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Characterisation of *Escherichia coli* in poultry and their interaction with phytochemicals

A thesis submitted towards a Doctoral of Philosophy

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DECLARATION

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

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) and commensal *E. coli* are often found in the intestinal tract of poultry. APEC can cause several types of disease manifesting at various developmental stages in the life cycle of poultry. The goals of this study were to investigate the relationship between APEC and commensal *E. coli*, and to elucidate whether specific dietary components such as plant extracts (thymol and carvacrol) may be implemented to control APEC. Genotypic and phenotypic diversities were estimated in 200 *E. coli* isolates from poultry of which 100 were from healthy turkey, 35 from healthy chicken, and 65 APEC strains were isolated from infected chicken. The genetic data indicated high diversity among *E. coli* isolates whereas phenotypic diversity association with pathogenicity was unclear.

The antimicrobial activity of thymol and carvacrol against *E. coli* had a significant impact on reducing bacterial growth, biofilm formation, and motility. Moreover, thymol reduced conjugation, and induced morphological changes in *E. coli*. An *E. coli* strain was adapted to tolerate high concentration of thymol, and its metabolic profile detected by NMR analysis showed slowed growth with a shift from respiration to fermentation as indicated by increasing lactate and pyruvate family amino acids. Genome sequencing of the tolerant strain showed a mutation in the *acrR* gene encoding a suppressor of the AcrAB-TolC efflux pump suggesting that overactivation of the AcrAB efflux pump increased thymol clearance. The impact of thymol on the composition and activity of caecal microbiota was assessed by *in-vitro* batch culture. 16S rRNA sequences were used to identify caecal microbiota and metabolic profiles were characterised by ^{1}H-NMR spectroscopy. Thymol was associated with increases in lactic acid and a growth shift favouring commensal gut bacteria. In conclusion, supplementation with thymol may exert a positive effect on intestinal microbiota if used *in-vivo*.

List of Abbreviations

$(NH_4)_2SO_4$	Ammonium Sulphate
16S rRNA	16S ribosomal RNA
^1H NMR	Proton Nuclear Magnetic Resonance
ADH	Amino acid Decarboxylations
Ado	Adonitol
All	Allantoin
Amp	Ampicillin
APEC	Avian Pathogenic *E. coli*
API	Analytical Profile Index
APV	Avian Pneumo-Virus
Ara	Arabitol
ATP	Adenosine Triphosphate
Bp	Base pair
C	Cluster
CFU	Colony Forming Units
CIT	Citrate utilization
CLSI	Clinical and Laboratory Standard Institute
CLT	Chick Lethal Toxin
D value	Discriminatory index
DAEC	Diffusely Adherent
ddH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleotide Triphosphate

Dul	Dulcitol
E. coli	*Escherichia coli*
EaggEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMB	Eosin Methylene Blue
EOs	Essential Oils
EPEC	Enteropathogenic *E. coli*
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
FT-IR	Fourier-Transform Infrared
FT-IR	Fourier-Transform Infrared spectroscopy
G	Gram
GEL	Gelatine Hydrolysis
GM	Greater Wax Moth
GRAS	Generally Recognised as Safe
GSDU	Genomic Services and Development Unit
H	Hours
H_2O_2	Hydrogen Peroxide
H_2S	Hydrogen Sulphide production
H_2SO_4	Sulphuric acid
HCL	Hydrochloric Acid
HUS	Haemolytic Uraemic Syndrome

Ino	Inositol
Iss	Increased serum survival
JM109	*E. coli* K12 strain
K_2HPO_4	Dipotassium Hydrogen Phosphate
Kbp	Kilobase Pairs
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate.
L	Litre
l^{-1}	Millie litter
LB agar	Luria-Bertani agar
LB broth	Luria-Bertani broth
LD 50	50% Lethal Dose
LDC	Lysine Decarboxylase Enzyme
LTs	Heat-labile enterotoxins
M	Molar
M9	Minimal Salts Medium
MAR	Multiple Antibiotic Resistant
MBC	Minimum Bactericidal Concentrations
McC	MacConkey agar
Mel	Melitzitose
MES	Multidrug Efflux Systems
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
MIC	Minimum Inhibitory Concentration
Min	Minute

mM	Millie Molar
MS	Mass Spectrometry
NA	Nutrient Agar
Nal	Nalidixic acid
NB	Nutrient Broth
NCCLS	National Committee for Clinical Laboratory Standards guidelines
NGS	Next Generation Sequencing
NMEC	Newborn Meningitis causing *E. coli*
NMR	Nuclear Magnetic Resonance
ODC	Ornithine Decarboxylase
OH	Hydroxyl group
ONPG	O-Nitrophenol/D-Galactopyranoside
OPLS	Orthogonal Projections to Latent Structure
OTUs	Operational Taxonomic Units
PBS	Phosphate Buffered Saline
PCA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
Pmol	Pico Molar
Pro	Proline
QRDR	Quinolone Resistance Determining Regions
Raf	Raffinose
rRNA	Ribosomal Ribonucleic Acid
S	Second
Sal	Salicin
SCFA	Short Chain Fatty Acid

SD	Standard Deviation
SEM	Scan Electron Microscopy
SHS	Swollen Head Syndrome
Sor	Sorbose
SS	Salmonella Shigella Agar
Stx	Shiga Toxin
suc	Sucrose
TAE	Tris-Acetate
TDA	Tryptophan Deaminase
tsh	Temperature-sensitive hemagglutinin
UK	United Kingdom
UoS	University of Surrey
UPEC	Uropathogenic *E. coli*
UPGMA	Unweighted Pair Group Method for Arithmetic Averages
URE	Urea Hydrolysis
V/V	Volume/Volume
VL	Viande-Leuvre medium
VP	Na Pyruvate- acetoin production
VTEC	Vero Cytotoxin *E. coli*
WGS	Whole Genome Sequence
WHO	World Health Organization

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CHAPTER ONE: LITERATURE REVIEW

Bacteria can live in water and soil as well as in living organisms, including plants, animals and humans. Many bacteria do not harm humans, but rather survive in humans as commensals and many of these are regarded as highly beneficial to the host by stimulating immune developing, suppressing pathogens and contributing to nutrition by generating dietary components such as vitamins and certain amino acids (Conlon and Bird, 2014), for example. *Escherichia coli (E. coli)* is a Gram negative, facultatively anaerobic bacterium that can live in many different environments. It is a common commensal of the gastrointestinal tract of humans and animals. However, certain *E. coli* are significant zoonotic pathogens such as the Vero cytotoxin carrying types of *E. coli* (VTEC) which causes several symptoms of disease such as diarrhoea, vomiting and fever (Berg, 2004), these can be transferred to humans by direct or indirect methods, for example; eating some products of food, both plant and animal derived contaminated with *E. coli*.

1.1 *Escherichia coli (E. coli)*: description, disease and diversity

E. coli is a prokaryote cell; a prokaryotic cell usually contains a single circular chromosome (DNA) which is not enclosed in a nucleus. In 1885 a German bacteriologist called Dr. Theodor Escherich discovered this bacterium. He identified it in stool specimens collected from babies with enteritis; a disease that can cause stomach pain, nausea, vomiting and diarrhoea (Manning, 2010). The significance of *E. coli* as a human pathogen has been identified since its discovery, and the organism has been associated with a range of disease presentations such as diarrhoea, haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) surgical wound infection, septicaemia, meningitis, dysentery, bladder and kidney infections, and pneumonia. Some of these diseases are fatal especially in the very young, the elderly and the

immunosupressed. Different strains of *E. coli* are associated with different clinical outcomes (Parry *et al.*, 2002) and these will be described very briefly here.

E. coli is a straight, rod-shaped, Gram-negative bacterium. It can live on a broad variety of substrates. It is often motile by its flagella (Darnton *et al.*, 2007). *E. coli* is easy to cultivate on ordinary laboratory media, and there are many formulations of selective and differential laboratory media that allow optimal survival and growth of specific *E. coli* strains. For example, Eosin Methylene Blue (EMB) agar medium allows the differential growth of Gram negative bacteria, including *E. coli*, from intestinal inoculations. The *E. coli* colonies will be differentiated by color in comparison to other bacterial species in the inoculum. Another example is MacConkey's (McC) medium that is similar to EMB medium as it differentiates Gram negative, lactose fermenting bacteria based on their color formation. *E. coli* is defined as fermenting lactose and therefore would give a distinct pinkish color when grown on McC medium. However, only 98% utilize lactose (Edwards and Ewing, 1986) and some mutate to use it and were originally called *Escherichia mutabile*.

E. coli is a facultative anaerobe growing well in aerobic or anaerobic conditions. End products of growth on glucose are lactate, ethanol and acetate or organic acids with gas, usually hydrogen and carbon dioxide. *E. coli* reduces nitrates to nitrites and is also oxidase negative and catalase positive. Since many pathways in mixed-acid fermentation produce hydrogen gas, so is the case when *E. coli* lives with hydrogen-consuming organisms, such as methanogens or sulfate-reducing bacteria (Ingledew and Poole, 1984). The optimal growth temperature of *E. coli* is at 37°C, but in some cases it can grow and divide at temperatures of up to 46°C, but can survive at a higher temperature of 55°C (Fotadar *et al.*, 2005).

E. coli comprises a large number of types that have been isolated and characterized based on serological findings. Serology tests three groups of antigens to classify *E. coli*. These

antigens are; the lipopolysaccharides layer O antigen that comprises three parts, the outer immunogenic oligosaccharide polymers, the inner core phosphorylated oligosaccharides, and the lipid A endotoxin. The other two antigen groups are the capsular K antigen, and the flagellin H antigen. There are 181 designations for the O antigen, 60 different K antigens, and 56 H antigens from which a serotype profile of a given *E. coli* strain can be designated. For example, VTEC O157 possesses 'O' antigens 157, and 'H' antigen 7 (Bell and Kyriakides, 1998). Together they compose the serotype O157:H7. Sub-classification of several serotypes of *E. coli* is based on distinguishable exotoxins produced by *E. coli*, an example of exotoxins are the verocytotoxins which damage the intestinal tract, and in some people cause serious kidney failure. *E. coli* that cause diarrhoea can also be categorized on the basis of the mechanisms by which they cause disease (Berg, 2004). Currently the pathogens within the species are divided into eight groups as shown in **Table 1.1** (Parry *et al.*, 2002; Kausar *et al.*, 2009; Bidet *et al.*, 2007; Bell and Kyriakides, 1998) although these 'divisions' are under constant review and debate.

The mechanisms of disease in all the strains depend upon their genetic composition and virulence factors that may be encoded on the chromosome or extra chromosomal mobile genetic elements such as plasmids and bacteriophages: one consequence of this is that many virulence traits can be transferred from one serotype of *E. coli* to another, which frequently occurs in bacterial populations.

Table 1. 1: Serogroup and disease association of six types of *E. coli*

Virulence type	Sero-group examples	Disease association
Enteropathogenic (EPEC)	O18ab, O18ac, O26, O44, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, O158.	Enteritis in infants Diarrhoea in children under 6 months. Traveller's diarrhoea
Enterotoxigenic (ETEC)	O1, O6, O8, O11, O15, O25, O27, O63, O78, O114, O115, O148, O153, O159.	diarrhoea, vomiting and fever Traveller's diarrhoea
Enteroinvasive (EIEC)	O11, O28ac, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O164, O167, O173.	Shigella-like dysentery
Enterohaemorrhagic (EHEC)	O2, O4, O5, O6, O8, O15, O18, O22, O23, O26, O55, O75, O91, 103, O104, O105, O111, O113, O114, O117, 118, O121, O128ab, O145, O153, O163, 0157, O168	Shigella-like dysentery, stools contain blood and mucus Bloody diarrhoea (HUS)
Enteroaggregative (EaggEC)	O51, O78, O111	Persistent diarrhoea in children
Diffusely adherent (DAEC)	O75, O126	Persistent Childhood diarrhoea
Uropathogenic *E. coli*(UPEC)	O20,O131,O25,O101,O60,O8,O9, O156,O6,O153,O128,O2,O115,O136,O141,O89,O54,O21,O132,O12, O13,O15,O79,O152	Urinary tract infections
Extraintestinal Pathogenic *E. coli* (ExPEC)	O1,O2,O12,O14,O15,O16,O18,O45, O78,O83	Neonatal meningitis

Pathogenic *E. coli* are not only harmful to humans, but they are also harmful to farm animals and poultry. *E. coli* diseases in farm animals are similar to those of humans; however, they are referred to as colibacillosis. Colibacillosis is of two types enteric and systemic, the former primarily colonizes the intestine and secretes toxins that may have localized or dispersed effects. Systemic colibacillosis, however, involves aggressive *E. coli* strains that can survive and invade extra-intestinal tissues to generate a systemic pathological condition in the

animal. Different types of toxins determine the type of *E. coli* strain responsible for disease; these are classified as depicted in **Table 1.2**.

Table 1. 2: Heterogeneous *E. coli* groups causing disease in farm animals and poultry (Wray and Woodward, 1979)

E. coli group	Toxin/factor	Farm animal disease
Enterotoxigenic *E. coli* (ETEC)	colonization factors (*fimbriae*) and enterotoxins (ST, LT)	Diarrhoea, septicaemia
Enteropathogenic *E. coli* (EPEC)	virulence sequences (*LEE, locus of enterocyte effacement)*	lethal diarrhoea in young mammal
Shiga toxin producing *E. coli* (STEC)	Shiga toxin and *cytotoxic necrotizing factors*	may cause diarrhoea, and in young pigs it is associated with oedema disease
Avian pathogenic *E. coli* (APEC)	virulence factor insufficiently known	Airsacculitis, peritonitis, polyserositis and septicaemia

Aside from major threats to farm animals' health and survival, *E. coli* infections pose serious economic losses to farmers and a health threat to consumers. Animals excreting the pathogen without showing any clinical symptoms can go undetected and end up being consumed by humans. Mildly infected patients may suffer from abdominal pain or watery diarrhoea and symptoms can become more severe, including bloody diarrhoea, hemorrhagic colitis and haemorrhagic uremic syndrome.

1.2 Commensal *E. coli*

Commensal *E. coli* strains as mentioned above, are present in the intestines and faeces of warm-blooded animal hosts (Berg, 1996) including humans (Blyton *et al.*, 2013). They are found in the gut microbiota that has diverse bacterial species, for example in poultry gut microbiota ranges from 10^7 to 10^{11} bacteria per g of gut content (Apajalahti *et al.*, 2004). Every mammal is colonized with *E. coli* (Finegold *et al.*, 1983), it is present more at the first day of

bird life, and decreases thereafter (Lu *et al.*, 2003). Whereas in humans, it is estimated that there are 10^{21} of *E. coli* cells in the total human population which equates to 10^{9} per human (Conway and Cohen, 2015), it is also one of the early colonisers of human infants and is a lifelong colonizer of adults (Palmer *et al.*, 2007). Commensal *E. coli* are considered to be a factor in supporting digestion and providing protection against enteric pathogens by effectively contending within microbiota populations, stimulating immune responses and obstructing colonisation of pathogenic agents. In addition, commensal *E. coli* produce vitamins K and B12 that are required by the mammalian host (Schierack *et al.*, 2009; Bentley and Meganathan, 1982; Lawrence and Roth, 1996). Commensals are little understood, and besides serotype, they also can be classified according to their phylogeny (as can the pathogens) which is the inferred evolutionary history based on genome sequence data. A problem for classification is the fact that 'similar' bacteria can be commensal and pathogenic, and differentiation becomes difficult as will be discussed later in this thesis.

1.3 Avian pathogenic *E. coli* (APEC)

Avian Pathogenic *E. coli* (APEC), a subgroup of Extraintestinal Pathogenic *E. coli* (ExPEC), is found in the normal microflora of the intestinal tract of healthy poultry, including chickens, turkeys, ducks and other poultry species, but that can lead to infection and disease that causes major economic losses in the poultry industry around the world (Gross, 1994; Landman and van Eck, 2015; Ewers *et al.*, 2003). APEC can cause several types of diseases manifesting at various ages in poultry, broiler (meat) poultry and in egg-laying mature poultry. The most common diseases are respiratory tract infection-colibacillosis, yolk sac infection, swollen head syndrome, septicemia, polyserositis and cellulitis serum resistance. In young turkey birds, a disease called turkey osteomyelitis complex causes several types of symptoms such as soft tissue abscess, green stained liver, and osteomyelitis of the proximal tibia (Dziva and Stevens, 2008; Cunha *et al.*, 2014). The first phase of infection by APEC is colonization

of the trachea and air sacs, the second stage of infection is observed as colonization of the liver and pericardium followed by bacteremia.

1.3.1 Colibacillosis

Colibacillosis caused by APEC strains can affect day-old chicks, broilers and egg-layers. Colibacillosis is the most important disease affecting avian production worldwide, and is one of the main causes of economic losses in the poultry industry (Elfadil *et al.*, 1996; Lutful Kabir, 2010). Lesions related to colibacillosis mostly consist of airsacculitis, peritonitis, polyserositis and septicemia (Vandekerchove *et al.*, 2004). In general, colibacillosis is considered as a secondary disease perhaps after prior exposure to respiratory viral infections or Mycoplasma infections, however, it can also be a primary cause of disease (Cheville and Arp, 1978; Zanella *et al.*, 2000). Colibacillosis is initiated in the upper respiratory tract; this commonly happens after a primary infection with an immune suppressive disease caused by different pathogens such as Newcastle virus, infectious Bronchitis virus or *Mycoplasma* (Gross, 1991). However, these primary infections could increase the susceptibility of poultry to APEC strains owing to the declination of the upper respiratory cells and environmental influences, such as high concentrations of ammonia, and contaminated dust in poultry premises, which contribute to their respiratory stress (Nakazato *et al.*, 2009). In addition, this infection referred to as air sac disease generally happens in birds of 2 to 12 weeks old. The majority of the cases occurring in birds of 4 to 9 weeks old, and mortality rates reach as high as 20% (Dho-Moulin and Fairbrother, 1999). Usually death is the end of colibacillosis, although some birds might totally recover, or retain some residual disease with such sequelae as meningitis, swollen eye, osteoarthritis and coli granuloma that is characterized by multiple granulomas in liver, duodenum and cecum (Nakazato *et al.*, 2009).

1.3.2 Swollen head syndrome

Swollen head syndrome (SHS) affects poultry and especially chicken and turkey species which are natural hosts. SHS is generally seen after the 4th week of poultry life. SHS was first described in South America (Morley and Thomson, 1984) and was considered to be caused by coronavirus and *E. coli*. SHS is a complicated infection, usually a multi-factorial disease; where the primary etiological agent is an avian pneumo-virus (APV) and secondary to it is usually an *E. coli* infection. Respiratory and nervous pathological signs including gelatinous oedema in the skin of the head and peri-orbital tissues characterize SHS. The first clinical sign of infection is sneezing, and in some cases profuse tear secretion. The inflammatory exudate is initially transparent, but becomes opaque afterwards (Pattison *et al.*, 1989; Hafez and Lohren, 1990; Nunoya *et al.*, 1991). Arns and Hafez estimated that SHS disease is an important poultry disease in several countries (Arns and Hafez, 1992). SHS is said to cause significant losses in the poultry industry. For example, SHS is responsible for mortality of 3-4% of the birds and also for reduction of 2-3% of the egg production in Southern Africa (Morley and Thomson, 1984).

1.3.3 Cellulitis

Cellulitis in broiler chickens is an acute inflammation of connective tissues of the overlying chicken skin. It is characterized by the presence of subcutaneous fibrino-necrotic plaques and pus in the abdominal area. Cellulitis does not seem to affect the growth of poultry but results in complete or partial discard of the carcass at processing (Messier *et al.*, 1993). APEC causes cellulitis in broiler chickens (de Brito *et al.*, 2003; Jeffrey *et al.*, 2002). The lesion is initiated by a break in the integument, followed by a bacterial infection. Bacteria such as APEC adhere to the deeper and superficial tissue layers of the skin that appears to be important in the development of lesions and may be promoted by type 1 fimbriae (Gyles, 2010). The

epidemiology of cellulitis remains unclear, nevertheless it is associated with losses in the poultry industry (Elfadil *et al.*, 1996). Some studies of *E. coli* strains isolated from cellulitis lesions expressed many virulence-associated factors similar to those presented by strains isolated from other colibacillosis lesions and from faeces (Ngeleka *et al.*, 1996; Jeffrey *et al.*, 2002; de Brito *et al.*, 2003). However, other studies presented a positive relationship between APEC and ExPEC, mostly UPEC and Newborn meningitis causing *E. coli* (NMEC), proposing that some APEC strains could be considered probable zoonotic agents (Moulin-Schouleur *et al.*, 2007; Ewers *et al.*, 2004; Johnson *et al.*, 2008b) although there is debate about this association.

1.4 APEC virulence-associated factors properties.

There are many virulence-associated factors that add to the pathogenicity of organisms by providing a survival advantage to cope with hostile environments within hosts and cause infection. Several investigations discovered pathogenic mechanisms specific to APEC strains. The most important APEC virulence factors include bacterial capsule that is composed of polysaccharides to enable the avoidance of host immunogenic protection; multiple adhesins such as fimbriae that mediate adherence to cells and tissue surfaces; hemolysins; iron acquisition systems; colicins; serum resistance and various cytotoxins. Moreover, formation of biofilm on surfaces is considered important also.

1.4.1 Bacterial capsules

K antigen is not present on all strains, it depends on the presence of terminal lipid A-core of the outer membrane lipopolysaccharide or its absence. The difference between O and K antigen serotypes is their sugar composition, the linkage specificity in the polysaccharides, and the substitution of non-carbohydrate residues with other macromolecules (Whitfield and Roberts, 1999). Capsular antigens are considered virulence factors because they increase the

pathogenicity of bacteria by protecting the bacteria against non-specific host defenses during the early phase of infection (Jann and Jann, 1992). Capsular antigens consist of acid polysaccharides made up of repeating oligosaccharide units and are divided into two groups based on their chemical characteristics, biochemistry and genetics (Jann and Jann, 1992). Group I express capsule antigens at all growth temperatures. Members of group I belong to *E. coli* sero groups O8, O9 and O20, and their capsule composition is determined by chromosomal genes close to the *his* locus and in some strains close to *trp*. Usually the capsular acid polysaccharides of group I have large repeating units of tetra to hexa-saccharides, and hexuronic acid and pyruvate are the most common acid components. While group II is only detectable above 25ºC and belong to many O-groups. Co-expression of group II capsular antigens with lipopolysaccharides is not regulated, and chromosomally determined by the *kps* gene cluster, which is close to the *serA* gene. These polysaccharides have di- or tri-saccharide repeating units, their acidic components are more diverse than group I, hexuronic acids, *N*-acetylneuraminic acid, 3-deoxy-*manno*-D-octulosnic acid, mannosaminuroinic acid or phosphate as possible representatives (Sussman, 1997). Recently, developments in the biochemical characterization and molecular genetics of K antigens updated the classification system and divided capsules into four groups based on their biosynthetic criteria and genetics eliminating the reliance on serological identification and the use of polysaccharide structures as a predictive element (Whitfield and Roberts, 1999).

1.4.2 Adhesins

Respiratory tract infections caused by Gram-negative bacteria are instigated by molecular interactions between bacterial adhesins and complementary molecules on host cells called receptors. APEC adhesions include type 1 fimbriae and P fimbriae. Type 1 fimbriae known as common fimbriae of *E. coli*, is a hair like structure distinguished by its ability to bind to D-mannose containing residues on various surfaces. Type 1 fimbriae called also type 1 pili

are known to play an important role to encourage bacterial adhesion and grow as a biofilm (Martinez *et al.*, 2000; Schembri and Klemm, 2001). Fimbriae cause mannose-sensitive haemagglutination, which is the agglutination of guinea pig erythrocytes in the absence of mannose (da Rocha *et al.*, 2002). It also has the ability to recognize and bind to various human, animal mucosal and inflammatory cells (Sussman, 1997). While type I fimbriae is associated with upper respiratory tract colonization, P-fimbrial adhesion may be involved only in maintaining bacterial infection in birds (Mei *et al.*, 1997; Wooley *et al.*, 1998). Marc and co-authors (Marc *et al.*, 1998) established with the utilization of a *fim*-APEC mutant that type 1 fimbriae are not strictly required as a colonization factor for the development of avian colibacillosis.

The chaperone/usher dependent pathway assembles P-fimbriae forming these adhesive fimbriae. Chaperone/usher is one of three basic molecular mechanisms of presenting hundreds of different kinds of adhesive proteins and organelles that have been described in Gram-negative bacteria. Kallenius and co-authors (Kallenius *et al.*, 1981) associated P-fimbrial adhesins of *E. coli* strains with human urinary tract infections. The existence of pilus adherence in these pathogenic strains facilitates colonization of the avian respiratory and urinary tract, an event that seems to be a prerequisite for the expression of virulence. P-fimbriae cause mannose-resistant haemagglutination, and is encoded by the *pap* operon that contains 11 genes. Those genes encode regulatory, assembly, and structural proteins necessary to form an adhesive complex structure on the surface of *E. coli* (Sussman, 1997).

