may help prevent the proliferation of pathogenic bacteria and potentially AMR in the gut through mechanisms such as competitive exclusion, neutralisation of toxins, bactericidal activity or enhanced immune competence (Kabir, 2009; Fijan, 2014). *Lactobacillus fermentum* is an obligate heterofermentative and frequently isolated from fermented vegetables and this strain has been used as probiotics in animal feed (Reid and Friendship, 2002; Mikelsaar and Zilmer, 2009; Ale *et al.*, 2020). Effects of administering *L. fermentum* have included the improvement of health and immunity as well as growth performance in broiler chickens (Capcarova *et al.*, 2010; Bai *et al.*, 2013; Šefcová *et al.*, 2020).

Bai *et al.* (2013) observed that the administration of *Lactobacillus fermentum* and *Saccharomyces cerevisiae* improved the average daily gain and FCR during the starter phase (from 1 d to 21 d) and promoted the gut T cell immune system in broilers. Capcarova *et al.* (2010) reported that administering *L. fermentum* via drinking water decreased the triglyceride content in serum and improved total antioxidant status of broiler chickens. Šefcová *et al.* (2020) demonstrated that *L. fermentum* administration stimulated a humoral response to a *Campylobacter coli* infection in chicks and diminished proinflammatory cytokines.

However, little is known about the effect of administering *Candida famata* on bird productivity and health, but since *C. famata* is a commensal yeast found in the bird caecum, this yeast might have positive effect on chicken health. Coliforms adhere to the yeast wall (Ofek and Beachey, 1978; Eshdat *et al.*, 1981; Bagg and Silverwood, 1986), so an increase in the yeast population might reduce the population of coliforms in the gut. In this study, therefore, *L. fermentum* and *C. famata* were administered to broiler chickens throughout their life and it was hypothesised that this intervention might decrease the coliform population in the broiler caecum and reduce the prevalence of AMR and virulence of *E. coli* in the broiler caecum.

Whole genome sequencing (WGS) is useful analysis to determine the carriage of antimicrobial resistance gene (ARG) and genetic variations correlated with animal health (Joensen *et al.*, 2014; Solà-Ginés *et al.*, 2015; Hussain *et al.*, 2019). The use of WGS plays a significant role in public health by identification of antibiotics resistant genes and genomic sequencing type associated with foodborne outbreaks (Oniciuc *et al.*, 2018). In this study, it was used to investigate the change of multi-locus sequence type (MLST), genotypic AMR and virulence genes of *E. coli* with

bird age. In the previous study, it appeared that commensal *E. coli* (susceptible to antibiotic) outcompeted AMR and putative pathogenic *E. coli* as birds aged. This leads to the hypothesis that more commensal *E. coli* (that are sensitive to antibiotics) might evolve with increasing bird age. The aim of this experiment was therefore to determine the effect of a yeast (*Candida famata*) and a bacterium (*Lactobacillus fermentum*), administered in the drinking water, on the prevalence of phenotypic and genotypic AMR status and carriage of virulence genes by *E. coli* throughout the life of broiler chickens.

4.2 Material and Methods

4.2.1 Experimental design, birds and diets

The bird housing, management and slaughter conditions were as described in Chapter 3. A total of 220 chicks (day-old, male Ross 308, purchased from PD Hook, Cote, Oxfordshire) were used in a 35 d feeding trial. All chicks were weighed on arrival, wing tagged, blocked by liveweight, and then randomly placed in one of four brooder rings and fed a common starter diet (Table 4.1) until the birds were 14 days of age. Antibiotics were not administered during the experimental period. *Candida famata* (isolated from a chicken) and *Lactobacillus fermentum* (isolated from a pig; University of Surrey) were administered via the drinking water from borehole. The *Lactobacillus* strain was isolated from a pig in an experiment at the University of Surrey and *L. plantarum* was identified. However, characterising *Lactobacillus* species can be difficult, and this isolate had not been genetically sequenced. The characterisation of this isolate by API (reported in Chapter 5) suggests that it might in fact be *L. fermentum.* It was also observed that it produced a high concentration

of ethanol which is more characteristic of *L. fermentum* than *L. plantarum.* For the purposes of this thesis, therefore, it is referred to as *L. fermentum.* To be confident in its identification, the isolate would need to be characterised by whole genome sequencing.

Stock cultures of each isolate in *C. famata* and *L. fermentum* were incubated overnight in Yeast Extract Peptone Dextrose (YPD) broth or De Man, Rogosa and Sharpe (MRS) broth. Population density was then estimated by serial dilution and measurement of the optical density of the incubation medium at 600 nm. Cultures were then centrifuged at 5000 rpm for 5 min (Eppendorf centrifuge 5804R, Germany) and washed with sterile distilled water. An appropriate volume of the culture was then added to a measured volume of the drinking water in the hopper to attain the desired final concentration of *C. famata* and *L. fermentum*. Water was therefore provided either untreated (Control) or with a preparation of *C. famata* (CF; 10^7-10^9 CFU/ml water offered) or *L. fermentum* (LF; 10^5-10^8 CFU/ml water offered), or a combination of CF and LF (CFLF; total 10^6-10^8 CFU/ml water offered) in water hoppers two days each week (days 1, 4, 8, 11, 14, 18, 22, 25, 29 and 32). Water hoppers were replenished with fresh (untreated) water when required.

On day 14, all birds were weighed and blocks of birds were allocated (six birds per pen, all birds originating from the same brooder ring) to one of 24 pens, with six replicate pens per treatment. All birds were then fed a common grower/finisher diet. The ingredient and nutrient composition of the diets is shown in Table 4.1. The diets were fed in the form of a mash, and were manufactured by Target Feeds (Whitchurch, UK). Samples of each diet were analysed for crude protein, total starch, oil, sugar, methionine, lysine, and iron (Sciantec, York, UK). Lighting was via incandescent lights with 23 h continuous light per 24 h period for the first seven

days, followed by 18 h continuous light (6 h darkness) in each 24 h period. The birds were brooded according to the breeder's recommendations using infrared lights to provide supplementary heat when necessary.

Table 4.1 Ingredient and nutrient composition (as-fed basis) of chickens' diets.

	Starter (0-14 d)	Grower/ Finisher (15-35 d)
Ingredient composition (g/kg)		
Barley	40	40
Wheat	500	550
Soya bean meal	320	265
Rapeseed meal	42	42
Soya bean oil	50	65
L-lysine HCl	4	1
DL-methionine	3.45	2.42
L-threonine	2.05	2.02
Sodium bicarbonate	2.5	2.5
Salt	2	2.5
Limestone	11	8.56
Poultry vitamins/minerals*	2	2
Dicalcium phosphate (QPRDC)	20	16
Titanium dioxide	1	1
Nutrient composition (g/kg)		
ME (MJ/kg)	9.7	9.9
Crude Protein	219	210
Total Starch	244	265
Oil A	47.4	49.1
Sugar	46.8	43.3
Methionine	8.9	6.8
Lysine	16.1	11.3
Iron (mg/kg)	147	173

ME: Metabolisable Energy

* The vitamin/mineral premix supplied per kg of starter diets: vitamin A 6750 IU, vitamin D_3 2500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Se 0.125 mg, Mo 0.25 mg.
The vitamin/mineral premix supplied per kg of grower/finisher diets: vitamin A 5000 IU, vitamin D_3 2500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Se 0.125 mg, Mo 0.25 mg.

4.2.2 Sample collection

On day 1, a total of 16 birds were randomly selected and sacrificed by cervical dislocation. The whole intestinal tract (from proximal duodenum to ileo-caecal junction) and the yolk sac were taken and placed on ice for transportation. These were analysed for the determination of the population size (CFU/g) of yeasts and bacteria (coliforms and *Lactobacilli*), and for the determination of antimicrobial resistance and presence of virulence genes of coliforms. On days 8 and 14, four birds from each brooder ring were randomly selected and sacrificed and contents from the duodenum, ileum and caecum were taken and weighed, for the same determinations as above. Coliforms were not counted in the small intestine samples as earlier observations (Chapter 3) had detected very low populations in these segments of the gut. One isolate of *E. coli* was taken from the cultured caecal sample of each bird for the determination of antibiotic resistance (all birds) and the presence of virulence genes (with the exception of CFLF birds, as no significant interaction between CF and LF had been observed).

After the birds were allocated to their grower/finisher pens, one bird from each pen was randomly selected and sacrificed on days 22, 29 and 35. Samples of digesta from the duodenum, ileum and caecum were again taken and placed on ice for transportation and analysed as before. An isolate of *E. coli* was taken from each caecal sample for the determination of antibiotic resistance and (with the exception of CFLF birds, for the same reason as before) the presence of virulence genes.

4.2.3 Bird performance

Birds were weighed individually on days 1, 14 and 35. Mean body weight increase was then calculated on a pen basis during both the starter period and grower/finisher period. Feed intake was determined and FCR calculated on a pen basis during grower/finisher period.

4.2.4 Determination of viable *E. coli*, lactic acid bacteria and yeast populations and isolation of samples

The samples of whole intestine and yolk sac (from day-old chicks) and the samples of duodenal, ileal and caecal digesta (taken from the older birds) were weighed and serially diluted with phosphate-buffered saline (PBS, 0.01 M) and 100 µl of the suspension was then spread on De Man, Rogosa and Sharpe (MRS) agar (for the enumeration of *Lactobacillus* spp.), MacConkey agar (for the enumeration of coliforms, in caecal digesta samples only) and Sabouraud Dextrose Agar (SDA) (for the enumeration of yeast). Plates were incubated (37°C, 48 h) in an anaerobic incubator (Whitly MG1000 anaerobic workstation) for the *Lactobacillus* spp. cultures and aerobically incubated overnight at 37°C for the coliform and 30°C for yeast cultures. Colonies were then counted (Gallenkamp Colony Counter CNW 325 030Y, UK) to determine population sizes (CFU/g).

A single colony of *E. coli* was selected at random from each MacConkey plate and transferred to a separate, sterile microcentrifuge tube containing 500 µl of nutrient broth. These cultures were incubated at 37°C for 18-24 hours. 100 µl of the resulting *E. coli* suspension was transferred to a cryovial (Mast group, Mastdisks, UK) and stored at -80°C pending further analysis. A single colony of *Lactobacillus* sp. was

taken at random from each plate and transferred to a sterile microcentrifuge tube containing 1ml of 15% v/v glycerol (45 ml of glycerol plus 255 ml of MRS broth). These cultures were incubated anaerobically at 37°C for 18-24 hours. After that, the *Lactobacillus* sp. suspension was stored at - 80°C pending further characterization (Chapter 5).

4.2.5 Determination of antimicrobial resistance

In this experiment, AMR was originally estimated by the replicate plating method, unlike the disc diffusion assay used in the previous study. Replicate plating enables the identification of particular isolates that are resistant to one or more antibiotics. However, the replicate plating was unsuccessful and this was attributed to the presence of too much moisture in the plate or on the velvet after autoclaving. Thus, it was impossible to count the AMR *E. coli* colonies. The replicate plating assay was therefore halted and replaced with the selection of a single *E. coli* isolate from each plate that was then streaked on a series of plates with different antibiotics.

A sterile inoculation loop was inserted into the *E. coli* suspension in the cryovial and then streaked onto four MacConkey plates, each enriched with one of four antibiotics (ampicillin, nalidixic acid, tetracycline and chloramphenicol, each at a concentration of 20 µg/ml). Plates were then incubated (37°C, 16 h) and growth of colonies on each plate was then determined. When growth was observed on plates containing antibiotics, it was deemed that the colony that had inoculated the plate was resistant to that antibiotic.

4.2.6 Extraction of genomic DNA

The genomic DNA from 84 isolates of *E. coli* (taken from birds that received the control, CF or LF treatment, but not the CFLF treatment) were extracted to determine the presence of *E. coli* virulence factors and whole genome sequencing (WGS) analysis. The genomic DNA was extracted as described in Chapter 3. The purification of DNA was conducted as described in Chapter 3 and the 260:280 nm ratio was also recorded (1.82 $\pm$ 0.12). DNA stock were stored at -20˚C pending the analysis of virulence genes by PCR and WGS.

4.2.7 Whole genome sequencing (WGS) and data analysis

All extracts were submitted to Quadram Institute Bioscience to determine the genotype of individual *E. coli* isolates by WGS. Genomic DNA was normalised to 0.5 ng/µl with EB (10mM Tris-HCl). 0.9 µl of TD Tagment DNA Buffer (Illumina Catalogue No. 15027866) was mixed with 0.09 µl TDE1, Tagment DNA Enzyme (Illumina Catalogue No. 15027865) and 2.01 µl PCR grade water in a master mix and 3 µl added to a chilled 96 well plate. 2 µl of normalised DNA (1 ng total) was pipette mixed with the 3 µl of the Tagmentation mix and heated to 55ºC for 10 minutes in a PCR block. A PCR master mix was made up using 4 µl kapa2G buffer, 0.4 µl dNTP's, 0.08 µl Polymerase and 6.52 µl PCR grade water, contained in the Kap2G Robust PCR kit (Sigma Catalogue No. KK5005) per sample and 11 µl added to each well to be used in a 96-well plate. 2 µl of each P7 and P5 of Nextera XT Index Kit v2 index primers (Illumina Catalogue No. FC-131-2001 to 2004) were added to each well. Finally, the 5 µl of Tagmentation mix was added and mixed. The PCR was run at 72ºC for 3 minutes, 95ºC for 1 minute, 14 cycles of 95ºC for

10s, 55ºC for 20s and 72ºC for 3 minutes. Following the PCR reaction, the libraries were quantified using the Promega QuantiFluor® dsDNA System (Catalogue No. E2670) and run on a GloMax® Discover Microplate Reader. Libraries were pooled following quantification in equal quantities. The final pool was double-SPRI size selected between 0.5 and 0.7X bead volumes using KAPA Pure Beads (Roche Catalogue No. 07983298001). The final pool was quantified on a Qubit 3.0 instrument and run on a D5000 ScreenTape (Agilent Catalogue No. 5067-5579) using the Agilent Taestation 4200 to calculate the final library pool molarity.

The pool was run at a final concentration of 1.5 pM on an Illumina Nextseq500 instrument using a Mid Output Flowcell (NSQ® 500 Mid Output KT v2(300 CYS) Illumina Catalogue FC-404-2003) following the Illumina recommended denaturation and loading recommendations which included a 1% PhiX spike (PhiX Control v3 Illumina Catalogue FC-110-3001). Data were uploaded to Basespace (www. basespace.illumina.com) where the raw data was converted to eight FASTQ files for each sample. Using Bracken, all samples were reported as having 90%+ reads matching to an *E. coli* reference genome. ECtyper was used to get the O and H serotypes for the *E. coli* strains. Antibiotic resistance gene (ARG) were identified using ARIBA with the Resfinder database (version 3.1). The virulence genes were also identified using ARIBA, and the VFDB full database.

4.2.8 Virulence genotyping

84 DNA extracts taken from the *E. coli* isolates were analysed for seven avian pathogenic *E. coli* (APEC) virulence factors using PCR assays. The evaluation of

virulence genotyping was conducted according to the method described in Chapter 3.

4.2.9 Data analysis

The effect of the administration of CF, LF or interaction between CF and LF on bird performance (body weight gain, feed intake, feed conversion ratio) was determined by analysis of variance using the general linear model of Minitab (Minitab 17, Minitab Inc., PA, USA). The correlation between bird age and digesta site or administration of CF or LF and the interaction between these main effects on the population density of coliforms, lactic acid bacteria and yeasts was determined by ANOVA (mixed effects model). Fixed factors were bird age, digesta site and administration of CF, LF and CFLF, and pen was a random factor. Data were analysed as a 2 (+/-CF) x 2 (+/- LF) factorial design. Tukey's post-hoc test was used to compare means to observe significance at the level of $P < 0.05$. Chi-square analysis was used to determine the association between phenotypic or genotypic AMR and either the administration of CF and LF or bird age. The association between bird age and *E. coli* MLST was determined by Chi-square analysis. Associations between the carriage of virulence genes based on PCR and WGS and bird age and administering CF and LF were determined by Chi-square analysis. In addition, Chi-square analysis was conducted to determine the association between the carriage of virulence associated gene (VAG) and antimicrobial resistance (respectively, phenotypic AMR and VAG by PCR; carriage of antibiotic resistance gene (ARG) and VAG by WGS).

4.3 Results

4.3.1 Growth performance and population size of coliforms, lactic acid bacteria and yeasts in the gut

There was no significant effect of administering CF, LF and CFLF on body weight gain during starter period (see Appendix 2. The administration of CF, LF and CFLF did not affect bird performance (Table 4.2).

Table 4.2 Effect of the addition of *Candida famata* and/or *Lactobacillus fermentum* in the drinking water of broiler chickens on their performance.

	Control	CF	LF	CFLF	SEM	*P*-value		
						CF	LF	CF*LF
Grower/finisher period (day 14-35)								
Weight gain (g/bird/d)	82	80	78	79	1.8	0.777	0.223	0.591
Feed intake (g/bird/d)	126	122	123	120	2.8	0.201	0.453	0.967
Feed conversion ratio	1.54	1.52	1.58	1.52	0.02	0.070	0.370	0.322

CF: birds were treated with 10^8 CFU/ml *Candida famata* (CF) in their drinking water two days a week throughout their life; LF: birds were treated with 10^{5}-10^8 CFU/ml *Lactobacillus fermentum* (LF) in their drinking water two days a week throughout their life; CFLF: birds were treated with a mixture of CF and LF (10^6-10^8 CFU/ml) in their drinking water two days a week throughout their life. CF*LF: the interaction between CF and LF.

Even as day-old chicks, there was a large population in the whole intestinal tract of both *E. coli* (log_{10} 5.65 ± 0.35 CFU/g) and lactic acid bacteria (log_{10} 6.87 ± 0.13 CFU/g), which was larger than the populations observed in the yolk sac (log_{10} 4.27 ± 0.13 and 4.49± 0.08 CFU/g for *E. coli* and lactic acid bacteria, respectively). No yeast was detected in the yolk sac but some chicks (6 out of 16) had a small population of yeasts in the gut (log_{10} 3.18 ± 0.42 CFU/g) (see Appendix 3).

The administration of *C. famata* and *L. fermentum*, either alone or in combination, had no effect on the population of coliforms or *Lactobacilli*. Administering *L. fermentum* did not affect the population of yeasts either, but the administration of *C. famata* did increase the population density of yeasts (from 3.42 to 4.18 log_{10} CFU/g, $P < 0.001$). As birds aged, there was a general decline in the population density of coliforms from 8.71 to 7.97 log_{10} CFU/g between day 8 and day 35 ($P < 0.001$, Figure 4.1). The population density of lactic acid bacteria decreased from 8.84 to 7.80 log_{10} CFU/g as birds got older ($P < 0.001$, Appendix 4). There was also a significant ($P < 0.001$) interaction between the age of the bird and the site of the gut where digesta were collected with respect to the population density of *Lactobacilli*. The number of LAB was the highest (10.07 log_{10} CFU/g) in the ileum on day 14 of bird age and then the LAB population declined (7.80 log_{10} CFU/g) on day 35 of bird age. The population density generally increased from the duodenum through the ileum and was greatest in the caecum, and this difference between the different sections of the intestine was more apparent as the birds got older (Figure 4.2). There were some interesting interactions between bird age, site (duodenum, ileum and caecum) and the administration of CF or LF on the population size of yeasts. On days 14 and 22, the population density of yeasts was greater in the ileum (4.82 and 4.85 log_{10} CFU/g, respectively) than in the duodenum (3.55 log_{10} CFU/g). However, by day 29,

the 3.37 $\log_{10}$ CFU/g of yeast population in the ileum decreased and yeast counting was lower in the ileum than in the duodenum (4.29 $\log_{10}$ CFU/g). By day 35, the population density of yeasts was lower in all three sites compared with the density observed in those same sites when the birds were younger ($P < 0.001$**,** Figure 4.3). Administering *C. famata* increased the population of yeasts when the birds were younger (days 8, 22 and 29) but by day 35 the population density of yeasts was lower and was not affected by the administration of *C. famata* ($P < 0.001$, Figure 4.4). There was also an interaction between the administration of *C. famata* and digesta site on the yeast population (P=0.023). Administering *C. famata* increased the population of yeast in the duodenum and ileum but not in the caecum (Figure 4.5). In addition, there was an interaction between the administration of CF and LF (P=0.033, Figure 4.6). Administration of either *C. famata* (4 $\log_{10}$ CFU/g) or *L. fermentum* (3.26 $\log_{10}$ CFU/g) had no effect on the yeast population compared with the control (3.59 $\log_{10}$ CFU/g). However, if *C. famata* and *L. fermentum* (4.36 $\log_{10}$ CFU/g) were administered together, the yeast population increased.

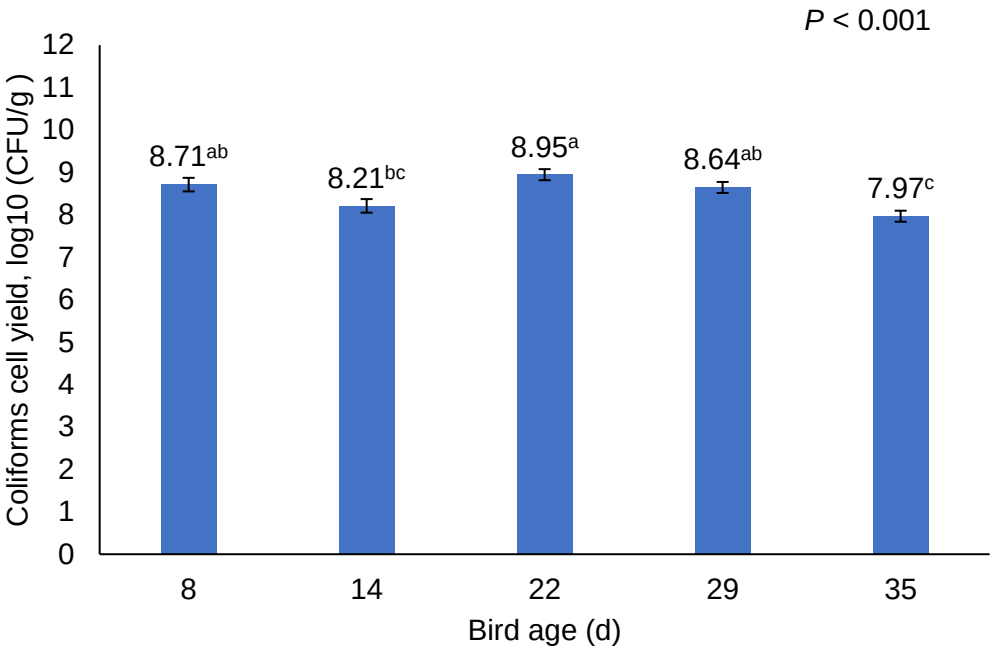


Figure 4.1 Effect of bird age on the population density of coliforms.

Values with different superscripts (a-c) are significantly different ($P < 0.05$).

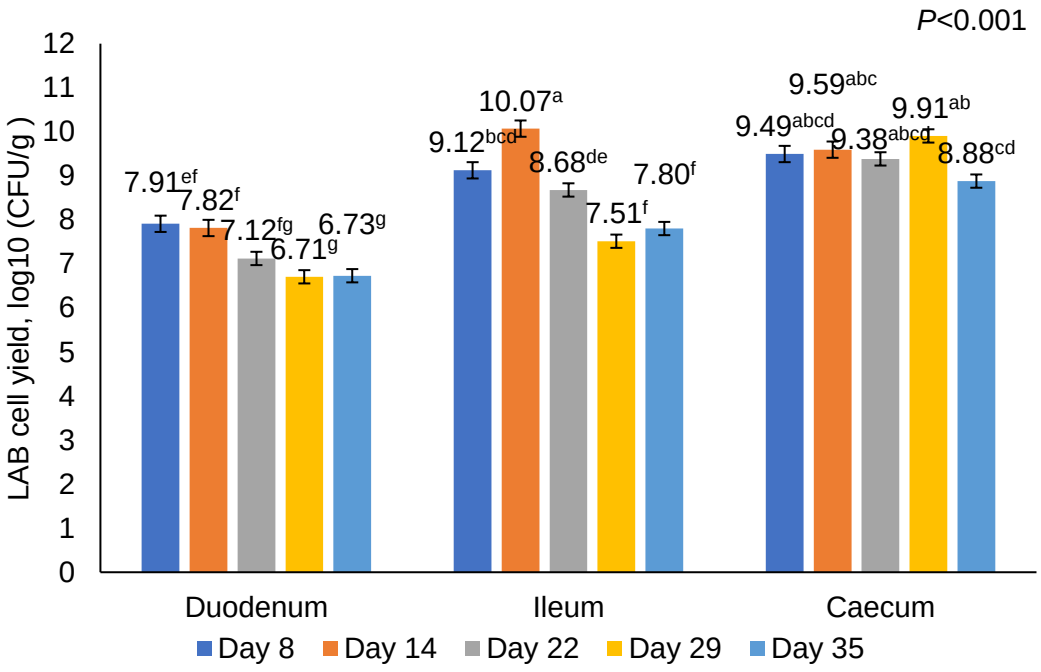


Figure 4.2 Effect of bird age and digesta site on the population density of Lactobacilli in broiler chickens.

Values with different superscripts (a-g) are significantly different ($P < 0.05$).

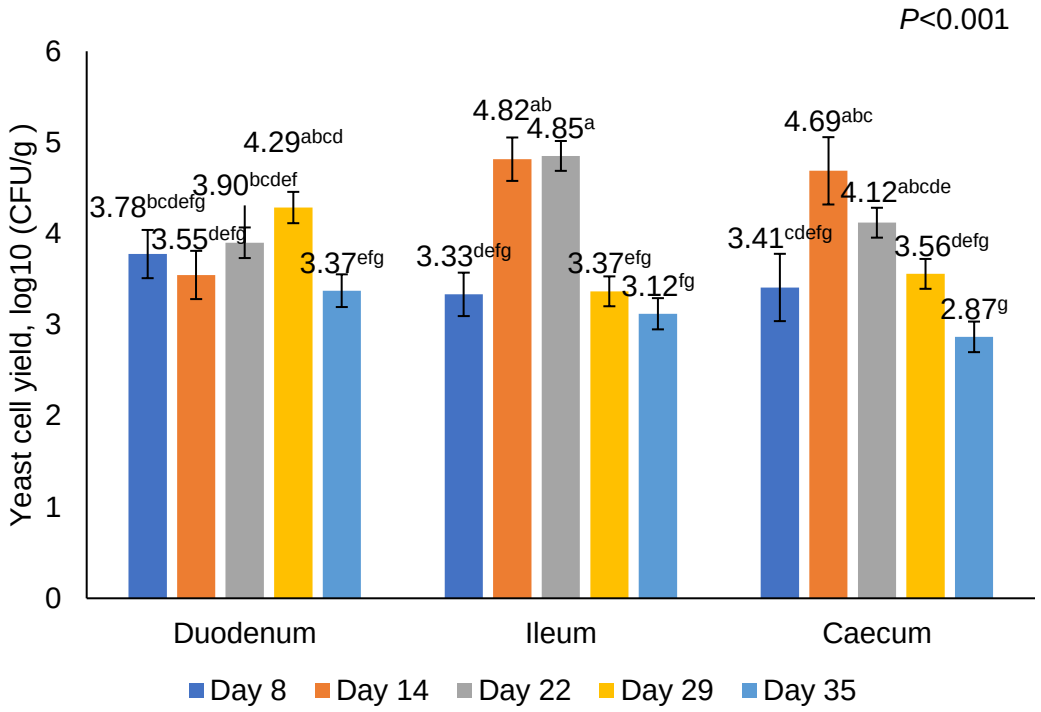


Figure 4.3 Effect of bird age and digesta site on the population density of yeasts.

Values with different superscripts (a-g) are significantly different ($P < 0.05$).

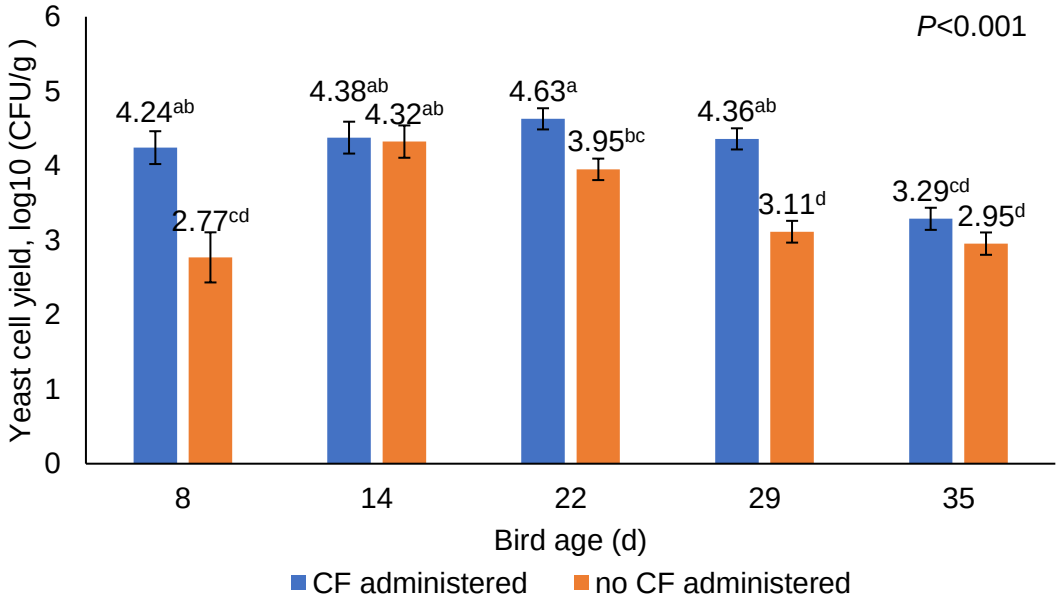


Figure 4.4 Effect of bird age and either of the group containing CF (CF and CFLF) or the group excluded CF (CON and LF) on the population density of yeasts.

CF: *Candida famata*; LF: *Lactobacillus fermentum*; CFLF: a mixture of CF and LF.

Values with different superscripts (a-d) are significantly different ($P < 0.05$).

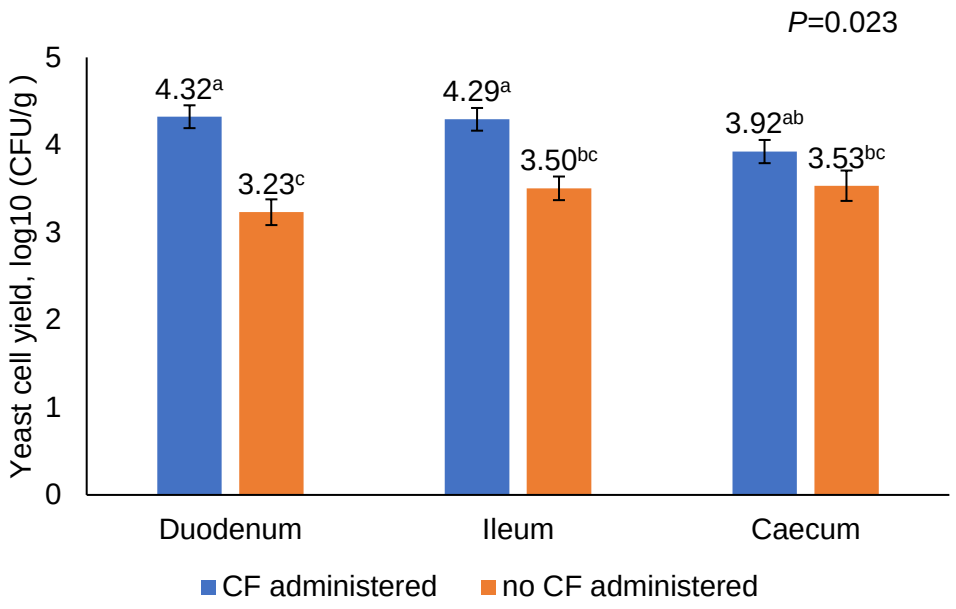


Figure 4.5 Effect of digesta site and either of the group containing CF (CF and CFLF) or the group excluded CF (CON and LF) on the population density of yeasts.

CF: *Candida famata*; LF: *Lactobacillus fermentum*; CFLF: a mixture of CF and LF.

Values with different superscripts (a-c) are significantly different ($P < 0.05$).

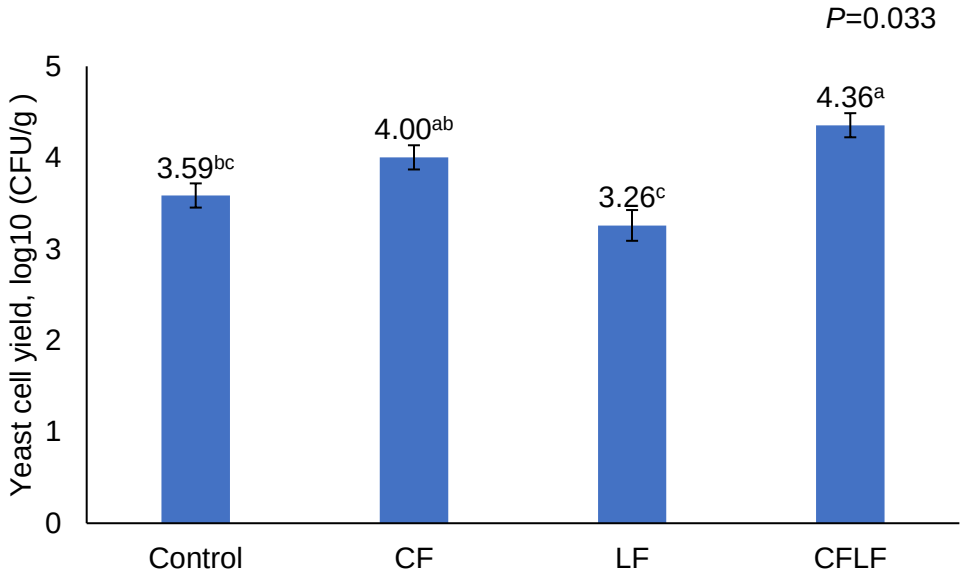


Figure 4.6 Effect of CF and LF treatment interaction on the population size of yeasts.

CF: *Candida famata*; LF: *Lactobacillus fermentum*; CFLF: a mixture of CF and LF.

Values with different superscripts (a-c) are significantly different ($P < 0.05$).

4.3.2 Antimicrobial resistance of *E. coli* from caecum

Figure 4.7 shows the proportion of caecal *E. coli* samples that were resistant to antimicrobials throughout the experimental period. Resistance to NA and CHL was low throughout the study. TET resistance was high (78.57%) at 8 d and then declined to 20.83% at 35 d (χ^2=30.03, $P < 0.001$) with no effect of treatment. AMP resistance was 100% at 1 and 8 d and then decreased to 37.5% at 35 d (χ^2=23.29, $P < 0.001$). The effect of the CF, LF and CFLF on the resistance of *E. coli* to ampicillin is shown in Figure 4.8. CF had a tendency (χ^2=6.21, P=0.102) to reduce it from 58% (CON), 65% (LF) and 52% (CFLF) to 32% (CF). As mentioned before, selecting a single *E. coli* isolate for the estimation of AMR test is a limitation in this study but does give some indication of the prevalence of AMR in the coliform population in these birds.

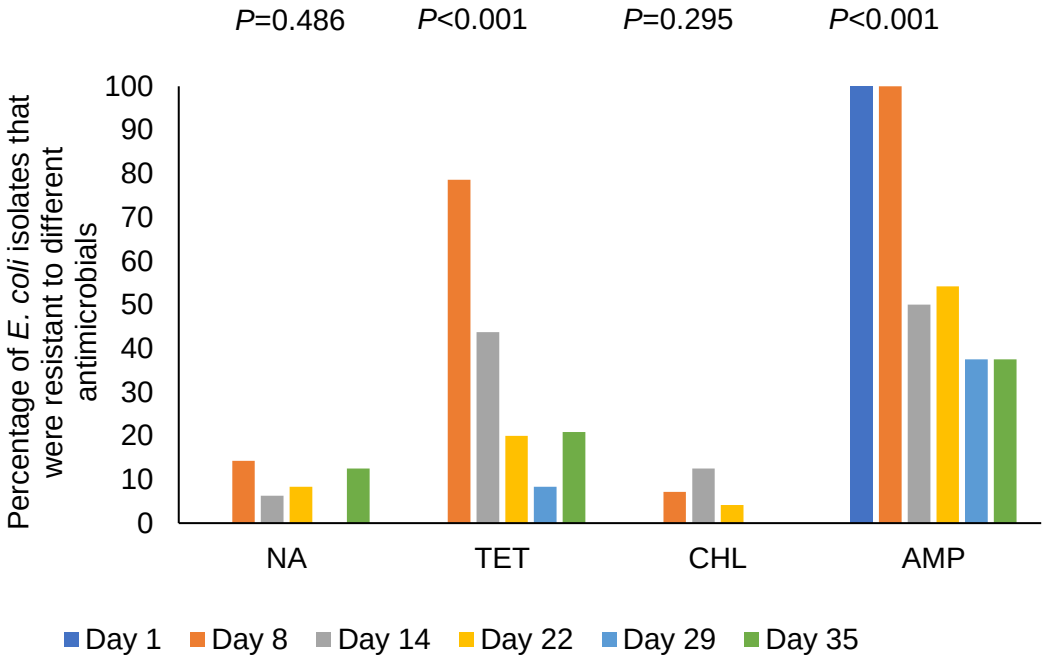


Figure 4.7 The percentage of *E. coli* isolates taken from birds of different ages (days of life) that were resistant to different antimicrobials.

NA: nalidixic acid; TET: tetracycline; CHL: chloramphenicol; AMP: ampicillin.

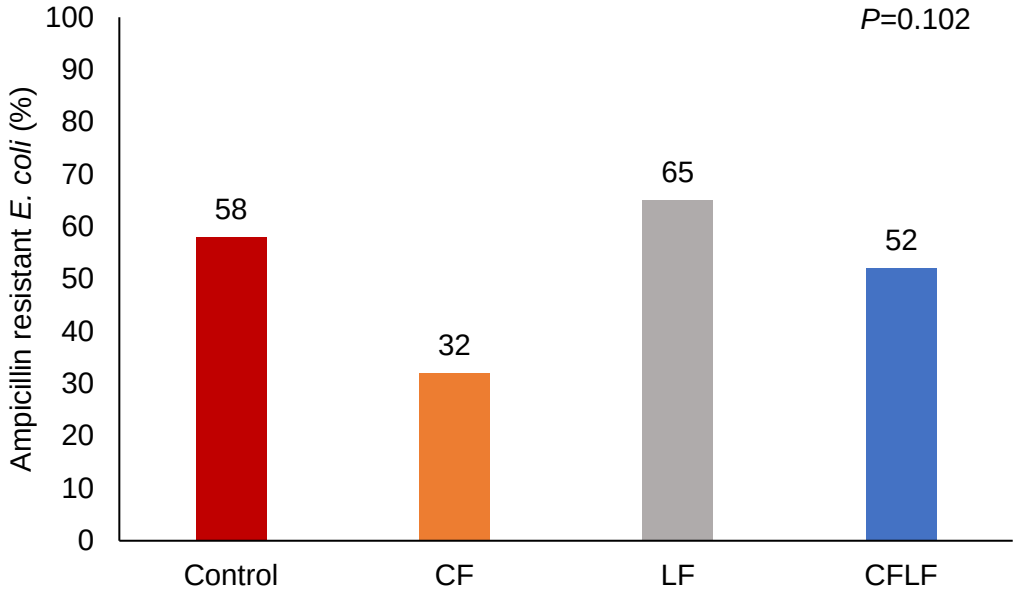


Figure 4.8 The effect of administration of either *C. famata* (CF), *L. fermentum* (LF) or both (CFLF) on the proportion of isolates of *E. coli* that were resistant to ampicillin (20 µg/ml).