In addition, curli fimbriae are the most common APEC adhesin, and are considered as a third category of *E. coli* surface organelles along with flagella and fimbriae. Curli fimbriae are described as a thin coiled, aggregative-like fibers on the surface of *E. coli*, and are composed of a single type subunit referred to as curlin (Olsen *et al.*, 1989). APEC strains associated with diarrheal sepsis are known to express curli (Olsen *et al.*, 1993). Curli fimbriae

are encoded by the *csg* gene cluster containing two different operons, and requires expression of both operons (Olsen *et al.*, 1989). One operon encodes the *csgA, csgB, and csgC* genes, and the second codes for *csgD, csgE* and *csgG* (Gophna *et al.*, 2001). However, curli expression is associated strongly with biofilm formation and adhesion to human proteins facilitating invasion of eukaryotic cells (Olsen *et al.*, 1989; Olsen *et al.*, 1993). Flagella beyond motility are required for biofilm formation by multiple pathogens, such as *Salmonella enterica* and pathogenic *E. coli*, it has been suggested to assist in overcoming surface repulsive forces and spreading of cells along a surface. Exopolysaccharides and surface antigens develop biofilm morphology (Danese *et al.*, 2000). That and extracellular features play important roles in allowing bacteria to adapt and adhere to surfaces (Friedlander *et al.*, 2015).

1.4.3 Iron sequestering system

Iron is an essential element for bacterial metabolism, survival and to achieve full virulence for bacterial pathogens (Mietzner and Morse, 1994). Iron assists vital roles in cellular activities, such as energy generation, peroxide reduction, oxygen and electron transport, and nucleotide biosynthesis (Skaar, 2010). Iron exists at low concentrations in extra-intestinal sites of infection. APEC strains survive and grow in low concentrations of iron availability, generally inside the host through the expression of iron acquisition systems. Only the ferrous iron (Fe^{2+}) transporter Feo is common to all commensal and pathogenic strains. Bacterial iron acquisition mechanisms have two ways to take up iron, direct and indirect (Gao *et al.*, 2012). The direct way of iron transport system is by either haem-containing protein, such as haemoglobin or haemopexin.

The indirect way for iron acquisition is based on a shuttle mechanism; it uses small-molecule compounds called siderophores as high-affinity ferric iron chelators (Williams and Griffiths, 1992), in which the iron exists in the ferric state (Fe^{3+}) in aerobic environments.

Almost all strains produce one or more siderophores, but no single siderophore is commonly produced by all. Two types of siderophores are known; fenolates and hidroxamate (Nakazato *et al.*, 2009). Aerobactin is an example of important hydroxamate siderophore that is encoded by a plasmid operon and contributes significantly to the virulence of ExPEC (Torres *et al.*, 2001) often located on Co1V plasmids whereas enterobactin, a fenolate and the strongest known siderophore that is present in all entero-invasive *E. coli* (EIEC) (Dall'Agnol and Martinez, 1999; Andrade, 2000). Yersiniabactin is another siderophore that is expressed by ExPEC strains (Fetherston and Perry, 1994), that has a preserved chromosomal gene island that encodes a biosynthetic gene cluster of *irp* and receptor *fyuA* (Carniel *et al.*, 1996).

1.4.4 Serum resistance

Serum immune components are an important defense mechanism in the host against foreign invasion that has a lethal effect on Gram-negative bacteria. Bacterial resistance to the serum complement system immune response is mediated by bacterial surface structures including the capsular antigen, lipopolysaccharides, and outer membrane proteins that correlate with virulence in most strains (Lynne *et al.*, 2007). Also, the presence of type 1 fimbriae is associated with serum resistance of APEC strains. In contrast, Mallata and co-authors (Mellata *et al.*, 2003) showed type 1 fimbriae did not appear to be important in serum resistance, at least for strain MT78. In 2000, Pfaff-McDonough and co-authors (Pfaff-McDonough *et al.*, 2000) reported the *iss* factor which increased serum survival of bacteria, is associated with APEC pathogenicity since the *iss* gene was found more frequently in pathogenic strains than non-pathogenic strains. In addition to the *iss* factor, the O78 polysaccharide, and the K1capsule antigen are virulence factors that increase bacterial serum survival (Mellata *et al.*, 2003).

1.4.5 Colicin

Colicins are encoded by plasmids that are carried in *E. coli* and related bacteria. The plasmids code for a toxin to kill other bacteria, also code for proteins that neutralize toxic proteins. These toxic colicins inhibit bacterial growth of the same or related species. Hardy found that colicins are composed of two units; the first unit provokes bacterial cell lesions, and the second unit protects the bacteria against their own colicins (Hardy, 1975). Colicins are encoded by genes located in *Col* plasmids (Nakazato *et al.*, 2009). Colicins la, lb, E1, E2, E3, I, K, B and V are the most prevalent among APEC (da Silveira *et al.*, 2002a), and the majority of APEC strains have colicin V plasmids (Wray and Woodward, 1979). Mutations in *colV* plasmids result in decreased virulence. Skyberg and co-authors suggested that some genes linked to *colV* plasmids are involved in the establishment of avian infections (Skyberg *et al.*, 2008).

1.4.6 Biofilm formation.

A biofilm is a complex aggregation of microbial cells marked by the excretion of a protective and adhesive matrix; it is recognised as a surface attached community with microbial cells interconnected by extracellular matrix polymeric substances. Biofilm can be either beneficial or harmful; it can benefit the bacterial population, while on the other hand it can cause serious problems to human health, the environment and industry. The majority of microorganisms form biofilms under various conditions (Sutherland, 2001). Residence in a biofilm community offers certain advantages to bacteria, one of which is the ability to acquire transmissible genetic elements, such as plasmids, at elevated rates (Davey and O'Toole G, 2000).

Among many biofilm associated bacterial pathogens only a few environmentally important bacterial species, such as *E. coli*, *P. aeruginosa*, *P. fluorescens* and few Gram-

positive bacterial pathogens are characterised extensively at the genetic level (Liu and Jansson, 2010). The extracellular polymeric substances produced by bacteria are necessary for the irreversible attachment of bacterial cells to environmental surfaces or tissue surfaces and these components include polysaccharides, extracellular DNA, proteinaceous compounds (Sutherland, 2001; Davey and O'Toole G, 2000), and cellulose (Zogaj *et al.*, 2001). Some bacteria switch between planktonic growth and a biofilm lifestyle in response to environmental changes. Bacteria growing in biofilms can withstand nutrient limitation and other stressful conditions, such as osmotic stress, mechanical stress, anoxia, pH changes, temperature, oxygen levels and exposure to antibiotics (Danhorn and Fuqua, 2007; Stanley and Lazazzera, 2004). Biofilms are habitually highly resistant to antibiotics with bacterial cells in biofilms tolerating up to 1000 times higher concentrations of antimicrobial agents than that needed to kill the corresponding planktonic bacterial cells (Hoyle and Costerton, 1991). Also, biofilm formation is a dynamic multi-stage process that includes initial attachment, microcolony formation, biofilm maturation, and ultimately dispersion (Padera, 2006; Costerton *et al.*, 1999). This is of particular interest to the poultry industry as the virulence and antimicrobial resistance of APEC (Delicato *et al.*, 2003), may be largely mediated by conjugative plasmids (Dozois *et al.*, 2000; Rodriguez-Siek *et al.*, 2005a). Thus, residence in a biofilm may enhance the ability of APEC to acquire plasmids, enabling it to efficiently cause disease and resist therapy to the detriment of animal and public health.

1.4.7 Conjugation

Conjugation is an important mechanism to transfer genetic information between bacterial strains horizontally. However, horizontal gene transfer can also occur through transformation and transduction. Conjugation can occur among distant bacterial organisms at a high frequency and is considered to be a major mechanism for creating new genetic traits in diverse environments (Hoffmann *et al.*, 1998; Ravatn *et al.*, 1998). In addition, there is

evidence of gene transfer from bacteria to eukaryotes (Doolittle, 1998) as well as animal to bacterial parasites (Wolf *et al.,* 1999). Moreover, bacteria residing in the gastrointestinal tract exchange genetic information between them, notably antimicrobial resistance is an increasing clinical problem (Sommer and Dantas, 2011). In fact, conjugation is classified as an essential contributor to the distribution of antimicrobial resistance and virulence factors in bacterial populations (de la Cruz and Davies, 2000). Lederberg and Tatum were the first to describe conjugation in *E. coli* in 1946 (Lederberg and Tatum, 1946) and later Hayes (Hayes, 1953) identified the F plasmid (fertility factor F), that for a long time was the only identified conjugation plasmid. Plasmids are self-replicating genetic entities separate from the chromosome that contain a specific subset of genes from the bacterial genetic pool and are often mobile genetic elements (Eberhard, 1990; Burrus *et al.*, 2002; Tatum and Lederberg, 1947; Bates *et al.*, 1998). A donor cell extends one or more projections pili that attach to a recipient cell and pull the two bacteria in close proximity, this pilus is a protein structure. (Griffiths *et al.*, 2000.).

1.5. Analysis of APEC virulence determinants.

Various nucleic acid–based techniques have been used as molecular biological tools to assess the clonal variability of many bacteria including *E. coli.* Such molecular techniques include pulsed-field gel electrophoresis (PFGE), plasmid profiling, and polymerase chain reaction (PCR)-based methods such as randomly amplified polymorphic DNA PCR (RAPD-PCR), repetitive extragenic palindromic sequence PCR (Rep-PCR), and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) (Versalovic *et al.*, 1991; Chansiripornchai *et al.*, 2001; Hollmen *et al.*, 2011). The use of new molecular genetic techniques and their applications to microbial ecology has established that only a small proportion of natural microbial diversity has been discovered. More genetic information of microorganisms may elucidate more the evolution of bacterial environment (Ramazanzadeh *et al.*, 2013).

Rep-PCR is a genotypic BST method that uses oligonucelotide primers complementary to repetitive sequences dispersed throughout the genome of *E. coli* (Versalovic *et al.*, 1991). Therefore, REP-PCR is considered a genomic fingerprinting technique that generates specific strain patterns obtained by the amplification of repetitive DNA elements present along the bacterial genome (Busch and Nitschko, 1999). REP elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (de la Puente-Redondo *et al.*, 2000). ERIC-PCR; also known as intergenic repetitive units' PCR, is based on DNA fingerprints that are specific to individual strains. It is a rapid method for molecular typing of *E. coli* strains, and has been described for most bacterial species such as Enterobacteriaceae family (Hulton *et al.*, 1991). The ERIC sequences are located in intergenic regions as palindromes of 127 bps (Sharp, 1997; Hulton *et al.*, 1991). The ERIC and REP-PCR techniques' approach is a rapid and highly reproducible methods (Mehta *et al.*, 2002), useful for surveying *E. coli* strains in complex samples and identifying genetic diversity (Ramazanzadeh *et al.*, 2013).

Many of these methods whilst still valid have been supplanted by whole genome sequencing and various analyses of gene content, SNPs, inferred multi-locus-strain typing (MLST) may be applied to this data rich source of information covering the entire genome rather than limited regions as generated by the other techniques. Cordoni and other have probably undertaken the most detailed analysis of APEC to date by genome analysis that challenges the use of virulence determinants and serotyping to classify and identifying 10 genomo-clusters (Cordoni *et al.*, 2016).

1.6 Modes of APEC control

1.6.1 Biosecurity

The prevention and protection of poultry from disease is important. Biosecurity is one of the most effective methods and consists of various measures such as concrete curtains around buildings, limited entry, disinfectant dips for boot and vehicle tyres, insect and bird control, filtered air supply and so on are used to prevent such introductions of disease-causing organisms into housed flocks. Biosecurity can reduce the magnitude of financial losses that may occur following infection (Gifford *et al.*, 1987; Waage and Mumford, 2008), and also can reduce the risk of introducing disease (Shane, 1993). Good biosecurity should be practiced at all times to help eliminate mycoplasma infections in primary breeding flocks. Moreover, it can help excluding other infections such as, laryngotracheitis virus infection, fowl cholera and the cycling of respiratory viruses (Stewart, 1987).

1.6.2 Vaccines

Vaccines are commonly administered veterinary medicines in poultry production to control some of the diseases. Vaccines are biological products that provide active acquired immunity to an individual disease and characteristically contain an agent which resembles a disease-causing microorganism, often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. Many kinds of vaccines are produced against many organisms, and are currently available against viral, bacterial and some parasitic diseases. For example, vaccine for Newcastle disease contains an attenuated virus which is a live but its ability to cause disease has been significantly reduced, but a combination of a live and inactivated Newcastle disease vaccine, administered simultaneously, is shown to provide better protection against virulent Newcastle disease vaccine and has been successfully used in poultry production (Senne *et al.*, 2004). Another vaccine was used in poultry is against

Salmonella infections, such as a novel trivalent inactivated *Salmonella* vaccine consisting of *Salmonella Enteritidis*, *Salmonella Typhimurium*, and *Salmonella Infantis* which can be an effective tool for controlling the *Salmonella* infections of different groups of serotype in chicken farms (Deguchi *et al.*, 2009). On the other hand, no vaccine can be 100% effective (Marangon and Busani, 2006), if the birds are vaccinated but exposed to large levels of the wild disease then the immunity generated by the vaccine can be overcome.

A wide range of methods of poultry vaccine administration is available, in both the hatchery and on poultry farms. The choice of method depends on the type of antigen in the vaccine and depends upon other factors such as the type of production, bird species, size of the flock, length of the production cycle, general health status, other vaccines to be applied and costs. Vaccines for poultry originate in two general forms: Modified (Live) usually given in the drinking water, or by aerosol spray or injection to protect poultry of all ages, and inactivated (Killed) given by injection, usually to older birds before the start of egg production to protect both the bird and its offspring. Generally, inactivated vaccines induce high and uniform levels of protection after administration of a live vaccine (Marangon and Busani, 2006). Viruses stimulate the development of vaccine induced immunity more than other types of microorganisms, so most successful poultry vaccines are against viral diseases such as; Marek's disease vaccine that is administered to chickens at the hatchery on the day they hatch. This vaccine is given medically under the skin at the back of the neck (Liu *et al.*, 2014). For the specific protection against APEC infection Poulvac *E. coli* can be used in poultry for active immunisation (Nagano *et al.*, 2012). Poulvac *E. coli* contains the live *E. coli*, given by spray vaccination at day one of age (Fernandes Filho *et al.*, 2013).

1.6.3. Antibiotics

Bacteria demonstrate two kinds of resistance to antibiotics, namely intrinsic that the species is resistant to an antibiotic even before its introduction, and acquired, that the species was originally susceptible to an antibiotic but later became resistant. Bacteria can acquire antibiotic resistance either by mutation or through exchange of genetic material among same or closely related species (Fernandez and Hancock, 2012; Nikaido, 1998). Resistance to several different antibiotics at the same time is even a more significant problem (Poole, 2012). In Gram-negative bacteria resistance arises through one of several biochemical mechanisms relating to inactivation of the antibiotic, often acquired resistance and enzyme dependent, and mutation that changes the site of activity of the antibiotic or related to reduced influx and enhanced efflux. By way of example, resistance in Gram-negatives to the β-lactamase antibiotic is enzymic whereby β-lactamases have presumably evolved to fight natural β-lactams produced by bacteria (Pucci and Bush, 2013) that cleave the lactam ring. However, the common administration of antibiotics has heavily influenced the development of β-lactamase-mediated resistance. One of the first β-lactamases able to confer resistance to β-lactams is known as TEM-1. This enzyme was found in *E. coli* isolated from a patient named Temoniera, and was detected and defined in the sixties after the widespread use of ampicillin in human medicine (Rawat and Nair, 2010; Von Salviati *et al.*, 2014). There are over 100 differing classes of β-lactamase that have been defined. TEM-1 and Amp C are the most commonly found, especially in poultry production

Certain bacteria can often become resistant to antimicrobials through a mechanism known as efflux (Poole, 2002; Nikaido, 1998) that occurs due to the activity of membrane transporter proteins widely known as multidrug efflux systems (MES). They are implicated in a variety of physiological processes other than efflux and identifying their natural substrates

and inhibitors is an active and expanding research discipline (Piddock, 2006; Fletcher *et al.*, 2010).

Several methods of antibiotic susceptibility testing exist, for example, quantitative methods, qualitative methods and automated susceptibility tests (Ambaye *et al.*, 1997; Jenkins and Schuetz, 2012). Quantitative methods assess the minimum amount of antibiotic that inhibits the visible growth of an isolate and a minimum inhibitory concentration (MIC) is determined. A bacterial isolate is subjected to various dilutions of an antibiotic, the highest dilution of antibiotic that has inhibited the growth of bacteria is considered as MIC, it can be made in broth or solid agar. Qualitative methods classify a bacterial isolate as sensitive, intermediate or resistant to a particular antibiotic such as disk diffusion method. The disc diffusion method is commonly used for which there are two types of disc diffusion method, the Kirby-Bauer and Stokes' methods: the Kirby-Bauer method is recommended by the Clinical and Laboratory Standard Institute (CLSI) (Hsueh *et al.*, 2010).

In the disc diffusion method a homogenous bacterial isolate at a specific bacterial cell density is spread on an agar plate and a paper disc containing a specific concentration of an antibiotic is placed on the agar surface and after incubation susceptibility is determined by observing the size of the resulting zone of inhibition surrounding each disc (Rolinson and Russell, 1972). This method has the advantage of simplicity, but for a given antibiotic disc several variables including inoculum, depth of agar, conditions of incubation, and medium composition as each markedly influences the size of the zone of inhibition. Unless these variables are carefully controlled, the results obtained may be misrepresentative (Rolinson and Russell, 1972; Noble and Davies, 1965).

Antibiotics are usually used to treat bacterial infections and different strains of *E. coli* have variable sensitivities. Antibiotics that are regularly used to treat *E. coli* infections include amoxicillin (in man) or ampicillin (in animals) and nalidixic acid although others may be used.

APEC strains showed high resistance against these and other commonly used antibiotics such as penicillin , erythromycin, tetracycline and nalidixic acid (Mendonca *et al.*, 2016; Oosterik *et al.*, 2014a) although resistance to amoxicillin, trimethoprim-sulfamethoxazole, kanamycin, Colistin are generally low (Mohamed *et al.*, 2014; Mendonca *et al.*, 2016; Cavicchio *et al.*, 2015) Antibiotic resistance is exceedingly common and there are many 'superbugs' resistant to all treatments largely due the profuse and inappropriate use of antibiotic treatments (Unemo and Jensen, 2017) Thus, it is extremely desirable to find other ways to control APEC and the use of natural antimicrobial agents such as plant extracts might offer some ecological and financial benefits.

1.6.4 Plant derived phytochemicals

Ancient civilisations recognised that some herbs and plant extracts could be used in the preservation of food (Hammer *et al.*, 1999; Abreu *et al.*, 2012) and for medicinal purposes (AlTurki, 2007). Plants synthesise a wide range of secondary metabolites, which are defined as substances produced by an organisms which are not part of its natural growth (Harborne, 1990) of which many have antimicrobial properties and of special interest because of their importance as pharmaceuticals, fragrances, industrial materials and cosmetics (Amaral and Silva, 2003). According to the World Health Organization (WHO), medicinal plants would be the greatest source to obtain a wide range of drugs with antimicrobial properties (Nascimento *et al.*, 2000). There are a great diversity of naturally occurring in medicinal plants but relatively few of the active components and their mechanisms of action in protecting against various diseases have been determined (Motaleb, 2011).

The emergence and spread of antibiotic resistance among pathogenic bacteria has been a rising problem for public health in recent decades (Chandra *et al.*, 2017). This increase in antimicrobial resistance has led to the study of plants products in search of new antimicrobials (Clardy *et al.*, 2006). These chemical substances or phytochemicals have the ability to control

and manage natural predators (Cowan, 1999; Tahara, 2007) and parasites (Palmer-Young *et al.*, 2017; Richardson *et al.*, 2015). Moreover, phytochemicals have strong antibacterial effects against bacterial strains of Gram-negative and Gram-positive bacteria (Sokovic *et al.*, 2010). Some previous studies reported that the supplementation of different plant extracts reduced APEC related diseases (Baydar *et al.*, 2004; Gutierrez *et al.*, 2008). Phytochemicals can be grouped by their chemical constituents and include alkaloids, terpenoids, saponins, phenolics and essential oil constituents (Kennedy and Wightman, 2011).

1.6.4.1 Phytochemicals- Alkaloids

Alkaloids are one of the largest groups of chemical compounds produced by plants. Normally, alkaloids are produced from the *Apocinaceae* and *Solanaceae* families. Many of these metabolic by-products are derived from amino acids and include an enormous number of bitter, nitrogenous compounds. Alkaloids often contain one or more rings of carbon atoms, usually with a nitrogen atom in the ring (Coley *et al.*, 1985). Alkaloids and extracts of alkaloid-containing plants have been used throughout human history as remedies, poisons and psychoactive drugs. The importance of the medicinal properties of alkaloids first came into existence when morphine was isolated from *Papaver somniferum*, which is generally used as a painkiller. Alkaloid was found to inhibit *S. aureus* and *E. coli* that are more susceptible than other selected bacterial strains from human sources at the concentration of 500 $\mu g\ l^{-1}$ (Gurrapu and Mamidala, 2017). Owing to their bioactivities, alkaloids are often toxic to herbivoures but have been exploited by specialized species of herbivores as defense compounds (Sugimoto and Hori, 2010)

1.6.4.2 Phytochemicals -Terpenoids

Terpenoids are the largest group of natural products and can be found in all classes of living things (Jiang *et al.*, 2016). Terpenoids are produced from *Asteraceae* and *Lamiaceae*

plant families (Sulsen *et al.*, 2017). Plant terpenoids are used for their aromatic qualities as terpinols produce the scent of plants and are called essential oils. They play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical applications. The chemical structure of terpinols is $(C_{10}H_{16})_n$ and arise as diterpenes, triterpenes and tetraterpenes (Gershenzon and Dudareva, 2007). The characteristic smell of *Eucalyptus*—a smell of cinnamon, cloves, and ginger—is due to the presence of terpenoids. Examples of well-known terpenoids include menthol, citral, camphor, salvinorin A in the plant *Salvia divinorum*, and the cannabinoids found in *Cannabis* (Chandra *et al.*, 2017). Terpenes and terpenoids may inhibit microbial cells and are used as an alternative strategy to control the shelf-life and safety of food (Lanciotti *et al.*, 2004).

1.6.4.3 Phytochemicals -Saponins

Saponins are a group of naturally occurring plant glycosides, characterized by their strong foam-forming properties in aqueous solution. They are one of the most important class of natural plant products consisting of the steroid aglycone with one or more sugar units attached at different positions (Osbourn *et al.*, 2011). There are more than 11 distinguished classes of saponins with, for dammaranes, tirucallanes, taraxasteranes, and steroids as examples (Challinor *et al.*, 2012; Man *et al.*, 2010). Their compounds have anticancer properties that inhibit tumors and suppressing their angiogenic induction by affecting the endothelial cells of blood vessels. They exert a broad range of pharmacological activities such as expectorant, anti-inflammatory, vaso-protective, antifungal and anti-parasitic (Sparg *et al.*, 2004; Sahu *et al.*, 2008).

1.6.4.4 Phytochemicals -Phenol group

Phenolics and polyphenols form one of the simplest groups of bioactive phytochemicals, consisting of a single of substituted phenolic ring (hydroxyl group OH) (Das

et al., 2010). This group seems to be toxic to microorganisms and the toxicity of phenol is related to the number and location of hydroxyl groups present on the phenol ring (Kyselova, 2011). Phenols inhibit enzymes by oxidising them through non-specific protein interactions or the reaction with sulfhydryl groups (Cowan, 1999). In addition, a phenolic compound able is to bundle with other compounds for example, organic acids and lipids (Kris-Etherton *et al.*, 2002). Generally, phenols are found in vegetables and at high concentrations in fresh fruit (Vinson *et al.*, 2001).

1.6.4.5 Essential oils

Essential oils (EOs) are aromatic oily liquids obtained from plant parts such as, flowers, buds, seeds and leaves (Kocić-Tanackov and Dimić, 2013). About 300 EOs are commercially important and are destined chiefly for the flavours and fragrances market (Van de Braak and Leijten, 1999). There has been an increasing interest in the development of effective natural antimicrobials such as EOs, as food preservatives and in-feed additives because of their desired antimicrobial activity. The antimicrobial properties of EOs have been well recognised for many years (Hammer *et al.*, 1999; Canillac and Mourey, 2004). Also, the action of EOs in model food systems or in real food is well recognised in the literature (Koutsoumanis *et al.*, 1998; Skandamis and Nychas, 2000). EOs contain a wide series of secondary metabolites that possess biological properties including having antibacterial and antifungal (De Martino *et al.*, 2009; Nostro *et al.*, 2007; Nazzaro *et al.*, 2013), anti-oxygenic (Ultee and Smid, 2001) and insecticidal properties (Konstantopoulou *et al.*, 1992). These oils are present as variable mixtures of primarily terpenoids, especially monoterpenes (C10) and sesquiterpenes (C15), also diterpenes (C20) may be present. The components in EOs have various targets but particularly in the membrane and cytoplasm in certain situations they may alter the morphology of the cells (Nazzaro *et al.*, 2013). Numerous studies have shown that EOs of oregano (*Origanum vulgare*), bay (*Pimenta racemosa*) (Hammer *et al.*, 1999), thyme (*Thymus vulgaris*)

and clove (*Eugenia caryophyllata* synonym: *Syzygium aromaticum*) (Dorman and Deans, 2000) are among the most active in this respect against strains of *E. coli*, and the most effective is oregano oil (Lambert *et al.*, 2001; Skandamis and Nychas, 2000). Chemical analysis of these oils showed the constituents to be principally carvacrol, thymol, citral, eugenol and their precursors (Salzer, 1977; Juliano *et al.*, 2000; Demetzos and Perdetzoglou, 2001).