4.3.3 The antibiotic resistant genes and identification of *E. coli* by whole genome sequencing (WGS)

The profile of ARG in *E. coli* isolates was significantly affected by bird age (Figure 4.9), with ARG (aminoglycoside, β-lactamase, trimethoprim, fosfomycin, sulphonamide, tetracycline) generally declining after 8 d of age. *β*-lactamases (primarily *bla*$_{TEM}$), were isolated from 80% of samples at 8 d, but 47% of samples at 35 d (χ^2=21.27, *P*=0.001). Tetracycline resistance genes (*tet*A, *tet*B and *tet*J) were present in 60% of isolates at 8 d and 23.5% of isolates at 35 d (χ^2=16.41, *P*=0.006). Nearly all samples isolated carried the multi drug transporter gene mdfA-1, and this was not affected by bird age or treatment. Administering CF or LF had no effect on the carriage of ARG in *E. coli* isolates. Figure 4.10 presents the multilocus sequence typing of *E. coli* by bird age. A total of 19 different multilocus sequence typing (MLST)

were identified, with a further 19/76 isolates being unknown. At 1 d old, most isolates were MLST 48, and this was the most common type (14/76) throughout the study and was isolated from birds throughout their life. Greatest diversity was observed when birds were 8-22 d old; when birds were 29 and 35 d old the most common isolates were MLST 48 (20% of isolates) and 1665 (25% of isolates) (*P*=0.001).

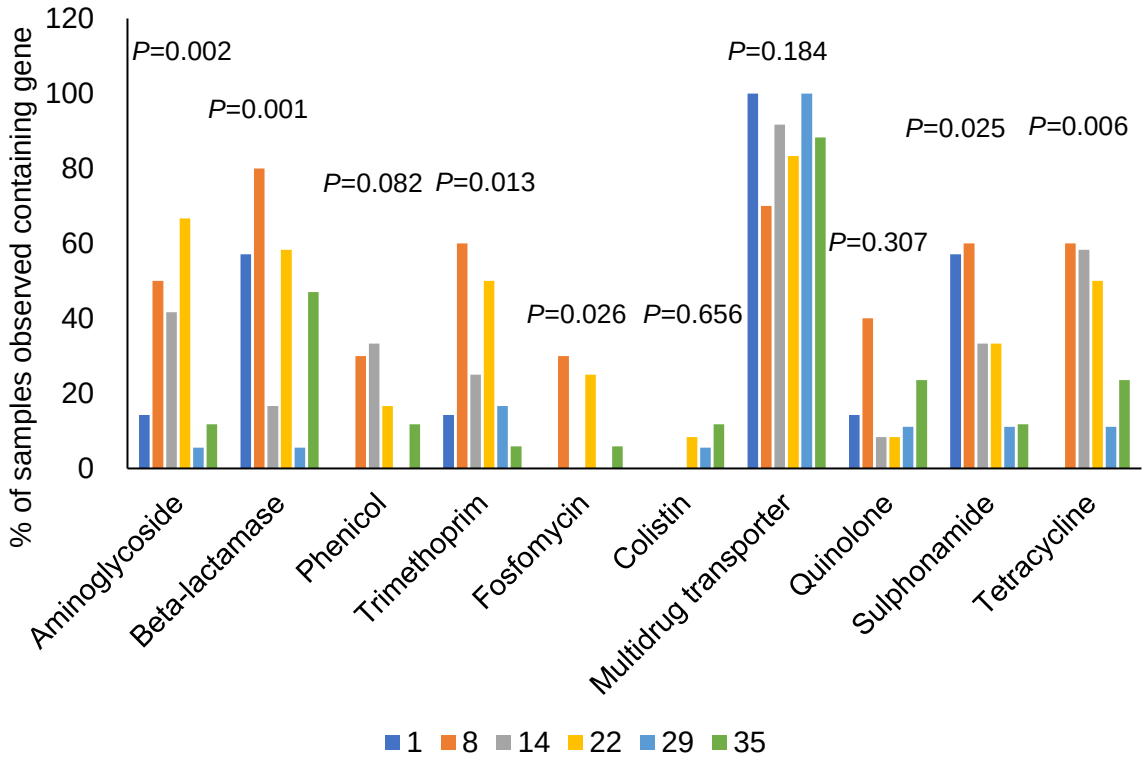


Figure 4.9 The effect of bird age on the carriage of different antibiotic resistance genes by isolates of *E. coli* taken from the caecum of broiler chickens.

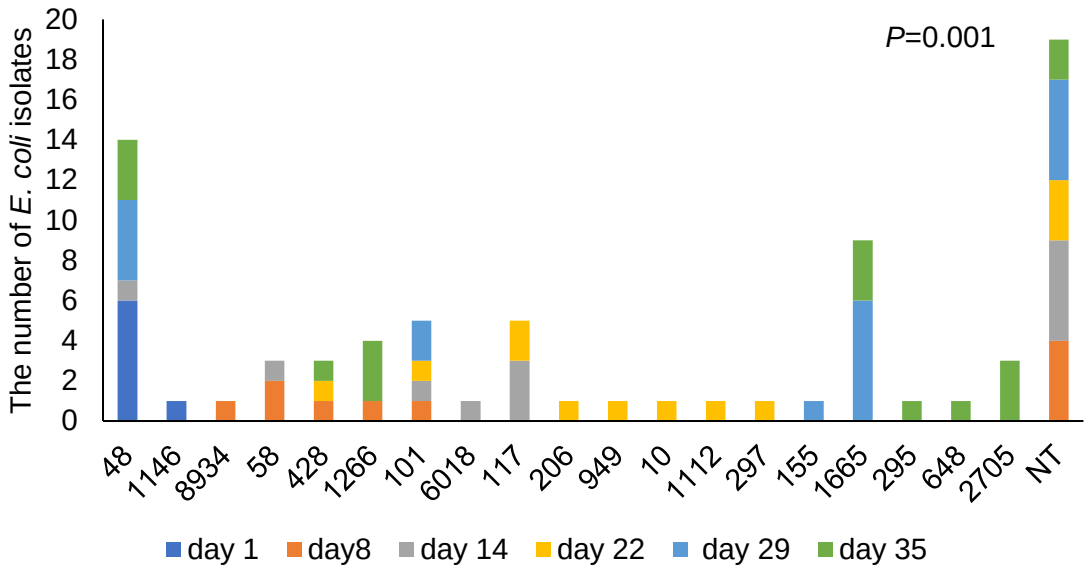


Figure 4.10 The effect of bird age (days of age) on the mutilocus sequence typing (MLST) identification of the *E. coli* isolate taken from the birds' caecum.
NT: MLST not identified.

4.3.4 *E. coli* virulence and its association with AMR

The proportion of virulence-associated genes in *E. coli* isolates detected by PCR and WGS analysis is presented in Figure 4.11. The proportion of isolates identified as carrying the different genes were: *iuc*D (26.19%), *tsh* (8.33%), *irp*2 (28.57%), *iss* (48.8%), *ast*A (8.33%) and *hly*A (35.52%) with no effect of treatment. None of the isolates carried the *pap*C by PCR. In the WGS analysis, there were many and various virulence genes identified in the *E. coli* isolates but the presence/absence of 11 virulence genes was determined, as these were the ones which have been most frequently identified with avian pathogenic *E. coli* (APEC). The percentage of isolates identified as carrying different genes were: *iuc*D (27.63%), tsh (1.32%), *irp*2 (38.16%), *ast*A (7.89%), *iut*A (26.32%) and *iro*N (50%) with no effect of treatment. None of the isolates carried the *pap*C, *iss*, *hly*A, *hly*F or *omp*T genes. The effect of bird age on the carriage of virulence-associated genes by *E. coli* isolates, by PCR

and WGS analysis, is shown in Figure 4.12. Isolates of *E. coli* taken from the youngest birds (days 1 and 8 of age) had a much higher incidence of *iuc*D (42.86% and 72.72%, χ^2=18.73, *P*=0.002), *irp*2 (0% and 81.81%, χ^2=24.12, *P* < 0.001), *iss* (71.43 and 90.9%, χ^2=17.83, *P*=0.003) and *hly*A (28.57% and 81.81%, χ^2=16, *P*=0.007) than in isolates taken from older birds by PCR analysis. Similarly, in WGS analysis, *iuc*D (60%, χ^2= 11.68, *P*=0.04), *irp*2 (70%, χ^2=19.38, *P*=0.002) and *iut*A (60%, χ^2=16.69, *P*=0.005) in *E. coli* isolates had a higher frequency on 8 day of bird age before declining as birds aged.

The association between the virulence genes and MLST is illustrated in Figure 4.13. MLST 48, 58, 428, 117 and NT in *E. coli* isolates had more *iuc*D, *irp*2, *iut*A and *iro*N rather than the other MLST. There was a tendency (χ^2=4.34, *P*=0.114) for birds which had been administered with LF (19.23%) to have *E. coli* with a lower incidence of the *hly*A gene compared to control (42.31%) and CF (44%) based on PCR analysis (Figure 4.14), but generally, administering CF or LF had no effect on the carriage of virulence associated genes in *E. coli* isolates. There was a significant association between phenotypic resistance by *E. coli* to tetracycline and the carriage of the genes *iuc*D (52%, χ^2=12.27, *P* < 0.001) and *irp*2 (52%, χ^2=9.573, *P*=0.002), and *iss* gene (68%, χ^2=5.25, *P*=0.022), and *tsh* (20%, χ^2=6.34, *P*=0.012) by PCR (Figure 4.15). The iron acquisition genes (*iuc*D, *irp*2 and *iut*A) were mainly associated with resistance to antibiotics classes in the WGS analysis (Table 4.3).

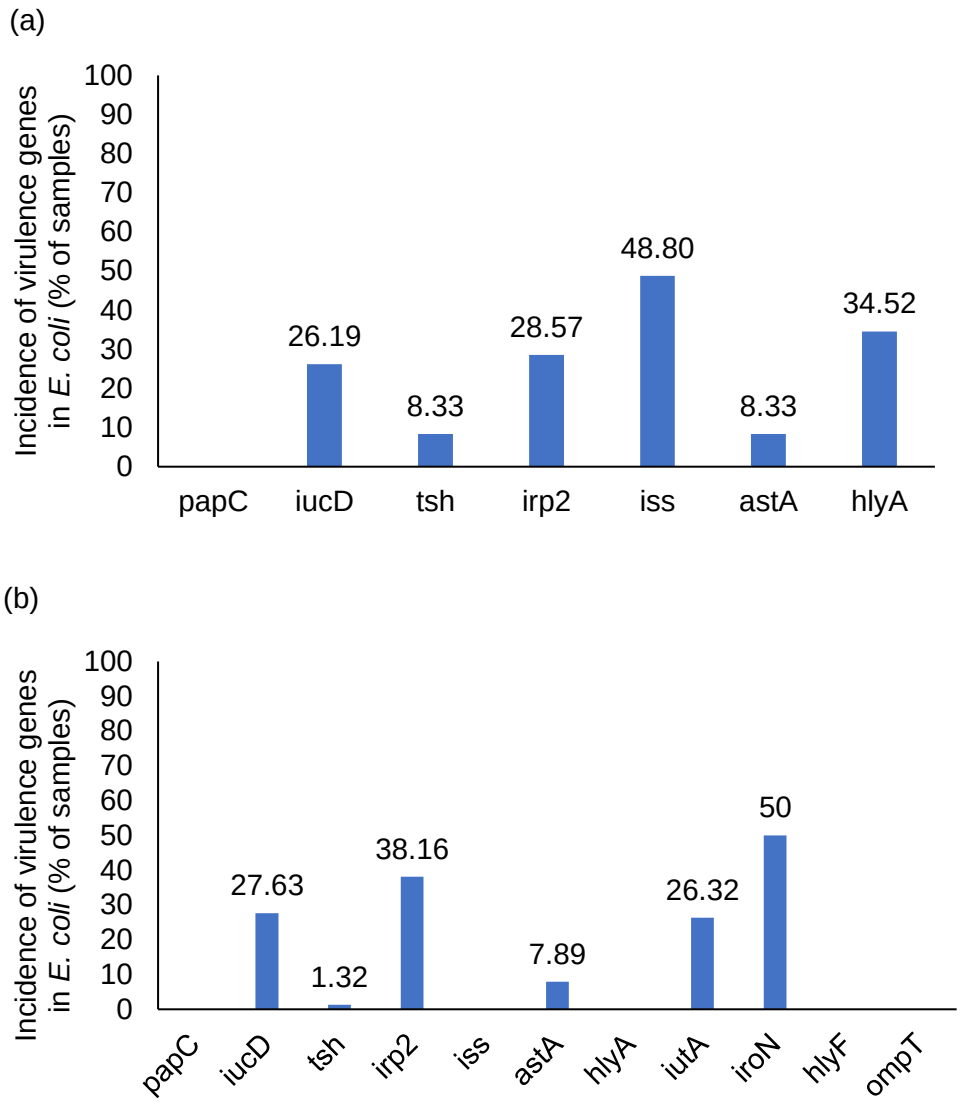
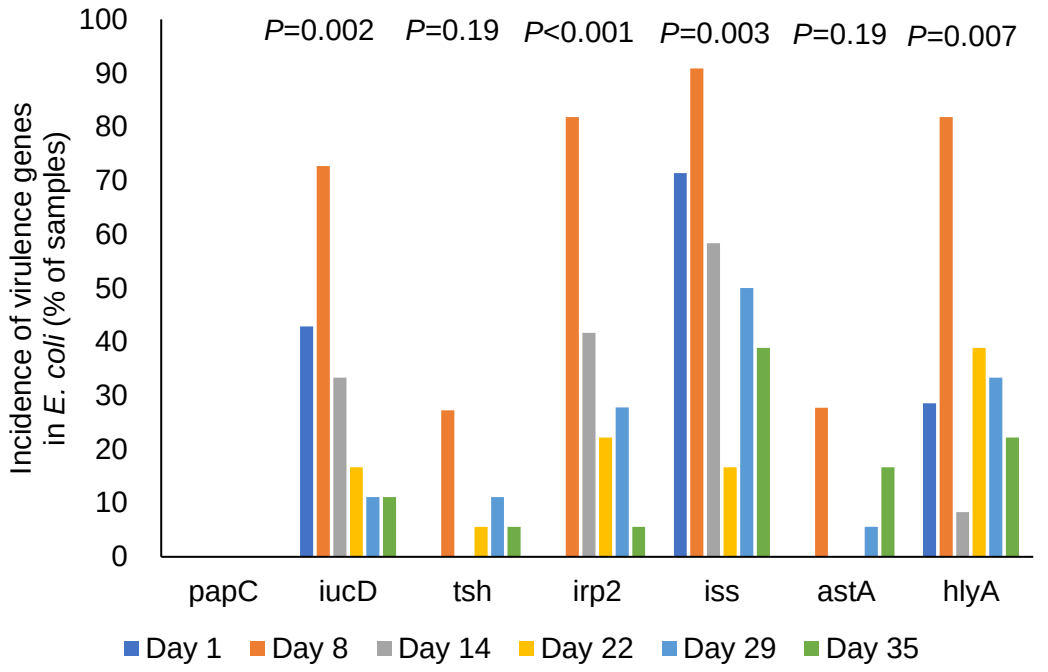


Figure 4.11 The proportion of virulence-associated genes in isolates of *E. coli* taken from the caecal digesta of birds analysed by (a) PCR and (b) WGS.

(a)



(b)

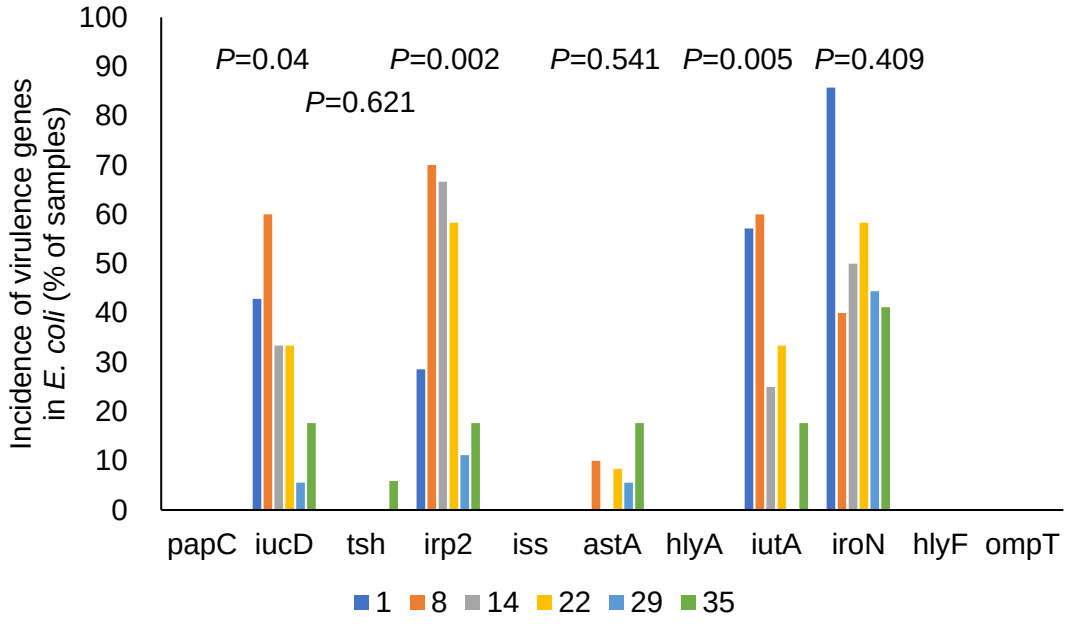


Figure 4.12 The effect of bird age on the carriage of virulence-associated genes in isolates of *E. coli* by (a) PCR and (b) WGS.

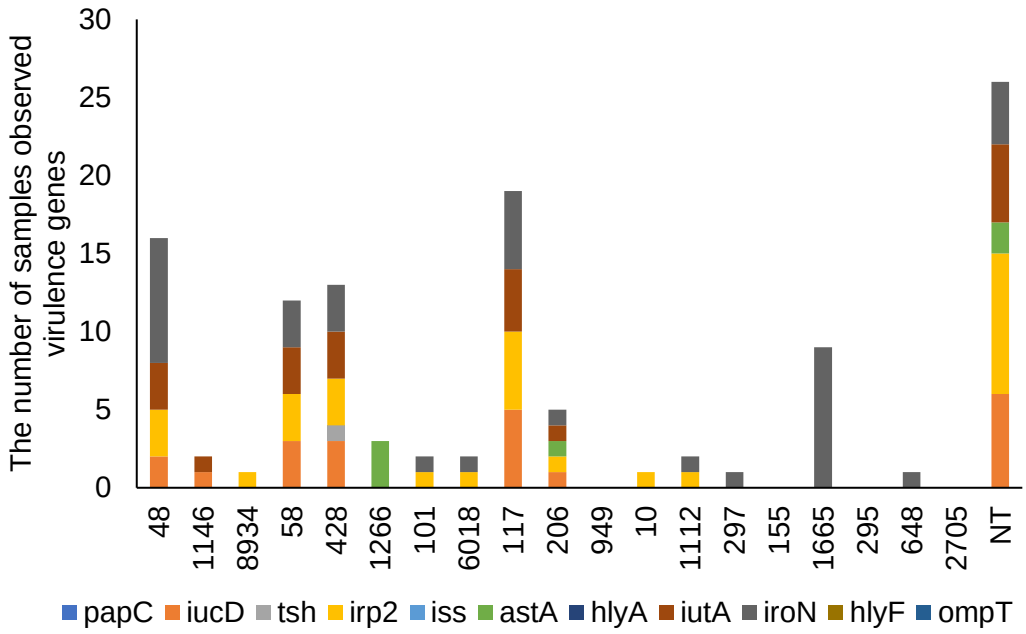


Figure 4.13 The association between MLST and virulence genes in *E. coli* isolates.

NT: MLST not identified.

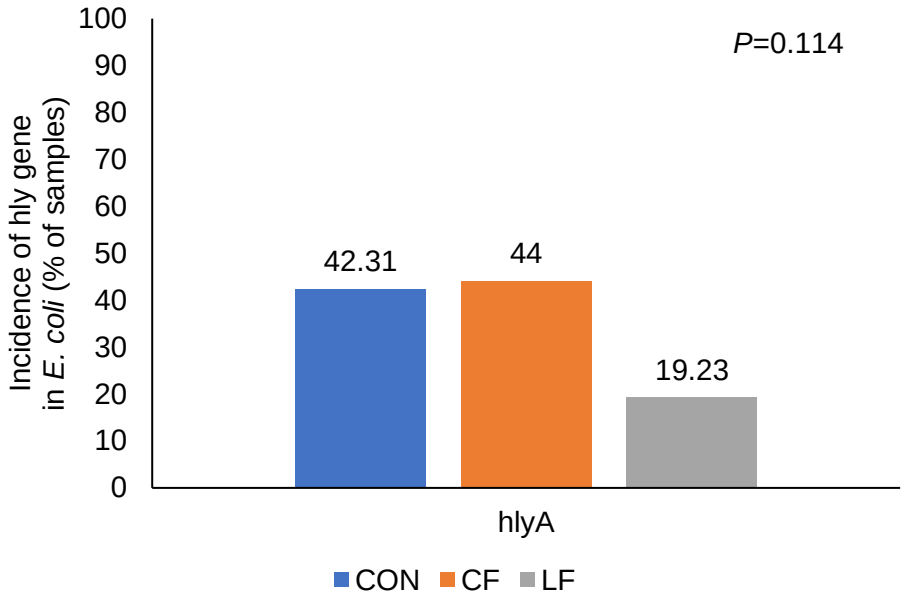


Figure 4.14 The effect of administering either *Candida famata* (CF) or *Lactobacillus fermentum* (LF) to broiler chickens on the carriage of the *hlyA* gene in isolates of *E. coli* taken from the bird's caecum.

CON: control; CF: *Candida famata*; LF: *Lactobacillus fermentum*.

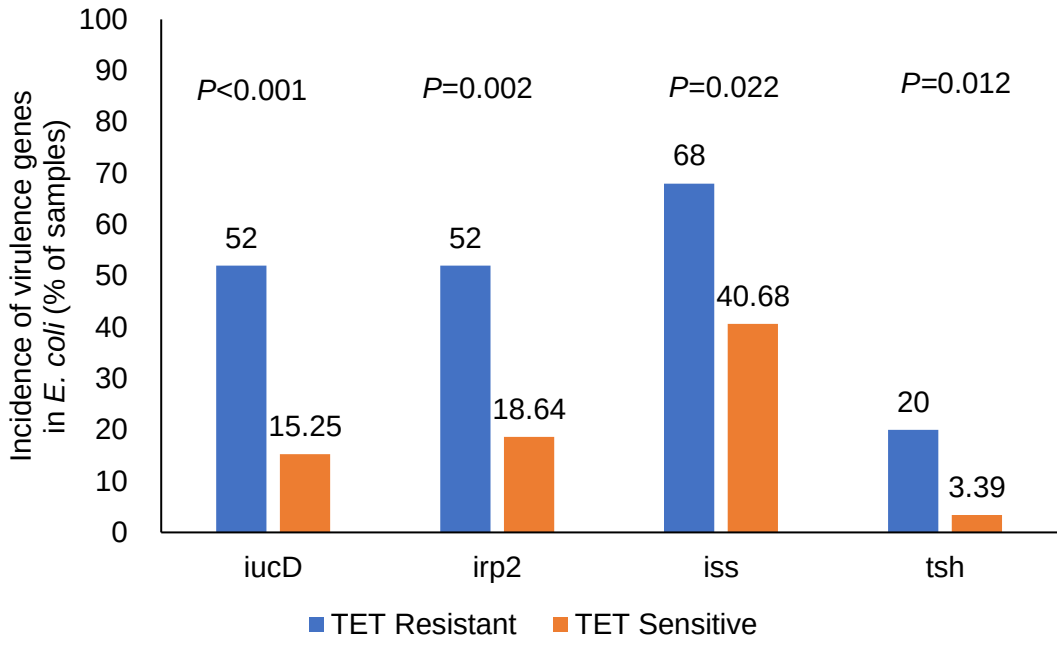


Figure 4.15 The association between the carriage of the *iuc*D, *irp*2, *iss* and *tsh* genes and the phenotypic sensitivity or resistance by isolates of *E. coli* to tetracycline (20 µg/ml).
TET: tetracycline.

Table 4.3 The association (*P*-values) by Chi-square analysis between the carriage of antimicrobial resistant genes and virulence associated genes as determined by WGS.

	*ast*A	*iuc*D	*irp*2	*iut*A	*iro*N
Aminoglycoside	0.489	<0.001	<0.001	<0.001	0.129
β-lactamase	0.022	0.003	0.218	0.001	0.348
Phenicol	0.294	<0.001	<0.001	0.002	0.328
Trimethoprim	0.684	0.001	0.001	0.001	0.118
Fosfomycin	0.033	0.007	<0.001	0.004	0.692
Colistin	0.192	0.03	0.009	0.269	0.304
Quinolone	0.976	<0.001	<0.001	<0.001	0.361
Sulphonamide	0.805	<0.001	0.004	<0.001	0.043
Tetracycline	0.981	0.001	<0.001	0.003	0.087

Significant associations (*P*<0.05) are highlighted in red.

4.4 Discussion

4.4.1 Growth performance and microbial population density

Unfortunately the starch and oil composition of samples analysed by Sciantec may have resulted in erroneous results. It was requested that the oil content be determined by acid hydrolysed ether extract, but ether extract was used instead. The starch data may have also been incorrect, due to dietary samples possibly not being ball milled prior to analysis. The calculated ME values were also too low. Therefore the data for dietary composition cannot be relied upon.

If beneficial microorganisms are to have any effect at all on the health and performance of the host animal, then they must colonise and proliferate within the gut (Angelakis *et al.*, 2013; Peng *et al.*, 2016). The absence of any effect of *L. fermentum* administration on the growth performance and the population density of coliforms and *Lactobacilli* in the gut would suggest that colonisation by *L. fermentum* had been unsuccessful. There was an increased yeast population in response to the administration of *C. famata*, and it was notable that this occurred mostly in the small intestine. However, as it was the small intestine in which the yeasts proliferated it is perhaps unsurprising that there was no associated effect on the caecal population of *E. coli.* The impact of *C. famata* on bird performance was also limited, with only a tendency (P=0.07) for feed conversion ratio to be improved. The optimal dose and route of administration therefore need further consideration to enable these species to colonise the gut and effect any changes in the microbiome.

One criticism of this study may be that the CF, LF and CFLF were administered intermittently in the water rather than continuously the feed. However, other studies (with admittedly different yeast and bacterial species) have demonstrated that

administering yeast and bacteria in water improved performance in broiler chickens. Karimi Torshizi *et al.* (2010) observed that the administration of nine different microbes (*Aspergillus oryzae*, *Lactobacillus acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. bulgaricus*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus thermophilus* and *Candida pintolopesii*) in water resulted in improved performance (in terms of bodyweight gain, feed intake and feed conversion ratio) compared with no microbes or administering them in the feed. Eckert *et al.* (2010) showed that intermittent *Lactobacillus* sp. treatment via drinking water significantly improved FCR and increased bodyweight compared with administration in feed or continuously in drinking water. A possible explanation for the beneficial effect of intermittent administration may be a consequence of the immunostimulatory properties of lactic acid bacteria. Administering yeast or lactic acid bacteria continuously would result in a sustained high state of immune stimulation and the energy for activation of the immune system might partition nutrients away from bird growth. Administering yeast or bacteria in the water intermittently may therefore be the optimum means of delivery, but in this study the dose (certainly of the *L. fermentum*) was insufficient to produce an observable effect on bird performance.

4.4.2 Phenotypic antimicrobial resistance (AMR) of *E. coli*

In this study, bacteria were isolated in samples of feed and in the excreta of day-old chicks. No bacteria were cultured from the sample of bedding (wood shavings). Coliforms isolated from the excreta (but not the feed) were resistant to ampicillin. This suggested that the coliforms colonising day-old chicks carried ampicillin resistance even though no antibiotic had been administered to the birds. Some other

studies have also observed antibiotic resistant *E. coli* isolated from poultry products and farm environments (Woolhouse *et al.*, 2015; Davis *et al.*, 2018; Vidovic and Vidovic, 2020).

There was a high degree of resistance by *E. coli* to tetracycline at 8 d and to ampicillin at both 1 and 8 d even though the birds were not exposed to antibiotics at any time in their life. A limitation of this study is that all birds assigned to a particular treatment were housed in a single pen until day 14, and so the subsequent replication that was achieved by placing them in replicate pens at day 14 could be considered pseudo-replication. In the subsequent study, birds were therefore allocated to one of 24 pens from day 1 of age. AMP and TET resistance then decreased as the birds aged. This is comparable to the finding of Roth *et al.* (2017) who also observed *E. coli* in faecal contents of day-old chicks were highly resistant to ampicillin, ciprofloxacin, streptomycin, sulfamethoxazole and tetracycline but that the prevalence of AMR *E. coli* by d 17 and 38 decreased (although only to ciprofloxacin, streptomycin and sulfamethoxazole). A high prevalence of AMR in day-old chicks was also observed by da Costa *et al.* (2011), who reported resistance to ampicillin, cefotaxime, tetracycline, streptomycin, gentamicin and enrofloxacin by *E. coli* isolates from cloacal samples from day-old chicks. The source of this high prevalence of AMR is unclear; broilers are vulnerable to environmental contamination because of the intensive systems in which they are reared (Wales and Davies, 2020), but *E. coli* (both commensal and pathogenic, potentially carrying antibiotic resistance genes) may transfer from breeding birds to chicks via the eggshell or egg yolk during oviposition (Rezaei Far *et al.*, 2013; Wales and Davies, 2020).

However, in the absence of any exposure to antibiotics, it is unclear why the prevalence of resistance then increased still further in the first week of life (although it did then decrease after that). Clearly there must be some other selective advantage for *E. coli* carrying antibiotic resistance genes when birds were young. In this study, antimicrobial resistance decreased after 8 d, and it appeared that resistant (more virulent) *E. coli* were replaced by robust, commensal *E. coli* which were susceptible to antibiotics.

The results of the present study negate the hypothesis that administering CF and LF, alone or in combination, might reduce the prevalence of AMR *E. coli*. However, the findings of this study suggest that the administration of CF tended to reduce the ampicillin resistance of *E. coli*. This finding is consistent with that of Roth *et al.* (2017), who added an organic acid additive to birds' feed and observed that this was associated with a reduced prevalence of ampicillin and tetracycline resistant *E. coli*. The production by bacteria of organic acids and other metabolites (or the addition of these compounds in the diet) may inhibit the growth of coliforms, particularly those carrying antibiotic resistant genes (Edens, 2003; Alagawany *et al.*, 2018). Further research is clearly needed to investigate the appropriate selection of beneficial bacteria to reduce the prevalence of resistant *E. coli* in broiler chickens.

4.4.3 Genotypic antimicrobial resistance and multilocus sequence typing (MLST) of *E. coli* by WGS

E. coli with a high prevalence of multidrug-resistant genes has been observed before, and associated with the carriage of these resistance genes on plasmids (Thorsteinsdottir *et al.*, 2010). Rafique *et al.* (2020) reported that *E. coli* isolated

from chickens was multidrug resistant (89.1%) with resistance to lincomycin (100%), streptomycin (81.5%), ampicillin (79.3%), ciprofloxacin (66.3%) colistin and quinolone (14.1%). Guo *et al.* (2019) identified genotypes (by WGS) with antimicrobial resistance in *E. coli* isolates from ready to eat food in Singapore. *E. coli* isolates were observed to carry the resistance genes to aminoglycoside (62.5%), β-Lactam (41.7%), quinolone/fluoroquinolone (45.8%), sulphonamide (45.8%), trimethoprim (45.8%) and tetracycline (70.8%). In this study, resistance genes to a range of antibiotic classes (aminoglycoside, β-lactams, trimethoprim, fosfomycin, sulphonamide and tetracycline) in caecal *E. coli* isolates were detected in young chicks and nearly all *E. coli* isolates (taken throughout the birds' life) carried the multidrug transporter (mdfA-1). However, the decline in phenotypic AMP and TET resistance as the birds aged was also observed in the genotypic characterisation. Interestingly, MLST of *E. coli* isolates which carried AMR genes were replaced by novel MLST isolates with few AMR genes as birds got older. The results of this research support the hypothesis, and this suggests that there was an evolution of the *E. coli* population, with commensal *E. coli* (which are susceptible to antibiotics) succeeding the more virulent (and resistant) strains that were dominant in the starter phase.

The characterisation and identity of *E. coli* isolates can be detected by whole genome sequencing (WGS). At 1 d old, most isolates were ST 48, followed by ST1146 and ST4618. There was wide diversity of *E. coli* strains between day 8 and day 22. When birds were 29 and 35 d old, the most common isolates were ST 48 (20% of isolates) and 1665 (26% of isolates). It seems that the great diversity and emergence of new (more commensal) *E. coli* strains emerged as the birds got older. ST 48 was found in the caecum of both day-old chicks and older birds, but the

serotype was different between chicks and older birds. It suggests that ST 48 might be well adapted to the chicken caecum, but that it is also subject to evolution. Strains of the same sequence type can have different capsular serotypes and properties (Silva *et al.*, 2006).

Solà-Ginés *et al.* (2015) reported APEC MLST included ST168, ST350, ST156 and ST23. Among avian faecal *E. coli* (AFEC) serotypes, ST10 was commonly observed followed by ST165. Gregersen *et al.* (2010) observed that nine different STs in *E. coli* isolates were identified from birds vaccinated against colibacillosis, while 17 different STs of *E. coli* isolates were identified in control birds. Vaccinated birds had a lower diversity compared with control group. ST 117, ST131 and ST428 were most prevalent, accounting for 50% of the isolates. ST117 led to salpingitis, peritonitis and septicaemia and ST428 was associated with salpingitis and peritonitis in birds. According to the previous study of the *E. coli* MLST database, nine STs (ST23, ST48**,** ST88, ST106, ST117, ST131, ST428, ST773 and ST847) have been correlated with human urinary tract infection (UTI). Ewers *et al.* (2009) showed that ST 95 isolated from chicken faeces was associated with septicaemia. Another ST 69 could cause human UTI. In this present study, various *E. coli* strains were observed, although no disease or death was observed in any of the birds. The prevalence and potential virulence of isolates was not associated with a particular MLST or serotype, and the pathogenic serotypes identified above were not detected amongst these isolates. None of the serotypes were identified as being of the APEC serogroups O1, O2 or O78, but the isolates identified in younger birds were clearly more potentially pathogenic than those identified in older birds. More benign MLST (48, 1665, 155, 295, 2705 and 1266) isolates were observed on 29 and 35 days of bird age. Further study is needed to confirm these findings, and then develop

strategies to support the establishment and proliferation of the more benign and commensal MLST in young birds. The study and investigation of association between serotype and characteristics of *E. coli* can contribute to surveillance studies in bird (and public) health and can prevent the proliferation of AMR bacteria.

4.4.4 *E. coli* virulence and its association with AMR

The carriage of seven virulence genes (*pap*C, *iuc*D, *tsh*, *irp*2, *iss*, *ast*A and *hly*A) were used in this study to differentiate between commensal and pathogenic *E. coli* in poultry by PCR. The *iss* gene was the most prevalent of the genes investigated, and *iuc*D, *irp*2, *iss* and *hly*A were also very prevalent in birds that were 8 d old before declining as the birds got older. Johnson *et al.* (2008) reported that five virulence genes (*iut*A, *hly*F, *iss*, *iro*N, and *omp*T) were associated with APEC isolates. In the WGS analysis, there were a lot of virulence genes identified in *E. coli* isolates and eleven virulence-associated genes (VAG) were selected and determined. They included the seven VAG evaluated by PCR and the minimal APEC predictors (*iut*A, *hly*F, *iro*N, and *omp*T) identified by (Johnson *et al.*, 2008). The *iro*N gene was the most prevalent followed by *iuc*D, *irp*2 and *iut*A in WGS. VAG results by both PCR and WGS showed a high prevalence of iron uptake related genes in *E. coli* isolates. However, *iss* and *hly*A genes were prevalent in isolates analysed by PCR but were not detected by WGS. The reason for this discrepancy between PCR and WGS result is unknown but may be an indication that these genes were carried on plasmids that were not extracted during the preparation of the samples for WGS. In both PCR and WGS analysis, young chicks were more susceptible than older birds to colonisation with putative pathogenic *E. coli,* but the VAG in *E. coli* isolates decreased after 8 day of bird age. This suggests that commensal and robust *E. coli*

competed with putative pathogenic *E. coli* and the latter were predominantly replaced as birds got older. This suggests that interventions should focus on trying to manipulate the microbial community of the young chick to maintain host health in the vulnerable starter period. There was little if any evidence that the administration of CF and LF, alone or in combination, affected the virulence of *E. coli* and so the hypothesis was largely rejected, although there was the suggestion that administering LF might be associated with a reduction in the carriage of the *hly*A gene in *E. coli* isolates. This may be worth further investigation.

In recent years there has been considerable interest in the detection of virulence genes in commensal and pathogenic *E. coli* isolates in poultry (Zhao *et al.*, 2009; Karami *et al.*, 2017). This study showed that MLST 48, 58, 428, 117 and NT in *E. coli* isolates had more iron uptake related genes (*iuc*D, *irp*2, *iut*A and *iro*N) than other serotypes identified. Janßen *et al.* (2001) noted that seven out of 17 virulence genes investigated were prevalent in isolates from birds that died of colibacillosis. The highest frequency of virulence genes was *fim*C (92.7%) followed by *iuc*D (88.7%), *tsh* (85.3%), *irp*2 (68%), *fyu*A (66%), *pap*C (30%) and *ast*A (17.3%). Associations between virulence and antimicrobial resistance have been observed before: As discussed in Chapter 3, APEC had a higher proportion of plasmid mediated multidrug resistance than their avian commensal counterparts (Johnson *et al.*, 2012). They also observed that certain virulence genes, including *ire*A, *ibe*A, *fyu*A, *cva*C, *iss*, *iut*A, *iha*, and *afa* were positively correlated with multidrug resistance (Johnson *et al.*, 2012). Szmolka *et al.* (2012) also reported that *tet*A (coding for tetracycline resistance) was highly correlated with the virulence genes *iro*N and *iss* in both commensal and avian pathogenic *E. coli*. In this study, phenotypic resistance to TET was associated with the carriage of the *iuc*D, *irp*2, *iss*

and *tsh* gene. In WGS analysis, the iron uptake related genes were associated with a range of antimicrobial resistance genes. This may provide a means of controlling the maintenance and spread of AMR *E. coli.* Withholding the use of antibiotics may not of itself be sufficient to control this spread since the prevalence of AMR *E. coli* in a poultry production setting is so widespread even when no antibiotics are administered. Altering the iron availability (in the gut) for *E. coli* may serve as a means of altering the selective advantage that APEC currently has, particularly in the starter period of the bird's life, encouraging instead the proliferation of commensal *E. coli* (with a lower resistance to antimicrobials).