1.6.4.5.1 Thymol and carvacrol

Thymol (2-isopropyl-5-methylphenol) and carvacrol (2-methyl-5-(1-methylethyl) phenol), shown in **Figure 1.1**. are part of a naturally occurring class of biocides, that has strong antimicrobial effects and have been classified as Generally Recognised as Safe (GRAS) (Costerton *et al.*, 1999; Burdock and Carabin, 2004). Their use in food has been approved and is legally registered in the Council of Europe as flavourings and foodstuffs (Europe, 2000; Hyldgaard *et al.*, 2012). Thymol and carvacrol are phenolic monoterpenes that are major components in the essential oils of *Origanum* and *Thymus*. Thymol is the isomeric form of the phenolic monoterpene carvacrol (Fachini-Queiroz *et al.*, 2012). Both thymol and carvacrol possess multiple biological properties such as anti-leishmanial, anti-inflammatory, antioxidant, anti-tumoral activities (Aeschbach *et al.*, 1994; Alam *et al.*, 1999; Robledo *et al.*, 2005) and anti-biofilm activity against *E. coli* 0157:H7 (EHEC) and other food borne pathogens (Kim *et al.*, 2016; Du *et al.*, 2015; Guarda *et al.*, 2011; Friedman *et al.*, 2002; Kang and Fung, 1999). Phenolic compounds are transported into the membrane and cause changes in the lipid-to-lipid and lipid-to-protein ratios in the membrane as well as in the membrane permeability and activity of membrane-bound proteins (Keweloh *et al.*, 1990; Sikkema *et al.*, 1995). The overall toxic effect of the phenolics on the cells is caused by distinct and complex mechanisms, such as narcosis, inhibition of growth, and the uncoupling of adenosine triphosphate synthesis (Escher *et al.*, 1997; Sikkema *et al.*, 1995).

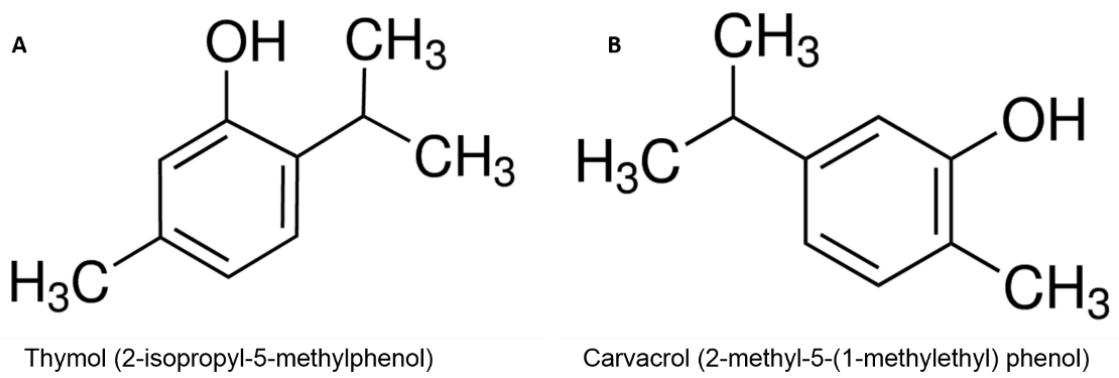

Figure 1. 1 Chemical structures of (A) thymol and (B) carvacrol.

1.7 General hypothesises

Avian Pathogenic *E. coli* (APEC) is the primary cause of economically important diseases of poultry, and commensal *E. coli* are often carried in the intestines of normal poultry, but little is known of the relationship between them and if there is any response to dietary changes that may be adopted as part of control measures against APEC. The first testable hypothesis is that commensals and avian pathogenic *E. coli* can be differentiated one from the other, as they possess differing genotypic and phenotypic characteristics. Thus, the first aim of this study is to isolate the resident *E. coli* in poultry and to characterise them both genotypically and phenotypically. However, it was also considered that simple technologies should be used as these would be more readily transferred to routine and diagnostic laboratories. This will in part give an overview of numbers of commensal and APEC strains and the differences between them (chapter 2).

The next testable hypothesis is that certain phytochemicals from some 'medical plants' may contribute to the control and management of APEC. These phytochemicals or extracts can be grouped by their chemical constituents into thymol and carvacrol and were used to carry out investigations into the antibacterial effects of plant extracts against *E. coli* isolates. This included determining MIC, and their effect on biofilm formation (chapter 3).

In addition, the use of phytochemicals to control and manage APEC, one additional concept is that some plant extracts/essential oils may reduce or control other biological properties of APEC and commensal *E. coli* such as plasmid gene transfer. The plasmidic spread of antibiotic resistance is another critical issue in animal production. Here, it is intended to use thymol to stop the conjugal transfer of plasmid mediated antibiotic resistance from one strain to another. The objective is to investigate the potential of phytochemicals to interfere with the various biological properties of APEC. This investigation is very contemporary and important to give a significant advantage to using phytochemicals to combat antibiotic resistance in food producing animals entering the human food chain (chapter 3).

Moreover, thymol resistance may arise in *E. coli* by exposure to gradually increasing thymol concentrations. If mutants arise the basis of the next hypothesis was that a combination of extracellular foot-printing metabolomics, morphological changes, and whole genome sequencing approaches of mutant and original strains would elucidate natural mechanisms of 'resistance' (chapter 4).

Finally, this series of investigations is largely observational but with potentially applications should *in vitro* batch culture fermentation studies indicate impact of thymol on caecal gut microbiota and its metabolism. The final testable hypothesis is that thymol may act upon the total population to change the profile of bacteria present. Thus, population profiling of batch cultures challenged will identify responses and the biochemical pathways affected (chapter 5).

1.8 Aims and objectives

- Isolate *E. coli* resident in poultry.
- Determine the genotypic and phenotypic characteristic of *E. coli* isolates.
- Investigate the diversity of *E. coli* populations in poultry.
- Adapt and train *E. coli* strains to tolerate high concentrations of thymol.
- Investigate thymol mechanism of action in *E. coli*.
- Evaluate the effects of thymol on chicken derived gut microbiota and assess the environmental conditions and thymol concentration that favours or limits the caecal microbiome.

Additional note: this introduction has not included all the possible topics that will be covered in this thesis but rather covered at a top level those key issues that provide a relevant background to help inform the approaches within each chapter. Thus, additional information will be provided as required in the introduction to each specific chapter when appropriate.

CHAPTER 2: GENOTYPIC AND PHENOTYPIC DIVERSITY BETWEEN COMMANSAL AND AVIAN PATHOGENIC *E. COLI*

2.1 Introduction

Escherichia coli is one of the most frequent causal agents of common bacterial infections for both human and animals (Allocati *et al.*, 2013) even though many *E. coli* may be non-pathogenic commensals. The pathogenic types may be classified into a number of specific pathotypes such as enterotoxigenic, enteropathogenic, enteroinvasive, or enterohaemorrhagic according to the presence of specific virulence factors (Nataro and Kaper, 1998). For brevity, all pathotypes have not been listed nor described in any detail here as for this thesis the focus is upon *E. coli* from poultry.

E. coli is present in the microflora of the intestinal tract of poultry where they normally inhabit the lower gastrointestinal tract. It may be assumed that many are non-pathogenic commensal types whilst others are pathogenic in poultry able to induce disease, commonly called colibacillosis that presents in a number of differing clinical pathologies. Isolates that come from diseased animals are named Avian Pathogenic *E. coli* (APEC). However, there is still considerable debate about the differentiation between commensal and pathogenic types because both, irrespective of definition into commensal or pathogenic groups, encode virulence determinants. The debate in the scientific literature regards the accurate definition of commensal and APEC types with the Nolan laboratory in the USA (Johnson *et al.*, 2008a) suggesting carriage of just five of the known 40 or so virulence determinants is sufficient for the *E. coli* isolate to be described as an APEC. The Woodward group (Cordoni *et al.*, 2016) have suggested carriage of seven to nine virulence determinants may be a more accurate definition of APEC. Given it is not possible to perform infection studies in poultry for every isolate made to confirm Koch's postulates and that even the pathogenic types reside in the gut

before inducing disease often as an opportunistic secondary infection to prior disease such as mycoplasmosis, accurate differentiation is at best complex.

Traditionally serotyping has been performed on isolates belonging to O1, O2 and O78 serotypes are regarded as pathogenic (Ewers *et al.*, 2003; Wang *et al.*, 2014). That said, this monothetic test is fallible and many other serotypes harbour APEC. Several of the more commonly reported virulence factors associated with APEC include increased serum survival (*iss*), production of aerobactin and K1 capsule, presence of type 1 and P fimbriae, and temperature-sensitive hemagglutinin (*tsh*) of the autotransporter group of proteins (Ewers *et al.*, 2004; Delicato *et al.*, 2003) and these may be used potentially to investigate isolates to differentiate tentatively between commensal and APEC strains when other tests such as serotyping and whole genome sequencing are not available. In addition, particular interest has yet to be shown in the study of the relationships between phenotypic relatedness and phenotypic variation of poultry isolates and there exist several simple phenotypic and genotypic methods that have been developed in recent years to differentiate the diversity in *E. coli* strains (Edwards and Ewing, 1986).

The greater wax moth larvae, *Galleria mellonella* is a simple and rapid invertebrate *in vivo* model that has been used as an infection model to describe and study microbial pathogenicity of several pathogenic bacteria, including enteropathogenic *E. coli* (Leuko and Raivio, 2012; Alghoribi *et al.*, 2014). This technique is a popular model for experimental bacterial studies because it meets the 3Rs requirements of replacement, reduction and refinement of animal studies. Home office restrictions requiring ethical approval do not apply, their short life span makes them ideal for high throughput studies and the innate immune system is potentially 'comparable' to mammalian studies (Tsai *et al.*, 2016). *G. mellonella* larvae are low cost to establish and easier to maintain when compared with other model hosts as they do

not require special laboratory equipment (Ramarao *et al.*, 2012). In addition, the larva is large enough to enable precise injection of pathogens (Vogel *et al.*, 2011). In this study, alternative *in vivo* model *G. mellonella* larvae were used to investigate the virulence characteristics of bacterial pathogenicity. The testable hypothesis was that turkey isolates were likely to be commensals and therefore harmless in an appropriate animal model compared with APEC. Here this hypothesis is tested using the Galleria model.

The aim of this study was to investigate the diversity of *E. coli* populations in poultry using a set of relatively simple and readily transferable tests that front-line diagnostic laboratories rather than specialist reference laboratories can undertake. The reason for this approach was pragmatic. Firstly, whole genome analysis which is readily available these days is, although reducing in cost, very expensive in terms of the sequencing itself and more significantly the time for detailed analysis. Secondly, until a pipeline for the bioinformatics is developed that calls APEC confidently, there remains still some debate surrounding the definition of APEC. Thirdly, the time taken to obtain results in the field where suspect disease is being investigated, the need is for simple, rapid and well-established methods of analysis. In addition, to the best of our knowledge no research has focused upon the use of simple metabolic tests to assess differences in commensal and pathogenic types. Thus, using basic PCR and simple growth characteristics, the aim was tackled using Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) to determine genotypic diversity, PCR analysis of a selection of APEC virulence factors determinants, and the phenotypic tests included susceptibility testing to some antimicrobial agents and carbon and energy sources utilization to study their similarities and differences between the isolates.

2.2 Materials and Methods

2.2.1 E. coli isolation and storage

2.2.1.1 Microbiological media

The microbiological media listed in **Table 2.1** were used for the different steps of the experiments performed and were procured from Sigma-Aldrich UK.

Microbiological media were sterilized by autoclaving at 121°C for 20 min except SS agar which was boiled. Solid media containing agar were cooled to 50°C before pouring 20 ml in sterile disposable Petri dishes (Nunc). Prepared plates were stored in sterile bags upside down, refrigerated at 4^{o}C until use. Tubes containing autoclaved liquid medium (broth) were also cooled and stored refrigerated at 4^{o}C until use. The manufacturer/supplier details for all medium are in **appendix 1**.

Table 2. 1: microbiological media

Medium	Comments
Tryptophan Broth	Medium to perform the indole test to confirm *E. coli* presence.
Nutrient Broth (NB)	General growth broth
Luria-Bertani broth (LB)	General growth broth
Salmonella Shigella Agar (SS)	Selective and differential medium widely used to isolate Gram-negative from faeces.
MacConkey agar (McC)	Selective media for *E. coli*
Eosin Methylene Blue (EMB)	Selective media for *E. coli*
Nutrient Agar (NA)	General media
Luria-Bertani agar (LB)	General media

Minimal Salts Medium (M9)

Minimal medium was prepared in 500 ml volumes for each component. The salt solution was prepared in 5X concentrated form as follows: - in 1 L of distilled water the following was dissolved 105 g K_2HPO_4, 45 g KH_2PO_4, 10 g $(NH_4)_2SO_4$ and 5 g sodium citrate then autoclaved. To prepare the 500 ml of minimal medium 100 ml of autoclaved salts solution was added with 15 g of purified agar and autoclaved to 374 ml ddH_2O. After cooling autoclaved minimal medium to 50˚C, 1 ml of 1M $MgSO_4$ and 10 ml of 20% carbon source substrate prepared by dissolving in sterile distilled water and filter sterilizing were added separately in multiple preparations.

2.2.1.2 E. coli isolation and storage

Sixty-Five APEC were provided by University of Surrey (UoS). Each of these isolates had been recovered from diseased birds and specifically from the tissues that were heavily infected with the *E. coli* (personal communication Prof R. M. La Ragione).

Samples were taken from commercial poultry guts from studies performed at the University of Reading CEDAR farm, Arborfield, Reading, RG2 9HX. Thirty-five swab samples were from chicken cloaca and 100 swab samples were from turkey caeca (see **appendix 2**). The samples were taken from a number of different studies in which the birds were from hatcheries that serve the poultry industry and were therefore representative of the birds that enter the poultry meat production industry. The swabs with approximately one gram of gut luminal contents were placed in test tubes containing NB. Swabs from chicken were spread on McC agar and incubated at 37°C for 18-24 h aerobically. A well isolated single pink colony was picked from the McC plate and sub-cultured on selective media SS agar and EMB to confirm the isolation of *E. coli* by lactose fermentation and acid production shown as dark

blue-black colonies with metallic green colonies. To further confirm the isolation of *E. coli* the indole test and Api20E (bioMerieux, UK Limited) strips test were used following manufacturer's instructions (see below). Confirmed and pure *E. coli* stocks were grown on NA plates and then stored as cryobank at -80°C. To do this, each isolate was inoculated in the cryobank tube (Mast group, Mastdisks, UK) with a suspension equivalent to McFarland 3 or 4 standard prepared by mixing a sweep of several colonies from a fresh NA plate culture that had been spread liberally across in NA plate and grown overnight at 37ºC.). Inoculated cryobank tubes were stored at -80˚C.

Swabs from turkey were thoroughly mixed in NB and dilutions (100 µl) were spread on thoroughly dried McC plates followed by incubation at 37°C for 18-24 h aerobically. Lactose positive colonies were pink to red which indicated the presence of *E. coli*. Well isolated single pink colonies were sub-cultured on selective media EMB and SS agar to confirm the isolation of *E. coli*. Confirmation testing and storage was as described above. Finally, APEC swabs were streaked onto suitable medium (NA or LB agar) and cultured for 18-24h at 37°C aerobically. Following incubation, a single colony was re-streaked into McC or EMB agar for confirmation testing and storage as described above.

2.2.1.3 API-20E test

The API-20E test kit for the identification of enteric bacteria (bioMerieux,UK Limited UK Limited) provides an easy way to inoculate and read tests relevant to members of the Family *Enterobacteriaceae* and associated organisms. A plastic strip holding twenty mini-test tubes was inoculated with a saline suspension of a pure culture (as per manufacturer's directions). This process also rehydrates the desiccated medium in each tube. A few tubes are completely filled (CIT, VP and GEL), and some tubes are overlaid with mineral oil such that anaerobic reactions can be carried out (ADH, LDC, ODC, H_2S, URE). After incubation in a

humidity chamber for 18-24 h at 37°C, the colour reactions were read (some with the aid of added reagents following manufacturer's instructions), and the reactions (plus the oxidase reaction done separately) were converted to a seven-digit code which is called the analytical profile index, from which name the initials "API" are derived. The code was fed into the manufacturer's identification book to determine genus and species where possible.

2.2.1.4 Indole test

The indole test is a commonly used simple biochemical test to differentiate Enterobacteriaceae and other genera. It was used to differentiate between *E. coli* and other organisms such as *Enterobacter* and *Klebsiella* (Winn 2006). Also, it was used to determine the ability of an organism to deaminate tryptophan to form the compound indole. Indole production is detected by Kovac's reagent that was procured from Sigma-Aldrich UK: the reagent is 4 (p)-dimethylamino-benzaldehyde which reacts with indole to produce a red coloured compound. After incubation of tryptophan broth inoculated with 2-3 colonies of *E. coli* and incubated at 37°C for 18 to 24 h, four drops of Kovács reagent were added directly to the tube (Harley, 2005). A positive indole test was indicated by the formation of a pink to red colour in the reagent layer on top of the medium within seconds of adding the reagent.

2.2.2 Genotypic tests

2.2.2.1 Extraction of Genomic DNA

Isolated cultures of *E. coli* strains grown for 18-24 h in Nutrient Broth (NB) were used for DNA extraction. Genomic DNA was extracted using QIAGEN Puregene yeast/bact. Kit B and standard protocols from fresh samples of Gram-negative bacteria cultures were followed. 1000 µl of overnight culture was pelleted in 1.5 ml microcentrifuge tube at 14,000xg for 2 min, then incubated at 80˚C for 5 min after adding 300 µl of cell lysis solution on the pellet. RNase A solution (1.5 µl) was mixed by gentle inversion 25 times then incubated for 15-60 min at

37˚C, followed by 1 min on ice. The tube content was vortexed vigorously for 20 s at high speed after addition of 100 µl protein precipitation solution, the mixture was centrifuged 14,000xg for 3 min. The supernatant was transferred to a clean 1.5 ml microcentrifuge containing 300 µl of isopropanol procured from Sigma-Aldrich UK, and mixed by gently inverting 50 times. The mixture was centrifuged at 14,000xg 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 centrifuge at 14,000xg for 1 min, and again the supernatant was discarded and allowed to air dry for 5 min. 100 µl of DNA hydration solution was added and vortex for 5 s, incubated at 65˚C for 1 h, followed by incubation overnight at room temperature with gentle shaking. The DNA concentration was determined with a ND-1000 Nanodrop spectrophotometer (Nano Drop technologies, USA). The Nanodrop tube was cleaned by pipetting 2 µl of dH_20 onto it and wiping with Whatman filter paper. The Nanodrop was blanked with 1 µl of DNA hydration solution. One µl DNA solution was added to the measuring stage and the DNA concentration was recorded in ng/µl, the concentration 260:280 nm ratio and 260:230 nm was recorded of 1.8 ± 0.15. DNA stock were prepared as 100 ng/µl and stored in -20˚C for PCR test.

2.2.2.2 ERIC-PCR

E. coli isolates were fingerprinted using the ERIC-PCR. ERIC-PCR specific primers (ERIC1, 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC2, 5′ AAGTAAGTGACTGGGGTGAGCG-3′) (Maluta *et al.*, 2012; Ramazanzadeh *et al.*, 2013) were synthesized by Eurofins Genomics UK.

Each ERIC-PCR reaction was carried out in a total volume of 50 µl of Promega PCR mixture comprised of 100 ng of *E. coli* DNA, 1 µl (25 pmol) of each primer, 1 µl (200 mM) of dNTP mixture, 10 µl of 5X buffer solution, 4 µl (25 mM) of $MgCl_2$, 1 µl (1.0 U) of *Taq* DNA

polymerase. Double distilled water was added to the mixture to make a final volume of 50 µl. The reactions were carried out in 0.2 ml microcentrifuge tubes (sigma-Aldrich, UK). PCR amplification was carried out using a 48-well MJ Mini Thermal Cycler (Bio-Rad thermal) with the following protocol; initial denaturation at 94˚C for 2 min, followed by 35 cycles of denaturation at 95˚C for 30s, annealing at 52˚C for 30s, extension at 72˚C for 1 min, and a final extension step at 72˚C for 5 min.

The amplicons were examined by electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) in 1.5% TAE agarose gels stained with 0.5 mg/ml of ethidium bromide all of them procured from Sigma-Aldrich UK. DNA ladders, 1-kpb and 100bp, from QIAGEN UK were used and an electric field of 50 V was applied during electrophoresis for 45-60 minutes. The images were captured using G: BOX Chemi-XR5, GeneSye. The data was analysed visually according to similarities between the banding patterns of strains and grouped accordingly into groups. Briefly, DNA band sizes were determined using the detect band button of NTSYS pc software Version 2.1b. The overall analysis of similarities between strains was based on banding patterns. The presence of a given band was coded as 1 and the absence of a given band was coded as 0. To perform ERIC fingerprint analyses, a binary matrix of band presence scored as 1 or absence scored as 0. These scores were entered in to the SAHN program of the NTSYS-pc software Version 2.1b for the construction of dendrogram based on simple matching coefficient and UPGMA (Unweighted Pair Group Method for Arithmetic Averages) in cluster analysis to determine the genetic relatedness of the *E. coli* strains. Clustering was defined at a coefficient of 0.089 and the number of clusters and singletons produced were used to calculate the discriminatory index (D value) as described by Hunter and Gaston (Hunter and Gaston, 1988).

2.2.2.3 Virulence genotyping

All *E. coli* samples were analysed for eleven avian pathogenic *E. coli* (APEC) virulence factors using PCR assays based on the most virulence determines associated with different diseases on poultry. Target genes and their descriptions are summarized in **Table 2.2** with their primer sequences. All PCR assays were performed with 25 µl of Promega PCR mixtures containing 2 µl (100 ng) of template DNA, 2 µl (1.0 U) of Taq DNA polymerase, 5 µl 5X PCR buffer, 4 µl (25 Mm) MgCl2, 1 µl dNTPs mix (200 mM) and 1 µl (25 pmol) of primer pairs, and appropriate volumes of double-distilled water. The reactions were carried out in 0.2 ml microcentrifuge tubes (Sigma-Aldrich UK). The PCR amplification was carried out using a 48-well MJ Mini Thermal Cycler (Bio-Rad) with the following protocol; initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C, for 30s, annealing at 58°C for 30s, extension at 68°C for 3 min, and the final extension step at 72°C for 5 min. Nuclease-free water was used as negative control in each run. Analysis of the amplified products was performed by electrophoresis (50 V for 1 h) with a 1.5% agarose gel stained with ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). 100pb and 1Kbp DNA ladders were used. Gel images were captured using G-BOX Chemi-XR5, (Syngene), connected to a computer. The analysis of specific band sizes presents for different virulence genes tested were scored as present (+) or absent (-).

Table 2. 2: 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				
fimH	Type 1 fimbriae adhesion	AGAACGGATAAGCCGTG GCAGTCACCTGCCCTCCGGTA	508	(Zhao *et al.*, 2009)
papC	P-fimbriae, Pyelonephritis associated pili	TGATATCACGCAGTCAGTAG CCGGCCATATTCACATA	501	(Sanger *et al.*, 1977; Janben *et al.*, 2001)
csg	Regulator of the curli fimbriae operon	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC	200	(Knobl *et al.*, 2012)
crl	Curli fiber gene	TTTCGATTGTCTGGCTGTATG CTTCAGATTCAGCGTCGTC	250	(Knobl *et al.*, 2012)
tsh	Temperature-sensitive hemagglutinin	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; Janben *et al.*, 2001)
irp2	Iron- repressible protein associated with yersinabactin synthesis	AAGGATTCGCTGTTACCGGA AACTCCTGATACAGGTGG	413	(Dozois *et al.*, 1992; Schubert *et al.*, 1998)
Protectins				
iss	Increase serum survival	ATCACATAGGATTCTGCC CAGCGGAGTATAGATGCC	309	(Ewers *et al.*, 2005)
kps (k1)	Capsule polysaccharide	TATAATTAGTAACCTGGGGC GGCGCTATTGAATAAGACTG	927	(Knobl *et al.*, 2012)
astA	Enteroaggregative heat-stable toxin	TGCCATCAACACAGTATATC TCAGGTCGCGAGTGACGG	116	(Franck *et al.*, 1998)
Miscellaneous				
cva/cvi	Structural genes of colicin V operon	TCCAAGCGGACCCCTTATAG CGCAGCATAGTTCCATGCT	598	(Ewers *et al.*, 2007)

2.2.3 Phenotypic testes

2.2.3.1 Carbon source utilisation

Two hundred strains of isolated *E. coli* were tested for their ability to utilize 11 substrates as sole sources of carbon and energy by the following method, these eleven substrates are known to be highly variable in the *E. coli* population as a whole (Edwards and Ewing, 1986; Woodward and Charles, 1983). Two to three colonies of each strain were inoculated on minimal agar. Minimal Salts Medium (M9) was prepared as per section 2.3.1.1. The following carbon sources were used: sucrose (suc), raffinose (raf), inositol (ino), adonitol (ado), arabitol (ara), dulcitol (dul), allantoin (all), proline (pro), sorbose (sor), melitzitose (mel), and salicin (sal). Twenty ml of media were poured per sterile Petrie dish and allowed to solidify at room temperature. Eight strains *E. coli* were streaked on to 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; the clear growth scored as positive (+), absence of discernible growth was scored negative (-). Mutability that is defined here as rare colonies on a background of very weak or no growth was scored also (M).

2.2.3.2. Antimicrobial susceptibility test

In this experiment 135 isolated samples (from chicken and turkey see appendix 1) and 65 samples APEC provided from university of Surrey were examined for their antimicrobial sensitivity. Antimicrobial sensitivity was investigated using the disk diffusion method (Bauer *et al.*, 1966) with some modification in NA media (Mast group, Mastdisks, UK) according to the standards and interpretation criteria described by National Committee for Clinical Laboratory Standards NCCLS/CLSI guidelines (Hsueh *et al.*, 2010). The following antibiotics were used: nalidixic acid (10µg), amikacin (30µg), ampicillin (10µg), streptomycin (10µg), colistin sulphate (10µg), chloramphenicol (30µg), and trimethprim (5µg). Overnight culture

samples were adjusted for turbidity with a McFarland Standard Tube 0.5 (McFarland standard of 0.5 Bacterial lawns). To do this, overnight cultures containing approximately 1 to 2 x 10^8 CFU/ml were adjusted with fresh sterile nutrient broth by measuring absorbance at 600nm using a spectrophotometer with a 1 cm light path and matched cuvette using an absorbance of 0.06 that is equal to 0.5 McFarland. Within 15 minutes after adjusting the turbidity of the inoculum suspension, 100 µl of *E. coli* were pipetted onto the nutrient agar plate, and spread over the surface. The plate was left for 5 minutes to allow the culture to soak into the agar. Each disc was placed by sterile forceps on a surface of agar plate, 6 discs per plate with the space between the discs around 24-30 mm, and the distance to the edge no less than 1cm. The disc was tapped lightly with the forceps to make sure it adhered to the agar when the plate is inverted. The plates were incubated at 37°C for 18-24 hours and susceptibility was determined by measuring the zone of inhibition. The diameter of the zone of growth inhibition around each disc were measured and compared with zones of inhibition of standard controls according to standards of the NCCLS/CLSI.

2.2.4 Statistical analysis

To perform ERIC fingerprint analyses, a binary matrix of band presence scored as 1 or absence scored as 0. These scored were entered in the SAHN program of the NTSYS-pc software Version 2.1b for the construction of dendrogram based on simple matching coefficient and UPGMA (Unweighted Pair Group Method for Arithmetic Averages) in cluster analysis to determine the genetic relatedness of the *E. coli* strains.

For the purpose of statistical analysis of variance was performed on each substrate for which growth was positive for different isolates. To determine the diversity between all characteristics tested by genotypic and phenotypic was used a multivariate analysis of Principal Components Analysis (PCA). The basic statistical correlation between all characteristics tested

was determined by using statistical programme of Minitab version 17 software. A p-value of <0.05 was taken to indicates statistical significance.

2.2.5 Galleria model.

2.2.5.1 Identification of 50% lethal dose (LD 50) in G. mellonella larvae infection of virulence

In vivo treatment assays using the Greater Wax Moth *G. mellonella* model were done by Dr Jonathan W. Betts at the University of Surrey. *E. coli* strains were grown in Luria Bertani broth for 16 h at 37˚C. The cultures were washed and diluted in PBS, for each strain ten *G. mellonella* larvae were injected with 10 µl of 10^2, 10^3, 10^4 and 10^5 CFU/larva into the left proleg (Hornsey and Wareham, 2011). Larvae were then incubated at 37˚C in the dark and the dilution that killed 50% of the larvae (LD 50) for each replicate was determined after each 24 h. In addition, control inoculation was performed with PBS to measure lethal effects. Larvae were monitored over 0, 24, 48, 72 and 96 h and survival outcome were determined; larvae were considered dead when no response was observed following touch. Larvae melanisation was taken as an indicator of morbidity and was quantified based on the reverse scoring method of (Tsai *et al.*, 2016; Betts *et al.*, 2017), whereby a score of 4 indicated total melanisation of the larva, 2 equalled melanin spots over the larva, 1 equalled discolouration of the tail and a score of 0 equalled no melanisation. All *in vivo* experiments were carried out in triplicate on three separate occasions. Survival analysis and statistical significance were determined using the log-rank test and the Kaplan–Meier survival. The LD50 curves and melanisation scores were plotted using GraphPad Prism 6.0 software (San Diego, CA, USA) at a significance level of P = <0.05.

2.3 Results

2.3.1 E. coli isolated and storage

The total number of confirmed *E. coli* isolates that were made and stored was 135, 35 from chicken and 100 from turkey. In addition, 65 isolates provided by the UoS from infected chicken described as APEC samples were also re-confirmed and stored (**appendix2**)

2.3.2 Genotypic diversity

2.3.2.1 ERIC-PCR

The ERIC-PCR genotyping method results show the different number of bands present, different sizes, and characteristic band patterns of *E. coli* strain DNA fingerprints. In total, 200 isolates taken from three sources including 135 *E. coli* isolates from poultry (100 from turkey and 35 from chicken) and 65 strains isolated from infected chicken classified as APEC strains (**appendix 2**). The amplification products of ERIC-PCR generated distinct banding patterns that were differentiated by agarose gel electrophoresis based on molecular weights and the number of observed bands using ERIC–PCR profiles. One hundred and seventy-five fingerprinting patterns were determined among 200 isolated strains. The sizes of the PCR products ranged from slightly less than 150bp to > 3000bp with products ranging from 450-1250bp most commonly encountered (**Figure 2.1A-C**). Out of the 200 isolates strains 175 (87.5%) were grouped in 31 groups which shared banding patterns indicative of similar origin of dissemination, and 25 (12.5%) isolates displayed a single profile. Complex patterns of fingerprints have been obtained from all isolates.

A dendrogram based on simple matching coefficient and UPGMA of DNA bands was constructed by NTSYSpc version 2.2 with clustering defined at a coefficient of 0.089. **Figure 2.2** is a dendrogram showing the genetic diversity and relatedness of the 200 *E. coli* strains. At

a coefficient of 0.089, 31 clusters and 25 singletons at a D value of 0.848 were identified. The index indicates that if two strains were sampled randomly from the population, then on 84.8% of occasions they would fall into different type. *E. coli* strains in the same cluster are genetically closely related to each other and more homogenous, whereas clusters consisted of 2 or more *E. coli* strains (**Table 2.3**). Cluster 1 was the biggest cluster in the dendrogram having 76 *E. coli* strains and all strains in this cluster were commensals as shown by later tests and most of them were isolated from turkey and only 5 strains isolated from chicken. Similarly, *E. coli* strains in cluster 2 to cluster 10 were related to each other more than those in clusters 11 and 12. Whereas those strains were mixed turkey and chicken commensal *E. coli* isolated. Clusters 13, 14 and 15 were more related to each other than other clusters. *E. coli* strains in cluster 16 to 22 are more related to each other however, cluster 16 had 3 strains isolated from turkey and one from chicken, while other strains in the clusters 17 to 22 they were isolated from APEC strains. Furthermore, *E. coli* strains in clusters 23 to 31 were similar, clusters 23, 24 and 26 were related to strains in clusters 29, 30 and 31 and differ to cluster 27 and 28 which related to each other. And also, all strains in those clusters contained APEC strains with 3 commensal strains isolated from turkey in the last cluster 31. Twenty-five singleton (single isolates) *E. coli* strains belonging to these groups are heterogenous and show more distant relation to other *E. coli* strains at a coefficient of 0.089, although all singleton groups were isolated from chicken (12), and APEC (12) strains except the last strain was isolated from turkey.

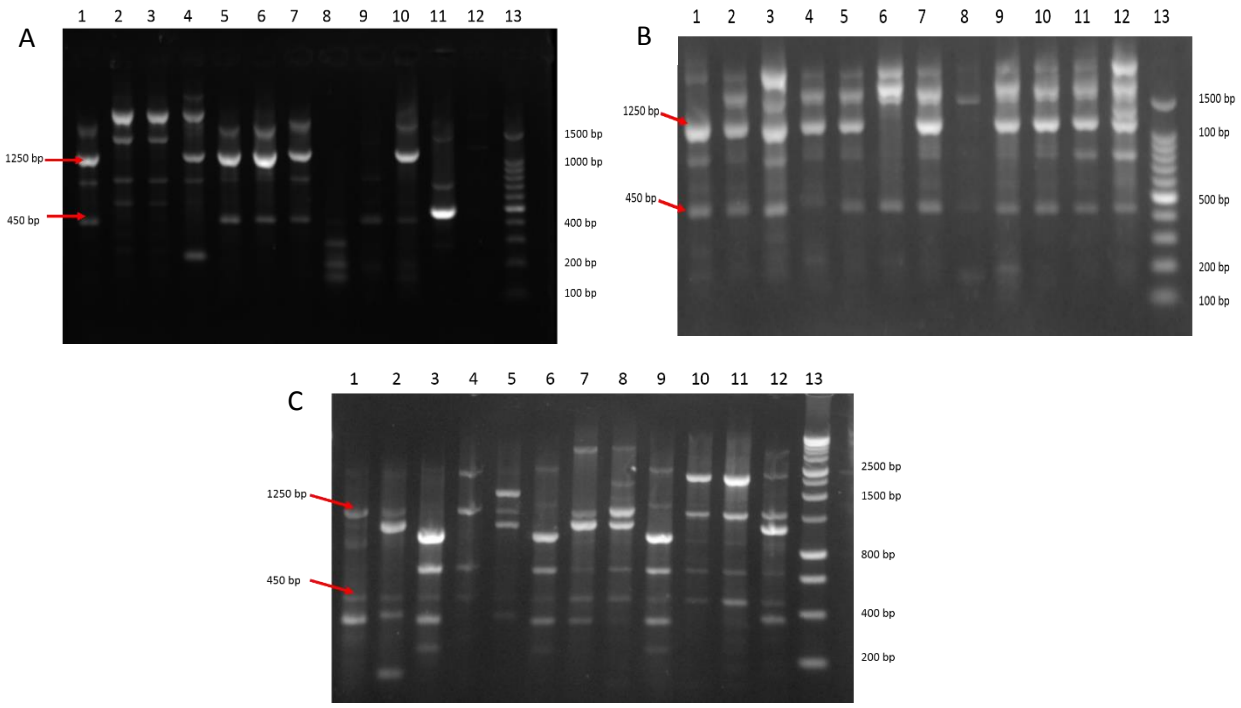

Figure 2. 1 Examples of ERIC-PCR profiles of E. coli isolates from (A) turkey, (B) chickens and (C) APEC, Lanes 1-12. Lanes 13 indicate ladder, arrow indicate 450-1250bp.

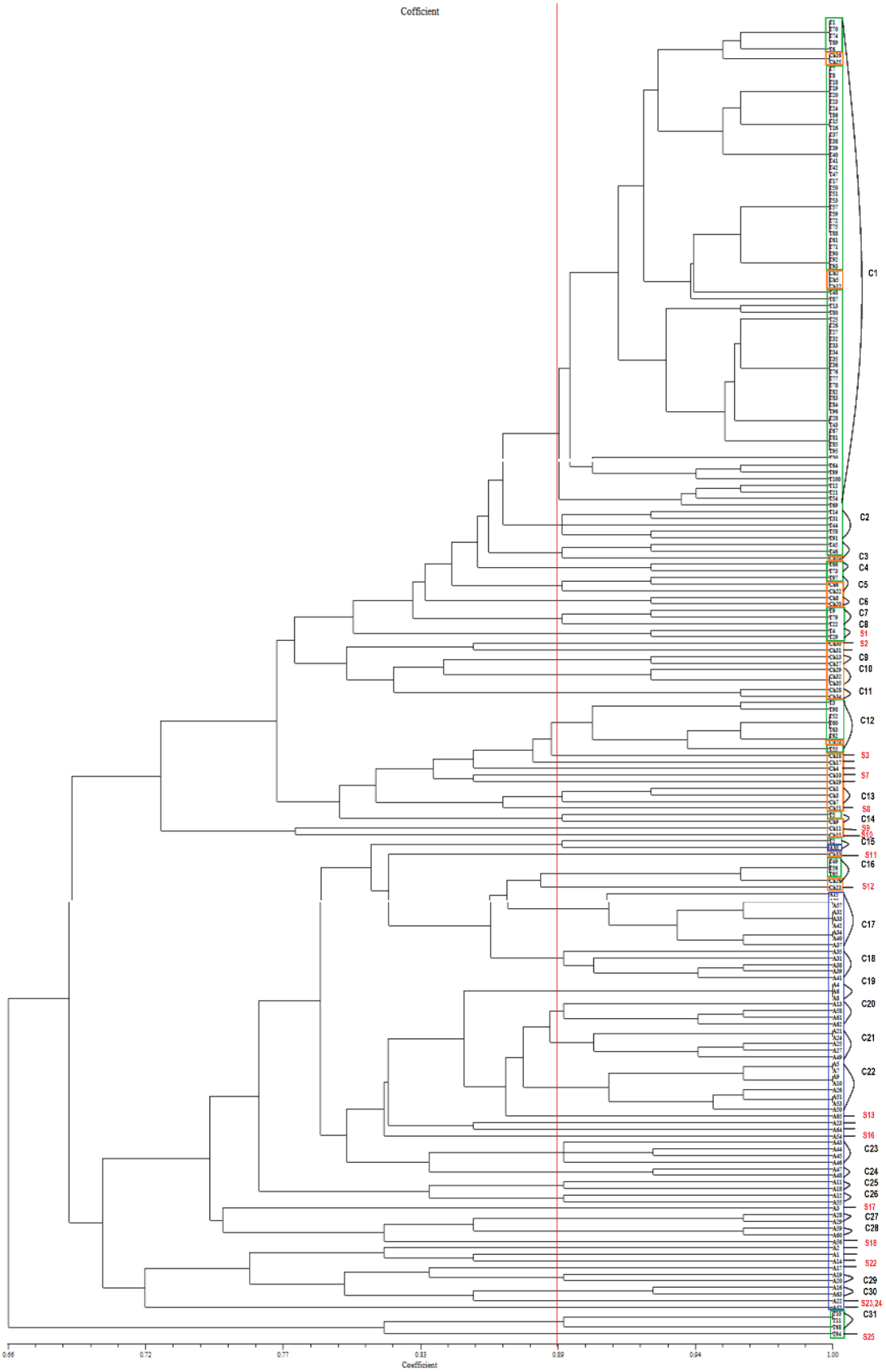

Figure 2. 2 Dendrogram of genomic similarity of 200 *E. coli* strains using ERIC-PCR results. C1-C31= *E. coli* cluster 1-31, S1-S25= *E. coli* singleton 1-25, T=turkey (green), Ch=chicken (orange) and A=APEC (blue).

Table 2. 3 Clustering and similarity of *E. coli* isolates and Number of strains in each cluster.

Cluster	Number of strains in each cluster	Isolated group		
		Turkey	Chicken	APEC
C1	76	71	5	-
C2	5	5	-	-
C3	3	2	1	-
C4	2	2	-	-
C5	3	1	2	-
C6	2	-	2	-
C7	3	3	-	-
C8	2	2	-	-
C9	2	-	2	-
C10	3	-	3	-
C11	2	-	2	-
C12	9	8	1	-
C13	3	-	3	-
C14	2	1	1	-
C15	2	1	-	1
C16	4	3	1	-
C17	8	-	-	8
C18	5	-	-	5
C19	3	-	-	3
C20	4	-	-	4
C21	5	-	-	5
C22	8	-	-	8
C23	4	-	-	4
C24	2	-	-	2
C25	2	-	-	2
C26	2	-	-	2
C27	2	-	-	2
C28	2	-	-	2
C29	2	-	-	2
C30	2	-	-	2
C31	3	3	-	-

2.3.2.2 Virulence genotyping

Two hundred *E. coli* strains were investigated for the presence of 11 virulence associated genes. The most frequently detected gene was *crl* that regulates the curli fimbrial operon found in all strains except one where it was not found in a turkey isolate (99.5%), followed by the Type 1 fimbrial adhesion gene *fimH* in 194 strains (97%), and 191 strains (95.5%) possessed the curli structural *csg* gene. In addition, 177 strains (88.5%) gave positive results for iron-repressible protein *irp2* gene; 148 strains (74%) for serum resistance *iss* gene; 123 stains (61.5%) were positive for the aerobactin *iucD* gene; 101 strains (50.5%) for colicin V operon - *cva/cvi* gene, and 97 strains (48.5%) for temperature-sensitive hemagglutinin *tsh* gene; 82 strains (41%) for enteroaggregative heat-stable toxin *astA* gene. Whereas 31 strains (15.5%) were positive for the *kps (k1)* gene encoding capsule and only 9 strains (5.5%) were positive for P-fimbriae *papC* gene (**Figure 2.3, & Table 2.4**).

Using the criterion that APEC should have 5 or more virulence associated genes to be classified as APEC (Ewers *et al.*, 2005), the results showed that 184 strains out of 200 strains in total could be classified as APEC strains. All strains isolated from chicken (35 strains) and APEC strains provided by (UoS) (65 stains) were APEC strains in addition to 84 of 100 strains isolated from turkey (**table 2.5**). Sixteen strains isolated from turkey possessed less than 5 genes as follows, 13 strains had 4 genes, two strains had 3 genes and one strain had 2 genes. In addition, there were no strains containing 9, 10 or 11 genes found in strains isolated from turkey. Thus, there were less APEC strains (84%) strains isolated from turkey than from chicken (100%). One strain possessed all 11 virulence factors associated genes from APEC strains and other high numbers of virulence factors was 10 genes detected in 2 strains (1%) one from APEC and other from a chicken strain. Highest numbers of strains had 7 genes in 51 strains (25.5%), followed by 8 genes in 43 strains (21.5%), and 6 genes detected in 39 strains (19.5%). However, if the criterion of 7 or more genes were used to classify APEC then 119

strains would be defined as APEC and less than half (37%) of the turkey isolates would be defined as APEC, 82.8% of chicken isolates and similarly in APEC isolated 81.5%.

If we consider the three common virulence genes (*crl, fimH* and *csg*) which were found in high percentage (**table 2.4**) (99.5%, 97% and 95.5%) respectively, almost all strains among the three-isolated source had those genes. If we remove those genes the result of the criterion that APEC should have 5 or more virulence associated genes to be classified as APEC as Ewers and his colleges consider, the results would be changed as shown in **Table 2.5**. Seventy stains out of 200 strains in total could be classified as APEC strains (35%). Thirteen strains isolated from turkey out of 100 strains possessed 5 genes (13%) and no turkey isolates contained more than five genes.

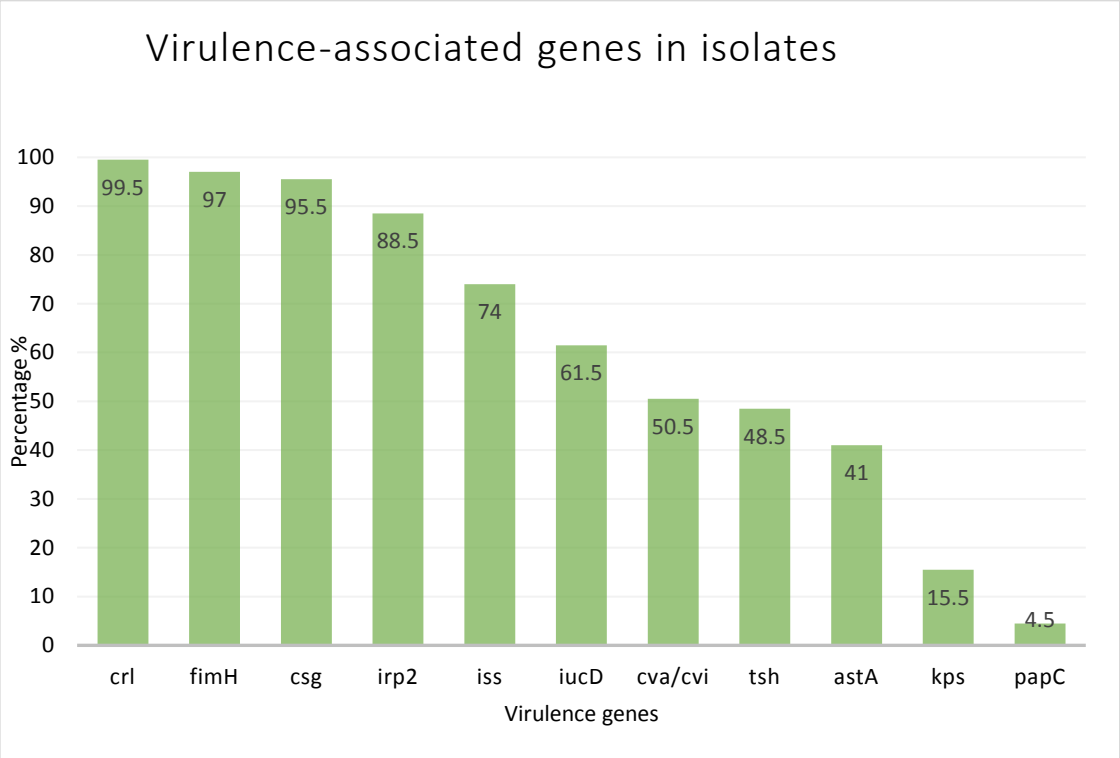

Figure 2. 3 Total detection rates of virulence-associated genes in isolates.

Table 2. 4 Prevalence of virulence-associated genes in APEC field strains, as detected by PCR.

Samples(n)	*crl*	*fimH*	*csg*	*irp2*	*iss*	*iucD*	*cva*	*tsh*	*astA*	*K1*	*papC*
Turkey 100	99	97	96	92	55	31	30	39	44	4	3
%	**99**	**97**	**96**	**92**	**55**	**31**	**30**	**39**	**44**	**4**	**3**
Chicken 35	35	34	35	30	32	32	19	20	27	7	1
%	**100**	**97.1**	**100**	**85.7**	**91.4**	**91.4**	**54.2**	**57.1**	**77.1**	**20**	**2.8**
APEC 65	65	63	60	55	61	60	52	38	11	20	5
%	**100**	**96.9**	**92.3**	**84.6**	**93.8**	**92.3**	**80**	**58.4**	**16.9**	**30.7**	**7.6**
total	199	194	191	177	148	123	101	97	82	31	9
%	**99.5**	**97**	**95.5**	**88.5**	**74**	**61.5**	**50.5**	**48.5**	**41**	**15.5**	**4.5**

Table 2. 5 Prevalence of virulence-associated genes in *E. coli* isolates. Total numbers of isolates positive for 11 virulence genes and total number of isolates positive for 8 virulence genes after removal of three common genes (*crl*, *fimH* and *csg*).

Total No. of virulence associated genes	Total numbers of isolates for 11 virulence genes				Total numbers of isolates for 8 virulence genes			
	Turkey (n=100)	Chicken (n=35)	APEC (n=65)	Total (n=200)	Turkey (n=100)	Chicken (n=35)	APEC (n=65)	Total (n=200)
0	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**1**
1	**0**	**0**	**0**	**0**	**12**	**0**	**0**	**12**
2	**1**	**0**	**0**	**1**	**25**	**1**	**2**	**28**
3	**2**	**0**	**0**	**2**	**24**	**4**	**8**	**36**
4	**13**	**0**	**0**	**13**	**25**	**8**	**20**	**53**
5	**22**	**1**	**3**	**26**	**13**	**11**	**20**	**44**
6	**25**	**5**	**9**	**39**	**0**	**10**	**13**	**23**
7	**25**	**7**	**19**	**51**	**0**	**1**	**1**	**2**
8	**12**	**11**	**20**	**43**	**0**	**0**	**1**	**1**
9	**0**	**10**	**12**	**22**	**0**	**0**	**0**	**0**
10	**0**	**1**	**1**	**2**	**0**	**0**	**0**	**0**
11	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**0**

To show the genetic divergence between *E. coli* strains generated by using ERIC-PCR profiles and virulence associated genes, Principal Component Analysis (PCA) was performed that considered the combination of all genotypic variables. Score plots between the first 2 components for which the eigenvalue for them were most significant (6.1 and 2.6, respectively) show the clear separation between presumptive commensal and APEC strains (**Figure 2.4**). However, the two sources of commensal *E. coli* strains showed some overlap but with some separation between them. In addition, strains from chicken were located between of turkey and APEC strains indicating a greater similarity between all chicken isolates that between presumptive commensals irrespective of poultry source.