4.5 Conclusion

This study shows that avian *E. coli* have a high prevalence of ampicillin and tetracycline resistance, as well as virulence associated genes in the young bird (day 8 of age). This did decline as the birds got older, and commensal strains became dominant. This result indicates that there must be some other selective advantage for *E. coli* carrying antibiotic resistance and virulence genes in young birds, as no antibiotics were administered to these birds. A variety of ARG were prevalent in young birds and most *E. coli* carried multi-drug transporter genes. In addition, diverse MLST *E. coli* strains were identified, but they seemed to be replaced by commensal types (which were susceptible to antibiotics) in older birds. Furthermore, the carriage of *iuc*D, *irp*2, *iss* and *tsh* genes was associated with tetracycline resistant *E. coli*. Antibiotic resistant genes were therefore associated with (among other things) iron uptake related genes. This finding suggests there might be a positive relationship between mechanisms for iron accumulation by *E. coli* and the resistance to a range of antimicrobials. This could offer a means of controlling the

spread of AMR, by encouraging the proliferation of isolates that do not rely on this iron uptake system.

The administration of beneficial bacteria requires those bacteria to colonise and proliferate in the gut of the host bird, and exert some influence on the growth of pathogens. Therefore, in the next chapter, LAB strains isolated from the caecum of this bird study were evaluated for their *in vitro* biological activity and production of antimicrobial compounds. These data could then be used to select potential beneficial bacteria for evaluation in future *in vivo* studies.

Chapter 5. *In vitro* identification and antimicrobial activity of lactic acid bacteria isolated from the broiler caecum

5.1. Introduction

Many researchers have evaluated the potential of indigenous bacteria from poultry (Nazef *et al.*, 2008; Musikasang *et al.*, 2009; Rajoka *et al.*, 2018). Lactic acid bacteria (LAB) have been commonly used to animals and LAB have been well known to produce various useful secondary metabolites which have been demonstrated *in vitro* to have antibacterial activity, and *in vivo* have improved animal productivity and health (Salomskiene *et al.*, 2019; Jha *et al.*, 2020).

There have been considerable studies on antibacterial activity of useful bacteria against pathogen. Salehizadeh *et al.* (2020) isolated bacteria (S08, S01, and S06) from broiler faeces and the isolates were identified as *Lactobacillus salivarius*, *Lactobacillus johnsonii*, and *Pediococcus acidilactici* by 16S rDNA gene sequences. The strains showed excellent adhesion to epithelial cells in the gut and antibacterial activity against *Salmonella typhimurium*. Oyewole *et al.* (2018) isolated and identified *Enterococcus faecium*, *E. faecalis*, *E. durans*, *Lactobacillus acidophilus* and *L. fermentum* from gut digesta in broilers. The isolates were tolerant to acidic pH and bile salts and inhibited the growth of pathogenic bacteria such as *E. coli*, *Salmonella typhimurium*, *Salmonella arizonae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *E. coli* O157:H7 and *Staphylococcus aureus*. Similarly, Rajoka *et al.* (2018) observed that strains of LAB (*Lactobacillus reuteri*, *Lactobacillus vaginalis*, *Lactobacillus johnsonii* and *Lactobacillus fermentum*) isolated from caecum contents were able to readily adhere to human colorectal adenocarcinoma cell lines

(Caco-2) and the isolates prevented the growth of potential pathogens (*Staphylococcus petrasii* subsp. Pragensis KY196531, *Salmonella enteric* sv. *typhimurium* CMCC (B) 50115, and *E. coli* ATCC 25922). Many researchers have reported the antibacterial activity of LAB against pathogenic bacteria, but there are few studies investigating the effect of LAB on the growth of AMR bacteria. This study attempts to evaluate the *in vitro* effect of LAB on the growth of *E. coli* (with and without the carriage of ARG and VAG) isolated from the chicken caecum. If a differential effect on *E coli*, depending on their virulence and AMR status, were to be demonstrated, then these LAB might be a means of preventing the proliferation of AMR bacteria.

Furthermore, little is known about the concentration and quantity of antimicrobial and beneficial compounds produced by bacteria isolated from the chicken gut. The compounds produced by LAB can be identified by analysing the supernatant of LAB cultures by Nuclear Magnetic Resonance (NMR) spectroscopy. NMR spectroscopy is a versatile technique that can be used to identify and quantify metabolites in samples (Ellinger *et al.*, 2013). It would be useful to identify and evaluate the association between antimicrobial activity for putative pathogenic, AMR *E. coli* and the concentration of compounds that are produced by different LAB strains. It is hypothesised that LAB isolated from the chicken gut might produce useful secondary metabolites (in addition to lactic acid) which might prevent the growth of AMR and putative pathogenic *E. coli*. The aim of this experiment was therefore to characterise LAB strains isolated from the broiler caecum in terms of their antibacterial activity against pathogenic and AMR *E. coli* (in both the cell free supernatant and neutralised supernatant of cultured LAB) and identify what antimicrobial compounds were produced by the different LAB isolates.

5.2 Material and Methods

5.2.1 Identification of lactic acid bacteria by analytical profile index (API)

Lactic acid bacteria (LAB) isolated from the caecal samples collected in the previous study (Chapter 4) were stored in microcentrifuge tubes with 15% v/v glycerol and MRS broth at -80°C pending identification and characterization of *in vitro* antimicrobial activity. 29 LAB isolates were selected for differentiation within the genus *Lactobacillus* and related genera by using the analytical profile index (API) 50 CHL test kit (bioMérieux, UK Ltd, Basingstoke, UK). The 29 LAB included five LAB isolates collected from chicks on day 1, a single isolate from each treatment (CON, CF, LF) from chicks on days 8 and 14, and two isolates for each treatment (from separate birds), when birds were 22, 29 and 35 days of age. The API 50 CHL test is based on a series of biochemical tests and can be used to identify lactic acid producing species based on their response to 49 different carbohydrates. The procedure for LAB identification was according to the kit manufacturer's instructions, with identification based on the biochemical profile obtained using the *apiweb*™ (V5.1) identification software and database (bioMérieux, UK Ltd, Basingstoke, UK).

5.2.2 Growth inhibition assay for *E. coli*

LAB isolates for further characterisation were selected based on there being high confidence in their identification (from the API characterisation) and that they were isolated from both CON and LF treatments on days 1, 8, 22 and 35 in the experiment reported in Chapter 4. Eight isolates of lactic acid bacteria selected by API test and the LAB9 isolate (*L. fermentum*; the LAB administered in the study in Chapter 4) were characterised for their antimicrobial activity.

This consisted of incubating different isolates of *E. coli* (also isolated from caecal digesta samples taken from the birds in the study reported in Chapter 4) with the cell-free supernatant of the LAB cultures, and also with these same supernatants after their pH had been neutralised. The characteristics of the selected *E. coli* isolates (based on their phenotypic AMR and carriage of virulence genes as assessed by PCR, see Chapter 4), were that five isolates (E2, E3, E4, E5, E6) carried ≥ 3 virulence genes with resistance to AMP (apart from E6, which was sensitive to AMP, TET, CHL and NA). These isolates were more virulent than E1, E7 and E8. The isolates with the greatest number of virulence genes were E4 and E5. E4 was resistant to both AMP and TET, while E5 was resistant to AMP, TET, CHL and NA. However, the E2 DNA was not extracted and genotypic characterisation was not evaluated. The *E. coli* isolates E1, E3, E5 and E6 carried ≥ 3 antimicrobial resistant genes while isolates E7 and E8 were susceptible to antibiotics as assessed by WGS analysis. Whole genome sequencing confirmed the findings of PCR analysis, in that the isolates E4, E5 and E6 were more virulent than the other isolates (E1, E7, E8). Isolates E7 and E8 were not susceptible to antibiotics, whereas the other isolates were. The growth of the different *E. coli* types in these supernatants was compared with *E. coli* growth in MRS and LB (Luria-Bertani) broth to estimate the extent of inhibition arising from the presence of antimicrobial compounds present in the cell-free supernatants.

5.2.2.1 Preparation of supernatant from Lactic acid bacteria and pH profiles

Two inoculation loopfuls from each selected LAB stock culture in cryovials were inoculated in 10 ml of MRS broth and incubated anaerobically at 37°C for 24 hrs. The optical density (OD) of the suspension was measured and recorded. The cell free supernatant of the LAB cultures was collected following centrifugation at 5000 rpm for 5 min and was then sterilized by passing it through a 0.2 µm micro filter.

A pH electrode was calibrated against pH 4.0 and pH 7.0 standards and rinsed with deionized water. The pH of the LAB culture supernatants was determined and a subsample (5 ml) was neutralised (to pH 6.8–7.2) by the addition of approximately 300-350 µl sodium hydroxide (1 M). The supernatants were then stored at -20°C pending the inhibition assay.

5.2.2.2 Inhibition assays (against pathogenic and AMR *E. coli*)

The eight *E. coli* isolates (selected from the same caecal samples as the LAB isolates) were stored in cryovials containing beads and cryopreservative-added broth at -80°C pending characterization of *in vitro* antimicrobial activity. A bead from the *E. coli* stock culture was cultured in 2.5 ml of MRS plus 2.5 ml of LB broth (1:1 ratio) and incubated at 37°C for 18-24 hrs with constant agitation (160 rpm). To determine the effect of cell-free supernatant and neutralised cell-free supernatant of LAB on growth inhibition of *E. coli*, two Greiner CELLSTAR 96-well plates were prepared. In the first plate, 100 µl of 1 in 10 dilution (MRS plus LB broth) of the *E. coli* suspension was added into individual wells of the 96-well plate. 200 µl of the cell-free supernatant of the LAB was added to each well in duplicate for each *E. coli*

isolate. In another (96 well) microplate, 100 µl of 1 in 10 dilution (MRS plus LB broth) of the *E. coli* suspension and 200 µl of the neutralised cell-free supernatant of the LAB was transferred in duplicate into each well. Duplicate wells of 100 µl of *E. coli* culture with 200 µl MRS broth were used as controls. The growth of *E. coli* was analysed by placing the microtiter plate into FLUOstar Omega multi-mode microplate reader (BMG LABTECH, Ortenberg, Germany) to measure the optical density of each well under anaerobic conditions (5% of CO_2 and 1% of O_2). Other incubation conditions were: temperature (42^0C), plates were continuously shaken (200 rpm), and absorbance (600 nm) was recorded each hour.

5.2.3 Proton nuclear magnetic resonance (^{1}H NMR)

The supernatants from the LAB cultures were analysed by ^{1}H NMR spectroscopy to identify and semi-quantitatively determine the relative concentration of compounds produced by the nine lactic acid bacterial strains, which might be related to the inhibition of *E. coli* growth. The method used was described by Dakheel (2018).

5.2.3.1 Preparation of phosphate buffer

To prepare the phosphate buffer solution (pH 7.0), disodium hydrogen orthophosphate (Na_2HPO_4, 2.88 g), sodium dihydrogen orthophosphate (NaH_2PO_4, 2.88 g), 2,2',3,3'-tetradeuteropropionic acid (TSP, 1mM, 0.0172 g, used as a reference in the NMR technique) and sodium azide (NaN_3, 0.0193 g) were weighed

into a 100 ml volumetric flask, dissolved and the solution made up to volume with deuterium oxide (D_2O).

5.2.3.2 Sample preparation for ^{1}H NMR analysis

The nine strains of lactic acid bacteria were anaerobically incubated at 37 °C in MRS broth for 24 hours. The cultures were then centrifuged at 5,000 rpm for 5 minutes, filtered and stored at -20 °C for a week. A sample of the supernatant (400 µl) was then transferred to a sterile microcentrifuge tube to which 200 µl phosphate buffer solution was added. Afterward, these samples were vortexed and centrifuged at 10,000 rpm for 10 minutes, and then 550 µl of the supernatant was transferred to a 5 mm internal diameter NMR tube.

5.2.3.3 ^{1}H NMR acquisition and processing

Samples were analysed with a Bruker Avance III 500 MHz spectrometer (Bruker, Rheinstetten, Germany), operating at 11.7 T, with a 5 mm double resonance broad band probe equipped with a z-gradient coil. NMR measurements were performed at 297 K. Standard 1D ^{1}H NMR spectra were acquired using a “NOESY-1D” pulse sequence with water suppression and a relaxation delay of 5 s. ^{1}H spectra were collected with 256 scans (and eight dummy scans) in 32k data points with a spectral width of 8012.820 Hz. Sample shimming was performed automatically on the D_2O signal. A line broadening factor of 0.3 Hz was applied prior to Fourier transformation of the Free Induction Decay (FID). After acquisition, spectra were manually phased, calibrated to the TSP at δ 0.00 ppm and baseline corrected using topspin 3.6.1

software (Bruker, Rheinstetten, Germany). Major peaks were assigned using an online open access Human Metabolome Database (HMDB) and manually integrated. Metabolites of bacteria isolated from samples of animals can be referred to compound information and NMR spectra using HMDB and many animal studies have used HMDB (Le Roy *et al.*, 2016; Yang *et al.*, 2016; Tonks, 2018). They were quantified relative to the TSP peak in each spectrum (relative concentration).

5.2.4 Data analysis

The percentage of *E. coli* growth inhibition by each LAB strain was estimated by dividing the difference in absorbance (at 600 nm) between the *E. coli* cultured with an LAB supernatant and *E. coli* incubated in MRS:LB broth in the absence of LAB culture supernatant (control), dividing by the control absorbance and then multiplying by 100. The supernatants from neutralised cultures stimulated growth rather than inhibiting it, and so the percentage of *E. coli* growth stimulation in these cultures, relative to the control, was determined by dividing the absorbance (at 600 nm) of these cultures by the absorbance at 600 nm of the control and then multiplying by 100. The effect of LAB strain and *E. coli* strain on percentage *E. coli* growth inhibition (in unadjusted media) or on percentage *E. coli* growth stimulation (in neutralised media) was determined by analysis of variance using the general linear model of Minitab (Minitab 19, Minitab Inc., PA, USA). Tukey's post-hoc test was used to compare means to observe significance at the level of $P < 0.05$.

5.3 Results

5.3.1 Identification of lactic acid bacteria by analytical profile index (API)

All strains were detected and identified as useful lactic acid bacteria except for two strains which could not be identified. Bacterial strains that were identified included *Lactococcus lactis lactis* 1, *Lactobacillus plantarum* 1 and *Lactobacillus paracasei* ssp. *paracasei* 1 with percentage confidence in these identifications >48.6% (in one instance) and between 64.1 and 95.8% for the other isolates (Table 5.1). The most frequently identified species was *Lactococcus lactis*, which was identified in 17 of the 27 LAB isolates that were identified.

The eight LAB strains that were subsequently characterised for *E. coli* inhibition and ^{1}H-NMR analysis of their culture supernatant (together with the *Lactobacillus fermentum* preparation used in Chapter 4) are summarised in Table 5.2. Data regarding the optical density of the culture following their overnight incubation, and the pH of the culture supernatant before and after neutralisation are also presented.

Table 5.1 Identification (by API system, bioMérieux, UK Ltd, Basingstoke, UK) of the *Lactobacillus* isolates taken from samples of caecal digesta of the birds in the experiment reported in Chapter 4.

Bird age (d)	Treatment	Identification	% Confidence
1	N/A	*Lactococcus lactis* ssp. *lactis* 1	95.8
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	92.8
1	N/A	*Lactococcus lactis* ssp. lactis 1	95.8
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	88.1
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	99.6
8	CON	*Lactococcus lactis* ssp. *lactis* 1	64.3
8	CF	*Lactococcus lactis* ssp. *lactis* 1	64.3
8	LF	*Lactococcus lactis* ssp. *lactis* 1	64.3
14	CON	*Lactococcus lactis ssp*. *lactis* 1	64.3
14	CF	*Lactococcus lactis ssp*. *lactis* 2	69.8
14	LF	*Lactobacillus fermentum* 1	97.3
22	CON	No result	-
22	CON	*Lactobacillus plantarum* 1	76.9
22	CF	*Lactobacillus paracasei* ssp. *paracasei* 1	99.9
22	CF	*Lactococcus lactis* ssp. *lactis* 1	64.3
22	LF	*Lactobacillus paracasei* ssp. *paracasei* 1	99.9
22	LF	*Lactococcus lactis* ssp. *lactis* 2	61.1
29	CON	*Lactococcus lactis* ssp. *lactis* 1	48.6
29	CON	*Lactococcus lactis* ssp. *lactis* 1	64.1
29	CF	*Leuconostoc mesenteroides* ssp. *mesenteroides*/*dextranicum* 1	69.2
29	CF	*Lactococcus lactis* ssp. *lactis* 1	90.0
29	LF	*Lactococcus lactis* ssp. *lactis* 1	93.2
29	LF	*Lactococcus lactis* ssp. *lactis* 1	64.3
35	CON	No result	-
35	CON	*Lactococcus lactis* ssp. *lactis* 1	72.7
35	CF	*Lactobacillus paracasei* ssp. *paracasei* 3	79.9
35	CF	*Lactococcus lactis* ssp. *lactis* 1	51.1
35	LF	*Lactococcus lactis* ssp. *lactis* 1	92.2
35	LF	*Lactobacillus brevis* 1	93.0
	Inoculation	*Lactobacillus fermentum* 1	99.3

N/A: not applicable; CON: control; CF: *Candida famata*; LF: *Lactobacillus fermentum*.

Table 5.2 The identification and optical density (OD) at 600 nm following overnight incubation, and of the pH of the culture supernatant before and after neutralisation of the LAB isolates selected for the *E. coli* growth inhibition assay.

Sample	Treatment	Day	Taxon	% Confidence	OD of suspension	pH	Neutral pH
LAB1	-	1	*Lactococcus lactis* ssp. *lactis* 1	95.8	1.08	4.8	7.24
LAB2	-	1	*Lactococcus lactis* ssp. *lactis* 1	95.8	1.06	4.6	7.33
LAB3	LF	8	*Lactococcus lactis* ssp. *lactis* 1	64.3	1.09	4.71	7.1
LAB4	CON	8	*Lactococcus lactis* ssp. *lactis* 1	64.3	1.43	4.34	7.08
LAB5	CON	22	*Lactobacillus plantarum* 1	76.9	0.89	4.73	7.9
LAB6	LF	22	*Lactobacillus paracasei* ssp. *paracasei* 1	99.9	1.06	4.76	6.96
LAB7	CON	35	*Lactococcus lactis* ssp. *lactis* 1	72.7	0.93	4.57	6.88
LAB8	LF	35	*Lactococcus lactis* ssp. *lactis* 1	92.2	0.87	4.56	7.77
LAB9	inoculation		*Lactobacillus fermentum*	99.3	1.4	4.73	6.94

CON: control; CF: *Candida famata*; LF: *Lactobacillus fermentum*.

5.3.2 Growth inhibition assay for *E. coli*

There was no significant difference (*P*=0.147) between LAB strains in the extent to which *E. coli* growth was inhibited when the culture medium was not neutralised. The mean inhibition was 53.2% (SEM 0.79%). There was no significant difference (*P*=0.796) between *E. coli* isolates in their growth after 24 h incubation in the absence of LAB culture supernatant. However, when incubated with the un-neutralised LAB culture supernatant, there was a highly significant ($P < 0.001$) difference between *E. coli* isolates in the extent to which their growth was inhibited. The probable pathogenic E5 was inhibited to the greatest extent (64%) while another commensal (E7) was inhibited least (41%). These data are presented in Figure 5.1. The lactic acid inhibited putative pathogenic *E. coli* (E4, E5, E6) to a greater extent (55%) than commensal *E. coli* (E1, E7, E8, 51%), *P*=0.02, Figure 5.2. E2 and E3 were excluded from this analysis because of uncertainty about their genotype and characterisation. The lactic acid inhibited antibiotic resistant *E. coli* (E1, E3, E5, E6, carrying ≥ 3 antimicrobial resistant genes) to a greater extent (56%) than antibiotic susceptible *E. coli* (50%, E7 and E8, *P*=0.002, Figure 5.3). E4 were excluded from this analysis because E4 carried two antimicrobial resistant genes.

When the LAB supernatants were neutralised, there was a highly significant difference ($P < 0.001$) both between LAB isolates and between *E. coli* isolates. The LAB isolates LAB5 (*Lactobacillus plantarum*) and LAB8 (*Lactococcus lactis*) were associated with nearly a threefold increase in the growth of *E. coli*, whereas isolates LAB3 (*Lactococcus lactis*), LAB4 (*Lactococcus lactis*), LAB6 (*Lactobacillus paracasei*), LAB7 (*Lactococcus lactis*) and LAB9 (*Lactobacillus fermentum*, original inoculum) only doubled growth. These data are presented in Figure 5.4. A putative pathogenic *E. coli* E4 and E5, and the commensal E7 were all stimulated in their

growth to a greater extent than the other isolates. The stimulation of growth of E8 (commensal) was not significantly different from that of the more virulent isolates (Figure 5.5).

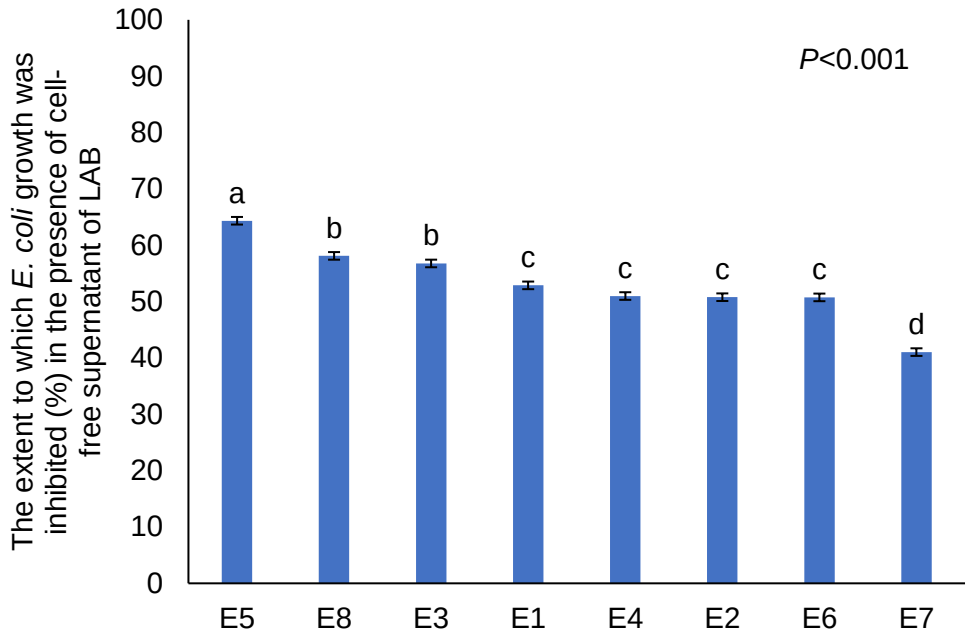


Figure 5.1 The effect of unneutralised cell-free supernatant of LAB on *E. coli* growth inhibition.

Putative pathogenic (carried ≥ 3 virulence genes): E4, E5, and E6; Commensal: E1, E7 and E8. Antibiotic resistant (carrying ≥ 3 antimicrobial resistant genes): E1, E3, E5 and E6; Antibiotic susceptible: E7 and E8.

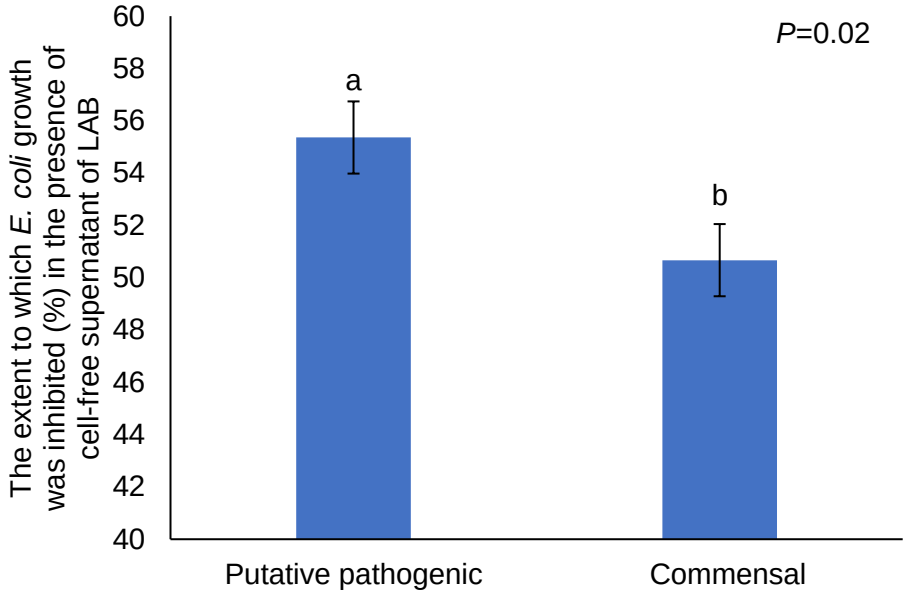


Figure 5.2 The effect of unneutralised cell-free supernatant of LAB on growth of putative pathogenic and commensal *E. coli*.

Putative pathogenic: E4, E5, and E6, Commensal: E1, E7 and E8.

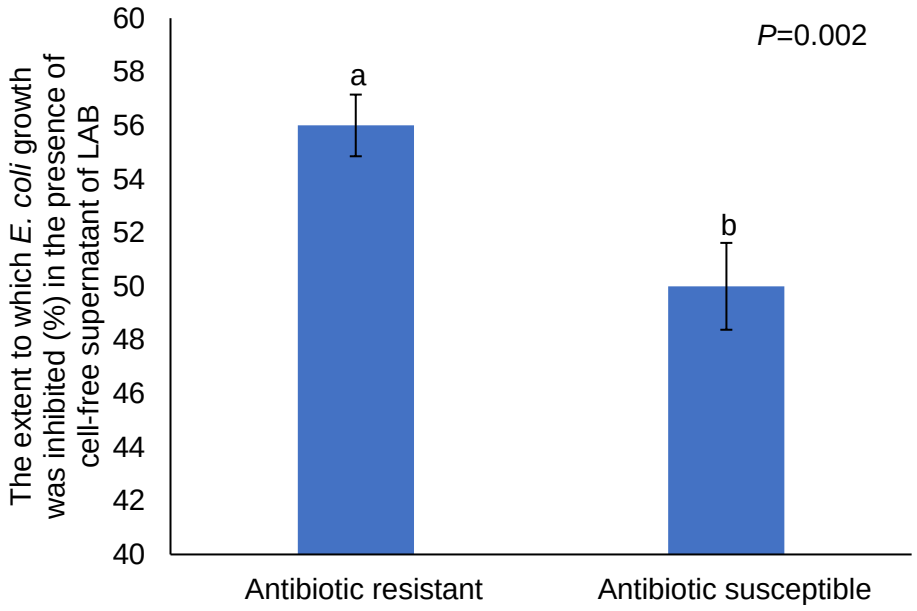


Figure 5.3 The effect of unneutralised cell-free supernatant of LAB on growth of antibiotic resistant and susceptible *E. coli*.

Antibiotic resistant: E1, E3, E5 and E6, Antibiotic susceptible: E7 and E8.

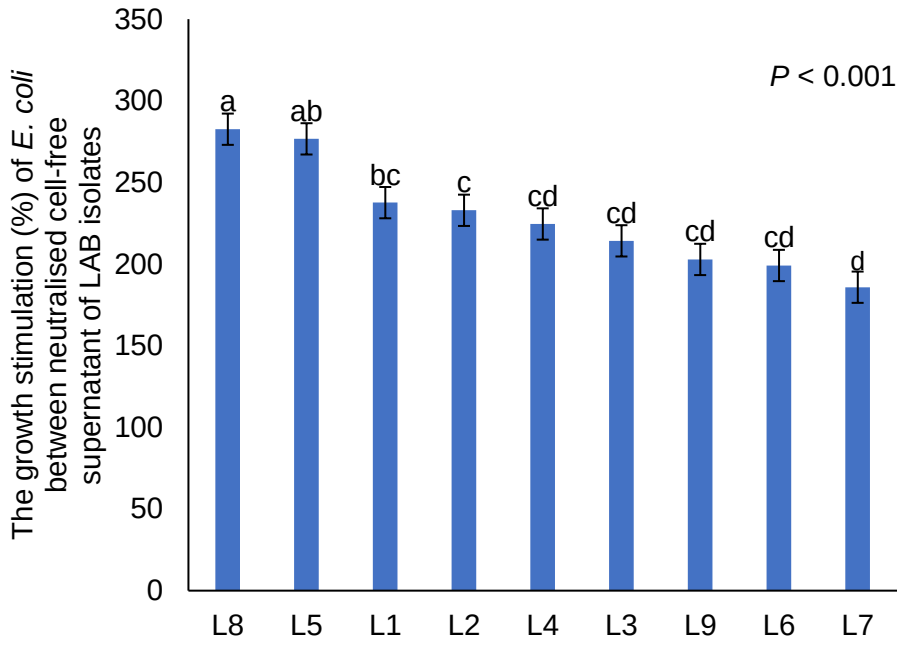


Figure 5.4 The growth stimulation of *E. coli* between neutralised cell-free supernatant of LAB isolates.

L1, L2, L3, L4, L7, L8: *Lactococcus lactis*; L5: *Lactobacillus plantarum*; L6: *Lactobacillus paracasei*; L9: *Lactobacillus fermentum*, original inoculum.

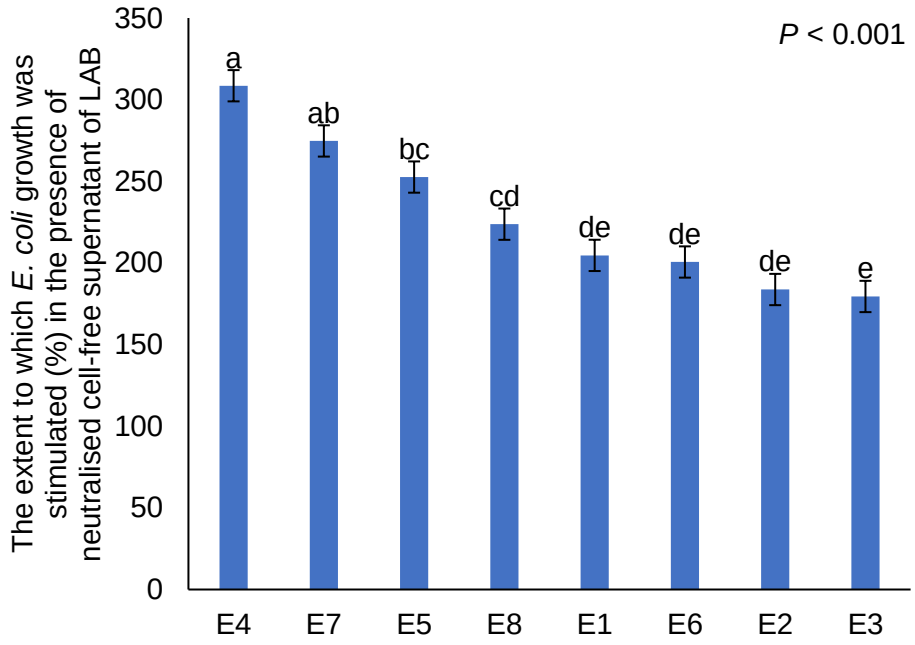


Figure 5.5 The growth stimulation of *E. coli* in neutralised cell-free supernatant of LAB.

Putative pathogenic (carried ≥ 3 virulence genes): E4, E5, and E6; Commensal: E1, E7 and E8.

5.3.3 Nuclear magnetic resonance (^{1}H NMR)

The identification and concentration of the LAB culture media are presented in Table 5.3. Data from these spectra of the LAB supernatants suggested that all LAB isolates produced lactic acid (as would be expected). Similar concentrations of acetic acid were also produced. However, as acetic acid was also observed in the control it is possible that the LAB were not the source of this fermentation product. *Lactococcus lactis* (LAB 4) produced relatively more lactic acid compared with the other isolates. The *Lactobacillus fermentum* (LAB 9), which was used as the inoculum in the bird experiment, also produced a high concentration of ethanol. This was not observed by any of the other isolates (Table 5.3 and Figure 5.6). Acetoin was also observed, at relatively low concentrations, in some of the supernatants, being numerically higher with *Lactococcus lactis* (LAB 1-4, 7 and 8), *Lactobacillus plantarum* (LAB 5) and *Lactobacillus paracasei* (LAB 6) compared with the control (Table 5.3). Interestingly, the two isolates with the numerically highest concentration of acetoin (LAB 5 and LAB 8) were the two strains associated with the greatest stimulation of *E. coli* growth in the neutralised supernatant.

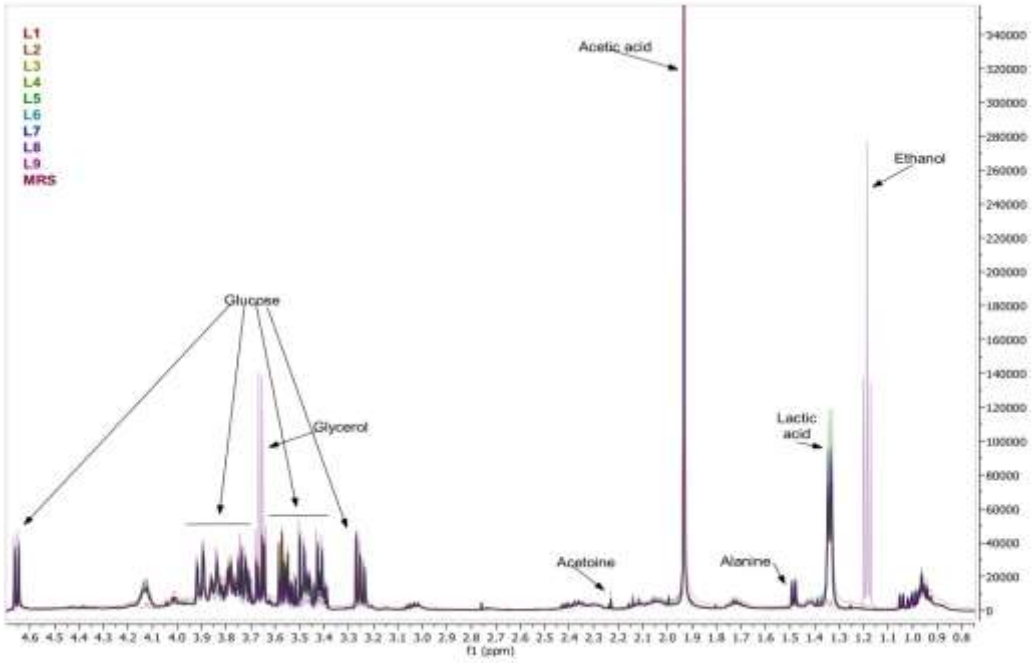
Table 5.3 The identification and relative concentration (arbitrary unit, AU) of compounds produced by LAB.

LAB	Taxon	Ethanol	Lactic acid	Acetoin	Acetic acid
LAB1	*Lactococcus lactis* ssp. *lactis* 1	0.038	36.586	0.520	42.760
LAB2	*Lactococcus lactis* ssp. *lactis* 1	0.041	42.722	0.475	46.638
LAB3	*Lactococcus lactis* ssp. *lactis* 1	0.046	37.393	0.475	42.287
LAB4	*Lactococcus lactis* ssp. *lactis* 1	0.224	50.274	0.316	42.192
LAB5	*Lactobacillus plantarum* 1	0.259	41.778	1.415	44.855
LAB6	*Lactobacillus paracasei* ssp. *paracasei* 1	0.188	36.642	0.483	44.365
LAB7	*Lactococcus lactis* ssp. *lactis* 1	0.039	38.828	0.198	44.803
LAB8	*Lactococcus lactis* ssp. *lactis* 1	0.044	42.370	1.227	46.098
LAB9	*Lactobacillus fermentum*	42.985	43.210	0.037	45.687
LAB10	Control (medium)	0.066	2.350	0.091	45.913

LAB: lactic acid bacteria

* Values highlighted in red are the highest concentration of each compound.

(a)



(b)

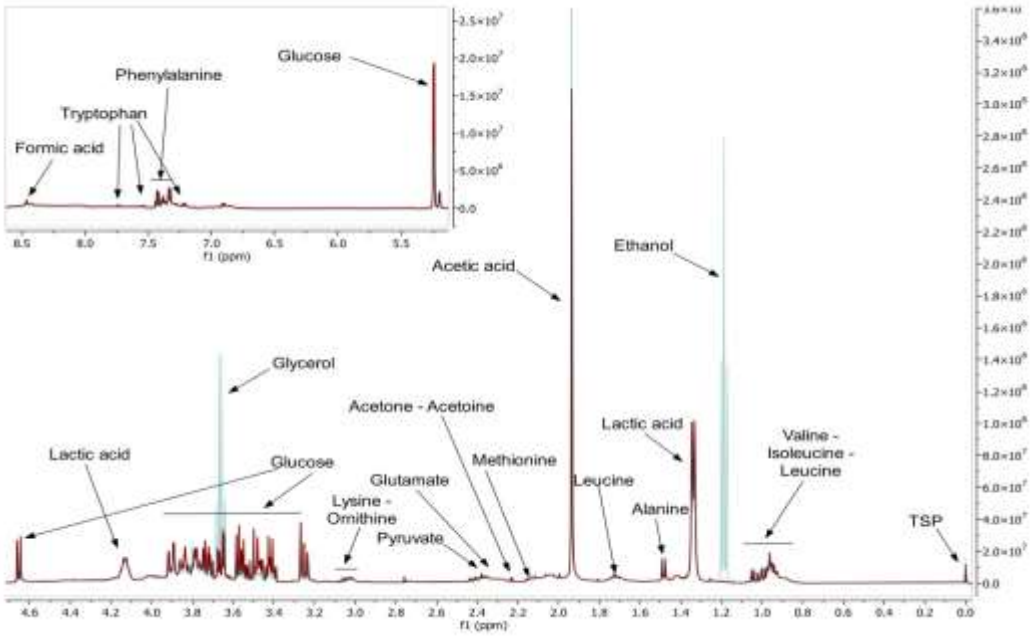


Figure 5.6 (a) ^{1}H NMR spectra of the culture media L1-9 and MRS (control), (b) ^{1}H NMR spectra of the culture media L4 (Red) and L9 (Green).

L1: *Lactococcus lactis* ssp. *lactis* 1; L2: *Lactococcus lactis* ssp. *lactis* 1; L3: *Lactococcus lactis* ssp. *lactis* 1; L4: *Lactococcus lactis* ssp. *lactis* 1; L5: *Lactobacillus plantarum* 1; L6: *Lactobacillus paracasei* ssp. *paracasei* 1; L7: *Lactococcus lactis* ssp. *lactis* 1; L8: *Lactococcus lactis* ssp. *lactis* 1; L9: *Lactobacillus fermentum*

5.4 Discussion

5.4.1 Identification of lactic acid bacteria isolated from the caecum

Lactococcus lactis ssp. *lactis* 1 was the predominant strain identified in the caecum and only one isolate of *Lactobacillus fermentum* was observed when LF treatment was administered via drinking water to broiler chickens. This finding suggested that administering *Lactobacillus fermentum* did not result in its successfully colonising and proliferating in the caecum. Lactic acid bacteria might be able to adhere to the mucosal wall rather than lumen, and it is possible that this is where the *L. fermentum* may have been found. Lin *et al.* (2007) reported that *L. fermentum* was very capable of adhesion to epithelial cells from chicken intestine. Yu *et al.* (2007) observed that *Lactobacillus reuteri* had excellent ability to attach to the crop and intestinal epithelial cells compared with *L. acidophilus*. This suggests that LAB can attach to and colonise mucous cells, and that samples of gut wall scrapings should have been taken for enumeration of LAB. It is also possible that there are differences between LAB strains in their ability to adhere to the gut wall. Another limitation in the characterization of the LAB isolates is the difficulty of obtaining a representative sample of the microbial community from the relatively small number of samples taken in the previous experiments. Characterising LAB using tests such as the API kit is also particularly difficult.