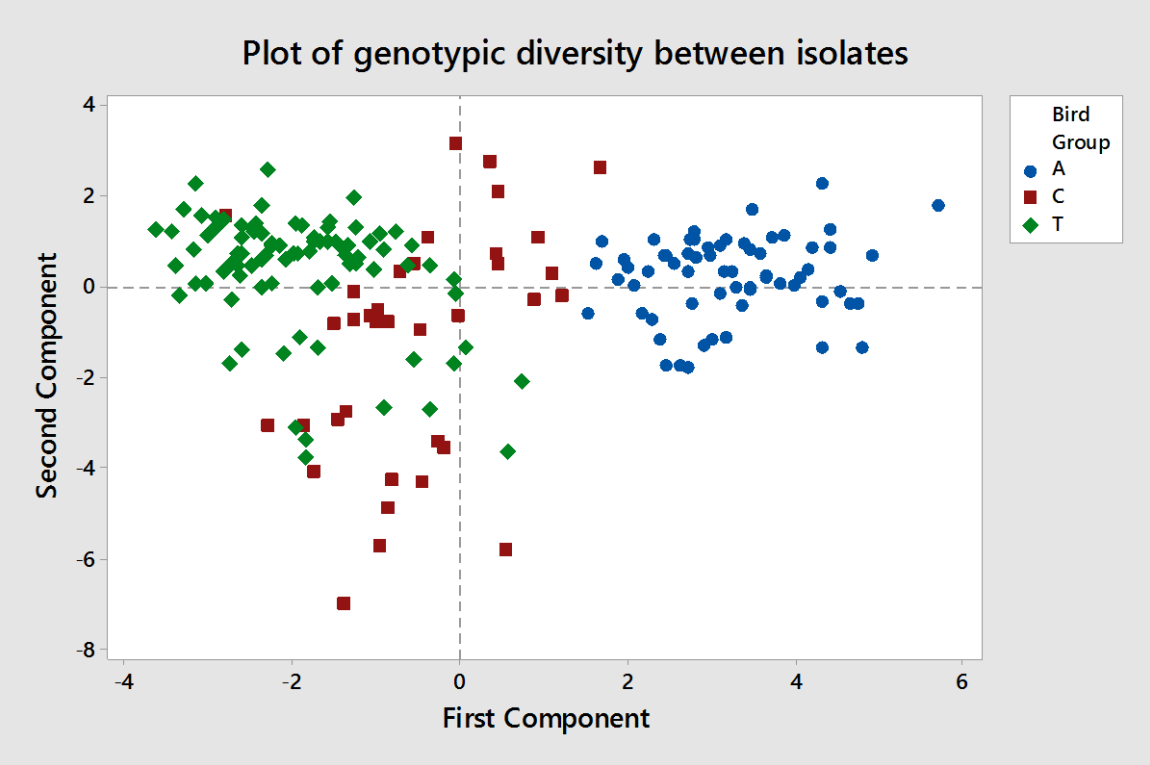

Figure 2. 4 Score plot of genotypic diversity between three sources isolated *E. coli*, A= APEC isolated (blue), C= chicken isolated (red) and T= turkey isolated strains (green). First component was virulence genes and second component was ERIC-PCR bands.

2.3.3 Phenotypic diversity

2.3.3.1 Carbon source utilisation

A large subset of the 200 isolates were able to utilise as a carbon and energy source dulcitol (38%), sucrose (37.5%), raffinose (36%) and sorbose (34.5%) irrespective of source of isolation. Fewer strains, notably those isolated from APEC, could utilize proline (7%), inositol (3%) 4 strains isolated from chicken and 2 from APEC, and salicin (1%) which isolated from chicken only. No strains could utilise either melezitose or allantoin (**Figure 2.5**). Most strains that could utilise raffinose were also able to utilise sucrose. Strains isolated from Turkey utilised adonitol and arabitol, while other strains from chicken and APEC could not. Six of the 11 carbon substrates were utilised by varying numbers of isolates within each of the three study groups with utilisation of 4 carbon substrates namely sucrose, raffinose, dulcitol**,** and sorbose was common **(Table 2.6**).

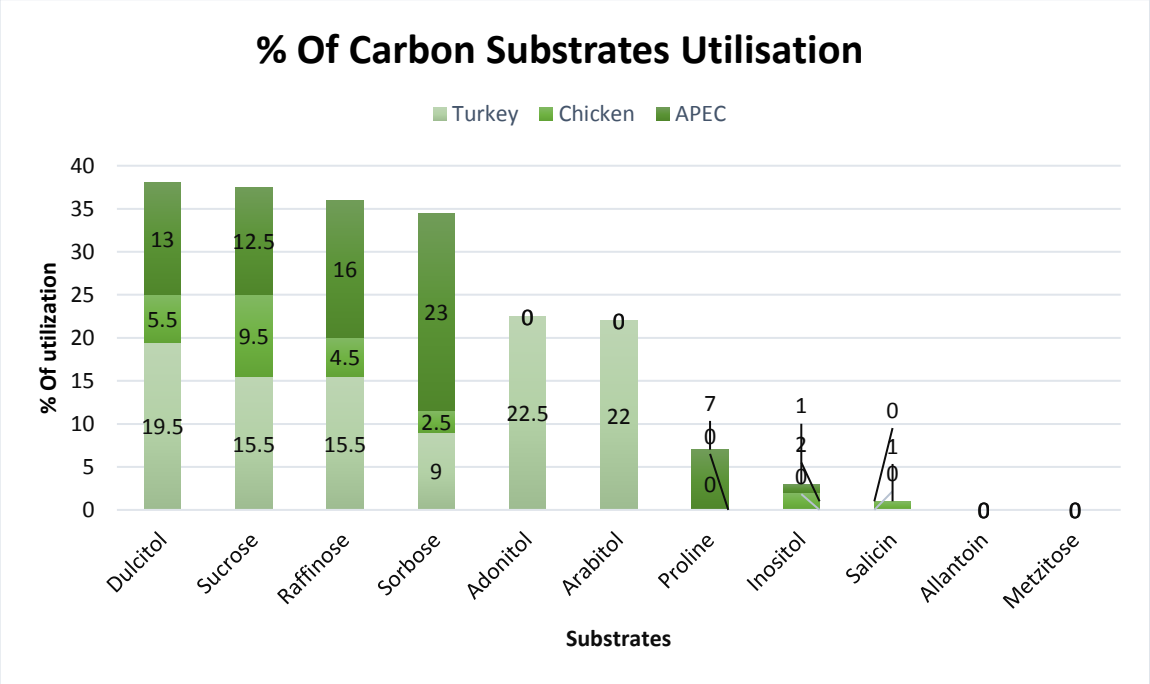

Figure 2. 5 Growth rates of strains grown minimal medium with eleven substrates.

Table 2. 6 Variation in the utilisation of 11 carbon substrates among 200 strains of *E. coli*.

isolation samples	n	% of strains fermenting carbon source											Total no. of sugars utilized tested
		suc	raf	ino	ado	ara	dul	all	pro	sor	mel	sal	
turkey	100	31	31	0	45	44	39	0	0	18	0	0	6
chicken	35	54.2	25.7	11.4	0	0	31.4	0	0	14.2	0	5.7	6
APEC	65	38.4	49.2	3	0	0	40	0	21.5	70.7	0	0	6
total	200	37.5	36	3	22.5	22	38	0	7	34.5	0	1	9

2.3.3.2 Antimicrobial susceptibility

The two hundred of *E. coli* isolates were tested for antimicrobial susceptibility against 7 antimicrobial agents using the disc diffusion method. In general, it was found that the isolates were susceptible to most antimicrobials tested. The total number of isolates susceptible to all of the antibiotics tested was 43.5%. The number resistant to at least one antibiotic was 23% and those that could be defined multiple antibiotic resistant (MAR, resistant to 3 or more antibiotics) was 12.5% (**Table 2.7**). In detail, samples isolated from turkey the frequency of susceptiblit to all antimicrobial was more than chicken and APEC isolated (53%, 34.2%, 33.8%) respectively. However, it was found that the APEC samples were resistant to the majority of antimicrobials tested compared to those isolated either from turkey or chicken, while resistance to the quinolone, nalidixic acid, and the ß-lactam, ampicillin, was noted in a relatively high frequency (36%-39%) irrespective of the source of the isolates tested **Table 2.8**.

Table 2. 7 Percentages of *E. coli* isolates (n=200) resistant to antimicrobial agents.

E. coli isolates source (n)	No. of isolated (% of total)					Total (n)
	0	1	2	3	4 & 5	
Turkey (100)	53 (53)	18 (18)	27 (27)	2 (2)	0	100
Chicken (35)	12 (34.2)	9 (15.7)	8 (22.8)	4 (11.4)	2 (5.7)	35
APEC (65)	22 (33.8)	19 (29.2)	7 (10)	7 (10)	10 (15.3)	65
Total (200)	87 (43.5)	46 (23)	42 (21)	13 (6.5)	12 (6)	200
%	43.5	23	21	6.5	6	100

Table 2. 8 Percentages of *E. coli* isolates, susceptible, intermediate and resistant to antimicrobial agents.

Antimicrobial agent (µg)	n = 200		
	susceptible	Intermediate	Resistant
Nalidixic acid (10µg)	122 (61%)	6 (3%)	72 (36%)
Amikacin (30µg)	184 (92%)	4 (2%)	13 (6.5%)
Ampicillin (10µg)	114 (57%)	7 (3.5%)	79 (39.5%)
Chloramphenicol (30µg)	184 (92%)	8 (4%)	8 (4%)
Colistin (10µg)	190 (95%)	4 (2%)	6 (3%)
Streptomycin (10µg)	165 (82.5%)	20 (10%)	15 (7%)
Trimethoprim (5µg)	174 (87%)	1 (0.5%)	25 (12.5%)

To estimate the phenotypic diversity between *E. coli* isolates a PCA was performed. The phenotypic characteristics used in this analysis were carbon source utilisation and antimicrobial susceptibility. Score plots are between the first 2 components for which the eigenvalues were 3.95 and 2.12 respectively. The results show that there is little separation between all *E. coli* isolates irrespective of origin (**Figure 2.6**). However, there are subsets of turkey and APEC origin that appear to be clearly separated from all other isolates. These data are at variance with the PCA for genotypic separation where clear distinction between groups was very apparent (**Figure 2.4**). Collectively the data suggest that the phenotypic tests used may not be that useful for differentiation between APEC and commensals.

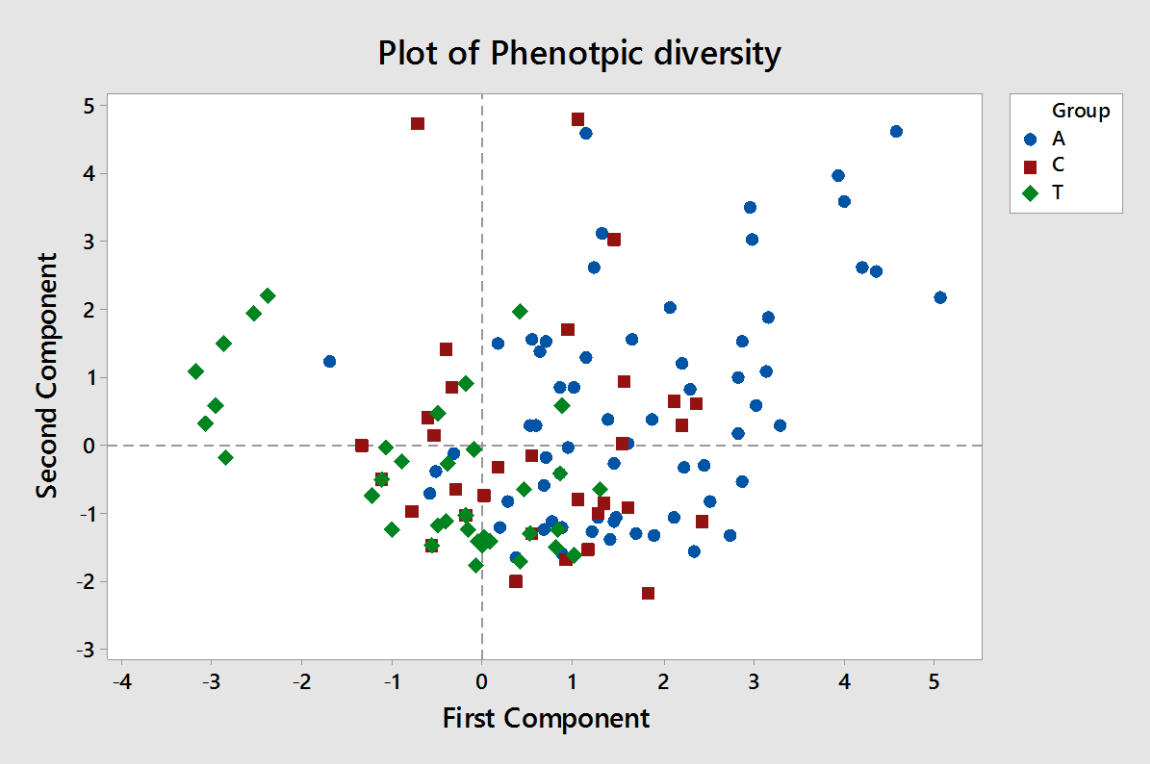

Figure 2. 6 Score plot of phenotypic diversity between three sources isolated *E. coli*, A= APEC isolated (blue), C= chicken isolated (red) and T= turkey isolated strains (green). Fists component was antimicrobial susceptibility and second was carbon source utilisation

The aim of this work was to investigate the similarity and diversity between presumptive commensal and APEC strains. A total of 200 strains were isolated from different three sources of poultry, presumptive commensal *E. coli* (turkey 100 and chicken 35) isolated from farm where there was high health status and APEC 65 strains isolated from infected birds. To estimate the diversity, we evaluated the correlation between the all characteristics we tested. **Figure 2.7** represents the plot of the phenotypic distance vs. the genotypic distance between the isolated strains for which the eigenvalue for the first 2 components were highly significant (8.63 and 3.06) respectively and showed that strains were grouped distinctly. As expected, turkey strains as well as the majority of chicken isolates (26) which were considered as presumptive commensal *E. coli* were clearly distant both genetically and phenotypically from APEC isolates. On the other hand, the minority of chicken isolates (9) shared some characteristics with APEC strains. Consequently, on the plots of the (PCA), based on the

distance of the 2 first components of the characteristics the eigenvalue for them were (3.95 and 2.12) respectively (**Figure 2.6**).

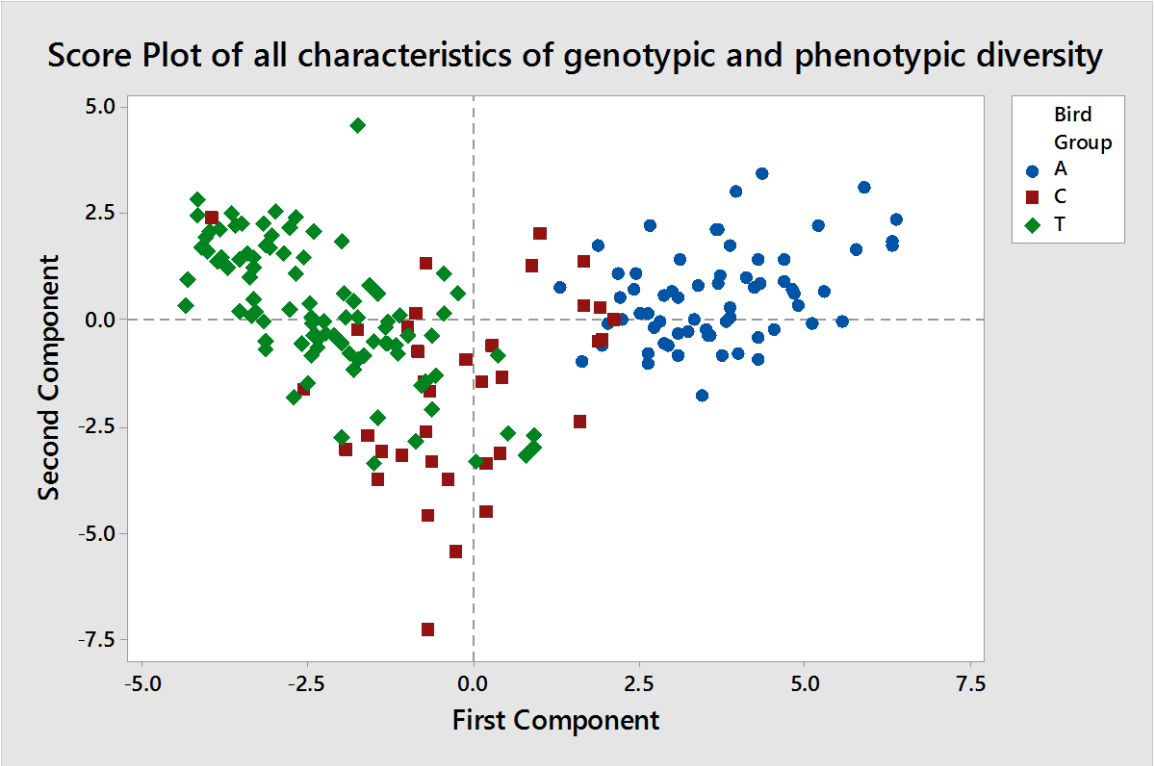

Figure 2. 7 Relationship between the phenotypic distance, and the genetic distance, resulting from comparisons between 200 E. coli strains, T=turkey (green), C=chicken (red) and A=APEC (blue). First component was genotypic characteristics and second component was phenotypic characteristics.

The relative diversity of isolates *E. coli* as assessed by both genotypic and phenotypic test is shown graphically in **Figure 2.8**. *E. coli* isolates that share traits are co-located on the graph. It would appear that some variables were co-located such that two groups (A and B) were formed and that these groups are more likely to share these traits, indicating a high correlation between them. Group A show clustering of a number of different antibiotic resistances. This may be anticipated because antibiotic resistances are often carried co-located on plasmids. Therefore, this clustering indicates acquisition of plasmids rather than any other trait. Group B were able to utilise proline, sucrose, raffinose, sorbose and dulcitol and carried *iucD, iss, cva/cvi* and K1 genes. There is a major differentiation between all isolates and those

that utilise arabitol and adonitol which as a group tended to be resistant to nalidixc acid and ampicillin. Also perhaps significantly, these lacked most virulence determinants. Is it possible that these are true non-pathogenic commensals and contrary to the analysis above these traits, namely use of arabitol and adonitol, may be used for differentiation?

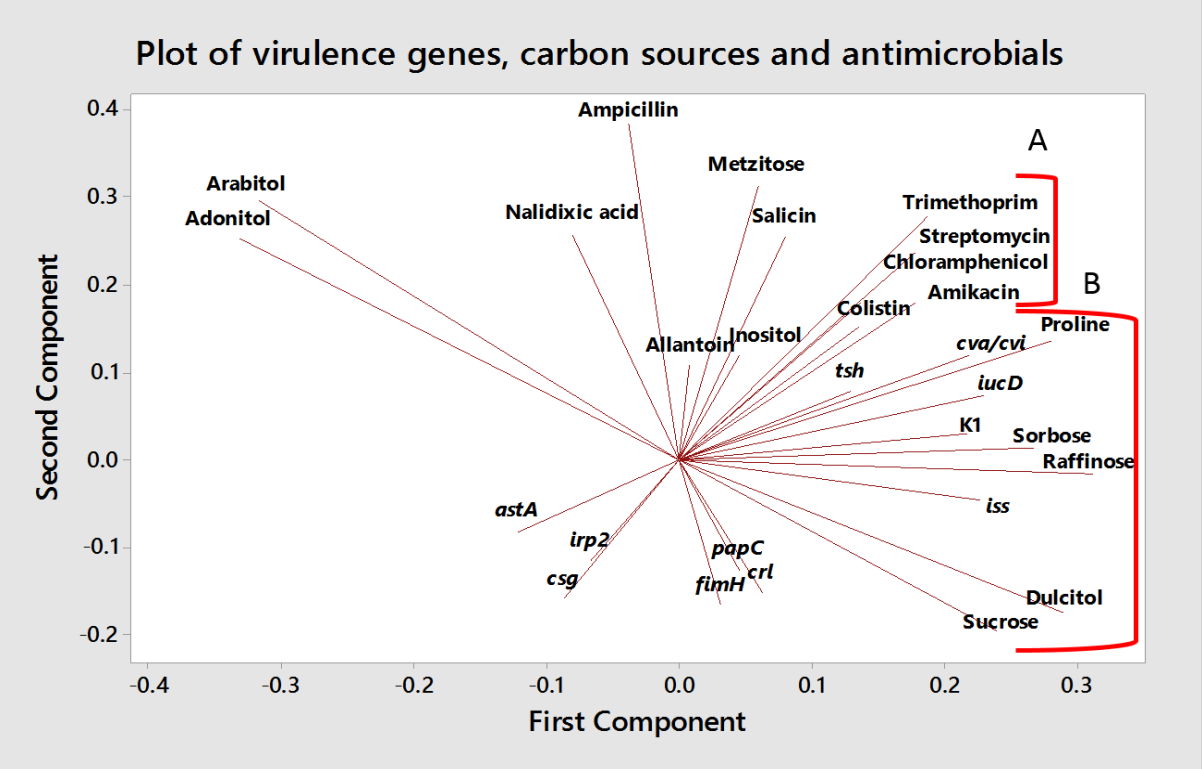

Figure 2. 8 Correlation between the virulence genes, carbon sources utilized and antimicrobials agent between 200 *E. coli* strains. First component was genotypic characteristics and second component was phenotypic characteristics

Significant correlations (p>0.005) between virulence genes and phenotypic characteristics in isolates *E. coli* were found between some variables (**Appendix 3**). For example, in **Table 2.9** correlation coefficients between utilisation of the carbon source arabitol and antimicrobials resistance nalidixc acid and ampicillin 0.307 and 0.195, whereas there was a negative correlation with resistance to amikacin, chloramphenicol, streptomycin and trimethoprim (-0.155, -0.151, -0.183 and -0.205, respectively). Also, arabitol was negatively correlated with many virulent including *iucD, iss, cva/cvi* and k1 (-0.234, -0.308, -0.201 and -0.231, respectively). Similar findings were shown for utilisation of adonitol as these two traits

are highly correlated (0.919). In addition, the correlation coefficients between arabitol and adonitol and other carbon sources tested, raffinose, dulcitol, proline, sorbose and sucrose were -0.367, -0.492, -0.239, -0.37 and -0.42. Conversely, utilisation of proline was associated with same of variables as arabitol and adonitol but with a positive Pearson correlation with *iucD, iss, cva/cvi and k1* (0.258, 0.195, 0.26 and 0.392). Such strong correlation between the virulence genes and phenotypic characteristics suggested that the presence or absence of some virulence genes might be a necessarily process associated with some carbon sources between the presumptive commensal *E. coli* and APEC isolates.

Table 2.9 correlation of basic statistical associations between adonitol, arabitol and proline with other carbon sources, antimicrobial resistant genes and virulence genes.

Carbon sources	Adonitol	Arabitol	Proline
Sucrose	-0.399 *	-0.420 *	0.343 *
Raffinose	-0.445 *	-0.367 *	0.335 *
Inositol	-0.066	-0.025	0.029
Adonitol	-	0.919 *	-0.299 *
Arabitol	0.919 *	-	-0.239 *
Dulcitol	-0.490 *	-0.492 *	0.295 *
Allantoin	0.007	0.004	0.009
Proline	-0.299 *	-0.239 *	-
Sorbose	-0.370 *	-0.371 *	0.284 *
Metzitose	0.005	0.123	0.170 *
Salicine	-0.058	-0.054	0.179 *
Antimicrobial			
Nalidixic acid	0.296 *	0.307 *	-0.191 *
Amikacin	-0.155 *	-0.155 *	0.268 *
Ampicillin	0.182 *	0.195 *	0.028
Chloramphenicol	-0.151 *	-0.151 *	0.179 *
Colistin	-0.068	-0.070	0.367 *
Streptomycin	-0.180 *	-0.183 *	0.232 *
Trimethoprim	-0.204 *	-0.205 *	0.263 *
Virulence genes			
tsh	-0.068	-0.064	0.164 *
iucD	-0.263 *	-0.234 *	0.258 *
irp2	0.082	0.052	-0.206 *
iss	-0.363 *	-0.308 *	0.195 *
astA	0.013	0.017	-0.082
cva/cvi	-0.209 *	-0.201 *	0.260 *
fim	-0.046	-0.040	-0.014
papC	-0.110	-0.110	0.016
crl	-0.124	-0.132	0.041
k1	-0.231 *	-0.231 *	0.392 *
csg	0.001	0.062	-0.162 *

*Correlation is significant at the 0.05 level (p-value).