Albazaz and Bal (2014) observed that *L. acidophilus*, *L. salivarius* and *L. fermentum* were the main species of *Lactobacillus* isolated from the chicken small intestine by RNA sequence, but these species were generally not identified in this study. This may be because of the different identification methods used between these two studies. In this study, the API test was used to identify LAB species as it is more

convenient, cheaper, easier and faster than genome sequencing. However, the identification of bacteria by API test might yield erroneous results, depending on the skill and experience of the experimenter (Khunajakr *et al.*, 2008). It is difficult to have complete confidence in the identification of LAB by the API test although characteristics of LAB can be identified and assumed. Future studies might determine the exact composition of specific LAB strains by genomic sequencing. The genetic sequencing can provide more reliable and informative identity for gut microbial population.

5.4.2 Antibacterial activity and metabolites of lactic acid bacteria

Lactic acid bacteria are well known for producing antimicrobial compounds and having strong antimicrobial activity (Røssland *et al.*, 2005). The organic acids such as lactic acid and acetic acid are the main antimicrobials produced from lactic acid bacteria and these acids can decrease the pH and inhibit the growth of pathogenic bacteria (Musikasang *et al.*, 2009; Šušković *et al.*, 2010). In this study, the untreated LAB supernatant inhibited *E.* coli growth to some extent, but there was no difference between untreated LAB supernatants in the amount of *E. coli* inhibition. When the supernatant was neutralised, this effect was abolished (and indeed, *E. coli* growth was stimulated). This study suggests that the acid produced by LAB is the main antimicrobial compound produced by LAB.

Putative pathogenic and AMR *E. coli* were inhibited to a greater extent than benign, antimicrobial sensitive *E. coli* strains and this indicates a possible means of using LAB to control APEC and AMR *E. coli* (even if they have no effect on the total *E. coli* population). However, this study used only a small number of *E. coli* isolates.

This experiment should be repeated with a much greater number of isolates, and with a greater range of AMR (and putative pathogenic) characteristics. It would also be useful to investigate a greater range of LAB and other microbes.

All the LAB supernatants had a high concentration of lactic acid and acetic acid according to the NMR analysis. However, acetic acid was detected in both the LAB supernatant and the control (MRS) medium. It seems likely, therefore, that the acetic acid probably came from the MRS medium, which contained sodium acetate trihydrate (5 g/l). *Lactococcus lactis* ssp. *lactis* 1 isolate (LAB 4) produced relatively more lactic acid than other isolates. *Lactobacillus fermentum* (LAB 9) produced overwhelmingly more ethanol (43 arbitrary unit, AU) compared to other strains (0.04-0.26 AU). *Lactobacillus plantarum* 1 (LAB 5) and *Lactococcus lactis* ssp. *lactis* 1 (LAB 8) had relatively higher concentrations of acetoin. In addition, neutralised cell-free supernatants of LAB 5 and LAB 8 stimulated relatively more *E. coli* growth. This suggests that acetoin (or other unidentified metabolites) promoted *E. coli* growth under neutralised conditions. However, in contrast to this finding, acetoin is known as an antimicrobial and aromatic compound (with a buttery flavour) produced by heterofermentative lactic acid bacteria (Romick and Fleming, 1998; Reis *et al.*, 2012; Lu *et al.*, 2016). Acetoin is produced by decarboxylation of α-acetolactate (Jyoti *et al.*, 2003; Šušković *et al.*, 2010; Audrain *et al.*, 2015), but it is unclear how much (if at all) acetoin affects *E. coli* growth. However, this present study indicated that other compounds (beside organic acids) produced by LAB had little inhibitory effect on *E. coli* growth.

Lactococcus lactis is a facultative anaerobe and homofermentative lactic acid bacteria which primarily generates lactic acid metabolised from glucose (Samaržija *et al.*, 2001). *Lactococcus lactis* also produces nisin (a bacteriocin) and antimicrobial

peptides (Šušković *et al.*, 2010; Perez *et al.*, 2014). Many studies have demonstrated the negative effect of bacteriocin produced by *Lactococcus lactis* on the growth of pathogenic bacteria (Henning *et al.*, 2015; Vijayakumar and Muriana, 2015). Vijayakumar and Muriana (2015) observed that untreated and neutralised bacteriocins from *Lactococcus lactis* inhibited *Listeria monocytogenes* 39-2. In addition, Enan *et al.* (2013) reported that the cell free supernatant of *Lactococcus lactis* Z11 isolated from Arabian yoghurt (Zabady) had strong antibacterial activity against *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*. However, it was sensitive to pepsin, trypsin and α-chemotrypsin but was heat stable (60-100°C, 5 min) and unaffected by lipase, amylase, organic solvents, and acid (pH range 2 to 7). The optimum conditions for bacteriocin activity was pH 6.5 and an incubation temperature of 30°C (Enan *et al.*, 2013). Similarly, Moreno *et al.* (2000) found that *Lactococcus lactis* bacteriocin was resistant to heat, a range of pH and sensitive to proteolytic enzymes. *Lactococcus lactis* subsp. *lactis* ITAL 383 and *Lactococcus lactis* subsp. *lactis* ATCC 11454 inhibited gram-positive microorganisms including *Listeria monocytogenes* and *Staphylococcus aureus*, while this strain did not affect the growth of gram-negative bacteria. The mode of action of nisin inhibited cell wall biosynthesis by binding lipid in the inner membrane (Stevens *et al.*, 1991; Zhou *et al.*, 2016; Li *et al.*, 2018). However, nisin was inactive to gram-negative bacteria such as *E. coli* and *Salmonella* because the outer membrane of gram-negative bacteria was impermeable and prevented nisin reaching and binding lipid II in the inner membrane (Stevens *et al.*, 1991; Li *et al.*, 2018). The isolates characterised in this study may have produced bacteriocin, but as it has limited if any effect on the growth of *E. coli*, this would explain why only the acids from the isolates appeared to exert any antibacterial activity (against *E. coli*).

Lactobacillus fermentum is known to be a heterofermentative bacteria (Ale *et al.*, 2020). Heterofermentative lactic acid bacteria decompose hexoses and produce metabolites and compounds such as organic acids, hydrogen peroxide, carbon dioxide, acetoin, ethanol, and aromatics including acetaldehyde and diacetyl (Millette *et al.*, 2004; Alomar *et al.*, 2008; Enan *et al.*, 2013). *Lactobacillus fermentum* is a potential bacteria and has excellent antimicrobial activity against pathogenic bacteria (Mikelsaar and Zilmer, 2009; Ale *et al.*, 2020). Lin *et al.* (2007) observed the supernatant of *L. fermentum* had antagonistic effect towards pathogens such as *E. coli*, *Salmonella*, *Shigella sonnei* and *Staphylococcus aureus*. Ramos *et al.* (2013) reported that *Lactobacillus brevis* SAU105 and FFC199 and *L. fermentum* CH58 had antagonistic activity against *Listeria monocytogenes* and *Staphylococcus aureus*. Bao *et al.* (2010) showed that *L. fermentum* F6 had a high tolerance to bile salts and prevented the growth of *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus typhimurium*, *Shigella flexneri* and *Escherichia coli*. de Souza Rodrigues *et al.* (2020) also demonstrated that *L. fermentum* TcUESC01 had anti-adherence activity and antimicrobial activity against *Streptococcus mutans* UA159, and was heterofermentative, producing mostly lactic acid (50%) but also phosphoric acid (8%).

The results of the present study confirmed the hypothesis. LAB produced useful antibacterial compounds such as lactic acid and ethanol and they inhibited the growth of putative pathogenic and AMR *E. coli* to a greater extent than commensal and antibiotic susceptible *E. coli*. Although there was no significant difference between the LAB isolates in terms of their inhibitory effects on *E. coli* growth, future studies in this thesis will focus particularly on LAB 4 and LAB 9. These isolates were associated with the highest relative concentration of lactic acid (LAB 4) and ethanol

(LAB 9) and produced relatively low concentrations of the (apparently) stimulatory acetoin. Therefore, *Lactococcus lactis* (LAB 4) and *Lactobacillus fermentum* (LAB 9) were identified as having the greatest potential value to control pathogenic and AMR *E. coli*.

5.5 Conclusion

In conclusion, this study demonstrated that the predominant LAB strain isolated from the chicken caecum is probably *Lactococcus*. Selected LAB isolates had strong antimicrobial activity against putative pathogenic and antimicrobial resistant *E. coli,* but this effect disappeared when the medium was neutralised, demonstrating that the antibacterial activity resulted from the production of lactic acid. The *Lactococcus lactis* isolate LAB 4 produced relatively more lactic acid compared with the other isolates, while the *L. fermentum* isolate (LAB 9) produced considerably more ethanol. These two bacterial isolates might (because of this antibacterial activity) have a role in improving the microbiome composition in broiler chickens if they could be administered in such a way as to become established in the chicken gut. The susceptibility of the growing chick to pathogenic and antimicrobial resistant *E. coli* has been demonstrated in Chapter 4. If interventions to encourage the early proliferation of commensal *E. coli* (without the resistance to antimicrobials) could be developed, this would make the growing chick less susceptible to antibiotic resistant disease. In the next chapter, therefore, these two isolates (LAB 4, *Lactococcus lactis*) and LAB 9 (*L. fermentum*) were selected and administered to young chicks via their drinking water to determine their effect on chick growth as well as the prevalence of antimicrobial resistant and pathogenic *E. coli* in the caecum of the broiler chicken.

Chapter 6. The effect of administering *Lactococcus lactis* ssp. *lactis* 1 and *Lactobacillus fermentum* 1 during the starter period on the prevalence of antimicrobial resistance and virulence genes of *E. coli* isolated from the broiler caecum

6.1 Introduction

There is considerable evidence that *Lactococcus lactis* and *Lactobacillus fermentum* have beneficial properties associated with the prevention of pathogen growth and maintaining a healthier gut microbiome (Capcarova *et al.*, 2010; Bai *et al.*, 2013; Enan *et al.*, 2013). Two species of lactic acid bacteria, *Lactococcus lactis,* LL and *Lactobacillus fermentum,* LF have been isolated from a chicken caecum and pig respectively and they have demonstrated a strong inhibitory effect on the growth of *E. coli in vitro* (Chapter 5). To better characterise the *E. coli* isolated from the caecum, whole genome sequencing (WGS) can be used to analyse the diversity and evolution of *E. coli* and identify the relation between certain *E. coli* sequence types and the carriage of AMR and virulence genes. The caecal microbiome is more diverse and abundant compared to the microbiome of other sections of the intestinal tract. Most fermentation of digesta occurs in the caecum by caecal microorganisms and the digesta is retained longer in the caecum compared with the rest of the gut (Bjerrum *et al.*, 2006; Rychlik, 2020). In addition, the population of coliforms in the caecum is high and *E. coli* are regularly studied as a representative indicator of AMR (Borda-Molina *et al.*, 2018; Davis *et al.*, 2018; Hesp *et al.*, 2019; Majewski *et al.*, 2020). The focus of this study was therefore on *E. coli* isolated from the caecum rather than other sections of the gut.

In the previous bird study (Chapter 4), it was observed that the birds were most susceptible to colonisation with coliforms resistant to both ampicillin and tetracycline at the end of the starter phase (around 8 d old). The phenotypic and genotypic antimicrobial resistance and virulence of *E. coli* in the chicken caecum then declined through the grower and finisher phases. Therefore, to confirm the findings of the previous study and determine exactly when the birds were at their most vulnerable, this research focused on the starter phase, monitoring changes in antimicrobial resistance and virulence associated genes of *E. coli* and determining the effect of administering lactic acid bacteria in the starter phase. It was hypothesised that the intervention of lactic acid bacteria might alter the AMR *E. coli* status in the young bird by advancing the maturation of the microbiome. This might prevent the proliferation of antimicrobial resistant and virulent coliforms in the starter period, such that the bird entered the growing and finishing phases with a less challenging gut environment, evidenced by a lower prevalence of AMR and virulence genes in the caecal *E. coli.* Therefore, the objective of the experiment reported in this chapter was to determine the effect of administering either *Lactococcus lactis* or *L. fermentum* in the drinking water of broiler chicks during their starter phase (1-11 d of life) on bird performance and the prevalence of antimicrobial resistant and virulence genes in *E. coli* isolated from the birds' caeca.

6.2 Material and methods

6.2.1 Experimental design, animals and diets

The bird housing, management and slaughter conditions were the same as described in Chapter 3. A total of 240 chicks (day-old, male Ross 308, purchased from PD Hook, Cote, Oxfordshire) were used in a 28 day feeding trial. All chicks were weighed on arrival, wing tagged, blocked by liveweight, and then randomly placed in one of 24 cages (ten chicks per cage) and fed a common starter diet until the birds were 14 days of age. *Lactococcus lactis* ssp. *lactis* 1 (isolated from a chicken in the previous bird study) and *Lactobacillus fermentum* 1 (isolated from a pig; University of Surrey) was administered via the drinking water. Stock cultures of each isolate were incubated overnight in De Man, Rogosa and Sharpe (MRS) broth. Population density was then estimated by serial dilution and measurement of the optical density of the incubation medium at 600 nm. Cultures were then centrifuged at 5000 rpm for 5 min (Eppendorf centrifuge 5804R, Germany) and washed with sterile distilled water. An appropriate volume of the culture was then added to a measured volume of the drinking water in the hopper to attain the desired final concentration of lactic acid bacteria. Water was therefore provided either untreated (Control) or with a preparation of *Lactococcus lactis* (LL; 10^7 CFU/ml water offered) or *L. fermentum* (LF; 10^7 CFU/ml water offered) via a nipple drinker on three days each week during the starter phase (1, 3, 5, 7, 9 and 11 d). There were eight replicate cages for each of these experimental treatments. The nipple drinker was replenished with fresh (untreated) water when required.

On day 14, all birds were weighed and then transferred to a floor pen (four birds per pen, all birds in each pen originated from the same cage), with eight replicate pens per treatment. All birds were then fed a common grower/finisher diet. The ingredient and nutrient composition of the diets is shown in Table 6.1. The diets were fed in the form of a mash, and were manufactured by Target Feeds (Whitchurch, UK). Samples of each diet were analysed for crude protein, total starch, oil, sugar, methionine, lysine, and iron (Sciantec, York, UK). Lighting was via incandescent lights with 23 h continuous light per 24 h period for the first seven days, followed by 18 h continuous light (6 h darkness) in each 24 h period. The birds were brooded according to the breeder's recommendations using infrared lights to provide supplementary heat when necessary.

Table 6.1 Ingredient and nutrient composition (as-fed basis) of chickens' diets.

	Starter (0-14 d)	Grower/ Finisher (15-28 d)
Ingredient composition (g/kg)		
Barley	40	40
Wheat (12.5% CP)	473	546
Soya bean meal (48% CP)	325	230
Rapeseed meal	42	40
Soya bean oil	50	55
L-lysine HCl	4	3
DL-methionine	3	2
L-threonine	2	2
Sodium bicarbonate	2.5	2.5
Salt	2	2.5
Limestone	10	9
Poultry vitamins/minerals*	4	4
Dicalcium phosphate (QPRDC)	17.5	14
Sunflower meal (expeller)	20	45
Titanium dioxide	5	5
Nutrient composition (g/kg)		
ME (MJ/kg)	10.4	10.8
Crude Protein	236	212
Total Starch	287	312
Oil A	41.9	41.6
Sugar	42.3	38.3
Methionine	6.0	4.7
Lysine	13.4	12.4
Iron (mg/kg)	109	126

ME: Metabolisable Energy

* The vitamin/mineral premix supplied per kg of starter diets: vitamin A 13500 IU, vitamin D_3 5000 IU, vitamin E 100 mg, vitamin B_1 3 mg, vitamin B_2 10 mg, vitamin B_6 3 mg, vitamin B_{12} 0.03 mg, nicotinic acid 60 mg, pantothenic acid 15 mg, folic acid 1.5 mg, biotin 0.25 mg, choline chloride 250 mg, Fe 20 mg, Mn 100 mg, Cu 10 mg, Zn 80 mg, I 1 mg, Se 0.25 mg, Mo 0.50 mg.

The vitamin/mineral premix supplied per kg of grower/finisher diets: vitamin A 10000 IU, vitamin D_3 5000 IU, vitamin E 100 mg, vitamin B_1 3 mg, vitamin B_2 10 mg, vitamin B_6 3 mg, vitamin B_{12} 0.03 mg, nicotinic acid 60 mg, pantothenic acid 15 mg, folic acid 1.5 mg, biotin 0.25 mg, choline chloride 250 mg, Fe 20 mg, Mn 100 mg, Cu 10 mg, Zn 80 mg, I 1 mg, Se 0.25 mg, Mo 0.50 mg.

6.2.2 Sample collection

On day 1, a total of 24 birds were randomly selected and sacrificed by cervical dislocation. The contents of whole intestinal tract (from proximal duodenum to ileo-caecal junction) and the yolk sac were taken and placed on ice for transportation. These were analysed for the determination of the population size (CFU/g) of *Lactobacilli* and coliforms, and for the determination of antimicrobial resistance and presence of virulence genes by coliforms. On day 3, 7, 9, 11 and 14, one bird from each cage was selected randomly and sacrificed. Digesta from the caecum were taken and placed on ice for transportation and these analysed for the same determinations as above. After the birds were allocated to their grower/finisher pens, one bird from each pen was randomly selected and sacrificed on day 28. Samples of digesta from the caecum were again taken and analysed as before.

6.2.3 Bird performance

Birds were weighed individually on days 1, 14 and 28. Mean body weight increase was then calculated on a pen basis during the starter and grower/finisher period. Feed intake was determined and FCR calculated on a pen basis during the grower/finisher period.

6.2.4 Determination of viable *E. coli* and lactic acid bacteria populations

The samples of whole gut digesta and yolk sac (from day-old chicks), and the samples of caecal digesta (taken from the older birds) were analysed for the determination of the population size (CFU/g) of *Lactobacilli* and coliforms. The

determination of population size of coliforms and lactic acid bacteria was as described in Chapter 4.

6.2.5 Determination of antimicrobial resistance and isolation of samples

Resistance to antimicrobials by the colonies of *E. coli* grown on MacConkey agar plates was determined by replicate plating using plates of MacConkey agar prepared with different antibiotics (ampicillin, nalidixic acid, tetracycline and chloramphenicol, each at a concentration of 20 µg/ml). Plates were then incubated (37°C, 18-24 h) and growth of colonies on each plate was then determined. When growth was observed on plates containing antibiotics, it was deemed that the colony in the parent plate was resistant to that antibiotic. The proportion of AMR was estimated from the number of colonies grown on the plate containing antibiotic as a proportion of the number of colonies grown on the parent plate.

A single colony of *E. coli* was selected at random from each MacConkey plate and transferred to a separate, sterile microcentrifuge tube containing 500 µl of nutrient broth. These cultures were incubated at 37°C for 18-24 hours. 100 µl of the resulting *E. coli* suspension was transferred to a cryovial (Mast group, Mastdisks, UK) and stored at -80°C pending further analysis. In addition, a single colony of *Lactobacillus* sp. was taken at random from each MRS plate and transferred to a sterile microcentrifuge tube containing 1 ml of 15% v/v glycerol (45 ml of glycerol plus 255 ml of MRS broth). These cultures were incubated anaerobically a 37°C for 18-24 hours. After that, the *Lactobacillus* sp. suspension was stored at -80°C.

6.2.6 Extraction of genomic DNA

The genomic DNA from 68 isolates of *E. coli* was extracted to determine the presence of *E. coli* virulence factors. DNA was extracted from seven yolk sac and 61 caecal digesta samples. These comprised seven samples (from day 1), three samples (one from each cage) for each treatment (on days 3, 5, 7, 11, 14 and 28). The same DNA extraction kit was used as described in Chapter 3.. In this chapter, 1.5 µl of RNase A solution was added and mixed by inverting 25 times. The extraction and purification of DNA followed the same procedure as described in Chapter 3. The purification of DNA was evaluated as described in Chapter 3 and the 260:280 nm ratio was also recorded (1.82 $\pm$ 0.07). DNA stock solutions were stored at -20˚C pending the analysis of virulence genes by PCR and WGS.

6.2.7 Whole genome sequencing (WGS) and data analysis

All extracts were submitted to Quadram Institute Bioscience to determine the genotype of individual *E. coli* isolates by WGS. The WGS and data analysis followed the same procedure as described in Chapter 4.

6.2.8 Virulence genotyping

68 DNA extracts taken from the *E. coli* isolates (61 of caecal and seven of yolk sac samples) were analysed for seven avian pathogenic *E. coli* (APEC) virulence factors using PCR assays. The evaluation of virulence genotyping was conducted as described in Chapter 3.

6.2.9 Data analysis

The effect of the administration of either LL or LF on bird performance (body weight gain, feed intake, feed conversion ratio) was determined by analysis of variance using the general linear model of Minitab (Minitab 19, Minitab Inc., PA, USA). The effect of bird age and administration of lactic acid bacteria, and interaction between these main effects on the population density of *Lactobacillus* spp. and coliforms and the proportion of phenotypic antimicrobial resistance was also determined by ANOVA (mixed effects model). Fixed factors were bird age and administering LAB and pen was a random factor. Tukey's post-hoc test was used to compare means to observe significance at the level of $P < 0.05$. Chi-square analysis was used to determine the association between antibiotic resistant genes (identified by whole genome sequencing) and *E. coli* MLST, bird age and treatment. The interaction between bird age and *E. coli* MLST was determined by Chi-square analysis. Associations between the carriage of virulence genes based on the PCR and WGS and bird age and administering LAB were determined by Chi-square analysis. In addition, Chi-square analysis was conducted to determine the association between the carriage of virulence associated gene (VAG) and antimicrobial resistance (respectively, phenotypic AMR and VAG by PCR; carriage of antibiotic resistance gene (ARG) and VAG by WGS).

6.3 Results

6.3.1 Growth performance and population size of coliforms and lactic acid bacteria in the caecum

There was no significant effect of administering either LL or LF on body weight gain during the starter period (see Appendix 5) or grower period (Table 6.2). Feed intake was close to the expected for birds of this age, but growth was lower and FCR consequently higher. This might suggest that the birds in study, while not displaying any clinical signs, were of sub-optimal health.

Even as day-old chicks, there was a large population in the gut of both coliforms (7.47 ± 0.53 $\log_{10}$ CFU/g) and lactic acid bacteria (7.66 ± 0.43 $\log_{10}$ CFU/g), which was larger than the populations observed in the yolk sac ($\log_{10}$ CFU/g of 2.88 ± 0.40) for *E. coli* (Appendix 6). There was no significant difference between treatments in the overall population of coliforms and lactic acid bacteria in caecal samples taken throughout the bird's life (Figure 6.1). The population density of coliforms (from 10.74 to 7.28 $\log_{10}$ CFU/g) and LAB (from 10.17 to 6.44 $\log_{10}$ CFU/g) declined between day 3 and day 28 of bird age ($P < 0.001$, Figure 6.2).

Table 6.2 Effect of LL or LF administration via drinking water on growth performance in broiler chickens.

	Control	LL	LF	SEM	*P*-value
Grower finisher period (day 14-28)					
Weight gain (g/bird/day)	63.8	58.3	58.2	1.79	0.061
Feed intake (g/bird/day)	101.7	97.9	98.0	3.12	0.623
FCR (Feed conversion ratio)	1.60	1.68	1.68	0.049	0.421

LL: bird were treated with 10^7 CFU/ml *Lactococcus lactis* (LL) in their drinking water three days a week during the starter phase; LF: bird were treated with 10^7 CFU/ml *Lactobacillus fermentum* (LF) in their drinking water three days a week during the starter phase.

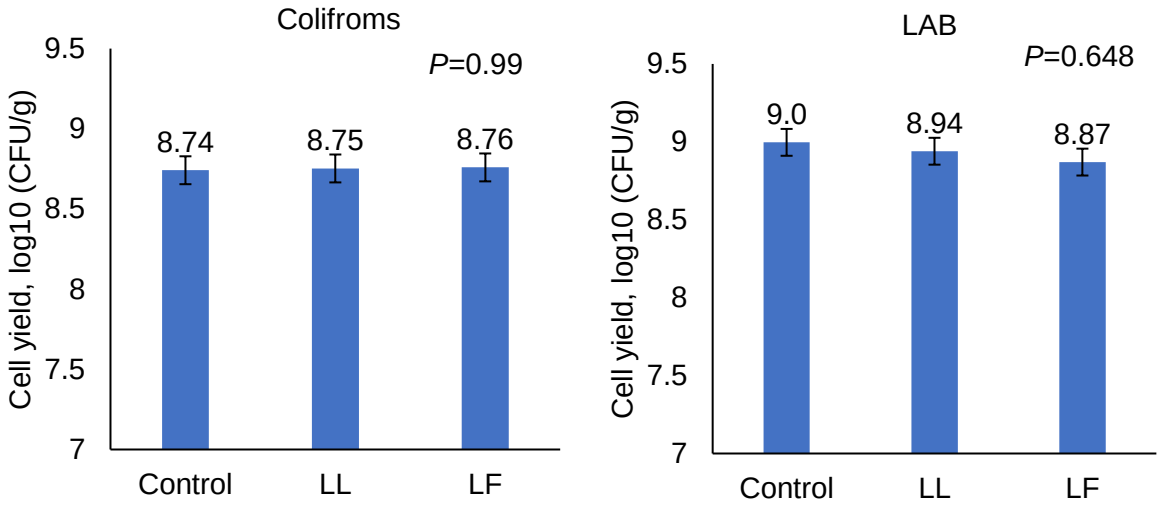


Figure 6.1 Population density of total coliforms and lactic acid bacteria (LAB) in caecal digesta taken from chickens from day 3 to 28 of age.

LL: *Lactococcus lactis*; LF: *Lactobacillus fermentum*

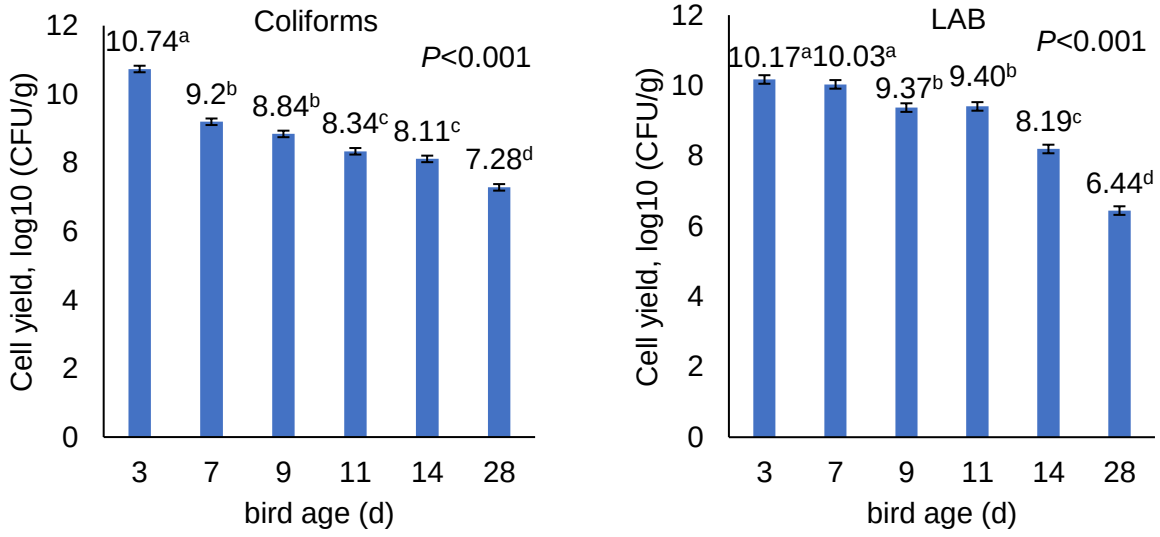


Figure 6.2 Effect of bird age on the population density of coliforms and lactic acid bacteria (LAB).

Values with different superscripts (a-d) are significantly different ($P < 0.05$).

6.3.2 Antimicrobial resistance of *E. coli* in caecal digesta

Figure 6.3 illustrates the prevalence of antimicrobial resistance in *E. coli* isolated from the birds' caeca. There was no significant effect of administering either LL or LF on the proportion of *E. coli* that were resistant to different antimicrobials. Little resistance to nalidixic acid or chloramphenicol was observed, but there was a high prevalence of resistance to both ampicillin and tetracycline. The percentage of antibiotic resistant *E. coli* based on bird's age is shown in Figure 6.4. This increased as birds aged from 7 d (60.43%) to 11 d (82.43%) (ampicillin, $P < 0.001$) or from 9 d (88.99%) to 11 d (98.82%) (tetracycline, $P=0.001$). Unlike the previous experiment, there was no decline in AMR by day 28. There was a high resistance to AMP and TET in *E. coli* isolates taken from both the caecum and yolk sac (seven samples) on day 1. A high resistance to AMP, TET and NA was also observed on day 3, but estimating the actual prevalence of AMR on days 1 and 3 was not possible because of the overgrowth of coliforms on the parent plates.

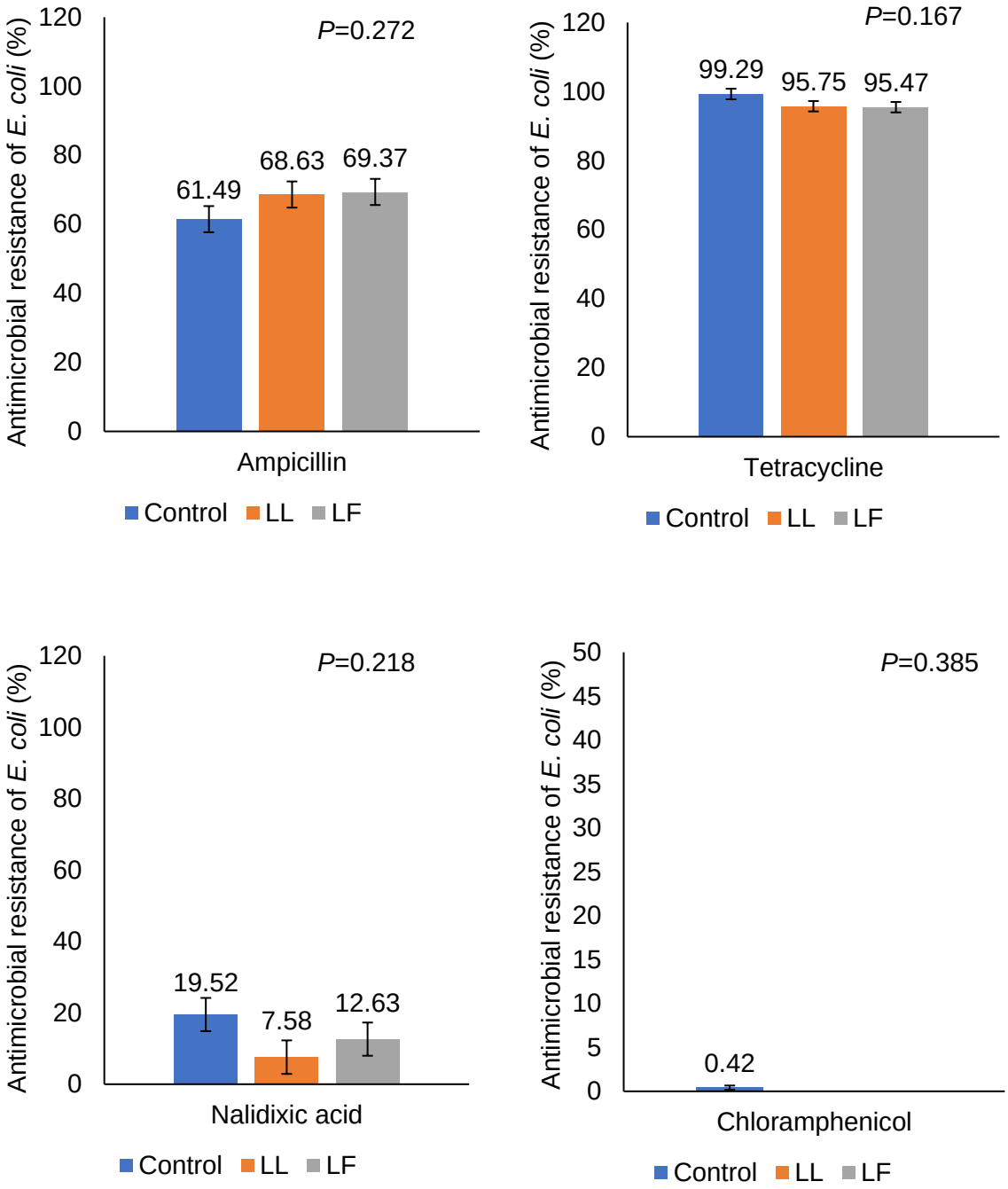


Figure 6.3 The effect of administration of either *Lactococcus lactis* (LL), *Lactobacillus fermentum* (LF) on the proportion of isolates of E. coli that were resistant to different antimicrobials (20 µg/ml).

LL: *Lactococcus lactis*; LF: *Lactobacillus fermentum*

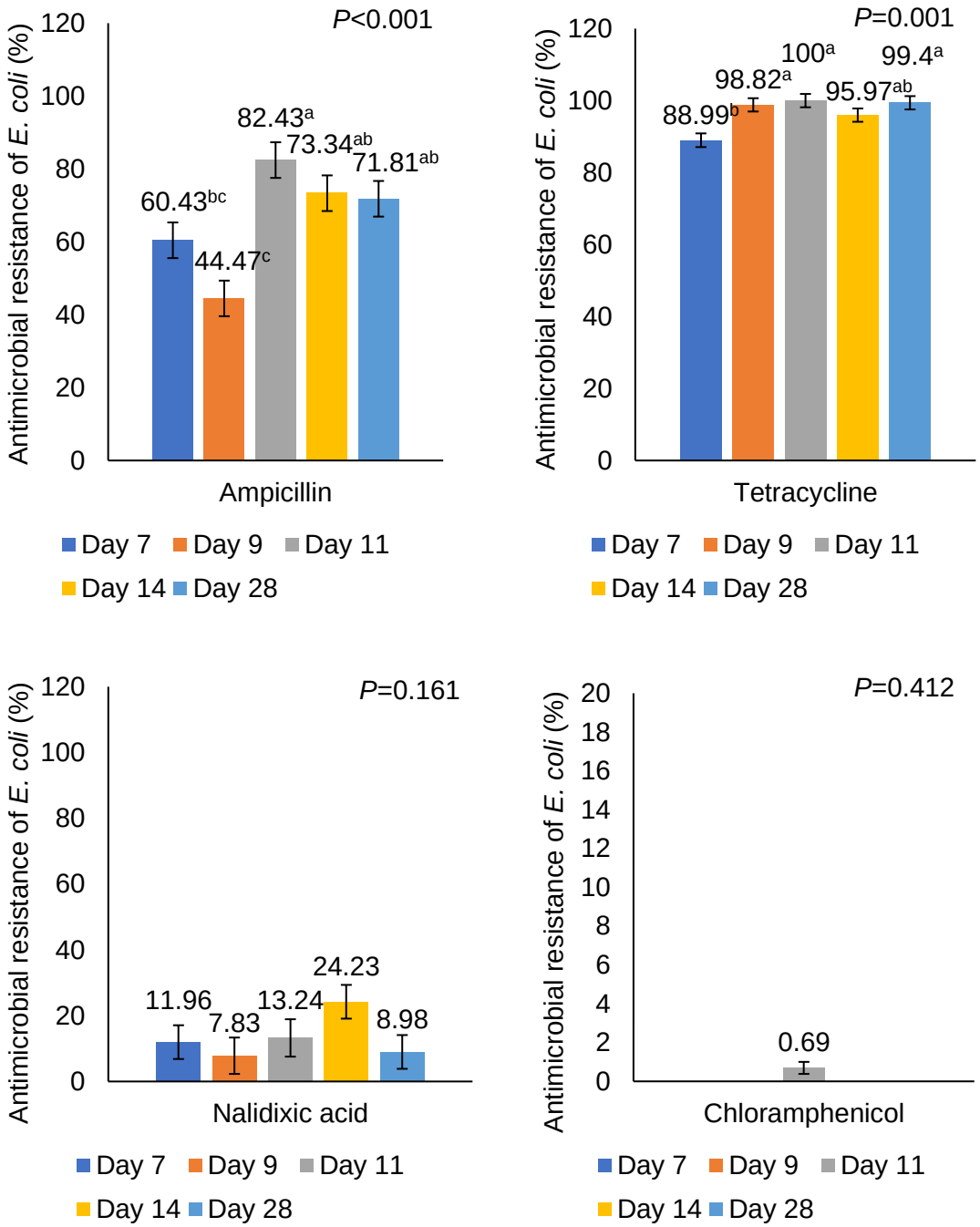


Figure 6.4 The percentage of *E. coli* isolates taken from birds of different ages (days of life) that were resistant to different antimicrobials.

Values with different superscripts (a-c) are significantly different ($P < 0.05$).

6.3.3 The antibiotic resistant genes and identification of *E. coli* by whole genome sequencing (WGS)

The genes coding for resistance to tetracycline in *E. coli* isolates by bird age is presented in Figure 6.5. The presence of multi drug transporter gene (mdfA1) was detected in all *E. coli* isolates. The presence of tetracycline resistant genes was detected in all samples on day 14 but this declined on day 28 (X^2= 25.46, $P < 0.001$). However, it is possible that some ARG were not detected by WGS because the extraction of DNA may not have included all plasmid DNA. Two genes coding for resistance to β-lactams (day 3), one to aminoglycoside (day 1), colistin (day 3), sulphonamide (day 1) and fosfomycin (day 3) were detected throughout the study. The administration of either LL (23.53%) or LF (22.22%) decreased the prevalence of tetracycline resistance genes compared to control (55.56%) (X^2= 5.67, P=0.059, Figure 6.6). The carriage of the *tet* gene in *E. coli* isolates was detectable but few other antibiotic resistant genes were detected by WGS. It is difficult to explain the reason of this result, but it might be related to a loss of plasmids or damage of DNA in the process of DNA extraction, or of DNA dilution for WGS analysis.

Figure 6.7 illustrates the multilocus sequence typing (MLST) of *E. coli* by bird age. A total of 11 different MLST were identified, with a further 7/59 isolates being unknown. At 9 and 11 d of age, most isolates were MLST 457, and this was the most common type (29% of isolates) throughout the study and was isolated from birds throughout their life. The second most common MLST was 1640, and it is possible that another four unidentified MLST (NT) on day 14 might be MLST 1640 as they were of the same serotype and all carried the tet gene. Greatest diversity was observed during the starter phase and new MLST were detected when birds were 28 d old (X^2= 122.40, $P < 0.001$). The association between the carriage of

tetracycline resistant gene and MLST is shown in Figure 6.8. MLST 1485 (eight out of nine *E. coli* isolates) and 1640 (four of 10 *E. coli* isolates) harboured the tet gene and this gene was mostly observed in the starter phase (X^2= 9.44, *P*=0.024).

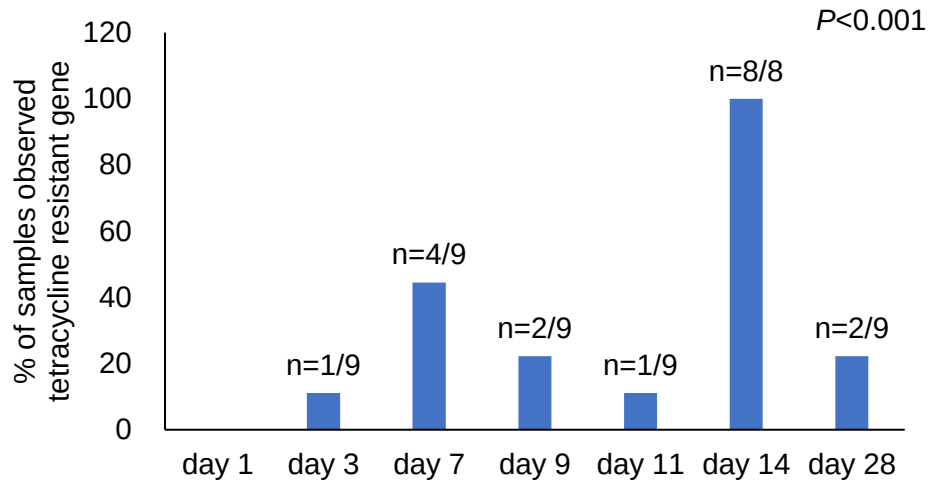


Figure 6.5 The effect of bird age on the carriage of tetracycline resistance genes by isolates of *E. coli* taken from the caecum of broiler chickens.

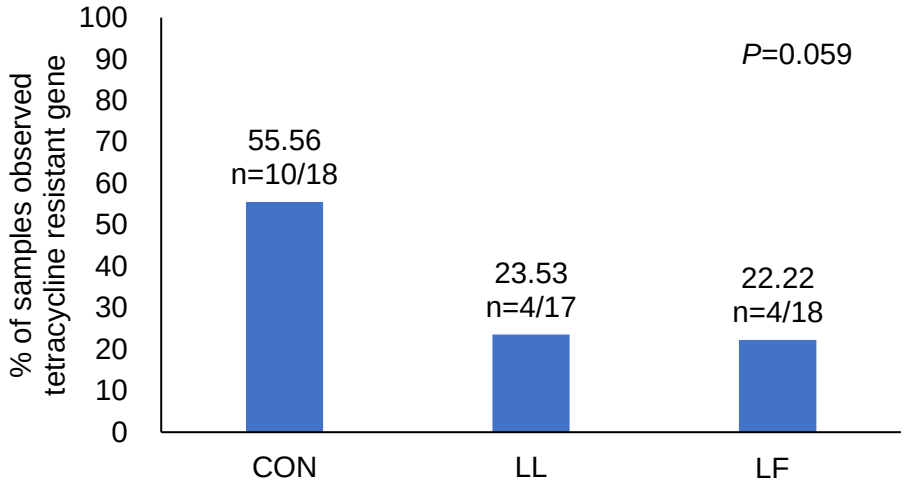


Figure 6.6 The effect of administering either *L. lactis* (LL) or *Lactobacillus fermentum* (LF) to broiler chickens on the carriage of tetracycline resistance genes by isolates of *E. coli* taken from the bird's caecum.

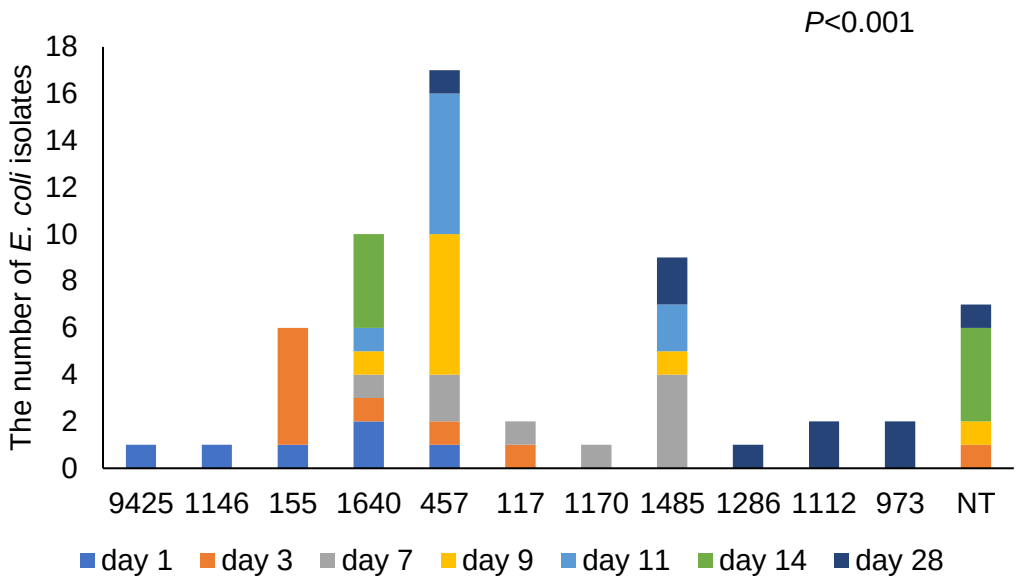


Figure 6.7 The effect of bird age (days of age) on the mutilocus sequence typing (MLST) identification of the *E. coli* isolate taken from the birds' caecum.

NT: MLST not identified

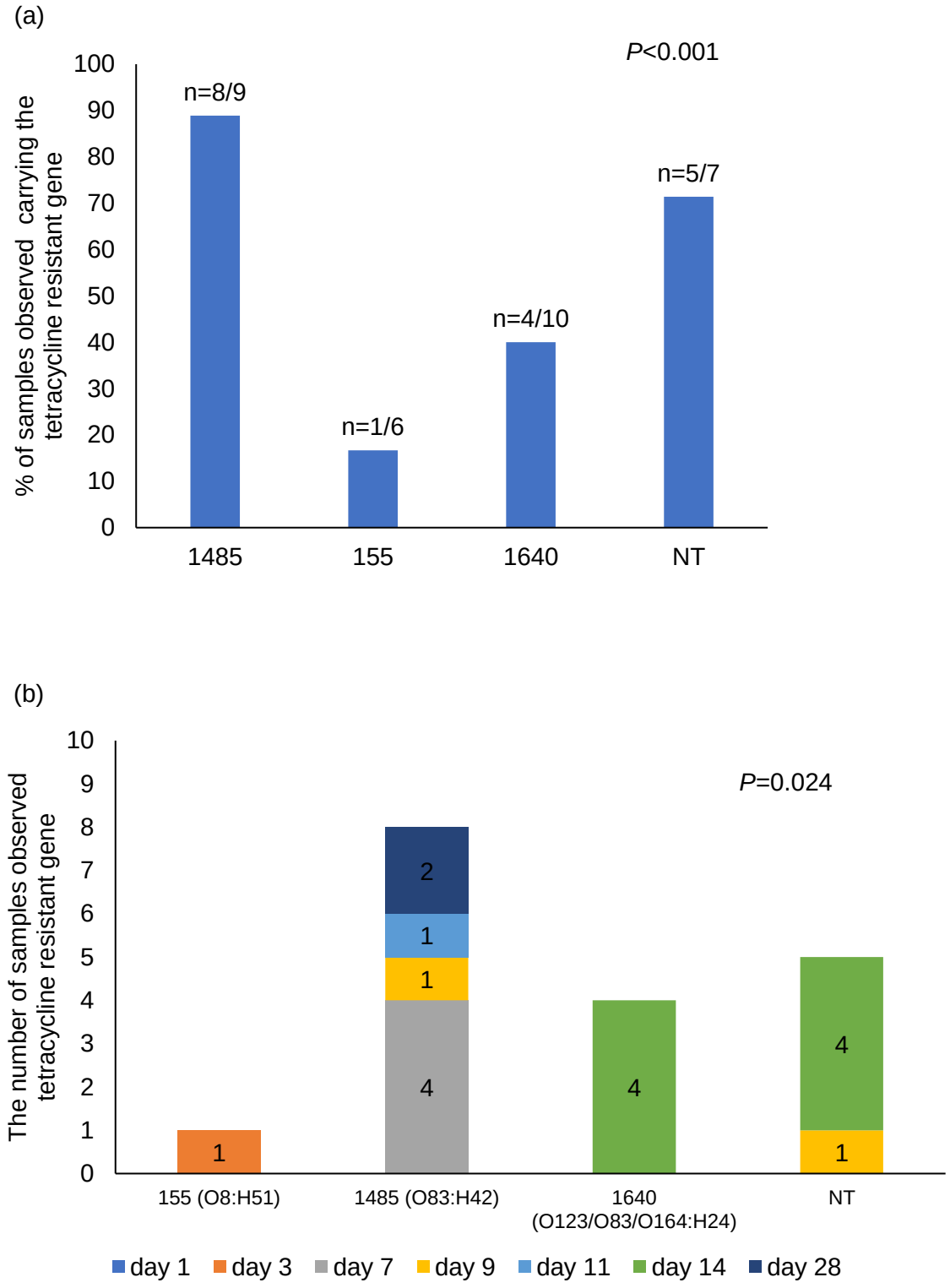


Figure 6.8 (a) The number of tetracycline resistant gene by MLST, (b) the association between the tetracycline resistant gene and MLST and bird age (days of age).

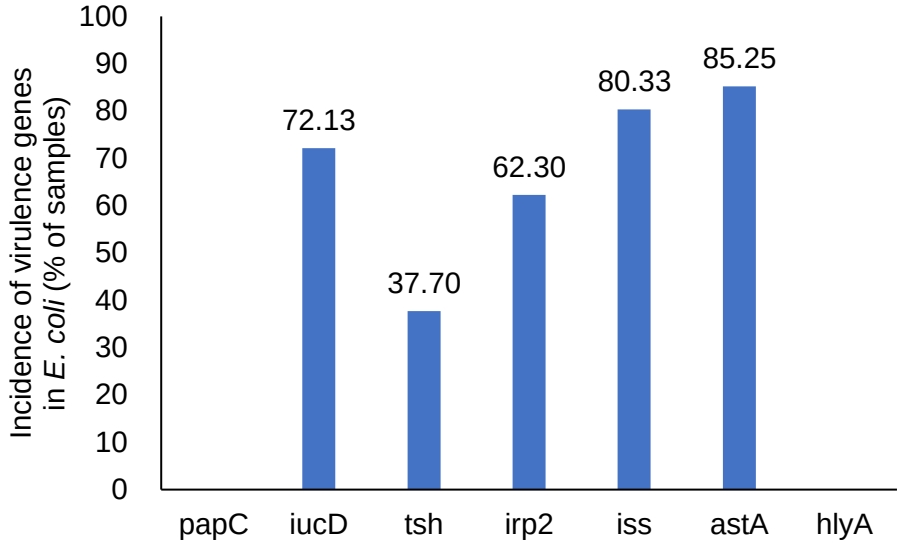
NT: MLST not identified.

6.3.4 *E. coli* virulence and its association with AMR

The proportion of virulence-associated genes in *E. coli* isolates detected by PCR and WGS analysis is presented in Figure 6.9. The proportion of isolates identified as carrying the different genes were: *iuc*D (72.13%), *tsh* (37.7%), *irp*2 (62.3%), *iss* (80.33%) and *ast*A (85.25%) with no effect of treatment. None of the isolates carried the *pap*C or *hly*A as determined by PCR. In the WGS analysis, there were many and various virulence genes identified in the *E. coli* isolates but the presence/absence of 11 virulence genes was determined. The percentage of isolates identified as carrying different genes were: *iuc*D (74.58%), *tsh* (1.69%), *irp*2 (57.63%), *ast*A (66.1%), *iut*A (77.97%) and *iro*N (84.75%). None of the isolates carried the *pap*C, *iss*, *hly*A, *hly*F or *omp*T genes. The effect of bird age on the carriage of virulence-associated genes by *E. coli* isolates, by PCR and WGS analysis, is shown in Figure 6.10. There was a low prevalence of virulence in *E. coli* isolates from the yolk sac. One *E. coli* isolate from yolk sac possessed *iuc*D and *ast*A genes and two *E. coli* isolates from yolk sac harboured the *iss* gene (out of seven yolk sac isolates). The other four isolates carried none of the virulence genes analysed by PCR. In the WGS analysis, two isolates from the yolk sac possessed the *iro*N gene and another isolate carried the *iuc*D and *iut*A genes. The prevalence of virulence genes (*iuc*D, *tsh*, *irp*2 and *iss*) except for *ast*A decreased to 33.33%, 22.22%, 11.11% and 44.44% respectively when birds were 28 days of age (by PCR analysis, P=0.014, $P < 0.001$, $P < 0.001$, P=0.001, P=0.001 respectively). Likewise, the virulence genes (*iuc*D, *irp*2, *ast*A, *iut*A and *iro*N) by WGS analysis declined to 33.33%, 11.11%, 33.33%, 33.33% and 44.44% respectively when the birds were 28 d old (P=0.022, P=0.002, P=0.008, P=0.006, and $P < 0.001$ respectively). The association between the virulence genes and MLST is presented in Figure 6.11**.**

MLST 457, 1640, 1485 and 155 possessed mainly *iuc*D, *irp*2, *ast*A, *iut*A and *iro*N during the starter phase. The Figure 6.12 illustrates the association with phenotypic antimicrobial resistance and virulence genes of *E. coli* by PCR. The carriage of the *irp2* gene was more associated with Amp resistant *E. coli* rather than ampicillin sensitive *E. coli* (67.86% and 0% respectively, X^2=9.00, *P*=0.003). *E. coli* resistance to NA was associated with the carriage of the genes *iuc*D (86.96%, X^2=4.04, *P*=0.045), *tsh* (65.22%, X^2=11.90, *P*=0.001), *iss* (95.65%, X^2=5.49, *P*=0.019) and *ast*A (95.65%, X^2=3.18, *P*=0.075) (Figure 6.13). The Figure 6.14 shows the association between the carriage of the tetracycline resistance gene and virulence genes by WGS. The tetracycline resistance gene was associated with the carriage of the genes *iuc*D (100%, X^2=8.83, *P*=0.003), *iut*A (100%, X^2=7.32, *P*=0.007), *iro*N (100%, X^2=4.66, *P*=0.031) and *ast*A (94.44%X^2=9.29, *P*=0.002) by *E. coli* as determined by WGS.

(a)



(b)

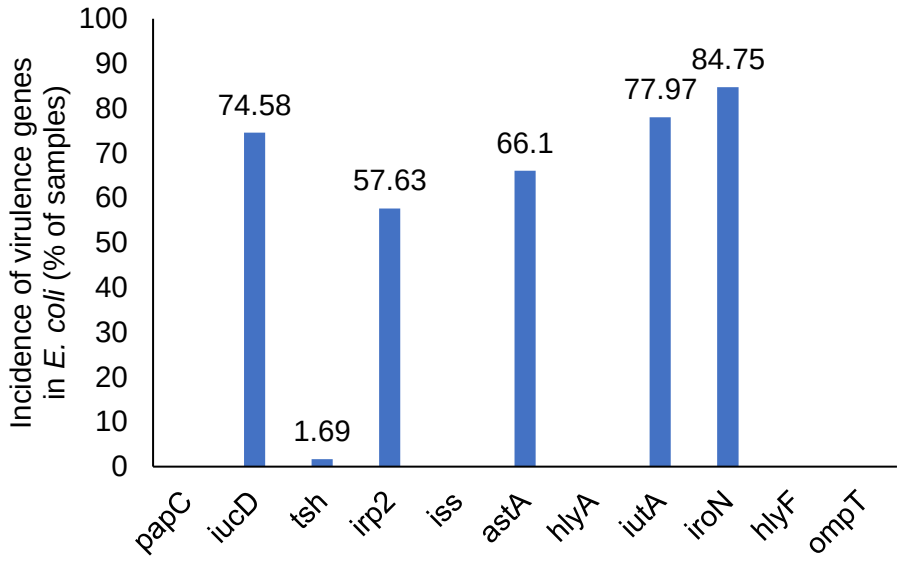


Figure 6.9 The proportion of virulence-associated genes in *E. coli* isolates taken from the caecal digesta of birds analysed by (a) PCR and (b) WGS.

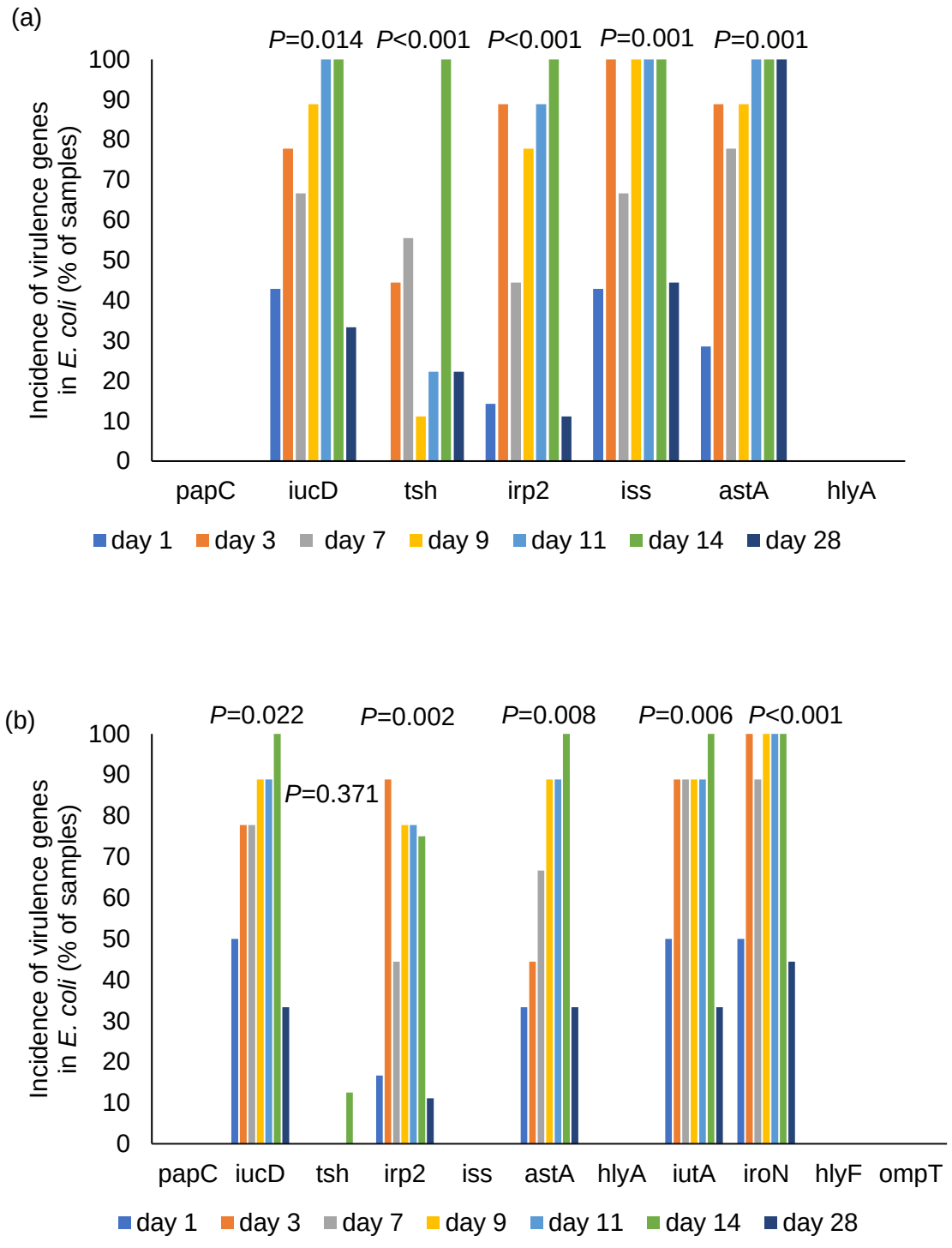


Figure 6.10 The effect of bird age on the carriage of virulence-associated genes in isolates of *E. coli* by (a) PCR and (b) WGS.

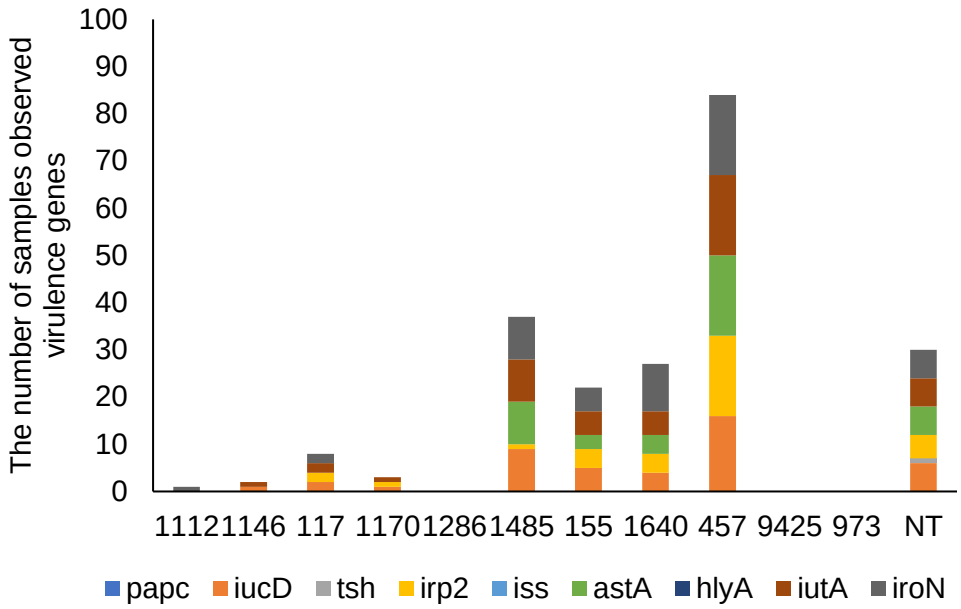


Figure 6.11 The association between MLST and virulence genes in *E. coli* isolates.

NT: MLST not identified.

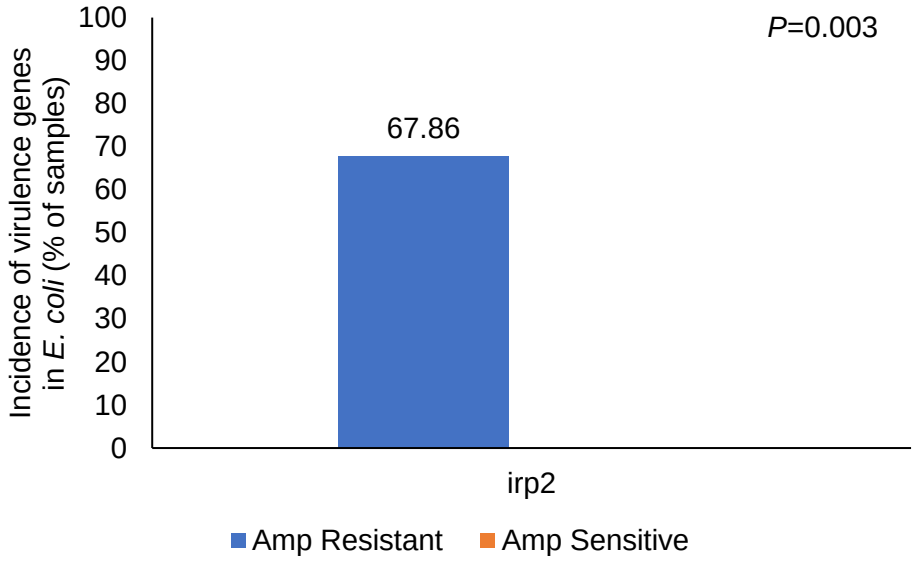


Figure 6.12 The association between the carriage of the *irp*2 gene and the phenotypic sensitivity or resistance by isolates of *E. coli* to ampicillin (20 µg/ml).

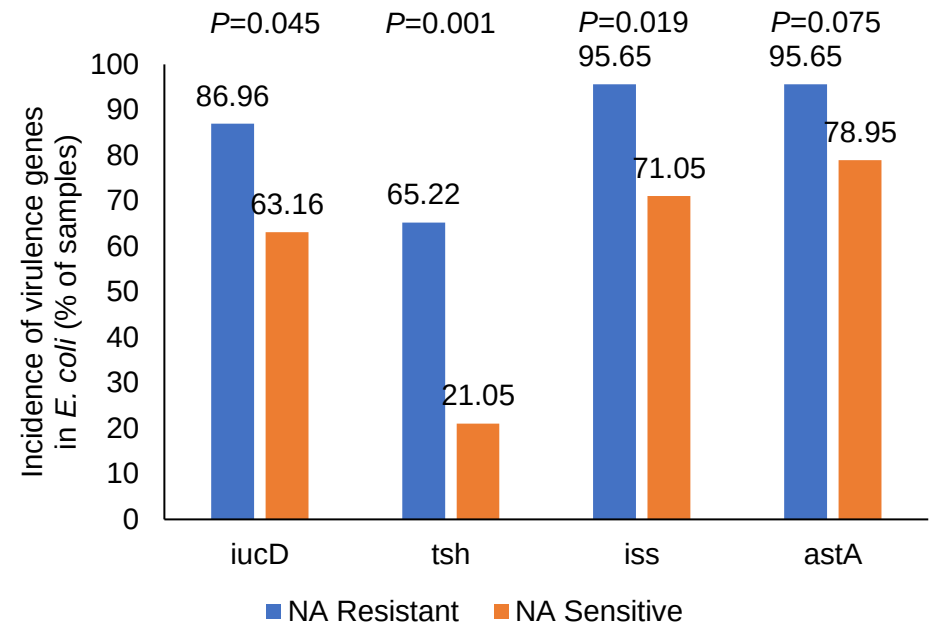


Figure 6.13 The association between the carriage of the *iuc*D, *tsh*, *iss* and *ast*A gene and the phenotypic sensitivity or resistance by isolates of *E. coli* to nalidixic acid (20 µg/ml).

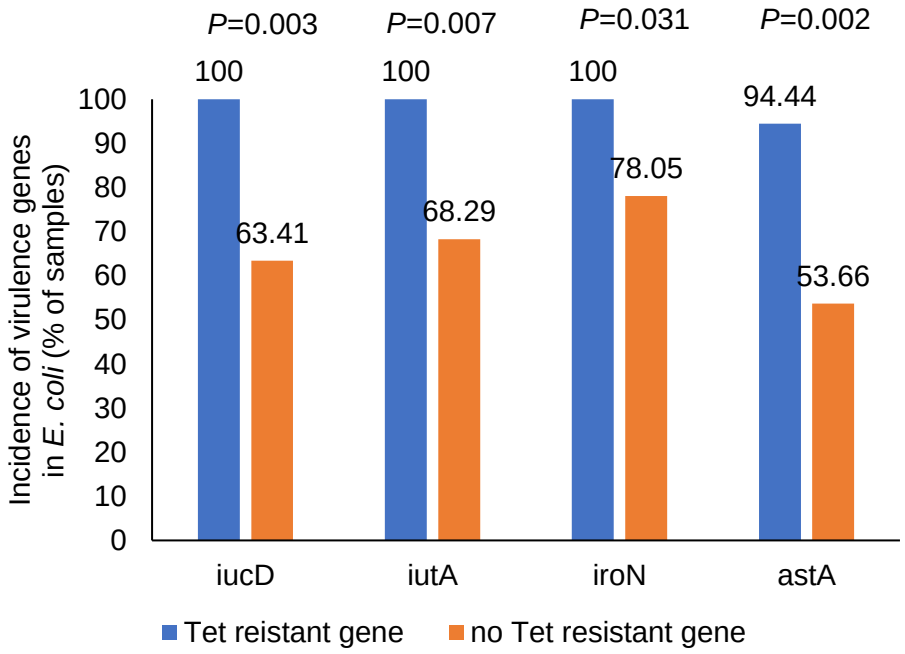


Figure 6.14 The genotypic association between the carriage of the *iuc*D, *iut*A, *iro*N and *ast*A gene and the tetracycline resistance gene in *E. coli* isolates by WGS.

6.4 Discussion

6.4.1 Growth performance and microbial population density

As discussed in Chapter 4, starch and oil data from Sciantec may have been incorrect.

The benefits and potential of lactic acid bacteria have been demonstrated *in vitro* for biological activity such as antimicrobial activity, and *in vivo* for improvement of immunity and effect on growth performance and animal health (Kabir, 2009; Musikasang *et al.*, 2009; Reis *et al.*, 2012; Reuben *et al.*, 2019). Murry *et al.* (2006) reported that administering 0.1% botanical probiotics (Feed Free^{TM}) containing *L. plantarum* and *L. salivarius* reduced feed intake and FCR from 21 to 42 days of bird age. The administration of beneficial bacteria increased the population of lactic acid bacteria and decreased the *Clostridium perfringens* population in the cloaca. Khan *et al.* (2011) observed that the administration of either the commercial probiotics protexin or biovet or yogurt via drinking water increased body weight and reduced FCR at days 28 and 39 of age. In addition, the cholesterol contents were reduced, and immunity was improved for broilers. However, in contrast to these findings, Olnood *et al.* (2015) reported that there was no significant effect of administering beneficial microbes on body weight gain, feed intake and FCR during the 6 weeks of bird life. However, the population of *Enterobacteria* in the caeca decreased on day 35 when either unidentified *Lactobacillus* sp. or *L. salivarius* or *L. johnsonii* was administered.

In this study, the administration of either *Lactococcus lactis* or *L. fermentum* during the starter phase had no effect on bird performance. These isolates had both demonstrated antibacterial activity *in vitro*, and so the lack of effect *in vivo* may

reflect the much more complex environment of the gut. These bacteria were not administered every day, and this may also have contributed to their lack of effect. The evaluation of beneficial bacteria efficacy for bird performance and gut microbiome is very dependent on factors such as the bacteria strain used, the method and frequency of administration, and the farm environment (Patterson and Burkholder, 2003; Khan *et al.*, 2011).

6.4.2 Phenotypic antimicrobial resistance (AMR) of *E. coli*

In this study, the high resistance to ampicillin and tetracycline was maintained throughout the birds' life. These results reflect those of Bezerra *et al.* (2016) who also found that all *E. coli* isolated from cloacal samples were resistant to trimethoprim-sulfamethoxazole and 95.4% to tetracycline, 91.4% to ciprofloxacin and 87.3% to ampicillin by disc diffusion method with birds of preslaughter age (35 to 38 days). Miles *et al.* (2006) reported that *E. coli* isolates from excreta samples of birds had a high frequency of resistance to tetracycline (82.4%), kanamycin (91.2%) and nalidixic acid (85.3%) in birds 42 days of age despite no exposure to antibiotics during the experimental period.

The high prevalence of tetracycline resistance was possibly associated with the prevalence of multi drug resistance genes (Johnson *et al.*, 2012; Szmolka and Nagy, 2013). Another possible explanation for this is that resistance genes are encoded with mobile genetic elements, such as plasmids (Wall *et al.*, 2016; Reygaert, 2018). These may confer resistance to antibiotic sensitive bacteria by conjugation, transformation and transduction, but also confer other selective advantages (Messaili *et al.*, 2019). The unexpected results might also be due to the fact that

these birds were ill as indicated by the high FCR (1.60-1.68) and low growth (60 g/d). The FCR of Ross 308 broiler birds from 14 d to 28 d would be expected to increase from 1.04 to 1.32, and birds between 14 and 28 d of age should grow at about 80 g/d (Aviagen, 2019). The poor growth and FCR observed in this study might indicate a subclinical infection (no clinical signs were observed) and this might have made the birds more susceptible to AMR infection and unable to demonstrate the reduction in AMR prevalence in caecal *E. coli* that was observed in the earlier experiments.

As observed, this finding is contrary to Chapter 3 and Chapter 4 as well as Diarra *et al.* (2007) and Roth *et al.* (2017) which showed a low prevalence of AMR as birds aged. These results may reflect a health issue affecting the birds in this study, but not the earlier experiments. It may also reflect the change in the way that phenotypic AMR prevalence was assessed. In this study, the phenotypic AMR test was assessed by replicating plating of all cultured coliforms while a single isolate was used for AMR by the disc diffusion assay or streaking on a plate with antibiotics in Chapter 3 and Chapter 4 respectively. Replicate plating for testing AMR provides a more robust estimate of the prevalence of resistance, but care is needed when conducting it to prevent contamination. The plates need to be completely dried and rigorous aseptic technique is required. The results of this study do not support the hypothesis that LAB administered during the starter phase might reduce the prevalence of AMR *E. coli* in the starter (and grower) period. Since AMR *E. coli* persisted throughout the study, this might suggest that the birds were challenged in some way, but also that the selective advantage of the AMR coliforms persisted in the caecum throughout the birds' life.

6.4.3 Genotypic antimicrobial resistance and multilocus sequence typing (MLST) of *E. coli* by WGS

One of the purposes of whole genome sequencing is to identify clonality of the strains and MLST and deduced O antigen type. Relating these data to the presence or absence of AMR features is informative and helpful for surveillance (Larsen *et al.*, 2012; Tyson *et al.*, 2018). Tyson *et al.* (2015) confirmed genotypic antimicrobial resistance in *E. coli* isolates from cattle by WGS. Frequently identified resistance genes were the sulfisoxazole resistance gene (sul2, 78.9%), streptomycin resistance genes (strA, strB, 75%), tetracycline resistance gene (tet(A), 71.1%) and chloramphenicol resistance gene (floR, 68.4%). In this study, a tetracycline resistance gene was identified in most *E. coli* isolates particularly when birds were 14 days of age. On the other hand, resistance to different classes of antimicrobials was rarely observed by WGS analysis. It is probable that extraction of DNA failed to give sufficient plasmid numbers for reliable sequencing. As most AMR are encoded by plasmids, this would result in an underestimate of overall AMR content in each isolate. While taking this potential loss of genetic material into consideration, it was notable that (unlike the phenotypic assessment) the administration of either LL or LF was associated (P=0.059) with a reduction in the carriage of tetracycline resistant genes. This finding should be treated with some caution. It was based on a single isolate of *E. coli* taken from each bird, and that isolate may not have been representative of the population. In addition, it is not clear why the lactic acid or any other compound secreted by the LAB inhibited the growth of coliforms carrying tet genes, but not other coliforms. However, if confirmed, this would be a great benefit of using these lactic acid bacteria to control the spread of tetracycline resistance.

The greatest diversity of *E. coli* strains was associated with bird age. MLST 457 (O11:H15) was the commonest type that was identified, followed by MLST 1640 (O124/O164:H25) and 1485 (O83:H42) particularly during the starter phase. Interestingly, the carriage of tetracycline resistant genes was significantly associated with MLST 1485 and 1640 during the starter phase, but only two MLST 1485 carried the tetracycline resistant gene in the grower phase. Many MLST which had contained resistance genes to tetracycline decreased in the grower phase, while new MLST which did not carry the tetracycline resistant gene appeared. Resistance to tetracycline in *E. coli* isolates was replaced by predominantly susceptible isolates at 28 day of bird age. At a genotypic level, therefore, the reduction in antimicrobial resistance which had been observed in earlier experiments was observed again. The evolution of *E. coli* strain was evidenced in that on day 28, three (two MLST 1485 and one 457) out of nine isolates were of the same type as in the starter phase but the other isolates were new (two MLST 1112 (O86:H27), MLST 973 (O11:H15), MLST 1286 (O16:H32), MLST 973 (O11:H15), NT (-:H11)).

It is worthwhile comparing the phenotypic and genotypic AMR to evaluate the accuracy of WGS for AMR prediction and comparison. Tyson *et al.* (2015) compared phenotypic and genotypic AMR in *E. coli* isolates taken from the caecum of cattle. There was an inconsistency in the assessment of resistance to streptomycin, with 81.3% of isolates carrying at least one ARG showing phenotypic resistance to streptomycin. However, phenotype and genotype were correlated for resistance to tetracyclines, quinolones and phenicols. This finding was also reported by Guo *et al.* (2019), who observed an association between phenotypic and genotypic AMR to ampicillin, chloramphenicol, trimethoprim and tetracycline. However, no such

association was observed for the resistance to colistin. In this study, there was an inconsistency between phenotypic and genotypic AMR, probably resulting from the loss of plasmids (with their associated genes) during either DNA extraction or the experimental process of WGS. This suggests that genotypic characterisation is more difficult and an elaborate skill is needed because of an inability to sequence low gene frequencies, or issues with mutations and technical difficulties associated with the technique. However, if genotypic AMR (by WGS analysis) is observed then this can be considered an accurate observation and provides reliable data for AMR prediction.

Little is known about the association between the carriage of antibiotic resistant genes and MLST in commensal *E. coli* isolates as bird age. In this study, MLST 973, 1112, 1286 did not carry any ARG and carried ≤1 VAG on day 28, and these could be identified as commensal and benign *E. coli*. It could be hypothesised that encouraging the proliferation of these MLST may reduce the prevalence of AMR. MLST 1485, 1640 and 155, on the other hand, all carried the tet gene. However, the data presented in Figure 5.8 demonstrate that a particular serotype may or may not carry the tet (or other ARG and VAG). This would indicate that these genes are not chromosomal, but rather carried on mobile genetic elements (such as plasmids) and so will be carried by some coliforms of a particular serotype while others (of the same serotype) do not carry these genes.

Many studies have demonstrated the association between certain MLST *E. coli* strains and poultry colibacillosis (Ewers *et al.*, 2009; Gregersen *et al.*, 2010; Papoušková and Čížek, 2020). Pires-dos-Santos *et al.* (2013) reported that ST 95 was mostly observed in Extra-intestinal pathogenic *Escherichia coli* (ExPEC) isolates in both human and poultry infections. This was followed by ST 131, ST 420

and ST 648 which were related to urinary tract infections (UTI) in humans. ST 69, ST73, ST95, ST131, ST648 are well known in public health, associated with a high prevalence of AMR and virulence genes (Dale and Woodford, 2015; Papoušková and Čížek, 2020). Colibacillosis observed in chickens aged between three and ten weeks was associated with the O1, O2 and O78 serogroup of *E. coli* (Ngeleka *et al.*, 2002; Dziva and Stevens, 2008; Hammoudi and Aggad, 2008). In the present study, these serotypes and serogroups were not observed, but rather MLST 457, 1640, 1485, and 155 were common during the starter phase and these possessed a high prevalence of *iuc*D, *irp*2, *ast*A, *iut*A and *iro*N genes (as determined by WGS). This serves to demonstrate the diversity that exists in pathogenic *E. coli* that may infect the chicken.

6.4.4 *E. coli* virulence and its association with AMR

The pathotype of *E. coli* strains can be determined by the variety of virulence associated genes they carry. However, the determination of what characterises APEC is a matter of debate (Josenhans and Suerbaum, 2002; Cross, 2008). As discussed in Chapter 4, Johnson *et al.* (2008) identified as minimal predictors of APEC the carriage of *iut*A, *hly*F, *iss*, *iro*N and *omp*T genes. In the present study, the carriage of seven virulence genes (*pap*C, *iuc*D, *tsh*, *irp*2, *iss*, *ast*A and *hly*A) were determined by PCR, which obviously did not include the determination of *iut*A, *hly*F, *iro*N and *omp*T which were identified by Johnson *et al.* (2008). However, the *iuc*D and *irp*2 genes are associated with the chelation, uptake and sequestration of iron, while *tsh* agglutinates erythrocytes and so all would be involved in similar functions to the *iut*A and *iro*N genes identified as characteristically APEC by Johnson *et al.* (2008). Of the seven genes determined by PCR in this study, only five (*tsh*, *iuc*D,

*irp*2, *iss* and *ast*A) were observed in these isolates. However, in WGS analysis, a large number of potential virulence genes were identified. Eleven were focussed on; the basis for this being that they included the seven that had been characterised by PCR and the additional minimal APEC predictors (*iut*A, *hly*F, *iro*N and *omp*T, Johnson *et al.* (2008)). Both PCR and WGS analysis confirmed that genes associated with iron uptake and sequestration were very prevalent in *E. coli* isolates. This would suggest that these characteristics confer a clear selective advantage to strains carrying these genes. The fact that the carriage of many of these genes is associated with resistance to ampicillin and other antibiotics is problematic, not least that the control of such virulent strains may not be controlled by antibiotic therapy. Young chickens are particularly susceptible to APEC, but the declining prevalence of virulence factors in *E. coli* by day 28 might indicate that, as observed in the earlier experiment, commensal *E. coli* might proliferate and outcompete the APEC strains as the birds got older. This finding supports the hypothesis that the prevalence of putative pathogenic coliforms decrease as birds age.