2.3.4 Galleria model

2.3.4.1 Selection of bacterial strains

Eight isolates of *E. coli* from a total of 200 isolated strains were examined in this study, 4 from infected chicken and other 4 from turkey. *E. coli* isolates from infected birds were classified as APEC isolates and the other isolated from turkey were presumptive commensal isolates. The isolates were selected on the basis of genotypic and phenotypic characteristics as determined previous. Two metabolic differences that were shown to be statistically significant in separating putative commensals from pathogens were; APEC's ability to utilised proline as a sole carbon and energy source, and for turkey isolates presumptive commensals the ability to utilise adonitol (ribitol) and arabitol as sole carbon and energy sources. From each group two isolate were fully sensitive to all 7 antibiotics tested, and the other two strains from each group were resistant to at least 2 antibiotics tested (**Table 2.10)**.

Table 2. 10 Variation of phenotypic (carbon substrates, antibiotic) and virulence-associated genes for 8 isolates *E. coli*, (+) positive and (-) negative.

Strains	Turkey				APEC			
	A	B	C	D	E	F	G	H
Carbon sources								
Suc	-	-	-	-	+	+	-	-
Raf	-	-	-	-	-	-	-	-
Ino	-	-	-	-	-	-	-	-
Ado	+	+	+	+	-	-	-	-
Ara	+	+	+	+	-	-	-	-
Dul	-	-	-	-	-	-	-	-
All	-	-	-	-	-	-	-	-
Pro	-	-	-	-	+	+	+	+
Sor	-	-	-	-	-	+	+	-
Met	-	-	-	-	-	-	-	-
Sal	-	-	-	-	-	-	-	-
Antibiotics								
Na	-	-	+	+	-	-	-	+
AK	-	-	-	-	-	-	+	-
Am	-	-	+	+	-	-	-	+
C	-	-	-	-	-	-	+	-
Co	-	-	-	-	-	-	-	-
S	-	-	-	-	-	-	-	-
T	-	-	-	-	-	-	+	+
Virulence genes								
Fim+	+	+	+	+	+	+	+	+
PapC	-	-	-	-	-	-	-	-
Csg	+	+	+	+	+	+	+	+
Crl	+	+	+	+	+	+	+	+
Tsh	-	-	-	-	+	+	+	+
iucD	-	-	-	-	+	+	+	+
irp2	+	+	+	+	-	+	+	-
iss	+	+	-	+	+	+	+	+
astA	-	-	+	+	-	-	-	-
cva	-	-	-	-	-	+	+	-
K+	-	-	-	-	+	+	-	-
Total genes (11)	**5**	**5**	**5**	**6**	**7**	**9**	**8**	**6**

2.3.4.2 Virulence in Galleria mellonella

The larvae were injected with a range of inoculum doses to determine the mortality rates. The Kaplan-Meier survival analysis for strains of each different type was used. Survival curves are shown in **Figure 2.9**. The survival outcome of the *E. coli* inoculated larvae varied, where B, C, F and H showed different mortality rates compared to A, D, E and G. Isolates of C were observed to be the least virulent in this model, the result show different response from each group. However, an interesting result to emerge from the data was the observation that there was a clear separation of pathogenic and less pathogenic *E. coli* among the tested isolate, but regrettably the isolates were not in the same group as anticipated in the testable hypothesis.

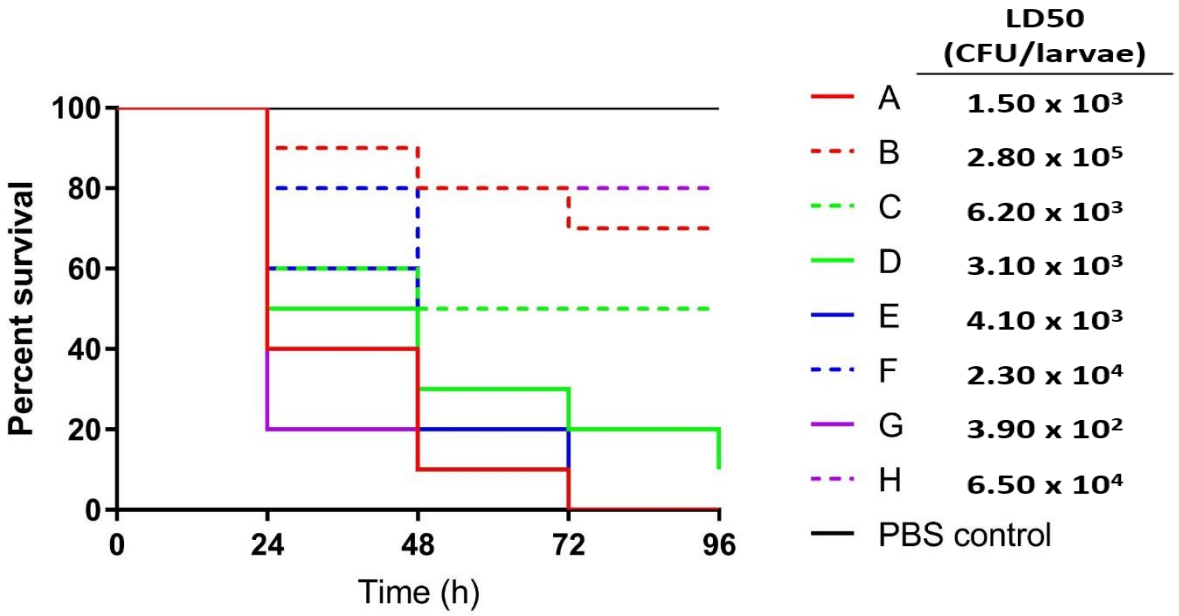

Figure 2. 9 Survival curves for *G. mellonella* larvae infected with 10^4 colony forming units (CFU)/larvae *E. coli* cells.

Our results show different mortality rates among strains *in vivo*, but no association between pathogenicity and proline utilisation was observed. Strain A was the most pathogenic isolate from turkey even though it had the fewest number of virulence genes (5 genes), and was fully sensitive to all antibiotics tested with LD50 (1.50×10^3). Strain C was the least pathogenic overall and was also isolated from turkey with resistance to nalidixic acid and ampicillin and a

low LD50 (6.20 x 10^3). The only thing that distinguishes strain C from the rest of the strains is that it does not have the *iss* gene. Moreover, strain F that is an APEC strain had the highest number of virulence determents (9 genes) and is fully sensitive to antibiotics; had a relatively high LD50 (2.30 x 10^4).

2.4 Discussion

2.4.1 Genotypic diversity

Regardless of intense research efforts aimed at controlling and eliminating avian pathogenic *E. coli*, relatively little is known about the diversity of the organism, its differences from commensal strains, and the mechanisms of spread and persistence in the poultry production environment. We were interested to know how and what distinguishes these pathogenic strains from their commensal counterparts. In the case of the studies described here that made use of chickens and turkeys from dietary investigation studies held at CEDAR, the University of Reading farm, as a source of *E. coli* from healthy animals for comparison with confirmed APEC from the University of Surrey Veterinary School. Both genotypic and phenotypic characteristics of the panel of isolates were investigated. The genetic tests used were ERIC-PCR which is a simple method for the analysis of chromosomal diversity among *E. coli* strains, and PCR analysis of APEC virulence determinants for 11 genes encoding known virulence factors. The phenotypic diversity tests used 7 antimicrobials to test for susceptibility and 11 carbon sources to test for carbon and energy source utilization. The eleven substrates are known to be highly variable in the *E. coli* population as a whole (Edwards and Ewing, 1986; Woodward and Charles, 1983).

The ERIC-PCR genotyping results demonstrated variable fingerprints in the isolates of *E. coli* that were tested in this study. Most of the currently used simple molecular biological

techniques that have been developed for genotyping for bacterial typing such as enterobacterial repetitive intergenic consensus ERIC-PCR, random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and repetitive bacterial DNA elements PCR (Maurer *et al.*, 1998; Versalovic *et al.*, 1991; Namvar and Warriner, 2006) can only give an indication of genomic similarity but do not relate to their evolutionary relationships. Other technologies such as MLST and whole genome SNP analysis provide greater depth of analytical power to assign isolates to evolutionarily related groups. Whilst this would be a useful adjunct if not a preferred option to the studies here, ERIC-PCR was used because the method is rapid, sensitive, repeatable and reliable, and therefore, it can be generally applied for the effective molecular differentiation of bacteria (Dorneles *et al.*, 2012; Guimaraes Ade *et al.*, 2011) and genetic diversity in *E. coli* isolates specifically (Ramazanzadeh *et al.*, 2013) in a cost effective manner. Furthermore, the aim was to assess similarity rather than evolutionary origin, however interesting that aspect would have been to follow.

Several studies have reported the use of ERIC-PCR for typing of poultry *E. coli* isolates (Ngeleka *et al.*, 1996; Dias da Silveira *et al.*, 2002; Namvar and Warriner, 2006), mostly discriminating between commensal *E. coli* and APEC isolates (da Silveira *et al.*, 2002b). The data presented here indicates the similarity in PCR fingerprinting profile existed from three sources with more homogenous within the group and high clusters were between presumed commensal isolates *E. coli* than APEC isolates. The ERIC-PCR typing method showed 137 patterns for 200 isolates. Twenty five (12.5%) of isolates displayed a single unique profiles whereas, 175 (87.5%) of them showed shared patterns which is indicative of similar strains of distribution in coefficient 0.089 was 0.848 D value, D value >0.9 is desirable and typing result can be interpreted (Hunter and Gaston, 1988). Interestingly, if we were to review the similarity dendrogram using a lower % relatedness for a cut off between profiles we see that three groups emerge and that each group is largely characteristic of APEC, chicken ‘commensals’ and

turkey ‘commensals’. ERIC-PCR profiles demonstrate genetic relatedness between *E. coli* strains. Therefore, isolates were similar between the birds as will be seen later, 31 profiles showed similar genotypes which indicate these strains were similar to one another. In previous studies describing the genetic diversity and clonal similarity of *E. coli* population by Selander and Levin, (1980), it was suggested the rate of genetic recombination in the natural population of *E. coli* is low but that the genetic structure of *E. coli* populations and the factors determining the amount of neutral gene variability in this bacterial species is significant (Selander and Levin, 1980). Different procedures other than ERIC-PCR as discussed earlier have been applied to study bacterial genetic exchange among *E. coli* isolates (da Silveira *et al.*, 2002a; Ewers *et al.*, 2004). The main objective here was not to understand the phylogenetic relations of the bacteria in the panel of isolates but rather to assess diversity and to attempt to establish any possible correlations with phenotypic behaviour as will be discussed below.

The genotyping of the 200 isolates revealed that a high percentage of them harbour virulence factors related to adhesion (*crl* 99.5%, *fimH* 97% and *csg* 95.5%) and iron acquisition (*irp* 88.5% and *iucD* 61%) followed by serum resistance (*iss* 74%), which are characteristic of the APEC pathotype.

Five adhesion virulence genes were tested (*crl, fimH, csg, tsh* and *papC*). The gene *crl* was the most prevalent, it was present in all isolates except one strain in turkey isolated (99.5%). Whether this was true or an artefact of PCR needs to be reassessed especially as (Knobl *et al.*, 2012) found it in all isolates (100%). Curli stimulates bacterial adherence to fibronectin, activation of plasminogen and chicken erythrocyte agglutination. The specific adhesion of type I fimbriae encoded by *fimH* was also highly prevalent (97%) in agreement with (Rossez *et al.*, 2014; Zhao *et al.*, 2009). The *fimH* adhesin is located on the very tip of the type I fimbria and enables colonization of the gut and the air sac of birds (Pourbakhsh *et al.*,

1997). Dozois and his colleagues discovered that *fimH* associated with *E. coli* causing avian septicaemia, also caused extra-intestinal disease in humans and mammals (Dozois *et al.*, 1992). This indicates that *fimH* has an important role in the pathogenesis of *E. coli* possibly in many species. In addition, the *csg* gene was also detected at a high rate (95.5%) and this gene is essential for the expression of curli fimbrial fibres being the major subunit protein. The most common virulence factors *fimH, crl* and *csg* were present in almost all of the isolates because they are important to colonization and adhesion in multiple host species (Antao *et al.*, 2009), so these may not be associated with APEC specifically but rather can be considered ubiquitous adherence systems for all *E. coli*. However, this does not preclude them from being considered as potential virulence determinants as they cause adherence which is important in the early stages of pathogenesis. Another important trait and adhesin detected in APEC, is the *tsh* protein that displays similarity to a subclass of the *IgA* protease family (Stathopoulos *et al.*, 1999; Dozois *et al.*, 2000), contributing to air sac colonization and is considered to be one of the major mechanisms of adherence to avian respiratory tract (Maurer *et al.*, 1998; Stathopoulos *et al.*, 1999). The prevalence of *tsh* may vary commonly in APEC isolates with 49.7% of strains positive for *tsh* as reported previously by (Dozois *et al.*, 2000) which is similar to the findings presented here (48.5%). In contrast high prevalence in APEC was shown by (Janben *et al.*, 2001) where 85.3% of isolates were positive but low percentiles of 28% and 13% were obtained respectively by (Knobl *et al.*, 2012). The gene *papC* that encodes P fimbriae was found in low rate in both presumptive commensals and APEC, only 9 isolates (4.5%) of 200 isolates, *papC* was the lowest of the tested virulence factors. P fimbriae, as indicated by the detection of *papC*, mediates adherence to internal organs and for colonisation of the air sacs, lungs, kidney, and blood (Pourbakhsh *et al.*, 1997; Stathopoulos *et al.*, 1999). P fimbriae are also associated with human urinary tract infection (Knöbl, 2011). Recent evidence suggests that APEC carrying P fimbriae are not related to those causing UTI in humans (La Ragione *et al.*, 2002). Several

studies showed that the prevalence of P fimbriae varied between 6.8% and 37% (Guastalli, 2013; Zhao *et al.*, 2009). This disparity may indicate that *papC* might be not an essential virulence trait for APEC (Vandekerchove *et al.*, 2005) or it might be confirmative virulence for certain sub-groups of APEC if it is present.

Both *irp2* and *iucD* are related to iron acquisition systems. Iron acquisition systems have been recognized to be associated with bacterial virulence especially in bacteria causing some disease such as septicaemia (Dho-Moulin and Fairbrother, 1999; Headley *et al.*, 1997). The *irp2* and *iucD* genes were detected in 88.5% and 61.5% of isolates tested, respectively, and have been linked with APEC previously (Zhao *et al.*, 2009). This investigation may indicate that having these two genes could be used as genetic marker for APEC strains. However, whilst in lower prevalence in isolates made from turkeys, both these genes were common in APEC and presumptive commensals isolated from chickens.

APEC virulence genes were attributed to the resistance to action of complement and bactericidal effects of serum. This resistance can be conferred by many cellular components like the capsular antigen and lipopolysaccharide. The *iss* gene encodes for increase serum survival was detected 74% in isolates, similar to studies by (Zhao *et al.*, 2009) who found 81%. However, (Knobl *et al.*, 2012) obtained a lower prevalence for *iss* from APEC isolated from infected poultry with 26%. The *iss* was detected in 38.5% of isolates made from diseased animals with colibacillosis (Delicato *et al.*, 2003). Furthermore, (Rodriguez-Siek *et al.*, 2005b) found the *iss* gene in low prevalence (18.7%) in isolates from healthy birds. The *iss* gene was identified as a plasmid carried gene which was associated with high pathogenicity strains and used this criterion for the differentiation of pathogenic strains. This indicates that *iss* might be a contributor to enhancing virulence and merely indicates other essential virulent traits are essential for disease.

The *astA* gene which encodes a heat-stable enterotoxin, considered to be widely distributed through different categories which found in diarrhoeagenic *E. coli* in both humans and animals. Also, the toxin was expressed by 38% of none pathogenic *E. coli* strains from asymptomatic children (Savarino *et al.*, 1996). In this study, the *astA* gene was detected in 41% of isolates, similar to the findings of Guastalli and his colleagues (2013) who showed 40.1% of strains were *astA* positive. In contrast, there were variations in the presence of the *astA* gene such as, 88.5 % and 20% of strains isolated from chicken affected with colibacillosis (Someya *et al.*, 2007; Ewers *et al.*, 2004).

Another virulent gene tested was the colicin V plasmid operon (*cva/cvi*) gene. Colicin V plasmids were found primarily among virulent enteric bacteria and have been shown to encode several virulence related properties in addition to colicin V (Vandekerchove *et al.*, 2005; Janben *et al.*, 2001). In this study 50.5% were positive for *cva/cvi* gene which is 26% were from APEC isolates and 15%, 9.5% isolates from presumptive commensal turkey and chicken, respectively. It is interesting that Vandekerchove *et al*. (2005) and Janben *et al*. (2001) suggest that *cva*/*cvi*, or Colicin V plasmids could be considered to be a defining feature of the APEC strains. Being present in only ~50 of isolates that were defined as APEC suggests this statement is contestable. Our data possessing *iss*, *iucD, tsh* and *cav/cvi* have been considered to be a defining feature of the APEC strains (Vandekerchove *et al.*, 2005; Johnson *et al.*, 2006). Thirty-one (15.5%) strains were positive for the *kps (k1)* gene encoding capsule production whereas previous reports identified a percentage ranging from 8 to 20% for APEC (Delicato *et al.*, 2003).

Collectively, the genotypic data demonstrate that a high degree of polymorphism exists among *E. coli* isolates that originated from different poultry sources when the respective bacterial genomes were analysed by the ERIC-PCR technique and virulotyping by PCR and of

importance to the rest of the studies, these data clearly indicate that the *E. coli* population in poultry is very diverse and most *E. coli* are potentially pathogenic. It is possible on the basis of this evidence to suggest that if the genomes are this variable, then there will be much phenotypic diversity also and this is discussed below. What is striking is the diversity seen even when using a relatively limited range of genetic tests. One discussion point could focus on the need for wide genomic studies such as those performed by Cordoni *et al*., (2016). However, the limitation of the Cordoni *et al*., study is the fact that it focused on APEC only rather than attempting any differentiation between APEC and presumptive commensals. The PCA plots suggest that presumptive commensal strains from chicken appeared to share similarities with and sit between turkey presumptive commensals and APEC in terms of shared factors. The turkey isolates generally possessed far fewer virulence determinants whereas chicken isolates showed greater diversity and therefore probably represented genuine commensals as well as APEC that at the time of isolation acted as gut commensals without inducing fulminant disease. The data also suggest that turkey isolates are more alike one with another and may therefore be adapted to the turkey as a host. Given *E. coli* related disease in turkey is relatively rare and is associated more with joint infection and unlike the various manifestations of colibacillosis, the data suggest two possibilities. First, these are genuine turkey related commensals lacking many virulence determinants or second, they are potential turkey pathogens but have as yet undefined virulence determinants that are different from those associated with chicken adapted APEC isolates.

Regarding specific genes associated with virulence of *E. coli* in avian species, previous investigations have indicated that the distribution of various virulence factors are useful markers for the detection and characterization APEC, and could therefore, be used in differentiation and diversity analysis of *E. coli* in poultry (da Silveira *et al.*, 2002a; Delicato *et al.*, 2003). The broadly accepted criterion that APEC may be defined as those *E. coli* of poultry

origin harbouring 5 or more virulence-associated genes as previously reported (Ewers *et al.*, 2005) could be used. According to these criteria, 184 strains out of 200 strains examined in total were classified as APEC, 16 strains isolated from turkey possessed less than 5 genes which were considered as commensals poultry. Interestingly, the APEC stains were confirmed pathogens in poultry and these as a group had more virulence genes than those from the chicken and turkey study described here. In addition, the majority of strains isolated from APEC had 7 and 8 genes. Is it possible that the '5' criterion is set too low and could be 7 or more, So if the criterion of 7 genes were used to classify APEC then 119 strains out of 200 strains would be defined as APEC. In a new study by Roussan and his colleges, typed APEC and showed they always harbour 5 to 8 virulence genes (Roussan *et al.*, 2014). Without performing Koch's postulates in live animal studies, the calling of an isolate as an APEC needs to be considered with care. Perhaps we need to consider isolates that possess 5 or more virulence determinants as potential pathogens if, and only if, the conditions enable them to become pathogens, namely these are opportunistic pathogens that need prior stress or infection for them to induce pathology. At the practical level then, we have to consider the vast majority of *E. coli* found in poultry may be capable of inducing pathology. If the hypothesis that the majority of *E. coli* can be pathogenic is true, then the management and dietary practices that maintain a healthy animal prevalent in the industry currently seem to prevent avian colibacillosis.

2.4.2 Phenotypic diversity

The foregoing discussion focused upon the genetic diversity of the isolates examined and concluded that the approaches used here, and that of many other authors, did not readily differentiate between APEC and commensal types. Another important consideration is whether or not there are other physiological or biochemical properties that contribute to or define commensal and pathogenic types. Serotyping has been used for the basic classification of *E.*

coli, which are defined by the combination of O (LPS) and H (flagella) antigens. Also, there are other phenotypic characteristics such as, antimicrobial resistance profiles, flagellar motility, carbon utilization and ability to form biofilm (Durso *et al.*, 2004; Yang *et al.*, 2004) amongst many others. In this study, we used antimicrobial resistance and carbon utilization. Whilst serotyping is used widely, the technique is reliant upon an appropriate source of sera that is now held by fewer and fewer reference laboratories worldwide. Also, the cost for serotyping within the UK at the one laboratory capable of serotyping animal isolates (APHA, Weybridge, UK) is prohibitive at £60 per isolate. For the same cost the entire genome can be sequenced and, as shown by Cordoni *et al.*, (2016) it is possible to infer serotype from the genes encoding LPS and flagella genes. Interestingly, Cordoni *et al.*, (2016) demonstrated that most isolates that could not be typed serologically and classified as untypable carried multiple LPS gene sets encoding two or more LPS types. That said, APEC isolates tend to belong to relatively few serogroups such as O1, O2 and O78. Reliance on a single test to define an isolate provides little assurance and so there is a need for the use of a wider range of simple tests and here the biochemical competence and antibiotic resistance characteristics were investigated.

Currently, *E. coli* are known to utilise a wide range of carbon and energy sources. However, within the diverse population of *E. coli* it is also known that there are several carbon and energy sources that are utilised variably. The studies of Edwards and Ewing in the 1960s gave a definitive picture of this variability and part of this study was to gain an understanding of which types of *E. coli* colonise avian species and whether there was any correlation with certain metabolic traits. It was reasoned that by understanding this there may be clues to controlling APEC by dietary intervention. By way of an example, L-sorbose is found in certain plant types and their presence in the diet may select for the 15% of *E. coli* isolates that can use this as carbon and energy source (Woodward and Charles, 1983). If commensals but not APEC utilise a particular substrate then these may be suitable feed ingredients that enrich commensals

and not APEC. Our results showed that a higher than anticipated utilisation rate for L-sorbose in APEC isolates was 46 out of 65 (70.7%), whilst in presumptive commensal isolates from turkey and chicken, 18% and 14% utilised sorbose, respectively. All isolates were tested and shown to differ in carbon substrate utilisation patterns. Of the 11 carbon sources tested, 2 were not utilised by any *E. coli* isolate whereas 9 were used variably. Interestingly, each of the three group of isolates could utilise variably only 6 substrates with the proportion of strains able to utilise a substrate dependent on the host from which they were isolated. This observation may indicate selection that was dependent upon what the animals were fed (Souza *et al.*, 1999). No isolates from any of three sources could utilise allantoin or melezitose and only 2 strains isolated from chicken could utilise salicin whilst inositol also utilized by 4 stains in chicken and 2 from APEC isolated strains.

Raffinose and dulcitol were frequently utilised by APEC isolates, 49.2% and 40% respectively. These substrates were also initially considered as probable discriminatory substrates for screening and identification of pathogenicity (Ratnam *et al.*, 1988; Durso *et al.*, 2004). *E. coli* isolates were more likely to use dulcitol and sucrose than other substrates (38%, 37.5%), which was similar to the findings of Durso and his colleges and they demonstrated that *E. coli* O157:H7 used dulcitol and sucrose more than were commensal *E. coli* (Durso *et al.*, 2004) moreover, *E. coli* O157:H7 was 100% positive for raffinose and dulcitol utilisation (Ratnam *et al.*, 1988). This indicates that raffinose and dulcitol might be utilised by APEC stains more than commensals *E. coli*. Interestingly the early work showed that there seemed to be a dependency upon the ability to utilise sucrose as a prerequisite to the utilisation of raffinose as there is a shared metabolic pathway and that the utilisation of both substrates is often plasmid mediated (Alaeddinoglu and Charles, 1979). Is it possible that dietary regimes that select for and enable transfer between strains of these metabolic capabilities contribute to pathogenicity also?