Little is known about the interaction between dietary intervention, management and bird age on the prevalence of virulence associated genes in *E. coli* isolates from the caecum in health poultry. Many studies have instead researched the comparison of virulence associated genes in *E. coli* isolates taken from either healthy chickens or those with colibacillosis (McPeake *et al.*, 2005; Paixao *et al.*, 2016). Unsurprisingly, it was demonstrated that various and specific virulence associated genes were more prevalent in pathogenic *E. coli* compared with commensal *E. coli* (Delicato *et al.*, 2003; Ghanbarpour *et al.*, 2011; Messaili *et al.*, 2019). Delicato *et al.* (2003) observed that the *csga* and *fim*H genes had the highest frequency in *E. coli* from both healthy and colibacillosis chickens. However, seven virulence genes (*iut*A, *tsh*,

iss, *cva*C, *pap*C, *pap*G and *fel*A) were observed more frequently in isolates from colibacillosis birds compared with healthy chickens. Ghanbarpour *et al.* (2011) discovered that the virulence genes *crl* and *fim*H were the most frequently observed virulence genes in both colisepticemic and healthy broilers, but that the combination of *crl, aer* and *fim*H was characteristic in colisepticemic birds. The *aer* gene was detected in 66.0 % of APEC and 24.1% of AFEC (Avian Faecal *Escherichia coli*) isolates, while the frequency of the *pap*C gene was 34.0% in APEC isolates and only 2.7% in AFEC isolates.

According to the studies referred to above, the *pap*C gene was mainly confined to APEC isolates, even though the frequency of the *pap*C gene was lower than other virulence genes (Delicato *et al.*, 2003; Ghanbarpour *et al.*, 2011). In this study, neither *pap*C nor *hly*A was detected in any *E. coli* isolates. In addition, no isolates carried the 'minimal predictor' set of APEC genes (*iut*A, *hly*F, *iss*, *iro*N and *omp*T) according to the WGS analysis. It could therefore be concluded that in this study there were no APEC isolated from the caecum of any of the birds, and indeed there was no evidence that any of the birds suffered from colibacillosis. There were, however, a number of isolates that carried four or more virulence genes and which may therefore have the potential to be pathogenic to the bird, but the combinations of virulence genes carried were different from those identified in the papers referred to above.

The relationship and association between antimicrobial resistance and virulence genes of *E. coli* is complex and has not been clearly explained. However, there has been increasing interest in this issue. This study showed that phenotypic resistance to AMP was associated with the carriage of the *irp*2 gene while resistance to NA was associated with the carriage of *iuc*D, *tsh*, *iss* and *ast*A. Unfortunately, it was not

possible to investigate similar associations between phenotypic resistance to TET and CHL. This was because in the case of TET, all the isolates were resistant, whereas in the case of CHL, all isolates were sensitive. In WGS analysis, the tetracycline resistance gene was correlated with *iuc*D, *iut*A, *iro*N and *ast*A. These findings provide possible insights into the selective advantage that AMR strains have, even in situations where the *E. coli* is not exposed to antibiotics.

Similarly, other studies have demonstrated an association between AMR and virulence genes in *E. coli*. The virulence genes *cva*C, *iss*, *iut*A and *tra*T were associated with phenotypic resistance to cefoxitin, gentamycin, kanamycin, streptomycin, tetracycline, ceftiofur and were linked with CoIV plasmids, which generally encode APEC (Johnson *et al.*, 2006a; Johnson *et al.*, 2006b). Messaili *et al.* (2019) reported that resistance to AMC (amoxicillin, a penicillin) was associated with *cva*B5' genes and TET resistance was correlated with the *tra*T gene in faecal *E. coli* samples from broilers. As discussed in Chapter 4, Johnson *et al.* (2012) identified that resistance to CHL (chloramphenicol) was correlated with both *iha* and *pap*; resistance to AMP was associated with *afa*, *iha* and *iut*A; and trimethoprim/sulfamethoxazole (SXT) resistance was associated with *afa* and *iut*A. Johnson *et al.* (2012) suggested that multidrug resistance was associated with plasmids and these plasmids were found among APEC isolates. In addition, multidrug resistance by *E. coli* was associated with the carriage of a range of virulence associated factors (Johnson *et al.*, 2012).

As discussed in Chapters 3 and 4, genes associated with iron uptake and sequestration might be more associated with AMR in *E. coli* isolates. Iron acquisition systems are stimulated by low concentrations of iron in the medium to enable microorganisms with these systems to accumulate iron. Genes associated with iron

uptake were highly prevalent in APEC isolates (Paixao *et al.*, 2016). The association between genes coding for iron uptake and those coding for multidrug resistance might be via linkage in mobile genetic material such as plasmids. It is possible that increasing the concentration of iron in the gut environment may be a means of preventing the proliferation of pathogenic (and by association, potentially AMR) coliforms.

6.5 Conclusion

There was no evidence in this study that the administration of lactic acid bacteria affected either growth performance or the population of coliforms and lactic acid bacteria in the caecum of broilers. Unlike earlier studies, the high (phenotypic) resistance to ampicillin and tetracycline by *E. coli* was maintained throughout the birds' life. However, genotypic tetracycline resistance in *E. coli* isolates was highest on day 14 but then decreased on 28 day of bird age. In addition, administering either LL or LF was associated with a reduction in the carriage of the *tet* (tetracycline resistant) gene, even though no such effect was observed on phenotypic resistance to TET. The prevalence of virulence genes (*iuc*D, *tsh*, *irp*2, *iss*) increased during the starter phase but had decreased when the birds were 28 days of age. In WGS analysis, a high prevalence of *iuc*D, *irp*2, *ast*A, *iut*A and *iro*N was observed in younger birds, but again these genes declined by day 28. MLST 457, 1640, 1485, and 155 were mostly detected in early bird life, and these serotypes had a high prevalence of *iuc*D, *irp*2, *ast*A, *iut*A and *iro*N genes. Strategies to reduce the prevalence of these potential pathogens in the young bird should be developed.

As observed before, there was a strong association between the carriage of the *irp*2 gene and ampicillin resistant *E. coli.* The resistance to NA was correlated with the

carriage of *iuc*D, *tsh*, *iss* and *ast*A genes. In WGS analysis, the carriage of tetracycline resistance gene in isolates was associated with the *iuc*D, *iut*A, *iro*N and *ast*A genes. In agreement with the previous studies, the young chicks' susceptibility to AMR and pathogenic *E. coli* has been demonstrated again (if not the complete recovery from AMR *E. coli* by 28 d). If a high concentration of iron was added to the diet, such that the iron concentration in the caecal digesta were increased, this might remove the competitive advantage of these pathogenic (and AMR) *E. coli*. In addition, the improvement of the gut environment during the starter phase may enable the proliferation of commensal coliforms. This is a potential avenue for future research, but it should be noted that the development of the gut microbiota and control of AMR and pathogenic bacteria are affected by a range of complex factors. Any such intervention would therefore require considerable thought and research, and would need to be considered alongside the management of other stress and environmental factors.

Chapter 7. General discussion

This thesis set out to determine the effects of dietary intervention and bird age on the prevalence of AMR *E. coli*, and the association between AMR and the carriage of virulence associated genes by *E. coli* in the broiler caecum. There was no evidence of any real benefit from any of the interventions investigated on the prevalence of AMR. As discussed, this might suggest that the yeast and LAB inocula that were administered did not colonise and proliferate within the caecum. However, there may still be value in further investigating their potential, as the amounts and periods of administration were small and short, and if higher doses were used there may be more obvious effects. High populations of yeast have been observed to decrease the number of coliforms in the gut as coliforms attach to the yeast wall (Eshdat *et al.*, 1981; Bagg and Silverwood, 1986). It is also true that all birds remained healthy throughout. If there had been more of an infectious challenge, then perhaps possible benefits of these interventions may have been more apparent.

In this thesis, a greater understanding of the changes in *E. coli* characteristics (AMR and pathogenicity) as the host bird aged has been developed. This next section will conclude the study by summarising the significant AMR *E. coli* research findings in relation to the research aims and questions and discuss the value and contribution of these findings. It will also review the limitations of the study and propose opportunities for future research.

7.1 The association between bird age and prevalence of AMR *E. coli*

The prevalence of AMR *E. coli* was more sensitive to the effect of bird age than administering yeast and LAB. All the *in vivo* experiments conducted in this study demonstrated a high prevalence of phenotypic resistance to ampicillin and tetracycline by *E. coli* in the broiler caecum. This was apparent in very young birds, and on occasion even day-old chicks. The selective advantage of this AMR is unclear since no antibiotics were administered throughout the birds' life. This must indicate that AMR *E. coli* have some other selective advantage beyond the carriage of (metabolically expensive) antibiotic resistant genes. Interestingly, in most cases, AMR *E. coli* were replaced with antimicrobial susceptible *E. coli* as the gut environment developed as the bird got older. This would suggest that the gut of the older bird might favour more benign *E. coli*, but the particular conditions in the older bird's gut that allow this change remains unclear.

Contrary to this finding, phenotypic AMR *E. coli* persisted throughout the bird's life in the experiment reported in Chapter 6. The method of evaluation of phenotypic AMR differed in the three bird studies, and perhaps part of the reason for this different observation was because of changes in the experimental method of assessing AMR. The persistence of a high prevalence of phenotypic AMR throughout the later study would suggest that the conditions of this experiment conferred a selective advantage for AMR *E. coli.* It is unclear what that advantage was, and why it was not present in the earlier studies. The birds in the Chapter 6 study grew much slower (and were smaller at 28 d) than the birds in the previous two studies. This poor performance may be indicative of an imbalance in the diet, a subclinical infection or some other environmental stress. Whatever the cause was, it was also associated with a persistence in the AMR prevalence of the *E. coli* in the

caecum, and so that stress may have maintained the conditions in the gut that supported the selective advantage of AMR *E. coli*. With hindsight, it would have been beneficial to collect samples of the caecal microbiome and caecal digesta (from all studies) to determine whether there were differences in the composition of the digesta and microbiome that may be associated with this persistence of AMR prevalence.

In the genotypic assessment of AMR, it was noted that *E. coli* isolates carried resistance genes to AMP and TET as well as a range of other antibiotics. As with the phenotypic assessment of AMR, it was observed that (in the earlier experiments) AMR declined as birds got older, adding support to the hypothesis that robust and commensal (antibiotic sensitive) *E. coli* outcompete AMR *E. coli* in older birds. It was also observed that different *E. coli* MLST, carrying fewer antibiotic resistant genes, emerged, and the *E. coli* population became more diverse as the birds aged (Chapter 4). In addition, the ST 48 isolate was identified in both day-old chicks and older birds, but characteristics of the serotype differed between day-old chicks (O?:H21, O4:H21) and birds that were 29 and 35 days of age (O113:H4). ST 48 in older birds carried only mdfA1 and so were more susceptible to antibiotics compared to ST 48 isolated in chicks. This suggests that it is not only the *E. coli* population that evolves as the bird ages, but within individual serotypes there may be evolution within the bird's life. It would suggest that new serotypes which are more benign and less AMR emerge as birds get older with the development of the gut environment. MLST 48, 1665, 295, 2705, 1266, 973, 1112, 1286 in older birds observed in Chapter 4 and 6 were more susceptible to antibiotics and were also less virulent. Therefore, the WGS study contributes to understanding of diverse MLST and evolution of benign *E. coli* as the birds aged. A strategy to promote the

proliferation of these MLST in future investigations could reduce the prevalence of AMR and presumptive pathogenic coliforms at an earlier age. This would have a positive impact on the poultry industry and public health by producing more resilient and healthy broiler chickens with less resistant AMR bacteria.

The correlation that was observed between phenotypic and genotypic AMR in Chapter 6 was not as good as that observed in Chapter 4. Although phenotypic tetracycline resistance persisted in Chapter 6, the carriage of tetracycline resistance genes in *E. coli* isolates declined after 14 days of bird age. In addition, there were few other antibiotic resistant genes detected. This may be because the *E. coli* isolated from older birds were in fact less resistant to antibiotics including tetracycline, but the phenotypic analysis suggests otherwise. A limitation of this study might be that only chromosomal DNA was successfully extracted, and plasmid DNA was inadvertently excluded. If this were the case, it would demonstrate the importance of plasmids (or other mobile genetic elements) in the transfer and carriage of antibiotic resistant genes. Preparing samples of DNA of an appropriate concentration and quality for WGS analysis was challenging. In the process of collecting, preparing, storing and analysing DNA, samples were repeatedly frozen and thawed and this might have caused DNA denaturation which would have a negative impact on genotypic sequencing.

Contrary to the original hypothesis that antimicrobial substances produced by beneficial microorganisms might inhibit the growth of *E. coli* carrying antibiotic resistance genes, administering either CF or LF (alone or in combination) did not have any effect on the high prevalence of antimicrobial resistant *E. coli*, although there was some, limited evidence that *C. famata* might decrease the resistance to ampicillin. There was no evidence, however, that the yeast and LAB intervention

affected phenotypic (or genotypic) AMR. This is in contrast to the findings of Saliu *et al.* (2020), who observed that administering either *Lactobacillus agilis*, *Lactobacillus salivarius* or commercial phytogenic feed additives alone or in combination reduced the prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* compared to control. However, only *L. agilis* reduced the transfer of the ESBL-carrying plasmid between *Enterobacteriaceae*. This demonstrates that it is possible to exploit LAB to produce conditions which limit the spread of AMR between bacterial species by horizontal gene transfer. As discussed before, the lack of any effect of the yeast and LAB used in these experiments may in part be because of the low doses that were used. However, it is worth bearing in mind that the activity of yeast and bacteria may be affected by the presence of bioactive compounds in the feed or additive.

Therefore, to take this study forward, interventions using novel beneficial microbes and bioactive compounds would need to be researched. This would include identifying their optimum dose, frequency and method of administration, and characterisation of their mode of action. Recently, interest in CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated) technology is increasing. CRISPR-Cas is a bacterial immune system and is extensively utilised for genome editing (Venter, 2019; Gholizadeh *et al.*, 2020). This has the potential ability to eliminate specific antibiotic resistant genes in bacteria and the development of genetic techniques such as CRISPR-Cas could help to overcome the AMR concern to public health in the future.

7.2 Association between AMR and other characteristics of *E. coli*

It was clear from the characterisation of *E. coli* isolated in all the experiments that young birds were particularly vulnerable to infection by virulent *E. coli*. Commensal *E. coli* that were less resistant to antimicrobials then became more predominant as the birds aged. Kemmett *et al.* (2013) also reported that the carriage of virulence genes in *E. coli* decreased as birds aged. 24% of *E. coli* isolates taken from day-old chicks carried five or more virulence genes, but this decreased to 1% of isolates when birds were 5 weeks old (Kemmett *et al.*, 2013).

Furthermore, phenotypic sucrose, dulcitol and sorbose utilisation by *E. coli* isolates was associated with the carriage of both iron acquisition genes and the *tsh* gene. This suggests that the availability of sucrose, dulcitol and sorbose might affect the competition between commensal *E. coli* and the virulent *E. coli* carrying iron uptake genes and *tsh*. Further investigation is needed to determine the mechanism by which atypical carbon source utilisation might be related to putative pathogenesis. If such a relationship does indeed exist, then manipulating the gut environment to alter the availability of different carbon sources might prevent the growth of virulent (and AMR) *E. coli*. This could be tested, initially *in vitro*, by the incubation of samples of caecal digesta with a series of different carbon sources (including sucrose, dulcitol and sorbose). The prevalence of AMR *E. coli* at the beginning and end of the incubation light give an indication of whether this approach encourages (or inhibits) the proliferation of AMR *E. coli.*

Many studies and this thesis showed the association between AMR and the carriage of particular virulence genes by *E. coli*, even if the mechanism of this association is unclear (Maynard *et al.*, 2004; da Silva and Mendonça, 2012; de Verdier *et al.*,

2012). It was noticeable that there was a high prevalence of iron uptake related genes in *E. coli*. Paixao *et al.* (2016) identified a strong association between APEC isolates and iron uptake genes. Iron is an essential element for growth and survival (Litwin and Calderwood, 1993; Nakazato *et al.*, 2009). Siderophore systems of *E. coli* are stimulated by low concentrations of iron and compete with iron sequestering mechanisms of the host. In addition, when the iron is limiting in the environment, the *E. coli* produces haemolysins which damages the haemoglobin in red blood cells to acquire the intracellular iron (Litwin and Calderwood, 1993; Paixao *et al.*, 2016). According to the observations presented in Chapter 3, 4 and 6, these studies confirmed a strong association between iron acquisition genes and antimicrobial resistance, and this might provide a clue to identifying the selective advantage of AMR *E. coli* in situations where no antibiotics are administered. It is hypothesised that manipulating iron supply could be a means of controlling *E. coli* that have this iron uptake ability and are AMR. If the availability of iron were higher, the selective advantage of the iron uptake systems would be removed, and this would benefit the more commensal (and coincidentally non-AMR) *E. coli* serotypes.

Supporting this hypothesis is the observation that the iron contents in the diets in both the starter and grower/finisher phase were higher in Chapter 4 than Chapter 6 (respectively, 147, 173 for Chapter 4 and 109,126 mg/kg for Chapter 6). The frequency of *iuc*D was lower in Chapter 4 throughout the birds' life compared with what was observed in Chapter 6. The carriage of the *irp*2 gene was also of lower prevalence in Chapter 4 during the starter phase. However, the *irp*2 had a lower frequency in Chapter 6 compared to Chapter 4 when birds were 4 weeks old. This might suggest that other factors, not just iron concentration, control the proliferation

of different *E. coli* serotypes. However, manipulating the concentration of iron in the birds' gut may help to inhibit the proliferation of pathogenic and AMR *E. coli*.

It is outside the scope of this thesis to continue this investigation, but this hypothesis could be tested using first *in vitro* and then *in vivo* experiments. An *in vitro* experiment would determine the growth of both commensal *E. coli* (susceptible to antibiotic) and AMR *E. coli* (with carriage of the iron sequestering genes) in media that had either a low or high iron concentration. A low iron concentration in the medium would encourage the proliferation of *E. coli* that carried the iron uptake related genes (and was resistant to antibiotics). What would be interesting to determine would be whether (in a mixed culture), with a high iron concentration, the colony that proliferated was the isolate that did not carry the iron uptake genes (and the antibiotic resistant genes as well). If this finding were confirmed *in vitro*, then a subsequent test *in vivo* would be to determine whether a high iron concentration in the digesta (perhaps achieved by the supplementation of the diet with iron) might encourage the proliferation of commensal *E. coli* at the expense of AMR *E. coli* carrying the iron uptake genes.

7.3 Conclusion and future works

The *E. coli* isolated from the caecum of broiler chickens had a high prevalence of resistance to AMP and TET in the starter phase even though antimicrobial agents were not administered at any time. Young chicks were particularly susceptible to colonisation with AMR and a putative pathogenic *E. coli*. However, antimicrobial sensitive and commensal *E. coli* became more dominant as the birds got older. Administering yeast and LAB had no effect on the prevalence of AMR or virulence

in *E. coli*, but there is scope for further investigation with higher doses of yeast and LAB, perhaps in combination with other bioactive compounds. There was good evidence of evolution of the *E. coli*, both within and between serotypes, as the birds aged. Furthermore, the *E. coli* that evolved were more commensal in nature and were more sensitive to antibiotics. Advancing the evolution of the *E. coli*, so that these more beneficial serotypes are dominant in the younger, more vulnerable, bird might have a significant impact on the health and performance of the UK (and global) poultry flock. An ongoing question throughout the thesis was what the selective advantage was for *E. coli* in carrying genes coding for antibiotic resistance when no antibiotics were administered. Part of the answer may lie in the associations that were observed between AMR and the carriage of genes coding for the sequestration of iron. The ability to utilise atypical carbon sources might also be involved. Confirming these associations, and the selective advantage that they confer, would be the next step in this investigation.

Providing an environment in the gut of the young chicken that would enable the more commensal (and antibiotic sensitive) *E. coli* to proliferate would then follow. This might be achieved by the identification of appropriate interventions that would alter the environment of the gut in the broiler chicken. These might include beneficial microbes, bioactive compounds and feedstuffs with a high concentration of particular carbohydrates. Manipulation of the concentrations of different minerals (including iron), while still ensuring that the supply of these minerals to the broiler chickens was optimised may also be beneficial. Ultimately, it would be valuable to identify and confirm particular interventions which controlled the proliferation of AMR and pathogenic *E. coli* especially in young chicks. This thesis provides a deeper insight into factors which can be utilised to explore the selective advantage

of AMR *E. coli* and develop a greater understanding of the evolution of *E. coli* in the chicken caecum.

These findings provide the following insights for future research:

- Mechanisms of persistence and dissemination of antibiotic resistant genes with mobile genetic elements between different bacterial communities should be identified.
- Confirm whether plasmids are the link between the carriage of AMR and virulence genes.
- Investigation of the selective advantage of AMR in *E. coli*, and consider the association between carbon source utilization, carriage of virulence associated genes and the evidence (genotypic and phenotypic) of AMR.
- Further research in this area should focus on identifying dietary and environmental management interventions that might encourage the proliferation of commensal *E. coli* in the bird's gut from an early age.
- In particular, it would be worth determining the association between the iron content of the feed and digesta and the profile of antimicrobial resistant and virulence genes in *E. coli* isolated from caecum.
- Develop relevant CRISPR-Cas technology and determine its effect on the maintenance of target AMR genes.

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Appendices

Appendix 1: Chapter 3

Effects of yeast via drinking water on body weight gain from d 1 to d 14.

	Control	*C. famata*	*S. boulardii*	SEM	*P*-value
Weight gain (g/bird/d)	27.86	27.45	27.44	0.48	0.783

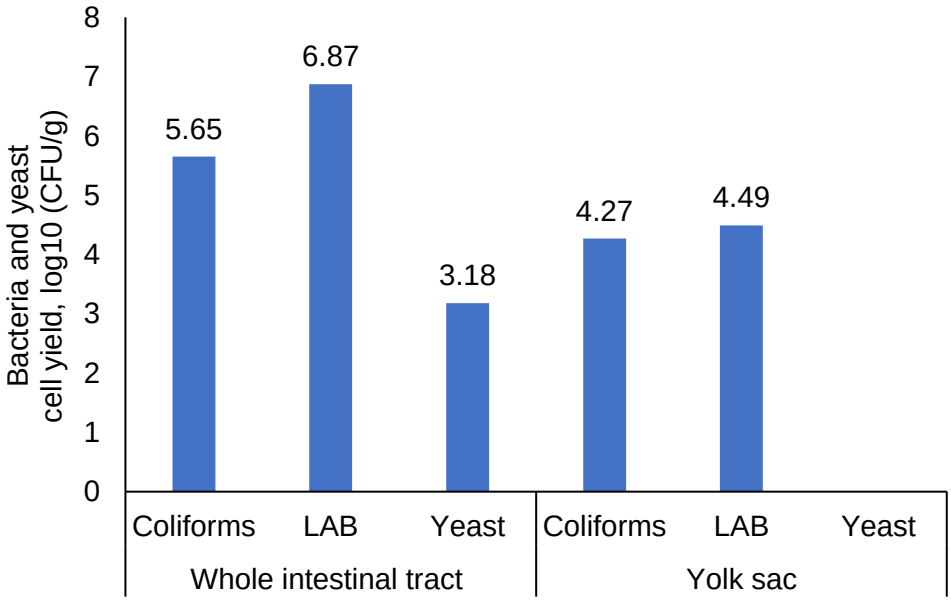
Appendix 2: Chapter 4

Effects of the addition of *Candida famata* and/or *Lactobacillus fermentum* on body weight gain from d 1 to d 14.

	Control	CF	LF	CFLF	SEM	*P*-value		
						CF	LF	CF*LF
Weight gain (g/bird/d)	29.34	27.82	28.33	28.16	0.61	0.169	0.577	0.268

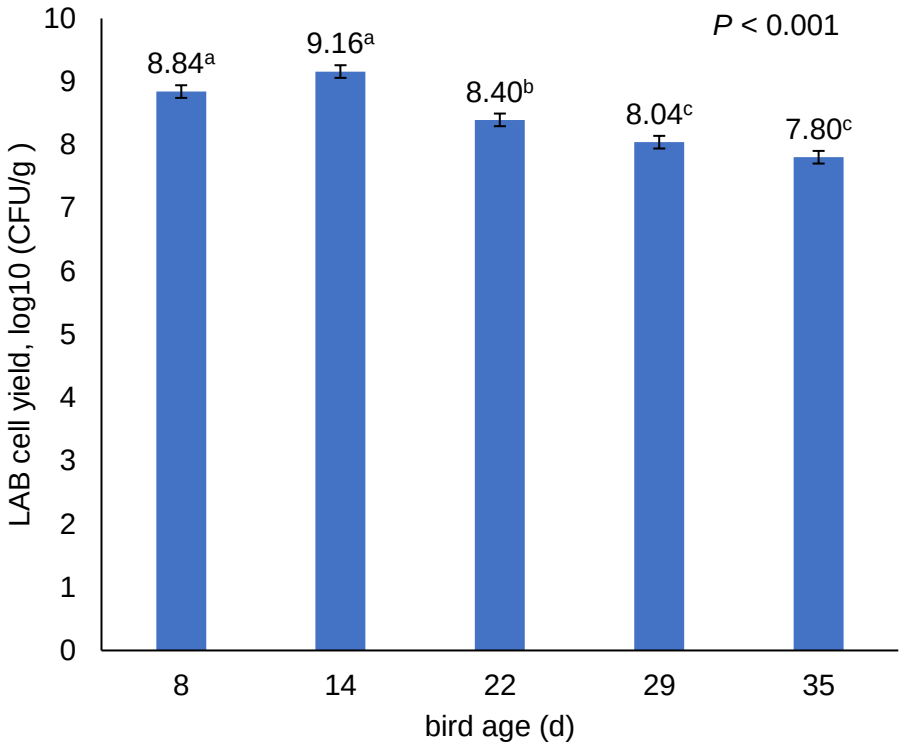
Appendix 3: Chapter 4

The population density of coliforms, lactic acid bacteria (LAB) and yeast from in the whole intestinal tract and yolk sac on day-old of chicks.



Appendix 4: Chapter 4

Effect of bird age on the population density of lactic acid bacteria in duodenum, ileum and caecum. Values with different superscripts (a-c) are significantly different ($P < 0.05$).



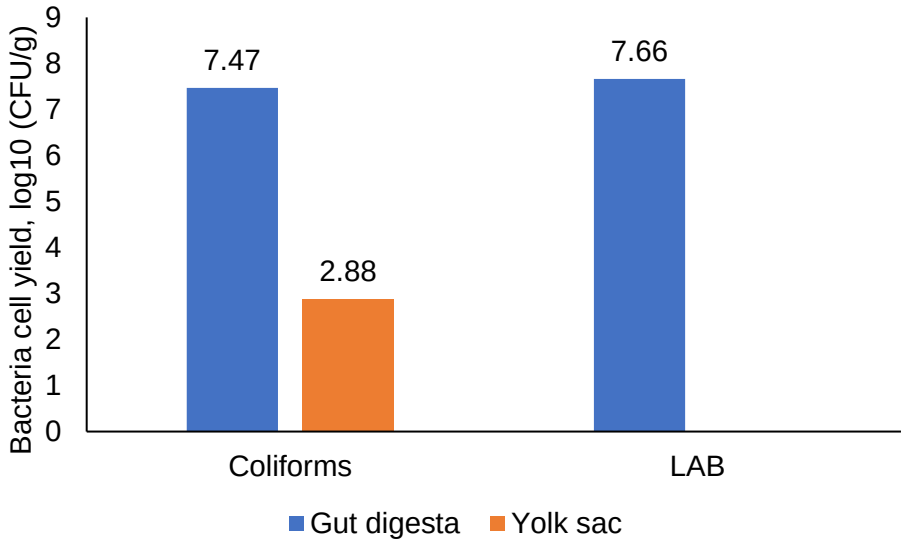
Appendix 5: Chapter 6

Effects of administering *Lactobacillus fermentum* or *Lactococcus lactis* on body weight gain from d 1 to d 14.

	Control	LL	LF	SEM	*P*-value
Weight gain (g/bird/d)	22.51	20.06	21.26	1.23	0.39

Appendix 6: Chapter 6

The population density of coliforms, lactic acid bacteria (LAB) in the gut digesta and yolk sac on day-old of chicks. LAB population in the yolk sac did not analyse because *E. coli* population and AMR *E. coli* was focused.



Appendix 7: The preliminary study of Chapter 2 has been accepted in *Animal* (Accepted 11 January 2022)

The effect of stocking rate and supplementary selenium on the fatty acid composition and peroxidisability of poultry muscle tissues

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Short title: Effect of stocking rate and selenium on poultry tissue peroxidisability

Abstract

It has long been established that selenium (**Se**) plays a crucial role in protecting biological materials from oxidative damage through the action of the selenoprotein glutathione peroxidase (**GSH-Px**), and the effectiveness of this protection is often dependent upon Se supply. More recent evidence has indicated that GSH-Px mRNA expression can be upregulated in response to potential oxidative damage risk, and that this upregulation is independent of Se supply. The current study aimed to determine the effect that Se supplementation, stocking rate and tissue fatty acid profile has on GSH-Px activity in breast and thigh tissue of commercial broilers. The study comprised a total of 168 Ross 308 broilers who were enrolled onto the study at 15 days of age. Prior to enrolment birds were brooded as a single group and received a starter diet containing no additional Se. The study was a 2 x 2 factorial design comprising of two levels of dietary Se (high Se, 0.5 mg/kg total Se, low Se background Se only), and two stocking rates (high stock 30 kg/m^2, and low stock 15 kg/m^2). At 15 days of age birds were blocked by liveweight and randomly allocated to one of the four treatments; there were six pen replicates per treatment. At 42 days of age one bird was randomly selected from each pen replicate, euthanased and samples of breast and thigh tissue harvested. GSH-Px activity, thiobarbituric acid reactive substances (**TBARS**), and fatty acid (**FA**) content of these tissues was determined. GSH-Px activity did not differ between tissue types but was greater in high Se birds ($P < 0.001$) when compared to low Se. TBARS concentrations were greater in thigh tissue ($P < 0.001$), and these thigh concentrations were greater with high Se birds ($P < 0.05$). There were marked differences between breast and thigh tissue in most determined FA ($P < 0.001$), with breast generally containing greater proportions of polyunsaturated FA (**PUFA**), so that breast tissue had a higher ($P <$

0.001) peroxidisability index (**PI**) than thigh. High Se resulted in lower proportions of 18:2 n-6, and a lower cis-9 18:1 concentration was reported across both tissues for the high stocking rate. A positive correlation between GSH-Px activity and peroxidability index in the thigh tissue of high Se birds (Pearson Correlation 0.668; *P* = 0.025) may indicate that increasing susceptibility to peroxidisation in lipid-rich tissues may also upregulate GSH-Px activity in Se-replete birds.

Key Words: Selenium, Glutathione Peroxidase, Poultry, Fatty Acids,

Implications:

Higher concentrations of unsaturated fatty acids in poultry meat may increase susceptibility to peroxidation, and this will have a negative impact on eating and keeping quality. This effect may be mitigated by optimising the selenium content of the poultry diet so that glutathione peroxidase (the selenium-containing enzyme which has an antioxidant action) activity can be maintained at optimal levels.

Introduction

The important role that selenium (**Se**) plays in preventing oxidative damage to biological tissues has long been recognised. This antioxidant action is mediated through a number of selenoproteins, proteins that are characterized by the presence of selenocysteine (**SeCys**) residues within their primary structure (Burk and Hill, 2015; Labunskyy *et al*., 2014). The most abundant selenoprotein in mammals is

glutathione peroxidase (**GSH-Px**), which reduces hydrogen peroxide and lipid hydroperoxides at the expense of glutathione. This family of selenoproteins comprises a number of known isozymes; Cytosolic (GSH-Px1), Gastrointestinal (GSH-Px2), Extracellular (GSH-Px3), Phospholipid Hydroperoxide (GSH-Px4), and GSH-Px6.

As with all selenoproteins expression and activity is dependent upon the Se status of the animal, and it has long been established that with declining Se status there is a commensurate reduction in selenoprotein expression and activity (Sunde, 2018). However, mechanisms exist whereby the Se status of some tissues, or the expression and activity of some selenoproteins, are moderated during periods of Se deficiency; the Se content of brain and testicular tissue are conserved during periods of Se deficiency through the action of apolipoprotein E receptor-2 (Burke *et al*., 2007; Olson *et al*., 2007), or selenoproteins of higher biological importance are expressed preferentially to those of lower importance (Behne *et al*., 1988). More recently a number of studies have shown that selenoprotein expression may be influenced/upregulated by factors other than simple Se supply/status that may reflect increased oxidative or peroxidation risk (Sneddon *et al*., 2003; Sunde and Raines, 2011; Toutat-Hamici *et al*., 2014).

Risk of tissue peroxidation increases with increasing levels of lipid unsaturation, and peroxidisability index (**PI**) has been used to express tissue susceptibility to oxidation based on its fatty acid (**FA**) composition (Witting and Horwitt, 1964). The total lipid and FA content of poultry muscle tissue has been shown to differ markedly between breast and thigh tissues, with thigh tending to have a greater total lipid content than breast tissue (Marion and Woodroof, 1964; Rymer and Givens, 2005). Despite this, differing FA profiles between tissue types could lead to certain tissues being at

increased risk of peroxidation. This increase in peroxidation risk may upregulate the activity of selenoproteins involved in protecting tissue from oxidative damage, but only when Se supply is adequate.

Broiler stocking rates have been shown to affect both bird performance and GSH-Px activity. Simitziz *et al*. (2012) and Li *et al*. (2019) have both reported that higher stocking rates reduce bird performance and increase the activities of the enzymes GSH-Px and superoxide dismutase. Li *et al*. (2019) also reported elevated concentrations of malondealdehyde (**MDA**), an indicator of lipid peroxidation, in the serum of high stocking density broilers. This elevation of oxidative stress in high stocking density birds may further compound the issue of peroxidability risk in lipid-rich tissues.

The aim of this study was to determine the relationship between FA profile and tissue GSH-Px activity in the breast and thigh muscle tissue of meat line birds, and the effects that supplementary dietary Se and stocking rate had on this relationship.

Materials and Methods

Animals and Diets

All animals used in this study were maintained at the Centre for Dairy Research (**CEDAR**), University of Reading. All experimental procedures and sampling were undertaken in accordance with the Animals (Scientific Procedures) Act, 1986, and at all stages of life, animals were kept in accordance with the Code of Recommendations for the Welfare of Livestock: Meat Chickens and Breeding Chickens (DEFRA, 2018). Diets were manufactured by Target Feeds (Whitchurch, Salop, UK) and were formulated to meet the breeder recommended age dependent nutritional requirements of birds throughout the study (Aviagen, 2014).

A total of 200 male Ross 308 day-old chicks (P.D. Hook Hatcheries, Oxon, UK) were brooded as a single group for the first 15 days of life. Upon arrival chicks were tagged and weighed, bedded on white wood shavings, received supplementary heat (in accordance with breeder recommendations), were offered *ad libitum* access to a starter diet mash (Table 1) that contained no supplementary Se, and clean fresh water. For the first seven days of life birds received 23 h continuous light followed by 1 h darkness in every 24-h period. From 8 d old, and for the remainder of the study, birds received 18 h continuous light followed by 6 h darkness in every 24-h period. Birds received no medication or vaccinations during the study.

The study was a two by two factorial design comprising two levels of dietary Se (low and high) and two levels of stocking density (low and high). The low dietary Se (Low Se) contained background Se (background Se estimated at 0.15 mg/kg) only and the high Se (High Se) contained background Se + 0.35 mg/kg supplementary Se in the form of Hydroxy-selenomethionine (Selisseo®, Adisseo, France). The high

stocking rate was intended to achieve approximately 30kg/m^2 by 42 d old (based on breeder performance targets), and the low stocking rate 15 kg/m^2. At 15 d old birds were weighed, blocked by live weight and randomly allocated to one of four treatments; a low Se diet and low stocking rate, high Se diet and low stocking rate, low Se diet and high stocking rate, or high Se and high stocking rate. There were six replicate pens per treatment. Feed offered and refused was recorded and feed intake calculated from these data on a pen basis. Birds were weighed individually on a weekly basis from 15 d old until study completion.

At 42 d old one bird was randomly selected from each pen, humanely killed by cervical dislocation. Samples of breast (*M. pectoralis major*) and thigh (*M. iliotibialis*) tissue were harvested, placed into a clearly labelled plastic bag, put immediately onto ice, and transported to the lab for determination of muscle GSH-Px activity. GSH-Px activity was determined using a commercially available kit (ab 102530, Abcam, Cambridge, UK) and was conducted in duplicate on fresh samples. Following determination of GSH-Px activity remaining samples were stored at -20^{o}C until FA analysis and determination of Thiobarbituric Acid Reactive Substances (**TBARS**).

FA were determined using a modified one-step method adapted from Sukhija and Palmquist (1988). Briefly, triplicate samples were homogenized, freeze dried and manually ground using a pestle and mortar. To 400 mg dried tissue 1 ml of internal standard (1 mg/ml methyl heneicosaenoate, H3265, Sigma Aldrich Company Ltd., Dorset, UK in toluene), 1 ml of toluene, and 3 ml 2% H_2SO_4 in methanol was added. Tubes were mixed thoroughly, sealed under nitrogen and incubated at 60˚C for 3 h. Tubes were left to cool to room temperature, after which 5 ml of 6% K_2CO_3 and 2 ml toluene was added and tubes mixed. Tubes were centrifuged at 2,500 rpm for

10 min, and the resulting upper phase was transferred to a tube containing 1.0 g Na_2SO_4, and left at room temperature for 1 h. Following centrifuging (5,000 rpm for 5 min), supernatant was transferred to vials. Resulting FA methyl esters (**FAME**) were analysed on a Bruker 450 gas chromatograph equipped with a flame ionisation detector, using a temperature programme (Kliem *et al*., 2013). Identification of FAME was completed using an external standard (GLC463, Nu-Chek Prep, MN, USA). All results were expressed as g/100 g FA. Peroxidisability index (**PI**) of tissue was calculated with the equation described by Erickson (1992): PI = (0.025 x $monoenes$) + (1 x $dienes$) + (2 x $trienes$) + (4 x $tetraenes$) + (6 x $pentaenes$) + (8 x $hexaenes$).

TBARS were determined in thawed samples using an adapted method of Subbarao and Richardson (1990). Briefly, duplicate samples were homogenised in 0.9% NaCl at a ratio of 1:2 tissue:total homogenate volume. To 40 µl each homogenate, 40 µl of 0.9 % NaCl and 40 µl of deionized H_2O was added, tubes were mixed and incubated at 37˚C for 20 min. Following incubation, 600 µl of cold 0.8 M HCl containing 12.5 % trichloroacetic acid, and 780 µl of 1 % thiobarbituric acid were added to each sample and boiled for 20 min. Samples were then cooled at 4˚C for 1 h before being centrifuged at 5000 g for 10 min. Absorbance of the resultant supernatant was read at 532 nm (Cecil CE2040 spectrophotometer) using an extinction coefficient of 1.56 x 10^5.

Physical performance data were analysed by ANOVA, using a general linear model (Minitab version 18 statistical software package; Minitab Inc. Pen State, Pennsylvania, US). Factors in the model included dietary Se (1 df), stocking rate (1 df), and the interaction between these terms. Data pertaining to tissue GSH-Px activity, FA profile, and TBARS concentration were analysed by ANOVA, using a

Mixed Model. Factors in the model included dietary Se (1 df), stocking rate (1 df), tissue type (1 df) and the interactions between these terms. Bird was used as a random factor within the model. Data are presented as least square means with SEM, and differences were deemed significant when $P < 0.05$. Pearson correlation coefficients between tissue PI and GSH-Px activity were determined (within dietary Se treatment) using the Minitab vs.18 statistical software package.