Forty-four commensal strains isolated from turkey utilised both adonitol (ribitol) and arabitol (22.5%) and one grew in adonitol alone. These two sugars (pentitols) are relatively abundant in nature (Mortlock, 1984) but it was commonly assumed by investigators that *E. coli* cannot utilise pentitols for growth. Whilst true for *E. coli* K12, this is not true for *E. coli* C that can grow at the expense of either adonitol or D-arabitol (Reiner, 1975). Reiner also investigated the origin of the genes responsible for pentitol catabolism in *E. coli*. Its assumed the utilisation of adonitol and D-arabitol in *E. coli* is chromosomally linked to *his*, therefore is not plasmid carried (Novick, 1969; Reiner, 1975). Furthermore, Woodward and Charles (1983) demonstrated that the genes for dulcitol (galactitol) utilisation and those for adonitol and arabitol utilisation are mutually exclusive at minute 44 on the *E. coli* genome. It seems likely that testing for growth on these pentitols may provide the easiest way to distinguish commensals *E. coli* strains from APEC strains if this correlation can be confirmed by further studies that make use of a much wider collection of *E. coli* isolates.

The ability to utilise proline as a sole carbon and energy source was limited to APEC strain only. However, only 21.5% were positive for this characteristic and it is questionable as to whether this will be a useful differential marker. However, it would be worth deeper analysis to see if this ability was correlated with other markers. It was noted that this marker was associated commonly with *cva/cvi, tsh, csg, irp2, iss, iucD* and K1 capsule formation suggesting a linkage with four major virulence determinants. Whilst a tantalising finding, this needs to be confirmed by further studies that make use of a much wider collection of *E. coli* isolates.

Antimicrobial treatment is one of most used control measures for reducing morbidity and mortality caused by APEC in poultry (Dho-Moulin and Fairbrother, 1999; Guerra *et al.*, 2003). The indiscriminate use of antimicrobials leads to the creation of more of resistant

isolates and they need to be used carefully in order to preserve their therapeutic usefulness in both animals and humans (Gyles, 2008). Among the 200 strains isolated from poultry, many were susceptible to most antimicrobials tested. The highest frequency of resistance was against ampicillin (39.5%) followed by nalidixic acid (36%). However, confirmed APEC isolates from infected chickens from the University of Surrey as a group showed the highest resistance to the majority of antimicrobials findings that were found to be in agreement with the observations of other reports that indicated a high percentage of resistance to the antibiotics in APEC (Kazemnia *et al.*, 2014; Salehi and Bonab, 2006; Mellata, 2013).

The prevalence of resistance against the ß-lactam was high in all *E. coli* irrespective of source and this finding is similar to those of (Normark *et al.*, 1980; Wang *et al.*, 2013). In this study, no attempt to define the mechanism of resistance was made but it is possible the encoding gene was *ampC* which is regarded as highly prevalent in poultry isolates (Lima-Filho *et al.*, 2013). The second most prevalent resistance was against nalidixic acid, a quinolone antibiotic, that usually develops due to exposure of quinolone antibiotics that select spontaneous mutations in the target site of DNA gyrase encoded by *gyrA*. The trend of high ampicillin and quinolone resistance in poultry implies these bacteria could be of public health concern as they also can be transferred to humans (van den Bogaard *et al.*, 2001). The isolates from turkeys carried the least resistance compared with both chicken groups although the APEC group was uniformly the most resistant. Collectively this data suggests that exposure to these antibiotics is still significant in the poultry sector despite attempts by the veterinary profession to move toward more responsible use. Perhaps use in the turkey sector is less than in broilers possibly. Given that no antibiotics were used in any of the CEDAR studies from which turkey and presumptive commensal chicken isolates were made suggests a heavy burden of resistance arising from the hatchery and the parent and grand-parent stock and/or the environment. Whilst antibiotic resistance is not considered a virulence factor *per se*, the higher

prevalence of resistance in APEC to commonly deployed antibiotics suggests that treatment of colibacillosis selects resistant APEC and the question arises as to whether resistance will contribute to pathogenesis by survival of APEC in the face of antibiotic treatment. Whilst *gyrA* mutations are chromosomal, most of the other resistances are likely to be plasmid mediated and therefore more ephemeral, moving rapidly between potentially unrelated host backgrounds.

To evaluate the extent of diversity and the links between the genotypic and phenotypic in the isolated strains, we correlated the phenotypic characteristics of 200 strains *E. coli* isolated with the genotypic characteristics. Altogether, we found that the genetic distance, based on ERIC-PCR bands and virulence factor genes were a useful indicator for determining the genotypic diversity of *E. coli* strains isolated from presumptive commensals in turkey and chicken grown in the same environment compared with APEC stains isolated from infected birds. The phenotypic distance, based on carbon source utilisation and antimicrobial resistance clearly showed diversity although correlations with pathogenicity were less clear. That said, there were tantalising pointers that use of dulcitol, sorbose, sucrose, raffinose and proline were more likely to be associated with APEC than presumptive commensals and conversely use of arabitol and adonitol may the correlated with commensals.

In our data, we found a significant negative correlation between utilisation of the carbon sources arabitol and adonitol with different virulence determinants tested such as, *iucD, iss, cva/cvi* and k1. In addition, the correlations between arabitol and adonitol with other carbon sources tested, raffinose, dulcitol, proline, sorbose and sucrose were also significant negatively correlated. Conversely, utilisation of proline was associated with same variables as arabitol and adonitol but with a positive Pearson correlation with *iucD, iss, cva/cvi and k1*.However, *iucD, iss* and *cva/cvi* are virulent genes considered as highly prevalence plasmid genes that were

found in 255 APEC isolated from turkey presenting airsacculitis (Cunha *et al.*, 2014). The *iss* gene was highly prevalent in *E. coli* isolates of known pathogenicity and serogroup were subjected to virulence genotyping and phylogenetic typing (Johnson *et al.*, 2008a). Johnson identified five plasmid genes, including *iss* associated with high pathogenicity strains and used this criterion for differentiation of pathogenic strains and 'faecal' *E. coli*. Thus, the presence of *iss* gene associated with this ability was more strongly correlated with pathogenicity. Similarly, the presence of *iucD* may be indicative of an APEC strain as discussed above especially as that gene was detected in 100% of (Someya *et al.*, 2007) and 78% detected by (Ewers *et al.*, 2004) APEC in recent studies. The capsule antigen-encoding gene *ksp* was frequently found in APEC strains in 51.3% in study done by (Ewers *et al.*, 2009). Thus, strong correlation between the virulence genes and phenotypic characteristics suggested that the ability to utilise some of uncommon carbon source such as arabitol and adonitol to discriminate between presumptive commensal *E. coli* and APEC.

The Initial classification of any bacterium has conventionally depended on the biochemical activities of the organism. Screening for the presence of a particular bacterium is often performed by selective media, and regular indicator media. Such methods depend on specific fermentative growth characteristics and resistance to antimicrobial compounds. Some biochemical properties have been exploited for the development of kits, which may be used to predict bacterial species, such as the analytical profile index (API, Biomerieux) and the Biolog system (Biolog Inc.). Generally, the majority of *E. coli* strains commonly ferment lactose, D-mannitol, melibiose, mucate and D-sorbitol but not D-adonitol, D-arabitol or cellobiose (Scheutz and Strockbine, 2001) that are used in API 20E test. In our result, it might be possible to use the adonitol and arabitol as positive and proline negative markers to differentiate between commensal *E. coli* and APEC.

2.4.3 Galleria model

The aim of this study was to correlate the genotypic and phenotypic characteristics in the 200 isolated strains with pathogenicity. The phenotypic distance, based on carbon source utilisation and antimicrobial resistance clearly showed not only diversity but also some correlations with pathogenicity as determined by virulence genes' PCR tests albeit less clearly. That said, there were interesting findings that isolates that use dulcitol, sorbose, sucrose, raffinose and proline were more likely to be associated with APEC than presumptive commensals, and conversely the use of arabitol and adonitol may be correlated with commensals. Indeed, utilisation of adonitol (ribitol) and arabitol is associated with non-pathogenic group C *E. coli* (Woodward and Charles, 1983).

For this study, the focus was on arabitol and adonitol that was hypothesised to be utilised by presumptive commensal strains whilst strains that utilised proline were APEC. *G. mellonella* larva was used as an *in vivo* model to determine the virulence of these two groups, presumptive commensals and APEC. Different studies have reported that the *G. mellonella* model is a powerful tool to consider the virulence of a range of bacterial and fungal pathogens (Desbois and Coote, 2012; Kavanagh and Reeves, 2004).

The *G. mellonella* innate immune systems shows a high degree of similarity to the mammalian immune system, and for that reason the use of *G. mellonella* is an attractive alternative to animal models for investigating pathogenicity (Wand *et al.*, 2013; Kavanagh and Reeves, 2004). Reports of relevance to our study include a report that pathogenicity of EPEC could be dissected using *G. mellonella* and that *E. coli* K12 was non-pathogenic (Leuko and Raivio, 2012) whilst another report indicated that this model was a valuable tool for examining virulence of UPEC strains (Alghoribi *et al.*, 2014). The results seem to show that *G. mellonella* model does not relate or correlate to poultry pathogenesis. Is it possible that factors other than those tested are involved in the pathogenesis of *E. coli* in the *G. mellonella*. As others have

reported, virulence in larvae was not correlated with serotype or phylogenetic group in uropathogenic *E. coli* (Alghoribi *et al.*, 2014). In addition, we used only 8 strains to analyses the differentiate between the isolates, and a bigger population would be more accurate and relative to other virulent determents. The testing was performed in triplicate and on three separate occasions, which suggests no need to repeat *G. mellonella* infection with the same strains to confirm these results. As mentioned above there may be other determinants that are of relevance to this model and therefore whole genome sequencing to compare these 8 strains genetically would possibly help to gain an understanding of their pathogenicity in this particular model. For example, in our case strain A and B were isolated from the same host with a high degree of phenotypic and genotypic similarity, and the *G. mellonella* model classified strain A as highly pathogenic while strain B was much less pathogenic. It would be possible to speculate about what factors came into play in the Galleria model but until comparative sequence analysis is performed it is probably wise to suggest that this model may not be appropriate for avian *E. coli*. If time, cost and HO licence were available then the obvious next step would be bird infection studies to test the hypothesis.

CHAPTER 3: THE IMPACT OF PHYTOCHEMICALS (THYMOL AND CARVACROL) AS ANTIMICROBIALS TO CONTROL *E. COLI*

3.1 Introduction

Some 10% to 15% of *E. coli* strains are opportunistic pathogens and/or belong to known pathogenic groups capable of causing food-borne disease (team, 2015). APEC cause significant economic losses in the poultry industry and are difficult to eradicate (Oosterik *et al.*, 2014b) possibly due to their ability to form biofilms and their associated resistance to disinfectants that enable survival and spread within production systems. The increased resistance of microorganisms to biocides (especially antibiotics) is a serious and evident worldwide problem that has encouraged research into the identification of new biocides with wide-ranging activity (Nazzaro *et al.*, 2013). Furthermore, cross-resistance between disinfectants and antibiotics can also lead to serious consequences for the public health (Russell, 2003). The excessive use of antibiotics in animal production as in human health care is resulting in increased resistance and emergence of "superbugs" resistant to nearly all available treatments (Murray, 1992; Franco *et al.*, 2009). Of considerable concern is the rapid transfer of the resistance genes by plasmids, transposons, gene cassettes and mobile genetic elements (Lester *et al.*, 1990; Winokur *et al.*, 2001; Folster *et al.*, 2017). In this context, significant efforts have been invested into research to find effective antimicrobial compounds for in-feed and surface decontamination which preserve the organoleptic properties of the food products (Dufour *et al.*, 2012; Negi, 2012). Novel products that act on new bacterial targets such as fatty acid biosynthesis or avoid the conventional mechanisms of resistance to current antimicrobials are also important (Sarker *et al.*, 2007; Tiwari *et al.*, 2009; Daglia, 2012). While synthetic antimicrobials are approved in many countries, the recent trend has been the use of safe natural preservatives and feed additives derived from micro-organisms, plants or animals (Rahman and Kang, 2009; Sharifi-Rad *et al.*, 2016). Many food preservation systems are used

to reduce the risk of outbreaks of bacterial food spoilage and food poisoning by using of chemical preservatives (Periago and Moezelaar, 2001; Techathuvanan *et al.*, 2014).

Plants produce an enormous array of secondary metabolites (phytochemicals) with medicinal properties that have been used as natural therapies traditionally for centuries (Hammer *et al.*, 1999; Abreu *et al.*, 2012). An important part of this diversity of phytochemicals is related to defence mechanisms of plants against attack by pathogenic microorganisms (Dangl and Jones, 2001). Essential oils (EOs) have a wide range of activities used for several purposes due to their variable content of antimicrobials. The antimicrobial activity of EOs is assigned to a number of a small terpenoid and phenolic compounds (Escudero *et al.*, 1985).

Thymol and carvacrol are phenolic compounds of EOs. The antimicrobial activity has been demonstrated against *E. coli* (Xu *et al.*, 2008; Reda *et al.*, 2015). These two components have a very similar chemical structure involving a system of delocalised electrons and a hydroxyl group, which makes it likely that they have a similar mechanism of antimicrobial activity (Ultee *et al.*, 2002). Nevertheless, the relative position of hydroxyl group on the phenolic ring not appear to influence the degree of antibacterial activity (Xu *et al.*, 2008; Lambert *et al.*, 2001). Hydrophilic ability is increased by hydroxyl group, which help them dissolve in to microbial membrane and damage them (Sikkema *et al.*, 1995).They possess a wide spectrum of antimicrobial activity, that has been the subject of several investigations *in vitro* (Dorman and Deans, 2000; Lambert *et al.*, 2001) and *in vivo* (Adam *et al.*, 1998; Manohar *et al.*, 2001). The mechanism of antimicrobial activity is related to their ability to disrupt the outer membrane, including permeabilisation and depolarisation of the cytoplasmic membrane, by reducing the pH gradient across the cytoplasmic membrane. The effect on the proton motive force leads to the depletion of the intracellular ATP subsequently leading to cell death (Ultee *et al.*, 2002; Xu *et al.*, 2008). Additionally interaction with the cell membrane causes leakage of cellular components, impairment of the energy metabolism and changes in fatty acids and

phospholipid composition (Ceylan and Fung, 2004). Once intracellular, the presence of the hydroxyl groups is related to inactivation of microbial enzymes (Di Pasqua *et al.*, 2007).

Motility and biofilm formation by bacterial pathogens are regarded as bacterial characteristics that have important roles in pathogenicity (Houry *et al.*, 2010; Chellappa *et al.*, 2013). *E. coli* is motile, and has the ability to form biofilms. Swimming and swarming motilities influence the biofilm development of *E. coli* (Verstraeten *et al.*, 2008). The formation of bacterial biofilms is initiated by cells transitioning from the free-swimming mode to growth on a surface (Petrova and Sauer, 2012). Pathogenicity involves the adhesion of bacterial to host cells, which is the first stage of *E. coli* infection. *E. coli* is also able to form biofilms on various biotic and abiotic surfaces. These biofilms show increased resistance to conventional antimicrobial agents and so in industrial environments such as farms, slaughter houses, food processing plants there is a high probability of *E. coli* biofilm formation which poses a substantial challenge, and methods of controlling these biofilms are urgently required. As mentioned above EOs can inhibit biofilm formation, one focuses of the studies presented here. The focus is upon those *E. coli* related to poultry production especially APEC and especially those that are multiply antibiotic resistant.

Another testable hypothesis in this study is whether by using EOs such as thymol that conjugation between the *E. coli* strains may be controlled. Gene transfer through conjugation is considered to be a major mechanism for the creation of a new genetic recombinants in diverse environments (Hoffmann *et al.*, 1998; Ravatn *et al.*, 1998). Indeed, conjugation is classified as an essential contributor to the distribution of antimicrobial resistance and virulence factors in bacterial populations (de la Cruz and Davies, 2000). With the recognition of antibiotic resistance as an emerging issue in the 1960s many new plasmids have been discovered and a greater understanding of the mechanisms of transfer have been defined (Burrus *et al.*, 2002;

Tatum and Lederberg, 1947; Bates *et al.*, 1998). Resistance to antimicrobials in bacteria is considered a global problem and as highlighted in the earlier results chapter resistance in poultry related *E. coli* isolates is very prevalent. Antimicrobials have been used widely to control bacterial disease in humans and animals. Their extensive use has increased the resistance of bacteria and is hypothesised to be the result of not only the selection and spread of resistant microorganisms, but also transfer of resistance plasmids. Plasmid transfer among bacteria provides a means for dissemination of resistance and asking the question whether EOs may inhibit such transfer is a highly valid question especially as the site of action of EOs is at the cell membrane where the pili are anchored that are required for primary stages of joining donor and recipient. Understanding the mechanism of transmission and resistance acquisition can contribute to the development of new strategies to control this phenomenon. Therefore, the inhibition of plasmid distribution through possibly inhibiting the conjugation process may be a convenient strategy to control transfer of antimicrobial resistant in bacterial pathogens. With this objective in mind, we proposed to use phytochemicals (phenolic compound, thymol) to test their ability to inhibit conjugal plasmid transfer between *E. coli* strains.

The present study set about investigating the inhibitory effects of using the phytochemicals thymol and carvacrol, which were believed to be the principal inhibitory components of EOs on *E. coli* growth, through evaluation of their impact of them on *E. coli* isolates by determining growth rate and MIC, biofilm formation, motility and conjugation. In addition, scanning electron microscope observations were performed to investigate the occurrence of surface damage on the treated *E. coli*.

3.2 Material and methods

3.2.1 Microbiological experiments

The microbiological media and chemicals used during the many different experiments described in this chapter were procured from Sigma-Aldrich UK.

3.2.1.1 Microbiology media.

Luria-Bertani (LB) broth and agar microbiological media were sterilised by autoclaving at 121°C for 20 min. LB medium containing agar was cooled to 50°C before pouring 20 ml in sterile disposable Petri dishes. Prepared plates were stored in sterile bags upside down, refrigerated at 4^{o}C until use. Tubes containing autoclaved LB and low salt LB broth were also cooled and stored refrigerated at 4^{o}C until use (**Appendix 1**).

3.2.1.2 Phytochemical stocks.

Thymol and Carvacrol, were dissolved separately in 50% (v/v) ethanol to give working stock solutions of 5 mg l^{-1}. The stocks were stored at 4˚C and used within 48 hours of making.

3.2.1.3 Antibiotic stocks.

3.2.1.3.a Ampicillin Stock

Ampicillin (Amp) 100 mg l^{-1} stock solution was made by weighing 1 g and dissolving it in 10 ml of sterile H_2O. Once dissolved the solution was filter sterilised through a 0.22μm filter with a syringe. Sterilised Ampicillin stocks were stored at -20°C in glass vials wrapped in silver foil.

3.2.1.3.b Nalidixic acid stock

Nalidixic acid (Nal) 10 mg l^{-1} stock solution was made by weighing 0.1 g and dissolving it in 10 ml of sterile H_2O to which 360 µl of 1M NaOH was added to achieve pH 11. Once dissolved the solution was sterilised through a 0.22µm filter and syringe. Sterilised Nalidixic acid stock was stored at -20°C in glass vials wrapped in silver foil.

3.2.1.4 E. coli strains

Susceptibility to the thymol and carvacrol was determined against the *E. coli* strains isolated from a selection of 50 poultry strains (15 chicken, 15 turkey and 20 APEC) with *E. coli* K12 strains JM109 and DH5α used for controls (**Appendix 4**). Those 50 strains were chosen based on the diversity of genotypic and phenotypic of isolated strains, and most of them possessed adhesion genes (*fimH, papC, csg, crl* and *tsh*). To determent if there has difference of thymol and carvacrol in response of presumptive commensal and APEC strains if they may be responsible for foodborne diseases and/or for spoilage of contaminated products. Stored *E. coli* strains at -80°C were normally grown from frozen on LB agar plates at 37°C for 16-18 hours. Liquid cultures were incubated aerobically shaking at 150- 200 rpm at 37 °C for 16-18 hours.

3.2.2 Effect of thymol and carvacrol on the growth of E. coli isolates

To investigate the effect of thymol and carvacrol on the growth of *E. coli* and determine the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs), studies were performed in triplicate in 96 well microplates. The working stock solution of thymol and carvacrol at a starting concentration of 5 mg l^{-1}, was serially diluted in twofold dilution made in fresh sterile LB broth covering the concentration from 3.125 – 1600 µg l^{-1} for both thymol and carvacrol. Of these dilutions, 200 µl was added to wells in triplicate of the Greiner CELLSTAR® 96-well plates (sterile, F-bottom with lid), according to the CLSI

M31-A3 guidance. Single colonies of strains of *E. coli* were streaked onto and grown on LB agar overnight at 37^{o}C. Colonies were suspended in LB broth, grown overnight at 37^{o}C, adjusted to an OD 600 = 0.02 (about $1x10^{8}$ CFU l^{-1}) of which 25 µl of this inoculum was added to each well of the microdilution plates, 3 replicas per strain for each dilution of phytochemical. To control the experiment, last column of wells was left an inoculated as a negative control. The 96-well plate was covered with a lid and placed in the FLUOstar Omega system (atmospheric control unit for microplate readers-BMG LABTECH, Germany) at 37˚C with orbital shaking (200 rpm) and run for 24 h with measurement spectrophotometrically (600 nm) recording every 1 h for optical density of growth bacterial culture. Immediately after the MICs were determined, the MBCs were assayed by transferring 5 µl from each culture with a compound concentration equal to or greater than the established MIC to LB agar plates. The MIC was recorded as the lowest concentration of phytochemicals at which no growth was detected in OD 600nm compared to positive control (culture only) which resulted in a significant decrease in inoculum viability >90% (Borges *et al.*, 2013). While the MBC was defined as the lowest concentration of phytochemical at which no growth could be observed after 24 h of incubation at 37°C, and where 99.9% or more of the initial inoculum was killed. Each experiment was performed in triplicate with three repeats for each phytochemical at different concentrations and the average MIC and MBC values were recorded.

3.2.3 Biofilm formation.

The effect of phytochemicals on the ability to establish biofilms was tested as described by (Naves *et al.*, 2008) with some modifications for fifty representatives of the panel of 200 strains described in chapter 2. Nine strains were selected for detailed studies and these represented those high biofilms forming isolates. Bacterial cultures were prepared exactly as described in section 3.3.2, but with LB broth 'low salt' rather than LB broth. To determine biofilm formation with phytochemicals (Zuroff *et al.*, 2010; Patel *et al.*, 2011), two

experimental approaches were used. First 'effect on biofilm formation', the wells of Greiner CELLSTAR® 96-well plates (sterile, F-bottom with lid) were filled with 200 µl of twofold dilutions of thymol and carvacrol separately made in LB Low salt broth to give a concentration range from 0.0 µg l^{-1} to 200 µg l^{-1}, a range up to the MIC but not beyond that of the strains selected for testing. Each well was inoculated with 25µl of overnight culture adjusted to an OD600 = 0.02 ($1x10^8$ cells/ml) and the plates were incubated for 24 h at 37˚C and then left for a further 4 days at room temperature 25˚C. The second approach 'effect on established biofilms' was similar to the above but with the addition of the phytochemicals after the initial 24 h incubation as follows. For the nine selected strains, 25 µl of an overnight culture was added to 200 µl of LB broth low salt in each well of a 96 well of Greiner CELLSTAR® 96-well plates (sterile, F-bottom with lid). Plates were incubated without shaking for 24 h at 37°C to enable bacteria to line the wells. The planktonic-phase cells were gently removed by pipette and the wells were washed twice with PBS and filled again with 250 µl of twofold dilutions of the thymol and carvacrol to give a concentration range from 0.0 µg l^{-1} to 200 µg l^{-1}. These plates were then incubated at room temperature for a further four days.

After the allotted incubation period, the plates were inverted and tapped onto ethanol soaked blotting paper to remove the well contents. Each well was washed twice with 300 µl of PBS (pH, 7.2), stained with 250 µl of 0.1% crystal violet obtained from Sigma Aldrich for 25-30 minutes and then washed twice with distilled water, Finally, 250 ul ethanol/acetone (90:10) was added to each well to lyse the biofilm cells and release cell bound crystal violet. The optical density was recorded for each well using an end point plate reader FLUOstar Omega system (atmospheric control unit for microplate readers-BMG LABTECH, Germany).

Biofilm measurements were calculated using the formula $SBF = (AB\text{-}CW)/G$, in which *SBF* is the specific biofilm formation, *AB* is the OD570 nm of the attached and stained bacteria,

CW is the OD 570nm of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values), and *G* is the OD 600 nm of cell growth in broth (Niu and Gilbert, 2004; Naves *et al.*, 2008). Biofilm determined using the SBF formulas (**Table 3.1**) semi-quantitatively the biofilm production classify in three categories the formula used was strong (S), moderate (M), weak (W) and negative (N).