Results

There were no effects of Se with respect to bird physical performance, with similar rates and efficiencies of gain between Se supplemented and unsupplemented birds (Table 2). There were effects of stocking rate ($P < 0.001$) whereby levels of feed consumption and rates of liveweight gain were greater in high stocked birds when compared to low. However, there were no effects of stocking rate on feed conversion ratio (**FCR**), nor were there any interactions between Se and stocking rate on any aspect of bird performance.

Predictably there were effects of Se supplementation on tissue GSH-px activity ($P < 0.001$), which was greater in birds supplemented with Se (Figure 1). Neither stocking rate nor tissue type had an effect on tissue GSH-Px activity. There were no effects of either stocking rate or Se on TBARS, although TBARS were greater in thigh tissue when compared to breast (Figure 2; $P < 0001$). There was an interaction between tissue type and Se ($P = 0.002$) whereby TBARS concentrations were greater in the thigh tissue of high Se birds when compared to low.

There were marked differences between tissue types with most determined FA (Table 3; $P < 0.05$). Generally, the proportion of most saturated FA (12:0, 14:0, 16:0) and *cis*-9 18:1 were greater ($P < 0.01$) in thigh tissue than breast, whereas proportions of 18:0 and most PUFA (18:3 n-3, 20:2 n-6, 20:3 n-6, 20:3 n-3, 20:4 n-6, 20:5 n-3, 22:4 n-6, 22:5 n-3 and 22:6 n-3) were greater ($P < 0.05$) in breast than thigh. Higher dietary Se resulted in higher ($P < 0.05$) proportions of 16:0 and *cis*-11 20:1 in both tissues, and lower ($P = 0.007$) proportions of 18:2 n-6. Stocking rate affected some FA proportions, with a higher rate leading to lower ($P < 0.05$) cis-9 18:1 across both tissues compared with the lower rate. The same was observed for 14:0 but only with the high Se diet ($P = 0.007$). There were interactions between Se and tissue type for 18:2 n-6 and 20:4 n-6, with the high Se diet decreasing the proportion of 18:2 n-6 ($P = 0.038$) but only in breast tissue, whereas the opposite occurred with 20:4 n-6 (only thigh tissue; $P = 0.027$). There were also interactions between Se, tissue type and stocking rate; in breast tissue (but not thigh tissue), a high stocking rate resulted in higher 20:4 n-6 concentrations, which was increased further by the high Se diet ($P = 0.033$). The opposite was true for *cis*-9 18:1, where the high stocking rate resulted in lower concentrations, made even lower by additional dietary Se ($P = 0.027$). Peroxidisability index was higher ($P < 0.001$) in breast than thigh tissue.

There was a positive correlation (Pearson $P = 0.025$, $r = 0.668$) between peroxidisability index and GSH-Px activity in thigh tissue from birds on the high Se diet, whereas this relationship was weakly negative (Pearson $P = 0.144$, $r = -0.446$) for breast tissue. There was no correlation between these measures for birds on the low Se diets (thigh Pearson $P = 0.932$, $r = 0.029$; breast Pearson $P = 0.382$, $r = 0.311$).

Discussion

The lack of effect of Se supplementation on bird physical performance is not unexpected and has been reported previously (Yoon *et al*., 2007; Juniper *et al*., 2011; Liu *et al*., 2015). Retarded growth is a recognised symptom of selenium deficiency, but the lowest dietary Se concentration used in these and the current study would not be low enough to result in the manifestation of symptoms associated with selenium deficiency. Consequently, increasing the dietary concentration of Se would be unlikely to result in any improvement in growth performance or efficiency or feed utilisation.

Increased stocking densities have often been reported to depress bird performance (Shawnawany, 1988; Feddes *et al*., 2002; Dozier *et al*., 2005), and issues tend to manifest themselves when birds start to reach heavier body weights at later stages of the production cycle (Dozier *et al*., 2005), with lower rates of feed consumption and live weight gain, with reduced efficiencies of gain. In the current study increased stocking is associated with improved bird performance; intakes and growth rates were greater in high stocking birds, although there was no improvement in feed conversion efficiency. This disparity between the current study and previous work may be a consequence of actual stocking densities at the end of the study. Anticipated stocking densities in the current study (15 and 30 kg/m^2 for low and high stocking, respectively) were based on breeder performance targets, however, at the end of the study actual stocking densities were 10.4 and 20.4 kg/m^2 for low and high stocking, respectively. It is likely that the threshold whereby stocking density (kg/m^2) starts to have adverse effects on bird performance was not reached; Dozier *et al*. (2005) reported that bird performance was adversely affected at stocking rates above 30 kg/m^2. Greater gains in high stocked birds could be

explained by increased feed intake, as feed conversion ratio did not differ between the two stocking rates. It is not clear why increased stocking rate increased intake behaviour and this warrants further investigation.

The higher GSH-Px activity observed in birds fed higher concentrations of Se is consistent with previous research (Haug *et al*., 2007; Huang *et al*., 2011; Haug *et al*., 2014). Haug *et al*. (2014) reported increased gene expression of GSH-Px4 in chicken breast tissue following supplementation of bird diet of 1.10 mg/kg Se compared with a diet containing 0.13 mg/kg Se. A similar effect was observed for GSP-Px1 and GSH-Px4 muscle gene expression when comparing two diets with less of a Se difference (0 vs 0.3 mg/kg Se; Huang *et al*, 2011).

There was evidence that lipid peroxidation was greater in thigh tissue from birds fed the high Se diet. This was unexpected, as it was proposed that the high Se diet would, if anything, result in lower TBARS formation, given the role of Se in cellular anti-oxidation mechanisms (Skřivan *et al*., 2012), and given the effect of dietary Se in the present study on tissue GSH-Px activity. Earlier studies report a decrease in tissue TBARS with increasing dietary Se (Skřivan *et al*., 2012) or no effect (Ryu *et al*., 2005). Leskovec *et al*. (2019) suggested the lack of effect of additional dietary Se on malondialdehyde in breast meat may have been due to the antioxidant capacity of the tissue being already optimal so that additional Se resulted in no further benefit. It may be that thigh tissue from High Se birds in the current study had a higher total lipid content than Low Se birds, although it is not clear why this would have occurred.

Overall tissue lipid content was not measured in this study but thigh tissue has a markedly higher lipid concentration than breast tissue (Rymer & Givens, 2005). A

higher total lipid content suggests a greater PUFA content, which are more susceptible to oxidation. This may have been the reason for increased TBARS formation for thigh tissue. Other studies report higher TBARS formation for thigh compared with breast tissue (Delles *et al*., 2016; Ahmed *et al*., 2017; Akbari Maghaddam Kakhi *et al*., 2017). As well as a higher overall lipid content, thigh tissue contains more myoglobin than breast, which can contribute to increased oxidative susceptibility through production of superoxide anions and hydrogen peroxides (Chaijan, 2008). Tissue differences in FA profile reflected the type of lipid present in each tissue. Breast meat contains a greater amount of lipids in the form of phospholipids (PL), whereas in thigh meat the predominant lipids are neutral lipids such as triacylglycerols (TAG; Hulan *et al*., 1989; Rymer & Givens, 2005). Generally, PL FA profile is characterised by a greater concentration of long chain PUFA, due to their structural importance in maintaining cell membrane fluidity (Wood *et al*., 2008). In contrast TAG tend to be richer in SFA and MUFA, and can be found within intramuscular adipocytes, whose numbers increase with increasing total lipid content of the muscle (Wood *et al*., 2008). Poultry meat studies have reported inconsistent patterns of FA distribution between breast and thigh, with some reporting similar proportions of SFA and PUFA but with thigh higher in MUFA (Ahmed et al., 2017), and others reporting breast with numerically higher SFA and PUFA and less MUFA (Funaro *et al*., 2014). The current study reported no difference between tissues for SFA, but PUFA were higher in breast, and MUFA was higher in thigh. Much of these discrepancies can be accounted for by differences between study diets, particularly dietary PUFA content. Thigh meat contained a higher proportion of medium chain SFA and cis-9 18:1, which is

consistent with thigh meat containing more neutral lipid such as TAG (Wood *et al*., 2008).

It is known that 18:3 n-3 tends to be stored in TAG lipids rather than PL (Gonzalez-Esquerra and Leeson, 2001; Betti *et al*., 2009), meaning that in poultry the thigh meat tends to have higher concentrations compared with breast (Rymer & Givens, 2005). The higher breast proportion of 18:3 n-3 in the current study is therefore unexpected. However, the higher breast proportion of the longer chain PUFA (n-6 and n-3) is consistent with these FA being preferentially stored in PL (Gonzalez-Esquerra & Leeson, 2001; Wood *et al*., 2008).

Given that thigh tissue appeared to be at greater oxidative risk than breast (demonstrated by TBARS results), and given that previous evidence suggests that a higher stocking rate was also thought to further increase oxidative stress (Simsek *et al*., 2009), it was hypothesised that GSH-Px expression and activity may be upregulated for factors promoting higher oxidative stress (Touat-Hamici *et al*., 2014), such as thigh vs breast, and high vs low stocking rate. It was also thought that these differences would have been greater when Se was limiting (Touat-Hamici *et al*., 2014). Results from the current study demonstrate that GSH-Px activity was unaffected by both of these comparisons, which might suggest that enzyme activity did not respond to oxidative pressure, possibly due to the oxidative pressure not being severe enough to elicit a response (TBARS of < 1 µg malondialdehyde/g sample have been found to not be associated with meat rancidity; Ripoll *et al*., 2011). However, PI can also be used as a measure of oxidative stress, taking into account the proportions of FA measured within a sample, and is weighted so that the higher the proportion of unsaturated FA, the higher the PI value. For this study, it was hypothesised there would be a positive relationship between tissue PI and GSH-Px

activity, due to the presence of unsaturated FA increasing activity and expression of GSH-Px to minimise oxidation. A positive correlation was only observed for thigh tissue for birds consuming the high Se diet, which supports the theory that GSH-Px activity may respond to oxidative stress, when Se is sufficient. A previous study reported a negative relationship between PI in lamb meat and GSH-Px activity (Gruffat *et al*., 2020), but this study did not report Se status of the animals. Results for PI should be interpreted with caution especially when comparing two different tissue types, as PI does not take into account overall lipid content.

Additional supplementary Se resulted in relatively minor and inconsistent changes in FA profile across both tissues sampled. Previous research has reported increases in tissue long chain PUFA concentration following Se supplementation; Haug *et al*. (2007) reported higher proportions of 20:5 n-3, 22:5 n-3 and 22:6 n-3 in thigh muscle following supplementation with 0.84 mg Se/kg diet, and Pappas *et al*. (2012) reported a similar pattern in breast meat following dietary supplementation with increasing Se (up to 3 mg Se/kg diet). In contrast, additional dietary Se of 0.2 mg/kg diet had no effect on the FA profile of breast meat (Leskovec *et al*., 2018). It is thought that dietary Se affects tissue PUFA indirectly by reducing the rate of peroxidation (Haug *et al*., 2007), rather than affecting the action/expression of elongases and desaturases responsible for their synthesis (Haug *et al*., 2014). In the current study, the high Se diet did increase some PUFA, but only in one tissue (for example 20:4 n-6 in thigh tissue). This was reflected in the lack of Se effect for peroxidisability index. The lack of an overall Se effect may be related to the Se dose used (0.5 mg/kg diet).

Conclusion

This study demonstrated that the presence of more unsaturated FA in meat can increase its oxidative susceptibility, but in turn this could also increase the activity of GSH-Px which would help counteract lipid peroxidation. Increasing the Se content of the bird diet also increases GSH-Px activity, and it may be that a threshold dietary Se level is required so that GSH-Px activity can respond to an increasing oxidative risk. However further research is required to confirm this.

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Declaration of interests

All authors have no conflict of interests.

Ethics statement

All experimental procedures and sampling were undertaken in accordance with the Animals (Scientific Procedures) Act, 1986, and at all stages of life, animals were kept in accordance with the Code of Recommendations for the Welfare of Livestock: Meat Chickens and Breeding Chickens (DEFRA, 2018).

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Table 1. Composition of the diets (g/kg as fed)

Ingredient	Starter (0-15 d)	Grower/ Finisher (15-42 d)	
		Low Se	High Se[1]
Barley	40	40	39
Wheat[2]	500	550	541
Soyabean meal[3]	320	265	261
Rapeseed meal	42	42	41
Soyabean oil	50	65	64
L-lysine HCl	4	1	0.9
DL-methionine	3.45	2.42	2.38
L-threonine	2.05	2.02	1.99
Sodium bicarbonate	2.5	2.5	2.5
Salt	2	2.5	2.5
Limestone	11	8.56	8.42
Poultry vitamins/minerals (Se free)[4]	2	2	2
Hydroxy - selenomethionine			16
Dicalcium phosphate	20	16	15.7
Titanium dioxide	1	1	0.9

[1] Selenium diet containing an additional 0.35 mg/kg supplementary Se in the form of Hydroxy-selenomethionine

[2] 12.5 % crude protein

[3] 48 % crude protein

[4] Target Feeds Ltd., Shropshire, UK.

Table 2. Effect of stocking rate (SR) and dietary selenium (Se) on bird physical performance.

	Low Se		High Se[1]		SEM	*P*–Values[2]		
	Low Stocking[3]	High Stocking[3]	Low Stocking	High Stocking		Se	SR	Se*SR
Feed intake (g/d)	325^{ab}	340^{a}	297^{b}	301^{b}	8.0	0.254	0.001	0.475
Live weight gain (g/d)	193^{ab}	202^{a}	173^{c}	178^{bc}	4.4	0.123	<0.001	0.645
Feed Conversion ratio	1.69	1.69	1.72	1.69	0.013	0.207	0.234	0.322

Different superscripts (^{a,b,c}) within row are significantly different ($P < 0.05$)

[1] Diet containing an additional 0.35 mg/kg supplementary Se in the form of Hydroxy-selenomethionine

[2] Effect of dietary selenium (Se), stocking rate (SR) or their interaction

[3] Low and High stocking rate were designed to result in 15 kg/m^2 and 30kg/m^2 by 42 d old (based on breeder performance targets)

Table 3. Effect of stocking rate (SR), dietary selenium (Se) and tissue (T; breast or thigh) on the fatty acid composition of tissue (g/100g total fatty acids) and peroxidisability index (least squares means).

	Breast				Thigh				SEM	*P*–Values[1]						
	Low Se		High Se[2]		Low Se		High Se									
	Low Stock[3]	High Stock[3]	Low Stock	High Stock	Low Stock	High Stock	Low Stock	High Stock		T	Se	SR	Se*SR	Se*T	T*SR	T*SR *Se
12:0	0.008^{b}	0.018^{ab}	0.009^{b}	0.016^{ab}	0.025^{a}	0.019^{ab}	0.023^{a}	0.019^{ab}	0.0032	<0.001	0.606	0.441	0.889	0.936	0.003	0.583
14:0	0.33^{bc}	0.34^{bc}	0.37^{ab}	0.31^{c}	0.38^{ab}	0.35^{bc}	0.40^{a}	0.37^{ab}	0.011	<0.001	0.088	<0.001	0.007	0.338	0.887	0.045
16:0	17.8^{b}	18.1^{b}	18.8^{ab}	18.3^{ab}	19.3^{ab}	18.4^{ab}	19.8^{a}	19.1^{ab}	0.36	0.001	0.019	0.075	0.517	0.980	0.131	0.352
18:0	9.4^{b}	9.9^{ab}	9.4^{b}	10.9^{a}	8.9^{b}	9.4^{b}	8.6^{b}	9.0^{b}	0.31	<0.001	0.670	0.001	0.362	0.063	0.203	0.287
18:1 *cis*-9	21.3^{bc}	20.9^{bc}	21.7^{bc}	20.0^{c}	24.9^{a}	22.9^{ab}	24.9^{a}	24.8^{a}	0.51	<0.001	0.321	0.004	0.667	0.106	0.966	0.027
18:1 *cis*-11	2.2	2.3	2.3	2.2	2.0	2.2	2.1	2.1	0.07	0.009	0.419	0.522	0.108	0.307	0.388	0.135
18:2 *cis*-9, *cis*-12	33.4^{bc}	33.1^{c}	32.2^{cd}	30.8^{d}	35.7^{a}	36.1^{a}	35.9^{a}	35.4^{ab}	0.51	<0.001	0.007	0.218	0.161	0.038	0.266	0.926
18:3 n-6	0.24^{ab}	0.26^{a}	0.27^{a}	0.21^{ab}	0.14^{b}	0.24^{ab}	0.28^{a}	0.20^{ab}	0.024	0.047	0.173	0.697	<0.001	0.072	0.398	0.118
18:3 n-3	2.4	1.7	1.9	2.1	1.8	1.2	1.4	1.6	0.32	0.027	0.940	0.250	0.071	0.898	0.981	0.795
20:1 *cis*-11	0.13^{b}	0.21^{ab}	0.27^{a}	0.16^{ab}	0.16^{ab}	0.24^{ab}	0.29^{a}	0.20^{ab}	0.031	0.185	0.029	0.682	<0.001	0.965	0.945	0.733
20:2 n-6	1.05^{a}	1.00^{a}	0.99^{a}	1.04^{a}	0.62^{b}	0.58^{b}	0.52^{b}	0.48^{b}	0.042	<0.001	0.061	0.511	0.390	0.099	0.457	0.416
20:3 n-6	0.80^{a}	0.77^{a}	0.77^{a}	0.81^{a}	0.47^{b}	0.49^{b}	0.44^{b}	0.38^{b}	0.029	<0.001	0.102	0.767	0.943	0.105	0.610	0.068
20:3 n-3	0.19^{a}	0.19^{a}	0.22^{a}	0.20^{a}	0.11^{b}	0.10^{b}	0.09^{b}	0.08^{b}	0.013	<0.001	0.777	0.228	0.293	0.058	0.943	0.531
20:4 n-6	6.2^{b}	6.4^{ab}	6.0^{b}	7.6^{a}	4.3^{c}	4.6^{c}	3.6^{c}	3.6^{c}	0.29	<0.001	0.432	0.011	0.134	0.002	0.086	0.033

	Breast				Thigh				SEM	*P*–Values[1]						
	Low Se		High Se[2]		Low Se		High Se									
	Low Stock[3]	High Stock[3]	Low Stock	High Stock	Low Stock	High Stock	Low Stock	High Stock		T	Se	SR	Se*SR	Se*T	T*SR	T*SR *Se
20:5 n-3	0.20^{abc}	0.34^{a}	0.30^{ab}	0.33^{a}	0.20^{bc}	0.18^{c}	0.23^{abc}	0.18^{c}	0.025	<0.001	0.603	0.836	0.500	0.797	0.027	0.958
22:2 n-6	0.30	0.27	0.28	0.32	0.29	0.27	0.26	0.25	0.046	0.440	0.880	0.865	0.521	0.477	0.759	0.680
22:4 n-6	1.80^{a}	1.78^{a}	1.77^{a}	1.99^{a}	1.09^{b}	1.05^{b}	0.99^{b}	0.94^{b}	0.095	<0.001	0.924	0.673	0.381	0.154	0.264	0.364
22:5 n-3	1.43^{a}	1.44^{a}	1.48^{a}	1.70^{a}	0.95^{b}	1.04^{b}	0.87^{b}	0.82^{b}	0.082	<0.001	0.943	0.249	0.772	0.010	0.376	0.149
22:6 n-3	0.86^{a}	0.91^{a}	0.88^{a}	0.96^{a}	0.58^{b}	0.63^{b}	0.45^{b}	0.49^{b}	0.050	<0.001	0.180	0.119	0.861	0.021	0.802	0.746
24:1 *cis*-15	0.03	0.04	0.04	0.05	0.02	0.03	0.05	0.04	0.013	<0.001	0.924	0.673	0.381	0.154	0.264	0.364
Total SFA[4]	27.5	28.4	28.7	29.6	28.6	28.2	28.9	28.5	0.54	0.999	0.061	0.541	0.989	0.274	0.076	0.957
Total cis-MUFA[4]	23.6^{bc}	23.5^{bc}	24.3^{bc}	22.4^{c}	27.4^{a}	25.4^{ab}	27.4^{a}	27.2^{a}	0.56	<0.001	0.326	0.006	0.979	0.162	0.890	0.021
Total n-6 PUFA[4]	44.1^{a}	43.5^{ab}	42.2^{ab}	42.9^{ab}	42.2^{ab}	43.3^{ab}	42.0^{ab}	41.4^{b}	0.62	0.014	0.004	0.701	0.776	0.742	0.707	0.064
Total n-3 PUFA[4]	5.16^{a}	4.57^{ab}	4.78^{ab}	5.29^{a}	3.67^{bc}	3.17^{c}	3.16^{c}	3.18^{c}	0.299	<0.001	0.850	0.503	0.051	0.313	0.625	0.484
Peroxidisability index[5]	93.5^{a}	91.3^{a}	89.1^{a}	97.4^{a}	73.2^{b}	76.5^{b}	70.0^{b}	70.1^{b}	2.38	<0.001	0.198	0.130	0.243	0.066	0.643	0.028

Different superscripts (^{a,b,c}) within row are significantly different ($P < 0.05$)

[1] Effect of tissue (T), dietary selenium (Se), stocking rate (SR) or their interactions

[2] Diet containing an additional 0.35 mg/kg supplementary Se in the form of Hydroxy-selenomethionine

[3] Low and High stocking rate were designed to result in 15 kg/m^2 and 30kg/m^2 by 42 d old (based on breeder performance targets)

[4] SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids

[5] PI = (0.025 x *monoenes*) + (1 x *dienes*) + (2 x *trienes*) + (4 x *tetraenes*) + (6 x *pentaenes*) + (8 x *hexaenes*) (Erickson, 1992).

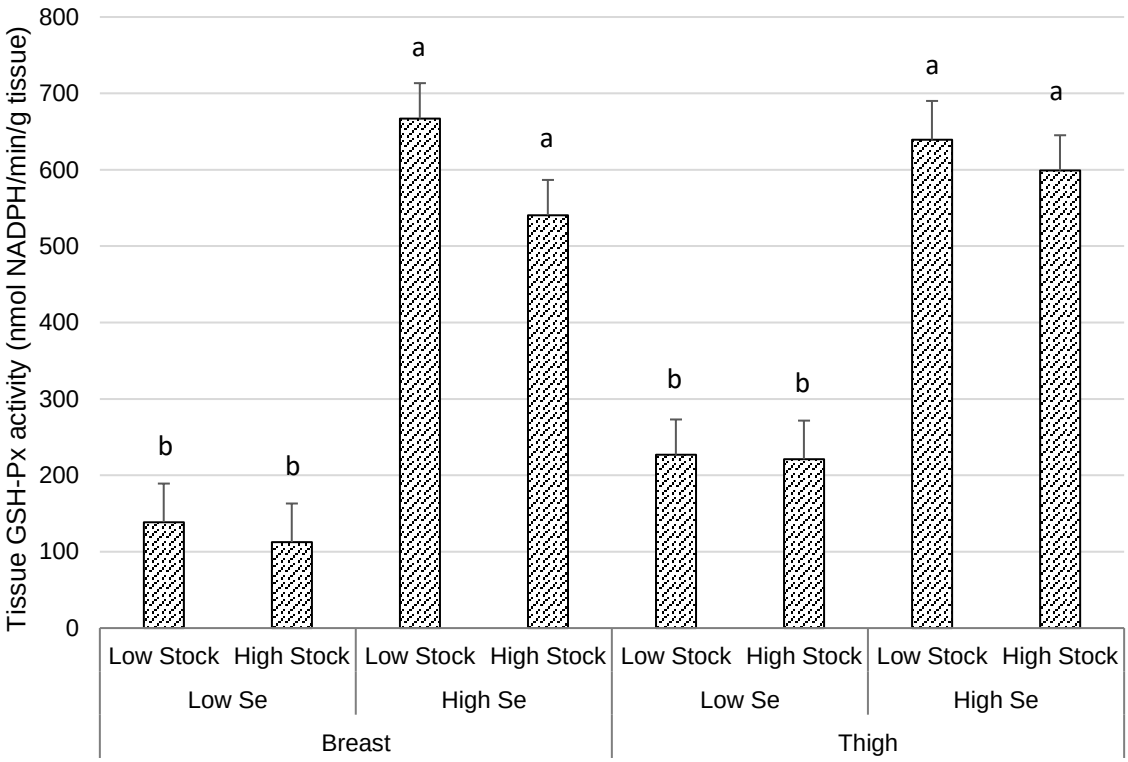

Figure 1. Glutathione peroxidase (GSH-Px) activity in breast and thigh tissue of poultry fed two diets differing in selenium content[1], stocked at either high or low density[2] (least squares means + SEM).

Bars with different letters are significantly different ($P < 0.05$)

[1] Diets containing background levels of Se (low Se) or an additional 0.35 mg/kg supplementary Se in the form of Hydroxy-selenomethionine (high Se)

[2] Low and High stocking rate were designed to result in 15 kg/m^2 and 30kg/m^2 by 42 d old (based on breeder performance targets)

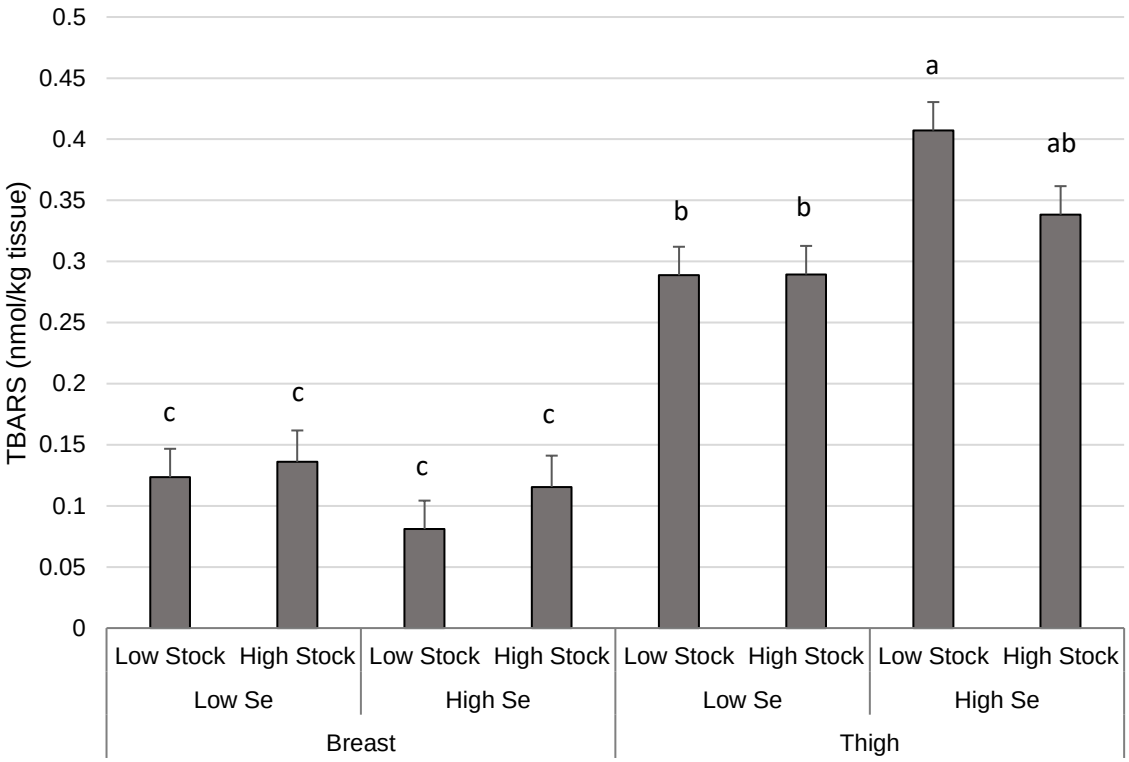

Figure 2. Thiobarbituric acid reactive substances (TBARS) concentration in breast and thigh tissue of poultry fed two diets differing in selenium content[1], stocked at either high or low density[2] (least squares means + SEM).

Bars with different letters are significantly different ($P < 0.05$)

[1] Diets containing background levels of Se (low Se) or an additional 0.35 mg/kg supplementary Se in the form of Hydroxy-selenomethionine (high Se)

[2] Low and High stocking rate were designed to result in 15 kg/m^2 and 30kg/m^2 by 42 d old (based on breeder performance targets)

Appendix 8: The study of chapter 3 has been accepted in *Animal* (Accepted 8 June 2021)

The effect of *Candida famata* and *Lactobacillus plantarum* on the number of coliforms and the antibiotic resistance and virulence of *Escherichia coli* in the gut of broilers

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Abstract

This study was undertaken to determine the effect of a yeast (*Candida famata*) and a bacterium (*Lactobacillus plantarum*), administered alone or in combination in the drinking water, on the population of yeast, *Lactobacillus* sp. and coliforms, and the prevalence of antimicrobial resistance (**AMR**) and virulence genes in *Escherichia coli* (***E. coli***) isolated from digesta samples taken throughout the life of broiler chickens. Male (Ross 308) day-old chicks (220) were used. *C. famata* (isolated from a chicken) and *L. plantarum* (isolated from a pig) were administered via the drinking water. Water was provided either untreated (**CON**) or with *C. famata* (**CF**; 10^8/ml), *L. plantarum* (**LP**; 10^5-10^8/ml), or a combination of CF and LP (**CFLP**; 10^6-10^8/ml) in water hoppers on two days each week for 35 d. Administering probiotics did not affect the growth performance in broiler chickens. No significant interactions were observed between main effects, and neither CF nor LP had any effect on the population size of *Lactobacillus* sp. or coliforms. The administration of *C. famata* increased the population density of yeasts in the small intestine at these ages. The population density of coliforms, *Lactobacillus* sp. and yeast decreased with age ($P < 0.001$). There was no significant effect of probiotics on the prevalence of phenotypic antimicrobial resistance and virulence genes in these studies. The prevalence of *E. coli* that was resistant to ampicillin and tetracycline, as well as carrying ≥3 virulence-associated genes was greatest at the end of the starter phase (around 8 days old), before declining through the grower and finisher phases. There was only limited evidence that administering either CF or LP affected either the antimicrobial resistance or the virulence of *E. coli* in the bird. However, tetracycline resistance in *E. coli* was associated ($P < 0.001$, $P < 0.01$, $P < 0.05$, and $P < 0.05$) with the carriage of the iron uptake systems of *E. coli* D (***iuc*D**), iron-repressible protein (***irp*2**), increased serum survival (***iss***) and temperature-

sensitive haemagglutinin (***tsh***) genes respectively, suggesting that the accumulation of iron and the genetic element conferring tetracycline resistance may be intertwined.

Keywords: Probiotics, *Escherichia coli*, Antimicrobial resistance, Virulence genes, Broiler chickens

Implications

Broiler chickens are particularly susceptible to infection by pathogenic and antimicrobial resistant coliforms in their starter phase. Although this may be overcome naturally as the birds age (and does not appear to be assisted by the administration of either yeast or *Lactobacillus* probiotics), this does leave a window of vulnerability in early life. It is possible that increasing the supply of iron in the gut lumen may encourage the evolution of more benign (and not antimicrobial resistant) coliforms.

Introduction

Antibiotics have been used to improve animal performance and prevent disease over several decades. However, the EU banned the use of antibiotics as growth promoters in 2006 because of the increase in antimicrobial resistance (**AMR**) arising from the misuse and overuse of antimicrobial agents (Castanon, 2007). Antibiotic use in UK broiler production has decreased substantially, but the growing prevalence of antimicrobial resistance by bacteria is a threat to both human and animal health (Dolejska *et al.*, 2012). Antimicrobial resistant bacteria from animal products can be transferred, directly or indirectly, into the human food chain and thereby threaten the human consumer (Landers *et al.*, 2012). *Escherichia coli* (***E. coli***), found in the

intestinal tracts of all animals and birds, has been widely used as a sentinel species for the determination of AMR (Jang *et al.*, 2017; Stromberg *et al.*, 2017). A high prevalence of antimicrobial resistant *E. coli* has been observed in day-old chicks in Austria (Roth *et al.*, 2017). Many researchers have demonstrated the presence of various antibiotic resistant genes in *E. coli* isolated from both caecal digesta and animal meats (Miles *et al.*, 2006; Diarra *et al.*, 2007; Smith *et al.*, 2007; Persoons *et al.*, 2010).

In addition to the carriage of AMR genes, some *E. coli* strains may also carry several virulence-associated genes which increase their pathogenicity and lead to disease. In poultry, the most common disease associated with *E. coli* is colibacillosis, but extraintestinal disease would include septicaemia, pericarditis, airsacculitis and perihepatitis (Delicato *et al.*, 2003; Dziva and Stevens, 2008). The carriage of these virulence genes may increase the selection advantage of the strain; in *E. coli* known virulence genes code for the products adhesins, invasins, toxins and iron acquisition systems (siderophores) (Paixao *et al.*, 2016).

Dietary interventions may alter the composition of the gut microbiome and the evolution of strains of *E. coli* (Olnood *et al.*, 2015). In doing this, the proportion of AMR bacteria in the bird and poultry meats may also be altered. Probiotics such as *Lactobacillus* sp. and yeasts may help prevent the proliferation of pathogenic bacteria and potentially AMR in the gut through mechanisms such as competitive exclusion, neutralisation of toxins, bactericidal activity or enhanced immune competence (Kabir, 2009; Fijan, 2014). The aim of this experiment was therefore to determine the effect of a yeast (*Candida famata*) and a bacterium (*Lactobacillus plantarum*), administered in the drinking water, on the prevalence of phenotypic AMR status and carriage of virulence genes by *E. coli* throughout the life of broiler chickens. Preliminary results from this experiment were presented in abstract form by Lee *et al.* (2020).

Material and methods

Experimental design, birds and diets

A total of 220 chicks (day old, male Ross 308) were used in a 35 d feeding trial. All chicks were weighed on arrival, wing tagged, blocked by liveweight, and then randomly placed in one of four brooder rings and fed a common starter diet (Table 1) until the birds were 14 days of age. No antibiotics were administered during the experimental period. *Candida famata* (isolated from a chicken) and *Lactobacillus plantarum* (isolated from a pig; University of Surrey) were administered via the drinking water. Stock cultures of each isolate in *C. famata* and *L. plantarum* were incubated overnight in Yeast Extract Peptone Dextrose (**YPD**) broth or De Man, Rogosa and Sharpe (**MRS**) broth. Population density was then estimated by serial dilution and measurement of the optical density of the incubation medium at 600 nm. Cultures were then concentrated by centrifugation (3 354 x g, 5 min) and washed with sterile distilled water. An appropriate volume of the culture was then added to a measured volume of the drinking water in the hopper to attain the desired final concentration of probiotic. Water was therefore provided either untreated (Control) or with a preparation of *C. famata* (**CF**; 10^7-10^9 cfu/ml water offered) or *L. plantarum* (**LP**; 10^5-10^8/ cfu/ml water offered), or a combination of CF and LP (**CFLP**; total 10^6-10^8 cfu/ml water offered) in water hoppers two days each week (days 1, 4, 8, 11, 14, 18, 22, 25, 29 and 32). Water hoppers were replenished with fresh (untreated) water when required.

On day 14, all birds were weighed, and blocks of birds were allocated (six birds per pen, all birds originating from the same brooder ring) to one of 24 pens, with six replicate pens per probiotic treatment. All birds were then fed a common grower/finisher diet (Table 1) in the form of a mash manufactured by Target Feeds

(Whitchurch, UK). Samples of each diet were analysed for starch, sugars, oil, crude protein and amino acids (Sciantec, York, UK). Titanium dioxide (1 mg/g) was incorporated in the diets as an indigestible marker, and feed samples (one per pen) were analysed for titanium dioxide (Short *et al.*, 1996). Lighting was via incandescent lights with 23 h continuous light per 24 h period for the first seven days, followed by 18 h continuous light (6 h darkness) in each 24 h period. The birds were brooded according to the breeder's recommendations using infrared lights to provide supplementary heat when necessary.

Bird performance, sample collection and determination of nutrient availability

Birds were weighed individually on days 1, 14 and 35. Mean body weight increase was then calculated on a pen basis (g/d) during both the starter period and the grower/finisher period. Feed intake was determined and feed conversion ratio (**FCR**) calculated on a pen basis. All birds were sacrificed by cervical dislocation on day 36, and one bird per pen was hung in a cold room (4^0C) for 24 h. It was weighed again, and the breast muscle was then removed (excluding skin and feathers). The breast muscle was weighed, and breast meat yield was then calculated (breast muscle weight/cold carcase weight).

On days 1, a total of 16 birds were randomly selected and sacrificed by cervical dislocation. The whole intestinal tract (from proximal duodenum to ileo-caecal junction) and the yolk sac were taken. These were analysed for the determination of the population size (cfu/g) of yeasts and bacteria (coliforms and *Lactobacilli*) and for the determination of antimicrobial resistance and virulence genes of coliforms. An isolate of *Lactobacilli* and yeast was randomly taken from the intestinal sample for the identification of *Lactobacilli* and yeast species.

On day 8 and 14, four birds from each brooder ring were sacrificed and contents from the duodenum, ileum and caecum were taken, weighed and cultured (in the same way as above) for the determination of yeast and bacterial population density. Coliforms were not counted in the small intestine samples as earlier (unpublished) observations had detected very low populations in these segments of the gut. One isolate of *E. coli* was taken from the cultured caecal sample of each bird for the determination of antibiotic resistance (all birds) and the presence of virulence genes (with the exception of CFLP birds, as no significant interaction between CF and LP had been observed). An isolate of *Lactobacilli* and yeast was randomly taken from the intestinal sample for the identification of *Lactobacilli* and yeast species.

After the birds were allocated to their grower/finisher pens, one bird from each pen was randomly selected and sacrificed on days 22, 29 and 35. Samples of digesta from the duodenum, ileum and caecum were again taken and the yeast and bacterial population densities were determined as before. An isolate of *E. coli* was taken from each caecal sample for the determination of antibiotic resistance and (with the exception of CFLP birds, for the same reason as before) the presence of virulence genes. An isolate of *Lactobacilli* and yeast was randomly taken from the cultured intestinal samples for the identification of *Lactobacilli* and yeast species. Samples of feed and a sample of ileal digesta taken from each bird on day 35 were also analysed for titanium dioxide (Short *et al.*, 1996) by determining the absorbance of the acid digest at 410 nm using a spectrophotometer (CECIL CE 2040, 2000 series) for the determination of dry matter availability.

Counting of viable cells and preparation of bacterial and yeast isolates

The samples of whole intestine and yolk sac (from day-old chicks) and the samples of duodenal, ileal and caecal digesta (taken from the older birds) were weighed and serially diluted with phosphate-buffered saline (**PBS**, 0.01 M) and 100 µl of the suspension spread on MRS agar (for the enumeration of *Lactobacillus* spp.), MacConkey agar (for the enumeration of coliforms, in caecal digesta samples only) and Saboraud dextrose agar (**SDA**, for the enumeration of yeast). Plates were incubated (37°C, 48 h) in an anaerobic incubator (Whitly MG1000 anaerobic workstation) for the *Lactobacillus* spp. cultures and aerobically incubated overnight at 37°C for the coliform and 30°C for yeast cultures. Colonies were then counted (Gallenkamp Colony Counter CNW 325 030Y, UK) to determine the population sizes (cfu/g).

The preservation of *Lactobacillus* sp. isolates was done by transferring the selected isolates to a sterile Eppendorf tube containing 1 ml of 150 g/l glycerol in MRS broth. These cultures were incubated anaerobically at 37°C for 18-24 hours. After that, the *Lactobacillus* sp. suspension was stored at -80°C. A single colony of *E. coli* and yeast was selected at random from each MacConkey and SDA plate respectively and transferred to a separate, sterile Eppendorf tube containing 500 µl of nutrient broth (*E. coli*) or YPD broth (yeast). These cultures were aerobically incubated at 37°C for 18-24 hours. 100 µl of the resulting *E. coli* and yeast suspension was transferred to a cryobank tube (Mast group, Mastdisks, UK) and stored at -80°C pending further analysis.

Characterisation of Lactobacilli and yeast isolates

Lactic acid bacteria (**LAB**) and yeast isolated from digesta samples were stored at -20°C for the identification of strains. A total of 29 LAB strains were selected from the caecum for identification of the genus *Lactobacillus* and related genera by using the API 50 CHL test kit (bioMerieux UK Ltd, Basingstoke, UK). A total of 24 yeast isolates were randomly selected for identification of yeast strains by using the API system (API 20C AUX strips, bioMerieux UK Ltd, Basingstoke, UK). Identification of *Lactobacillus* and yeast strains was made using the api*web*™ identification software (https://apiweb.biomerieux.com) with database V5.1 (API, bioMerieux UK Ltd, Basingstoke, UK).

Antimicrobial resistance and extraction of genomic DNA of E. coli isolated from caecal samples

A sterile inoculation loop was inserted into the *E. coli* suspension of a cryobank tube and then streaked onto four MacConkey plates, each enriched with one of four antibiotics (ampicillin, nalidixic acid, tetracycline and chloramphenicol, each at a concentration of 20 µg/ml). Plates were then incubated (37°C, 16 h) and growth of colonies on each plate was then determined. When growth was observed on plates containing antibiotics, it was deemed that the colony that had inoculated the plate was resistant to that antibiotic.

The genomic DNA from a total of 87 isolates of *E. coli* (taken from birds that received the control, CF or LP treatment, but not the CFLP treatment) were extracted to determine the presence of *E. coli* virulence factors. Genomic DNA was extracted using Pure-gene yeast/bact Kit B (Qiagen, Venlo, Netherlands) and standard protocols for fresh samples of Gram-negative bacterial cultures were followed, as described below

and by Alkandari (2017). Isolated cultures of *E. coli* were streaked on nutrient agar plates and incubated at 37°C overnight. Colonies of *E. coli* were then taken from these plates and transferred to a 1.5 ml sterile Eppendorf tube. These were then incubated at 80°C for 5 min after adding 300 µl of cell lysis solution on the pellet. The tubes were put on ice for 20 minutes. 100 µl of protein precipitation solution was added and vortexed vigorously for 20 s at high speed. The mixture was centrifuged at 12 045 x g for 3 min. The supernatant was transferred to a clean 1.5 ml Eppendorf tube containing about 700 µl of 99.5% ethanol and mixed by gently inverting 50 times. The mixture was centrifuged at 12 045 x g for 1 min, and the supernatant was carefully discarded. A total of 300 µl of 70% ethanol was added to the DNA pellet and inverted several times. The mixture was centrifuged at 12 045 x g for 1 min, and again the supernatant was discarded and the pellet was allowed to air dry for 5 min. 100 µl of DNA hydration solution was added and the mixture was vortexed for 5 s, incubated at 65°C for 1 h, followed by incubation overnight at room temperature with gentle shaking. This procedure was described by Alkandari (2017).

The purification of DNA was determined with a Nanodrop spectrophotometer (ND 2000, Nano Drop Technologies, USA). The quality of DNA was evaluated according to the method of Tonks (2018). The Nanodrop tube was cleaned by pipetting 1.5 µl of distilled water onto it and wiping with Whatman filter paper. The Nanodrop was blanked with 1.5 µl of distilled water. 1.5 µl DNA solution was added to the measuring stage and the 260:280 nm ratio was also recorded (1.82 $\pm$ 0.12). DNA stock solutions was stored at -20˚C pending the analysis of virulence genes by polymerase chain reaction (**PCR**, Alkandari, 2017).

Virulence genotyping

The DNA extracts taken from the *E. coli* isolates were analysed for seven avian pathogenic *E. coli* (**APEC**) virulence factors using PCR assays. The method used was described by Alkandari (2017). The virulence genes investigated (*pap*C, *iuc*D, *tsh*, *irp*2, *iss*, *ast*A, and *hly*A) are described in Table 2, with their primer sequences. PCR was performed using GE Healthcare Illustra™ PuReTaq Ready-To-Go™ PCR Beads (Thermo Fisher Scientific, UK) containing 2.5 units of recombinant PuReTaq DNA Polymerase, dATP, dCTP, dGTP, dTTP, stabilizers, bovine serum albumen and reaction buffer. PCR assays of *pap*C and *iuc*D were performed with 25 µl of PCR mixtures containing 5 µl of DNA extraction, each 1 µl (25 pmol) of forward and reverse primer pairs, and 18 µl of sterile double-distilled water. The other virulence genes (*tsh, irp*2, *iss*, *ast*A and *hly*A) were determined using 25 µl of PCR mixtures containing 5 µl of DNA, 1 µl (25pmol) each of forward and reverse primer pairs, $MgCl_2$ (1.25 µl, 10 mM) and 16.75 µl of sterile double-distilled water.

The PCR amplification was carried out using a 96-well MJ Thermal Cycler (Bio-Rad, UK) with the following protocol: initial denaturation at 94˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 58˚C for 30 s, extension at 68˚C for 3 min, and the final extension step at 72˚C for 5 min. Analysis of the amplified products was performed by electrophoresis (75 V for 1 h) with a 1.5% agarose gel stained with ethidium bromide in 0.5 X TBE buffer. 100 bp and 1 Kbp DNA ladders were used. Gel images were captured using G-BOX Chemi-XR5, (Syngene, UK), connected to a computer. The analysis of specific band sizes for different virulence genes tested were scored as present (+) or absent (-).

Data analysis

The effect of the administration of CF, LP or the interaction between CF and LP on bird performance (feed intake, body weight gain, feed conversion ratio, breast meat weight and yield) and nutrient availability was determined by analysis of variance using the general linear model of Minitab (Minitab 17, Minitab Inc., PA, USA) with CF, LP and interaction between these main effects as the only terms in the model. The effect of bird age, digesta site, administration of CF, administration of LP and the interaction between all these main effects were used in the analysis of population density of yeasts, coliforms and *Lactobacillus* spp. (determined by ANOVA, general linear model, Minitab as before). Significant differences were determined at the level of $P < 0.05$.

At each sampling time, the percentage of samples (from any one treatment) that were phenotypically resistant to each antibiotic was determined. Chi-square analyses were then used to determine the association between either the presence of phenotypic antimicrobial resistance or the presence of different virulence genes in *E. coli* and either the birds' age or its treatment (Control, CF, LP, CFLP). Associations between the carriage of virulence genes and the phenotypic expression of antimicrobial resistance were also determined by Chi-square analysis.

Results

Bird performance and characterisation of E. coli, Lactobacillus and yeasts in the gut

The administration of probiotics did not affect bird performance or dry matter availability (Table 3). Even as day-old chicks, there was a large population in the gut of both coliforms (log_{10} 5.65 ± 0.35 cfu/g) and lactic acid bacteria (log_{10} 6.87 ± 0.13 cfu/g), which was larger than the populations observed in the yolk sac (log_{10} cfu/g 4.27 ± 0.13 and 4.49 ± 0.08 for coliforms and lactic acid bacteria, respectively). No yeast was detected in the yolk sac but some chicks (6 out of 16) had a small population of yeasts in the gut (log_{10} 3.18 ± 0.42 cfu/g).

The administration of *C. famata* and *L. plantarum*, either alone or in combination, had no effect on the population of coliforms or *Lactobacilli*. Administering *L. plantarum* did not affect the population of yeasts either, but the administration of *C. famata* did increase the population density of yeasts (from 3.4 to 4.2 log_{10}cfu/g, $P < 0.001$). As birds aged, there was a general decline in the population density of coliforms ($P < 0.001$, Table 4). There was also a significant ($P < 0.001$) interaction between the age of the bird and the site of the gut where digesta were collected with respect to the population density of *Lactobacilli*. The population density increased from the duodenum, through the ileum and was greatest in the caecum, and this difference between the different sections of the intestine was more apparent as the birds got older (Table 4).

There were some interesting interactions between bird age, site (duodenum, ileum and caecum) and the administration of CF or LP on the population size of yeasts. On days 14 and 22, the population density of yeasts was greater in the ileum and caecum

than in the duodenum. However, by day 29, the yeast population was greater in the duodenum than in the ileum and caecum. By day 35, the population density of yeasts was lower in all three sites compared with the density observed in those same sites when the birds were younger ($P < 0.001$, Table 4). Administering *C. famata* increased the population of yeasts when the birds were younger (days 8, 22 and 29) but by day 35 the population density of yeasts was lower and was not affected by the administration of *C. famata* ($P < 0.001$, Table 4). There was also an interaction between the administration of *C. famata* and digesta site on the yeast population ($P < 0.05$). Administering *C. famata* increased the population of yeast in the duodenum and ileum but not in the caecum (Table 4). In addition, there was an interaction between the administration of CF and LP ($P < 0.01$, Table 4). Administering *C. famata* increased the population of yeast in the gut, and yeast counts increased even further, when both *C. famata* and *L. plantarum* were administered. Administering *L. plantarum* alone, however, had no effect on the yeast population.

The identification of the *Lactobacilli* and yeast isolates is presented in Table 5 and Table 6. A variety of *Lactobacillus* species were identified, with only two isolates (one of which was from a control bird) being identified as *L. plantarum*. The other positive *L. plantarum* identification was from a bird treated with LP. With respect to yeasts, a number of different *Candida* species were identified including *C. rugosa*, *C. zeylanoides*, *C. tropicalis* and *C. glabrata*. *Geotrichum candidum*, *Saccharomyces cerevisaea* and *Rhodotorula mucilaginosa* were also identified.

Antimicrobial resistance and virulence genotyping of E. coli from the caecum

Figure 1 shows the proportion of caecal *E. coli* samples that were resistant to antimicrobials throughout the experimental period. Resistance to nalidixic acid and chloramphenicol was low throughout the study. Tetracycline (**TET**) resistance was high (79%) at 8 d and then declined to 21% at 35 d ($P < 0.001$) with no effect of treatment. All of the samples taken from the gut of day-old birds were resistant to ampicillin (**AMP**) and this persisted to day 8. However, it then decreased to 38% at 35 d ($P < 0.001$). There was no effect of treatment on the resistance of *E. coli* to antibiotics.

The percentage of *E. coli* isolates (n=84) carrying different virulence-associated genes is also presented in Figure 1. The *pap*C gene was not detected in any of the samples. There was a significant association ($P < 0.01$) between bird age and the carriage of virulence genes by *E. coli.* Isolates of *E. coli* taken from the youngest birds (days 1 and 8 of age) had a much higher incidence of *iuc*D, *irp*2, *iss* and haemolysin *hly*A than in isolates taken from older birds. There was no significant association between treatment and the carriage of different virulence genes. There was a significant association between phenotypic resistance of *E. coli* to tetracycline and the carriage of the genes *iuc*D ($\chi^2=12.27$, $P < 0.001$), *irp2* ($\chi^2=9.573$, $P < 0.01$), *iss* ($\chi^2=5.25$, $P < 0.05$) and *tsh* ($\chi^2=6.34$, $P < 0.05$) (Figure 2).

Discussion

If probiotics are to have any effect at all on the health and performance of the host animal, then they must colonise and proliferate within the gut (Angelakis *et al.*, 2013; Peng *et al.*, 2016). The absence of any effect of *L. plantarum* administration on the population density of *Lactobacilli* in any segment of the gut, and the further observation that only one of the samples of *Lactobacilli* isolated from the chickens showed any evidence of them being related to the administered source would suggest that colonisation by *L. plantarum* had been unsuccessful. The only caveat to that analysis would be to note that the samples that were taken were of digesta (squeezed from the lumen of the intestinal section). Bacteria colonise the gut by adhering to the mucosal wall, and it is possible that the population adhering to the gut wall was different from the luminal population. However, Herias *et al.* (1999) observed no such distinction which would suggest that the colonisation of the gut by administered *L. plantarum* was indeed limited. This may explain the lack of effect of LP on bird performance and the populations of either *E. coli* or LAB. In contrast, Peng et al. (2016) administered a similar dose of *L. plantarum* to broilers but did so every day and in the feed (rather than water). They reported improvements in feed conversion ratio and a reduction in *E. coli* when broilers were aged 42 d. However, no effect on coliform populations was observed when the birds were at a more vulnerable age (day 21 of age).

There was an increased yeast population in response to the administration of *C. famata*, and it was notable that this occurred mostly in the small intestine. *C. famata* administration may therefore have stimulated the proliferation of yeasts, but it was other yeast populations such as *C. rugosa*, *C. zeylanoides*, *C. tropicalis*, *Geotrichum candidum*, *C. glabrata*, *Saccharomyces cerevisaea* and *Rhodotorula mucilaginosa* that also appeared to be stimulated. However, as it was the small intestine in which the

yeasts proliferated it is perhaps unsurprising that there was no associated effect on the caecal population of *E. coli.* Future research might investigate what effect, if any, the administration of *C. famata* had on the duodenal coliform population.

The impact of *C. famata* on bird performance was also limited, with only a tendency (*P*=0.07) for feed conversion ratio to be improved. The optimal dose and route of administration therefore need further consideration to enable these species to colonise the gut and effect any changes in the microbiome. Other studies (with admittedly different probiotics) have demonstrated that administering probiotics in water improved performance in broiler chickens. Eckert *et al.* (2010) showed that intermittent *Lactobacillus* based probiotic treatment via drinking water significantly improved FCR and increased bodyweight compared with administration in feed or continuously in drinking water. A possible explanation for the beneficial effect of intermittent administration may be a consequence of the immunostimulatory properties of probiotics. Administering probiotics continuously would result in a sustained high state of immune stimulation and the energy for activation of the immune system might partition nutrients away from bird growth. Karimi Torshizi *et al.* (2010) also observed that the administration of nine different probiotics (*Aspergillus oryzae*, *Lactobacillus acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. bulgaricus*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus thermophilus* and *Candida pintolopesii*) in water resulted in improved performance (in terms of bodyweight gain, feed intake and feed conversion ratio) compared with no probiotics or administering them in the feed. Administering probiotics in the water intermittently may therefore be the optimum means of delivery, but in this study the dose (certainly of the *L. plantarum*) was insufficient to produce an observable effect on bird performance.

There was a high degree of resistance of *E. coli* to TET at 8 d and to AMP at both 1 and 8 d even though the birds were not exposed to antibiotics at any time in their life. AMP and TET resistance then decreased as the birds aged, and these results reflect those of Roth *et al.* (2017) who also observed *E. coli* in faecal contents of day-old chicks was highly resistant to ampicillin, ciprofloxacin, streptomycin, sulfamethoxazole and tetracycline but that the prevalence of resistant *E. coli* by d 17 and 38 decreased (although only to ciprofloxacin, streptomycin and sulfamethoxazole). A high prevalence of AMR in day-old chicks was also observed by da Costa *et al.* (2011), who reported resistance to ampicillin, cefotaxime, tetracycline, streptomycin, gentamicin and enrofloxacin by *E. coli* isolates from cloacal samples from day-old chicks. *E. coli* (both commensal and pathogenic, potentially carrying antibiotic resistance genes) may transfer from breeding birds to chicks via the eggshell or egg yolk during oviposition. However, in the absence of any exposure to antibiotic, it is unclear why the prevalence of resistance then increased still further in the first week of life (although it did then decrease after that). Clearly there must be some other selective advantage for *E. coli* carrying antibiotic resistance genes.

In other research, AMR of *E. coli* isolated from caecal and faecal samples has been observed to continue to increase throughout the bird's life. The prevalence of coliform resistance to penicillins was high and reported to be increasing in intensively farmed broilers (Majewski *et al.*, 2020). Bezerra *et al.* (2016) observed that all *E. coli* isolated from cloacal samples were resistant to trimethoprim-sulfamethoxazole and 95.4% to tetracycline, 91.4% to ciprofloxacin and 87.3% to ampicillin with birds of preslaughter age (35 to 38 days). *E. coli* isolates from excreta samples of birds had a high frequency of resistance to tetracycline, kanamycin and nalidixic acid throughout the birds' life in a study reported by Miles *et al.* (2006). The high prevalence (frequency or density) of

resistant *E. coli* arose from genomic evolution as a result of successful competition with susceptible strains (Kolář *et al.*, 2001; Smith *et al.*, 2007), although if no antibiotics are administered, the basis for this successful competition is unclear. Another possible explanation for this is that resistance genes are associated with mobile genetic elements, such as plasmids. These may confer resistance to antibiotic sensitive bacteria by conjugation, transformation and transduction, but also confer other selective advantages. In this study, AMR declined after 8 d, suggesting that while chicks may have been infected with highly resistant *E. coli* (often APEC types), over the lifespan of the birds, these types were replaced by more robust non-antibiotic-resistant commensal types.

Roth *et al.* (2017) added an organic acid additive to the birds' feed and observed that this was associated with a reduced prevalence of ampicillin and tetracycline resistant *E. coli*. This finding is consistent with this study, as the administration of *C. famata* (which in addition to ethanol was also, *in vitro*, observed to produce ethyl acetate, data not shown) tended to reduce the ampicillin resistance of *E. coli*. Organic acids produced by probiotics or added directly to the diet may inhibit the growth of coliforms, particularly those carrying antibiotic resistant genes (Edens, 2003; Alagawany *et al.*, 2018). Further research is clearly needed to investigate the appropriate selection of probiotics to reduce the prevalence of resistant *E. coli* in broiler chickens.

The seven virulence genes used in this study are generally analysed to differentiate between commensal and pathogenic *E. coli* in poultry. The *iss* gene was the most prevalent of the genes investigated, but *iuc*D, *irp*2, *iss* and *hly*A were also very prevalent in birds that were 8 d old before declining as the birds got older. In recent years there has been considerable interest in the detection of virulence genes in

commensal and pathogenic *E. coli* isolates in poultry (Zhao *et al.*, 2009; Karami *et al.*, 2017). Janßen *et al.* (2001) reported that a high frequency of the genes *fim*C, *iuc*D and *tsh* was observed in APEC isolates.

According to Paixao *et al.* (2016), APEC isolates had a higher prevalence of the iron uptake-related genes and the serum survival genes compared with commensal strains of *E. coli.* This is because iron is an essential nutrient for *E. coli* and is of more limited availability in extraintestinal tissues compared with digesta. There is therefore a selective advantage for APEC to carry genes such as *iuc*D and *irp*2. What was interesting in this study was the association between *iuc*D, *irp*2, *iss* and *tsh* with tetracycline resistance. As observed before, it is unclear what selective advantage there is to the carriage of genes coding for tetracycline (or any other antibiotic) resistance when no antibiotics have been administered. However, it is possible that these virulence genes are carried on the same plasmid (or other mobile genetic element) as the genes coding for tetracycline resistance and the advantage (to APEC) is in the greater ability to scavenge and accumulate iron, with the resistance to tetracycline being a side benefit.

Associations between virulence and antimicrobial resistance have been observed before; Johnson *et al.* (2012) observed that APEC had a higher proportion of plasmid mediated multidrug resistance than their avian commensal counterparts. They also observed that certain virulence genes, including *iss*, were positively associated with multidrug resistance. Szmolka *et al.* (2012) also reported that *tet*A (coding for tetracycline resistance) was highly correlated with the virulence genes *iro*N and *iss* in both commensal and avian pathogenic *E. coli*. These findings suggest that there is a positive relationship between mechanisms for iron accumulation by *E. coli* and the resistance by *E. coli* to a range of antimicrobials including tetracycline. This may

provide a means of controlling the maintenance and spread of *E. coli* resistant to antimicrobials. The avoidance of antibiotics may not be sufficient to control this spread, since the prevalence of *E. coli* resistant to antibiotics is so widespread even when no antibiotics are administered. Altering the iron availability (in the gut) for *E. coli*, conceivably by altering the dietary iron content or removing any chelating agents, may serve as a means of altering the selective advantage that APEC currently has, particularly in the starter period of the bird's life. This might then encourage the proliferation of commensal *E. coli* (carrying fewer genes encoding either antibiotic resistance or virulence).

This study has confirmed that the strains of *E. coli* which are prevalent in the young broiler chicken (up to 8 days of age) are resistant to ampicillin and tetracycline and they also carry a number of virulence-associated genes. This poses a considerable threat to the bird when it is at a vulnerable stage of development. What was positive, however, was the evolution of the *E. coli* population as the birds aged. By the finishing stage, the dominant strains of *E. coli* were commensal and sensitive to antibiotics. The carriage of *iuc*D, *irp*2, *iss* and *tsh* genes was strongly associated with tetracycline resistant *E. coli*. Interventions to encourage the early proliferation of commensal *E. coli* (without the resistance to antimicrobials) would make the young growing chick less susceptible to antibiotic resistant disease. One such intervention may be through the manipulation of iron availability in the chicken gut.

Ethics approval

Animals in this study were handled according to the recommendations of the Code of Recommendations for the Welfare of Livestock: Meat chickens and breeding chickens (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/69372/pb7275meat-chickens-020717.pdf) and the Ross Broiler Management Handbook (http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf). No further ethical approval was required as no pain, suffering, distress or lasting harm was imposed.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request to the authors.

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Declaration of interest

None

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Table 1 The formulation and chemical composition (as-fed basis) of the chickens' diets.

	Starter (0-14 d)	Grower/ Finisher (15-36 d)
Formulation (g/kg)		
Barley	40	40
Wheat	500	550
Soya bean meal	320	265
Rapeseed meal	42	42
Soya bean oil	50	65
L-lysine HCl	4	1
DL-methionine	3.45	2.42
L-threonine	2.05	2.02
Sodium bicarbonate	2.5	2.5
Salt	2	2.5
Limestone	11	8.56
Poultry vitamins/minerals[1]	2	2
Dicalcium phosphate	20	16
Titanium dioxide	1	1
Chemical composition (g/kg)		
ME (MJ/kg)	9.7	9.9
Crude Protein	219.0	210.0
Total Starch	244.0	265.0
Oil	47.4	49.1
Sugar as Sucrose	46.8	43.3
Cystine	3.8	3.6
Aspartate	23.1	19.6
Methionine	8.9	6.8
Threonine	11.5	10.1
Serine	11.5	10.4
Glutamate	45.9	43.1
Glycine	9.6	8.7
Alanine	9.9	8.9
Valine	11.0	10.0
Iso-Leucine	9.9	8.9
Leucine	17.0	15.2
Tyrosine	4.8	4.3
Phenylalanine	11.2	10.0
Proline	16.3	15.5
Histidine	5.8	5.3
Lysine	16.1	11.3
Arginine	15.1	13.5

ME= metabolisable energy.

[1] The vitamin/mineral premix supplied per kg of starter diets: vitamin A 6750 IU, vitamin D_3 2500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Se 0.125 mg, Mo 0.25 mg.

The vitamin/mineral premix supplied per kg of grower/finisher diets: vitamin A 5000 IU, vitamin D_3 2500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Se 0.125 mg, Mo 0.25 mg.

Table 2

The description of the virulence factors investigated and the primers used for their detection by polymerase chain reaction with *E. coli* isolated from broiler chickens.

Gene	Description	Primer sequence	Amplicon size (bp)	References
Adhesins				
*pap*C	P-fimbriae, Pyelonephritis associated pili	TGATATCACGCAGTCAGTAG CCGGCCATATTCACATA	501	Sanger *et al.* (1977); (Janßen *et al.*, 2001)
tsh	Temperature-sensitive haemagglutinin	ACTATTCTCTGCAGGAAGT CTTCCGATGTTCTGAACG	824	(Dozois *et al.*, 1992; Ewers *et al.*, 2007)
Iron acquisition				
*iuc*D	Aerobactin synthesis, iron uptake chelate	ACAAAAAGTTCTATCGCTTC CCTGATCCAGATGATGCT	714	(Sanger *et al.*, 1977; Janßen *et al.*, 2001)
*irp*2	Iron-repressible protein associated with yersinabactin synthesis	AAGGATTCGCTGTTACCGGA AACTCCTGATACAGGTGG	413	(Dozois *et al.*, 1992)
Serum survival				
iss	Increase serum survival	ATCACATAGGATTCTGCC CAGCGGAGTATAGATGCC	309	(Ewers *et al.*, 2007)
Toxins				
*ast*A	arginine succinyl transferase A (Enteroaggregative heat-stable toxin)	TGCCATCAACACAGTATATC TCAGGTCGCGAGTGACGG	116	(Franck *et al.*, 1998)
*hly*A	α-haemolysin	GTCCATTGCCGATAAGTTT AAGTAATTTTTGCCGTGTTTT	1177	(Janßen *et al.*, 2001; Ewers *et al.*, 2004)

Table 3

Effect of the addition of *Candida famata* and/or *Lactobacillus plantarum* in the drinking water of broiler chickens on their performance and overall dry matter availability.

	Control	CF	LP	CFLP	SEM	*P*-value		
						CF	LP	CF*LP
Weight gain (days 1-14, g/bird)	381	362	368	366	7.9	0.169	0.577	0.268
Weight gain (days 14-35, g/bird/d)	82	80	78	79	1.8	0.777	0.223	0.591
Feed intake (days 14-35, g/bird/d)	126	122	123	120	2.8	0.201	0.453	0.967
Feed conversion ratio (days 14-35)	1.54	1.52	1.57	1.52	0.02	0.070	0.370	0.322
Breast weight (g)	418	397	492	394	32.1	0.078	0.283	0.251
Breast meat yield (%)	19.4	19.5	20.5	18.4	0.82	0.238	0.993	0.196
Dry matter availability (g/kg)	648	644	616	653	0.2	0.309	0.474	0.198

CF= birds were treated with 10^8 cfu/ml *Candida famata* (CF) in their drinking water two days a week throughout their life; LP= birds were treated with 10^5-10^8 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water two days a week throughout their life; CFLP= birds were treated with a mixture of CF and LP (10^6-10^8 cfu/ml) in their drinking water two days a week throughout their life. CF*LP= the interaction between CF and LP.

Table 4

Effect of treatment, digesta site and bird age on the mean population of *E. coli*, *Lactobacillus* spp., and yeast in samples of digesta taken from broiler chickens.

	Treatment[1]												SEM	*P*-value[2]							
	CON			CF			LP			CFLP											
	Site of digesta collection[3]																				
	DUO	ILE	CAE	DUO	ILE	CAE	DUO	ILE	CAE	DUO	ILE	CAE		A	S	CF	LP	CF*LP	A*S	A*CF	A*LP
E. coli (log_{10} cfu/g)																					
Bird age (d)																					
8			9.14			8.91			8.26			8.53	0.32								
14			8.19			7.60			8.53			8.53	0.32								
22			8.60			8.93			8.92			9.34	0.26								
29			8.46			8.68			8.69			8.75	0.26								
35			8.07			7.89			7.95			7.95	0.26	***		0.826	0.437	0.344		0.533	0.064
Lactobacillus spp (log_{10} cfu/g)																					
Bird age (d)																					
8	7.77	9.42	9.69	7.73	9.18	10.1	8.16	8.98	8.25	7.99	9.09	9.22	0.37								
14	7.39	10.5	9.36	8.41	8.52	9.49	8.25	10.8	9.90	7.21	10.5	9.63	0.37								
22	6.97	8.58	9.04	7.32	8.54	9.33	6.97	8.88	9.48	7.24	8.72	9.69	0.30								
29	6.77	7.61	9.90	6.66	7.85	9.94	6.78	7.06	9.83	6.61	7.54	9.95	0.30								
35	6.87	8.01	9.17	6.67	7.89	9.03	6.50	7.69	8.97	6.89	7.63	8.34	0.30	***	***	0.620	0.852	0.875	***	0.245	0.045
Yeast (log_{10}cfu/g)																					
Bird age (d)																					
8	3.00	3.17		5.14	4.05		2.48	2.10		4.72	3.95		0.40								
14	3.43	4.52		3.42	470		3.45	4.95		3.89	5.10		0.40								
22	3.59	4.09		4.12	4.97		3.45	4.93		4.46	5.42		0.32								
29	3.53	3.13		5.48	4.09		3.29	2.35		4.91	3.90		0.32								
35	3.86	3.41		3.06	2.93		2.06	2.24		4.05	3.83		0.36	***	0.435	***	0.502	0.013	***	***	0.046

[1] Treatment: CF= birds were treated with 10^8 cfu/ml *Candida famata* (CF) in their drinking water two days a week throughout their life; LP= birds were treated with 10^5-10^8 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water two days a week throughout their life; CFLP= birds were treated with a mixture of CF and LP (10^6-10^8 cfu/ml) in their drinking water two days a week throughout their life.

[2]Abbreviations: A= bird age, S= site of digesta sampling, CF= effect of CF treatment, LP= effect of LP treatment. *= denotes the interaction between main effects. There was no significant interaction (*P*>0.05) between site and either CF or LP. Three way and four way interactions were all not statistically significant (*P*>0.05). *** denotes P<0.001.

[3]Abbreviations: DUO= duodenum, ILE= ileum, CAE= caecum. Replication: n=4 when birds 8 and 14 d old, n=6 when birds 22, 29 and 35 days old.

Table 5

Identification (by API system, bioMerieux UK Ltd, Basingstoke, UK) of the *Lactobacillus* isolates taken from samples of caecal digesta of the broiler chickens.

Bird age, d	Treat	Identification	% C[1]	Bird age, d	Treat	Identification	% C
1	N/A	*Lactococcus lactis* ssp. *lactis* 1	95.8	22	LP	*Lactobacillus paracasei* ssp. *paracasei* 1	99.9
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	92.8	22	LP	*Lactococcus lactis* ssp. *lactis* 2	61.1
1	N/A	*Lactococcus lactis* ssp. *lactis* 1	95.8	29	CON	*Lactococcus lactis* ssp. *lactis* 1	48.6
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	88.1	29	CON	*Lactococcus lactis* ssp. *lactis* 1	64.1
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	99.6	29	CF	*Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* 1	69.2
8	CON	*Lactococcus lactis* ssp. *lactis* 1	64.3	29	CF	*Lactococcus lactis* ssp. *lactis* 1	90.0
8	CF	*Lactococcus lactis* ssp. *lactis* 1	64.3	29	LP	*Lactococcus lactis* ssp. *lactis* 1	93.2
8	LP	*Lactococcus lactis* ssp. *lactis* 1	64.3	29	LP	*Lactococcus lactis* ssp. *lactis* 1	64.3
14	CON	*Lactococcus lactis* ssp. *lactis* 1	64.3	35	CON	No result	-
14	CF	*Lactococcus lactis* ssp. *lactis* 2	69.8	35	CON	*Lactococcus lactis* ssp. *lactis* 1	72.7
14	LP	*Lactobacillus plantarum* 1	97.3	35	CF	*Lactobacillus paracasei* ssp. *paracasei* 3	79.9
22	CON	No result	-	35	CF	*Lactococcus lactis* ssp. *lactis* 1	51.1
22	CON	*Lactobacillus plantarum* 1	76.9	35	LP	*Lactococcus lactis* ssp. *lactis* 1	92.2
22	CF	*Lactobacillus paracasei* ssp. *paracasei* 1	99.9	35	LP	*Lactobacillus brevis* 1	93.0
22	CF	*Lactococcus lactis* ssp. *lactis* 1	64.3				

CON= control; N/A= not applicable; CF= birds were treated with 10^8 cfu/ml *Candida famata* (CF) in their drinking water two days a week throughout their life; LP= birds were treated with 10^5-10^8 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water two days a week throughout their life; CFLP= birds were treated with a mixture of CF and LP (10^6-10^8 cfu/ml) in their drinking water two days a week throughout their life. CF*LP= the interaction between CF and LP. CON= control; CF= *Candida famata*; LP= *Lactobacillus plantarum*. [1]%C: % confidence in identification.

Table 6

Identification (by API system, bioMerieux UK Ltd, Basingstoke, UK) of the yeast isolates taken from samples of deudonal, ileal and caecal digesta of the broiler chickens.

Bird age (d)	Treatment	Gut section	Identification
14	CON	Duodenum	*Candida rugosa*
14	CON	Ileum	*Candida rugosa*
14	CON	Ileum	*Geotrichum candidum*
14	CF	Duodenum	*Candida zeylanoides*
14	CF	Duodenum	*Candida tropicalis*
14	CF	Ileum	*Candida rugosa*
14	LP	Ileum	*Candida rugosa*
14	LP	Caecum	*Candida rugosa*
14	CFLP	Ileum	*Candida rugosa*
14	CFLP	Caecum	*Candida rugosa*
22	CF	Ileum	*Candida rugosa*
22	CF	Ileum	*Candida rugosa*
22	LP	Ileum	*Candida rugosa*
22	CFLP	Ileum	*Candida rugosa*
29	CF	Ileum	*Candida glabrata*
29	CF	Caecum	*Candida glabrata*
29	LP	Caecum	*Rhodotorula glutinis*
29	CFLP	Duodenum	*Candida rugosa*
35	CON	Duodenum	*Saccharomyces cerevisaea*
35	CF	Ileum	*Candida glabrata*
35	LP	Caecum	*Rhodotorula mucilaginosa 2*
35	CFLP	Duodenum	*Candida spherica*
35	CFLP	Ileum	*Saccharomyces cerevisaea 1*
35	CFLP	Caecum	*Candida pelliculosa*

CON= control; CF= birds were treated with 10^8 cfu/ml *Candida famata* (CF) in their drinking water two days a week throughout their life; LP= birds were treated with 10^5-10^8 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water two days a week throughout their life; CFLP= birds were treated with a mixture of CF and LP (10^6-10^8 cfu/ml) in their drinking water two days a week throughout their life.

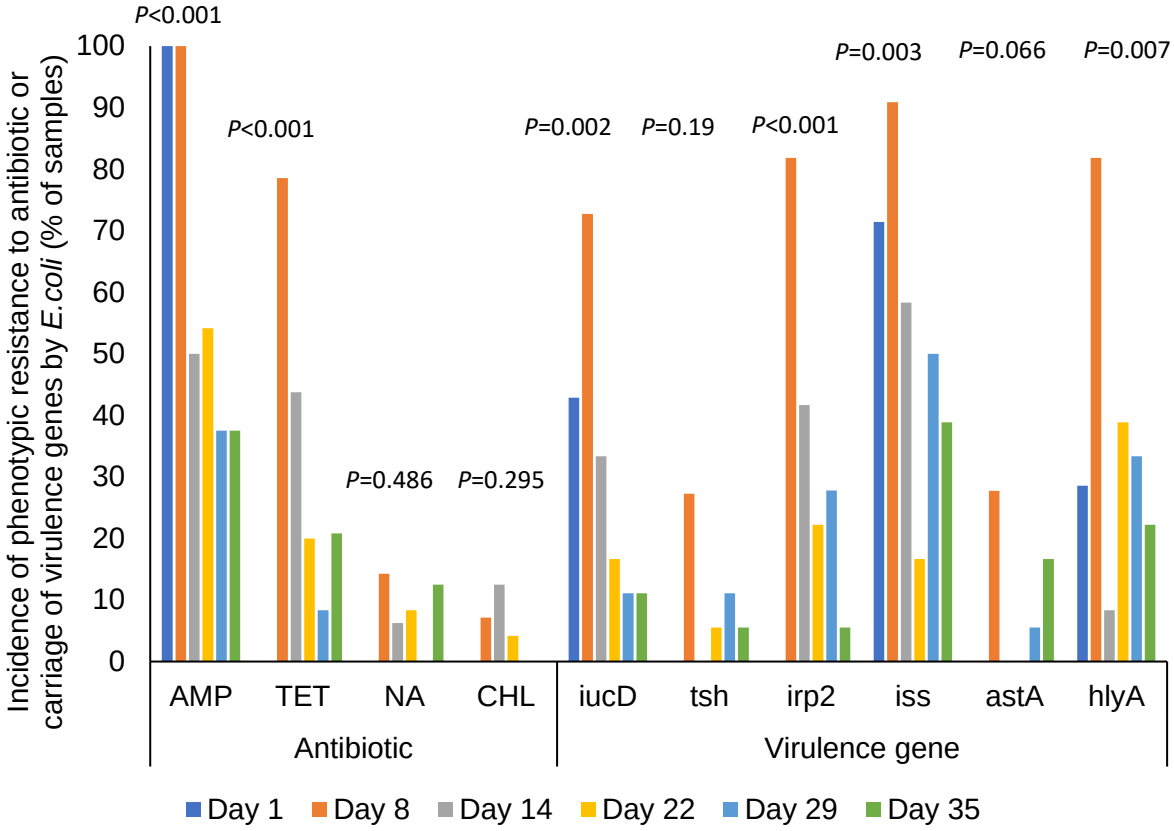

Figure 1. The percentage of *E. coli* isolates taken from broiler chickens of different ages (days of life) that were phenotypically resistant to different antimicrobials or carried different virulence genes.

AMP= ampicillin; TET= tetracycline; NA= nalidixic acid; CHL= chloramphenicol; For identification and description of genes, see Table 2.

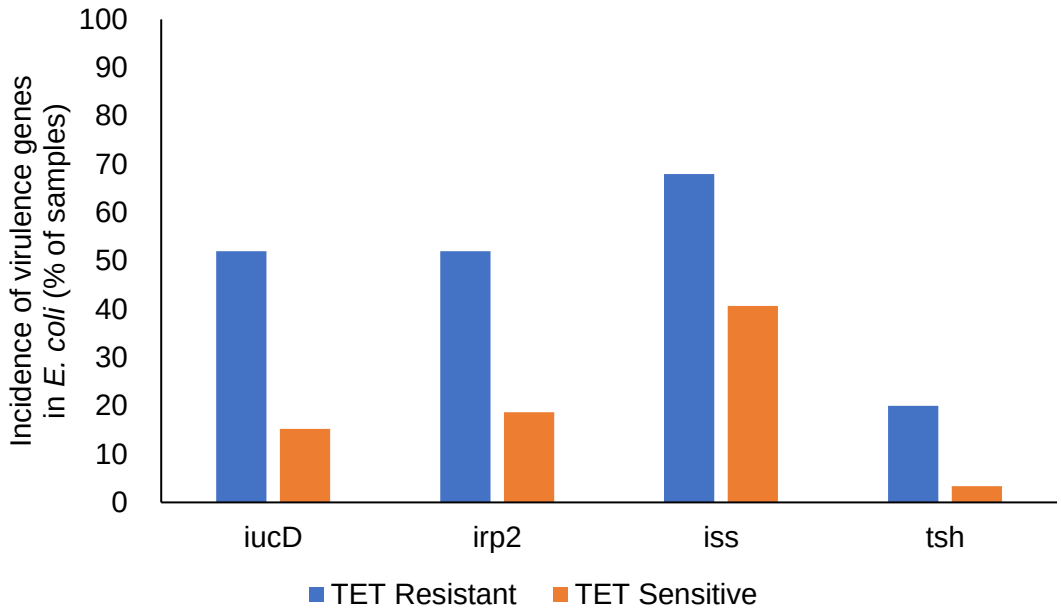

Figure 2. The association between the carriage of the genes coding for iron uptake chelate, iron-repressible protein, increased serum survival and temperature sensitive haemagglutinin and the phenotypic sensitivity or resistance by isolates of *E. coli* to tetracycline (20 µg/ml) in broiler chickens.

TET= tetracycline; For identification and description of genes, see Table 2.