Table 3. 1 Semi-quantitative classification of biofilm production using three different formulas. BF, biofilm formation; AB, stained attached bacteria; CW, stained control wells; SBF, specific biofilm formation; G, growth in suspended culture. All values are OD570nm, except G = OD600nm (Naves *et al.*, 2008)

Formula	**Strong (S)**	**Moderate (M)**	**Weak (W)**	**Negative (N)**
BF = AB - CW	≥ 0.300	0.200 – 0.299	0.100 – 0.199	< 0.100
BF = AB⁄CW	≥ 6.00	4.00 – 5.99	2.00 – 3.99	< 2.00
SBF = (AB - CW)⁄G	≥ 1.00	0.510 – 1.00	0.50 – 0.36	< 0.35

3.2.4 Motility

Motility was evaluated using soft-agar plates for which the basal medium was 5 ml of molten LB supplemented with 0.3% agar poured into Greiner CELLATAR® multiwell culture plates - 6 wells plates (TC treated with lid). To test the effect of phytochemicals on motility, the basal medium was supplemented separately with thymol and carvacrol (50-100-150µg l^{-1}), concentrations that are sub-MIC values. Plates were allowed to dry at room temperature overnight before use. The centre of each well was seeded with an overnight culture of one *E. coli* strain using a sterile inoculating needle. Swimming plates were incubated at 30°C for 24 h and at 37°C for a further 24 h, and the motility recorded after 6, 24 and 48 h. The diameter of growth zones was measured for both control and treatment plates.

3.2.5 Conjugation assay

APEC isolates resistant to ampicillin were used as donors whilst *E. coli* strain DH5α [F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k-, m_k+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1] was used as recipient a strain.

Stationary phase overnight cultures grown at 37ºC in LB broth were adjusted to OD=0.5 at 600 nm. Wild type APEC donor (100 µl) and DH5α as recipient (300 µl) were mixed with 100 µl of LB Broth in a sterile Eppendorf tube. Similarly, an additional two tubes of the mating mixture were prepared but supplemented with two concentrations of thymol (50 and 100 μgl^{-1}). The mating mixtures once prepared were pulse-centrifuged for 5sec in a bench top microfuge to enable greater cell to cell contact and the mating mixtures was incubated at 37˚C for 2 hours with very gentle shaking. After a very brief vortex to terminate conjugal transfer, the mixture was washed in PBS twice and samples of appropriate dilutions were spread on LB agar containing nalidixic acid (20 μgl^{-1}) and ampicillin (100 μgl^{-1}), also on plates contain nalidixic acid and ampicillin, separately. All plates were incubated overnight at 37˚C. Colonies grown on the plates were counted and the analysis of conjugation efficiency calculated, which is estimated as the number of transconjugants per the total number of donor bacteria. All tests were performed in triplicate with three repeats and controls were the donor alone and the recipient alone treated as for the mating experiment.

3.2.6 Effect of thymol on the morphology of E. coli as observed by SEM

Scanning electron microscope (SEM) observations were carried out as follows. After overnight incubation of the test strains in LB broth at 37˚C, the cell density was adjusted to OD 0.5 at 600 nm. The suspension was divided into three portions and thymol was added to two portions to achieve a concentration of 50 and 100 μgl^{-1}. The other portion was left untreated as a control. The samples were incubated at 37°C in a rotary shaker set at 200 rpm. After 2 hours,

the cells were harvested by centrifugation at 14,000xg for 2 minutes, washed twice and resuspended in phosphate buffer saline (PBS). 200 µl of each suspension was placed on poly-L-lysine-coated glass cover slips for 15 min on both sides. The adherent bacteria were fixed with a solution of 2.5% glutaraldehyde pH 7 for 15 min to fix the cells. After fixation, the samples were washed with water for 15 min, taken samples through by ethanol dehydration starting at 30% followed by 50%, 70%, 80%, 90% and finally 100%. Incubated in each ethanol solution was for 10 minutes but 1 hour in 100% ethanol. Dehydrated samples were placed in the Balzers critical point dryer (CPD 030), and metal coated in Edwards sputter coater (S15OB). All samples were observed with a field emission SEM equipped with a cold stage and a cryo-preparation chamber (Quanta 600F). the experiment was performed in triplicate.

3.2.7 Statistical analysis

In terms of growth inhibition, MIC determination and biofilm formation were analysed using the statistical program GenStat 16th Edition, analysis of variance by two-way and general ANOVA to assess the statistical significance value (confidence level >95%). A paired two-tailed Student's t-test was used to determine significant differences in SBF, motility between the control and samples supplemented with thymol and carvacrol, conjugation efficiency. The results were presented as the means standard deviation. Significance level for the differences was set at $p<0.05$.

3.3 Results

3.3.1 Antimicrobial activity of phytochemicals on E. coli isolates

3.3.1.1 Effect of thymol and carvacrol on growth rate

Thymol and carvacrol were active inhibitors of the growth of *E. coli*. Thymol was found to be the more effective with the lower MIC values than carvacrol (125-175 µg l^{-1} and 175-250

µg l^{-1} respectively). Examples of the inhibition profiles (growth curves) for thymol and carvacrol are shown in **Figures 3.1** and **3.2,** which clearly demonstrate the concentration dependent reduction of growth rate and yields which are significant (p= 0.001). Generally, at sub-MIC concentration range both thymol and carvacrol extended the lag phase of growth and both the growth rate and final cell density were reduced. Complete inhibition of *E. coli* was achieved with a thymol concentration 150 µg l^{-1}, and a carvacrol concentration of 250 µg l^{-1} for the test strain (strain 18) shown in **Figures 3.1** and **3.2.**

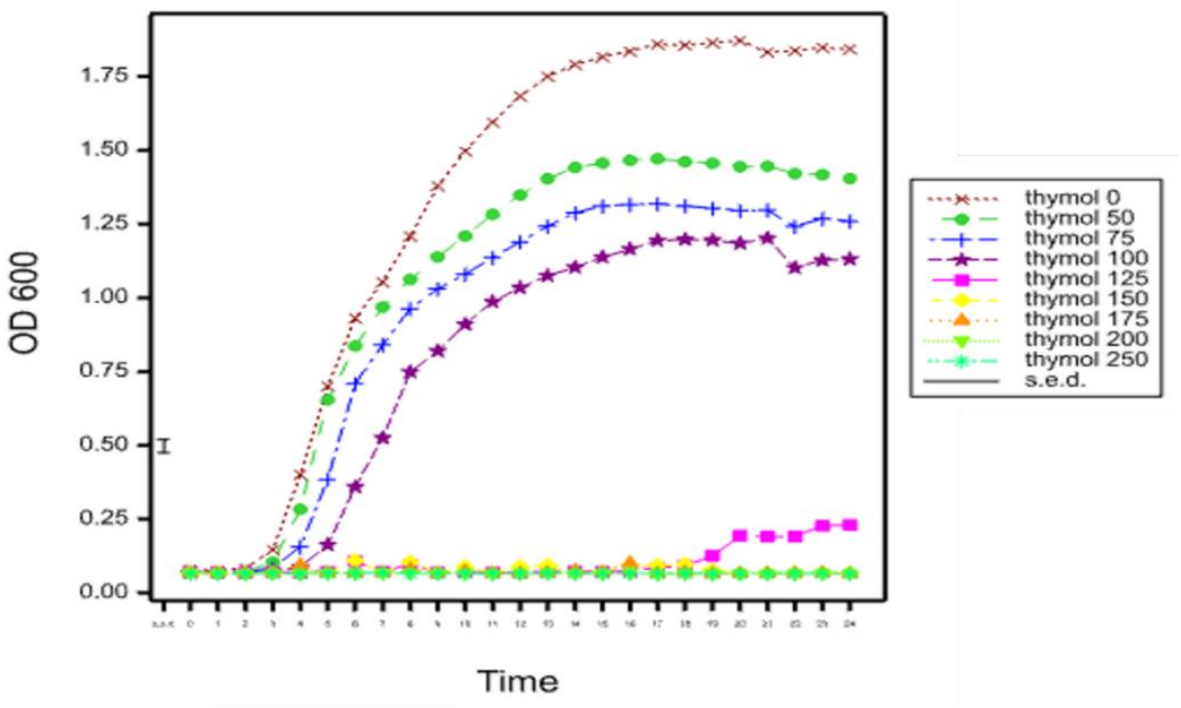

Figure 3. 1 The growth of *E. coli* strain 18 in presence of different concentrations of thymol between 0-250 µg l^{-1}. Average of SD bars showed in the graph at 0.5.

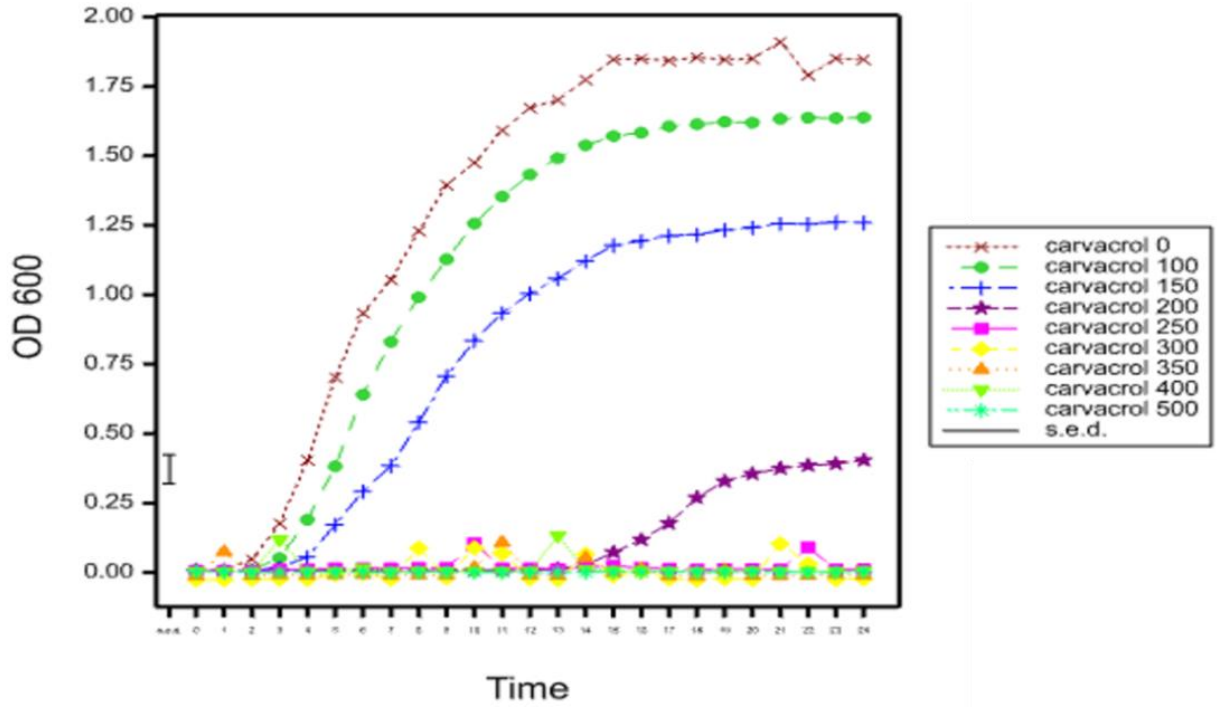

Figure 3. 2 The growth of *E. coli* strain 18 in the presence of different concentrations of carvacrol between 0-500 µg l^{-1}. Average of SD bars showed in the graph between 0.25-0.5.

3.3.1.2 MIC and MBC

The MIC was calculated as the lowest concentration that inhibited visible growth of *E. coli*. While the MBC was calculated as the lowest concentration at which no CFU were detected on solid medium. For these studies, the phytochemicals were serially twofold diluted to give a concentration range from 3.125 µg l^{-1} to 1600 µg l^{-1} for *E. coli* isolates and a control *E. coli* K12 laboratory strain JM109. **Figure 3.3** shows an example of the different responses between APEC isolates and control strain JM109 (p=0.001 for yield) over this concentration range of thymol. The results showed the MIC for both strains was 200 µg l^{-1}, although, the growth curves were different between the APEC isolates and JM109, the lag phase in control strains JM109 was extended whilst the log phase in APEC isolates was steeper. As was showed, APEC strain (strain 18) has higher growth rate than JM109 strain in all concentrations of thymol.

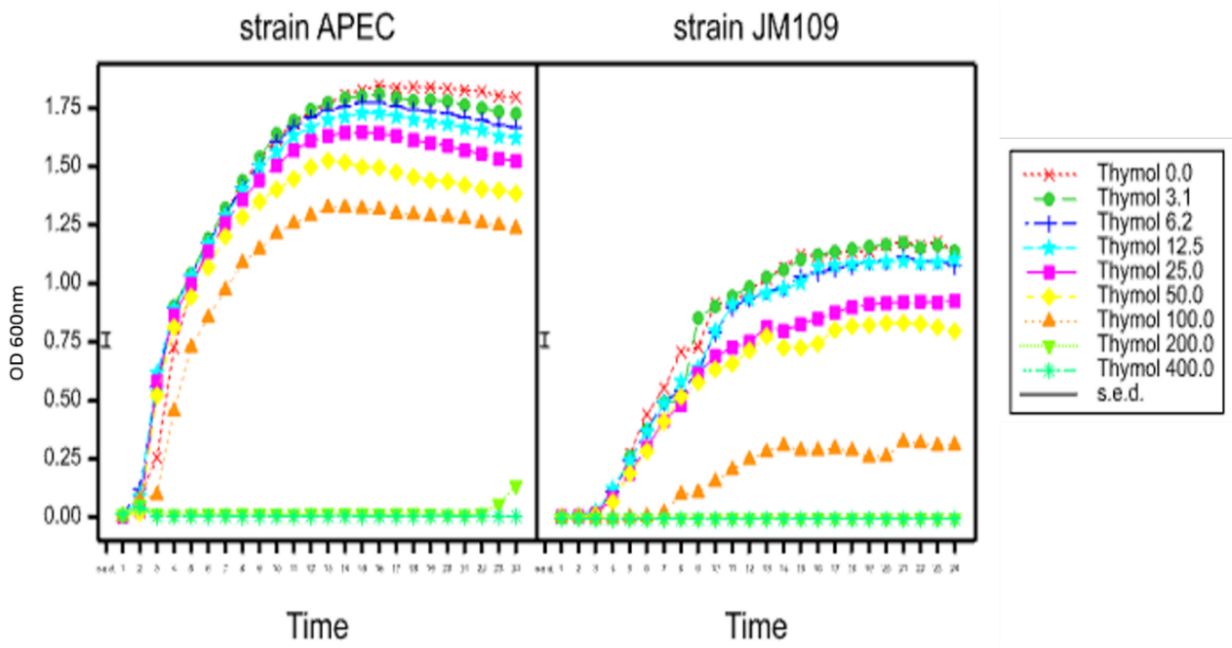

Figure. 3 Effect of different concentrations of thymol against APEC isolates and control strains JM109 between 0-400 µg l^{-1}. Average of SD showed in the graph at 0.75.

To determine accurately the MIC of thymol and carvacrol for all the strains described in chapter 2, the preliminary data described above was considered and the range of phytochemicals used to test inhibition of the growth of *E. coli* isolates was set at 50 µg l^{-1} intervals in the range 50 – 200 µg l^{-1} for thymol and 100 – 500 µg l^{-1} for carvacrol.

The variability in the concentration of thymol and carvacrol were evaluated and the MIC of both thymol and carvacrol were in the range 125-175 µg l^{-1} and 175-200 µg l^{-1}, respectively for all the strains (**Table 3.2**). The MBC values were in the range between 150-200 µg l^{-1} and 200-250 µg l^{-1} for both thymol and carvacrol, slightly higher than the MIC in the isolates *E. coli*, for more details see **Appendix 4**. There were no outliers that were either super-sensitive or super-resistant suggesting uniformity of response to these phytochemicals across the various isolates that were tested.

Table 3. 2 MIC and MBC of thymol and carvacrol for *E. coli* isolates

Isolates	Phytochemicals			
	Thymol		Carvacrol	
	MIC (µg l^{-1})	**MBC** (µg l^{-1})	**MIC** (µg l^{-1})	**MBC** (µg l^{-1})
Turkey	125-175	150-200	175-200	200-225
Chicken	125-175	150-200	175-200	200-225
APEC	125-175	150-200	175-200	200-225
JM109	175	200	175	200
DH5α	150	200	175	200

A concern that arose was that thymol and carvacrol stock solutions were dissolved in ethanol (50% v/v) because of their limited water solubility. Thus, the ethanol might also have a specific effect against *E. coli*. In order to assess this possibility similar growth curve experiments were done that used the 50% of ethanol instead of the phytochemicals stock solution in the same concentration. For example, the ethanol concentration for the MIC of thymol 125-175 µg l^{-1} was 2.8-3.9%, and carvacrol 175-200 µg l^{-1} was 3.9- 4.5%. Thus, growth curve studies were performed with ethanol added to the basal medium in a range of 0.0-5% (0.0- 200 µg l^{-1}). The results showed the lag phase of growth was extended albeit marginally with increasing ethanol concentrations (**Figure 3.4**) and the growth rate and final cell density were slightly reduced compared to the control. This data indicates that the concentrations of ethanol in the studies were at levels that did not cause inhibition. There was no significant effect of the ethanol in different amount used in the phytochemicals concentrations (p=0.27) (Oh and Marshall, 1993). **Figure 3.4** showed the effect of different concentrations of ethanol on two representative isolates.

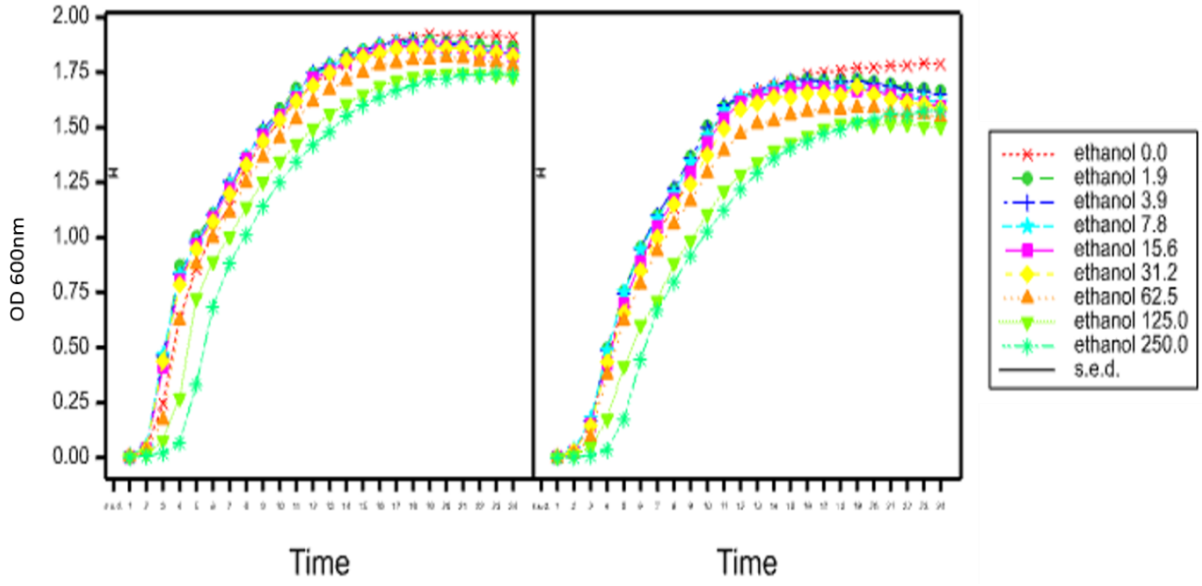

Figure 3. 4 Effect of ethanol in different concentrations on *E. coli*, the MICs of all strains tested were between 125-200µg l^{-1}. Average of SD bars showed in the graph at 1.25

3.3.2 The effect of phytochemicals on biofilm formation

Fifty of the *E. coli* isolates were evaluated for their ability to form biofilms in *vitro* (**Appendix 5**). LB low salt culture was selected as a control for growth as low salt is associated with biofilm formation, and different concentrations of thymol and carvacrol were added either for the entire incubation period or after the initial 24 h incubation (see materials and methods above). After testing the effect of thymol and carvacrol against the *E. coli* growth rate, the same concentrations of thymol and carvacrol were tested for inhibition of biofilm formation.

3.3.2.1 Effect on biofilm formation

First tests were done to assess the ability of the panel of strains to form biofilms and the OD values were determined using the SBF formulas (**Table 3.1**). Within the semi-quantitatively category of biofilm production, 18% of isolates were strong biofilm, 22% were moderate biofilm producer, whereas 60% were weak and negative biofilm producers from 50 isolates tested **Table 3.3**. Results recorded APEC isolates displayed greater ability to form (strong/moderate) biofilms (10/50, 20% isolates) in comparison to either turkey or chicken

(8%, 12%) respectively. However, chicken isolates showed higher relative propensity for strong biofilm formation including 4 strong (8%) and 2 moderate (4%) biofilm producing isolates. Turkey isolates had only 4 (8% of total) isolates able to form any biofilm (strong/moderate/weak). Of those 50 isolates originally tested, 16 that were strong to moderate biofilm formers were selected for tests to establish whether thymol and/or carvacrol inhibit biofilm formation.

Table 3. 3 Specific biofilm formation ability of the *E. coli* isolates.

E. coli isolates source (n)	No. of isolates (% of total) specific biofilm formation (SBF)			
	S	M	W	N
Turkey (15)	3 (20)	1 (6.7)	2 (13.3)	9 (60)
Chicken (15)	4 (26.7)	2 (13.3)	0	9 (60)
APEC (20)	2 (10)	8 (40)	0	10 (50)
Total (n=50)	9 (18)	11 (22)	2 (4)	28 (56)

Using the SBF definition, strong and moderate biofilm forming *E. coli* isolates representing turkey, chicken and APEC types were tested and all showed a concentration dependent reduction of biofilm formation (**Table 3.4).** These differences were statistically significant. The different *E. coli* isolates tested with different concentrations of thymol and carvacrol gave a diversity of OD values depending on the isolates, phytochemicals used and concentrations as anticipated. In general, the higher the concentration of either phytochemical, less biofilm was produced. Paired Student's t-test was performed on the observed optical density (595 nm) values to quantify and differentiate between the extent of biofilm formed by each *E. coli* isolate. Quantification of the intensity of crystal violet staining was used for the detection of the biofilm formed in the presence of the test components. An example of APEC isolate classified as strong SBF that has high OD value, biofilm decreased with increasing the concentrations of both thymol and carvacrol **Figure 3.5**. However, thymol was more active

than carvacrol (p=0.002), and biofilm production was completely inhibited at 150 µg l^{-1} thymol (p=0) which was the MIC of this isolates. While, carvacrol was reduced biofilm at higher concentration than thymol from 200 µg l^{-1} and the MIC of this isolates was 225 µg l^{-1} (p=0.001).

Table 3. 4 SBF mean values of *E. coli* isolates treated with different concentrations of thymol and carvacrol.

group	Isolates	control	Thymol µg l^{-1}				Carvacrol µg l^{-1}				
			100	125	150	175	125	150	175	200	225
Turkey	84	1.99	0.58	0.41	0.02*	-0.04	1.04	0.12	0.22	0.08*	0.08
		S	M**	W**	N**	N**	S**	W**	W	W	W
	3389	1.5	0.52	0.39	0.37	0.04*	0.67	0.04	0.2	-0.29	0.19*
		S	M**	W**	W	N**	M**	N**	N	N	N
	3208	2.29	0.75	0.82	0.95	0.47*	0.56	0.81	0.89	0.89	-0.27*
		S	M**	M**	M**	W**	M**	M**	M	M	N
	88-92	0.8	0.05	0.04	0.149	0.16*	0.067	0.11	0.24	0.09	0*
		M	N**	N**	N**	N**	N**	N**	N**	N**	N**
Chicken	Y195	1.25	0.06	0	-0.15	0.17*	0.28	0.3	0.14	-0.04*	-0.28
		S	N**	N	N	N	N**	N**	N**	N**	N**
	Y173	1.10	0.48	0.13	0.068	0.25*	0.24	0.26	0.07	0.1*	-0.37
		S	W**	N**	N**	N	N**	N**	N	N**	N**
	R3315	1.34	0.76	0.7	0.02	0.02*	0.76	0.37	0.09	0.08*	-0.06
		S	M**	M	N**	N	M**	W**	N	N	N
	Y175	1.97	0.16	0.14	0.13*	-0.41	0.5	0.85	0.7	0.06*	-0.03
		S	N**	N**	N	N**	M**	M**	M	N**	N**
	G286	1.98	0.55	0.49	-0.09*	0.04	1.26	0.7	0.42	0.25*	-0.06
		S	M**	W**	N**	N**	S**	M**	W**	N**	N**
	G228	1.94	0.69	0.78	-0.3*	0.06	0.91	0.74	0.45*	0.22	0.15
		S	M**	M**	N**	N**	M**	M**	W	N**	N**
APEC	12	0.91	0.1	0	0	0	0	0	0	0.05*	0
		M	N**	N**	N	N	N	N	N	N**	N
	19	1.55	1.05	0.8	-0.21*	0.03	1.5	0.7	0.51	0.1*	-0.01
		S	S**	M**	N**	N**	S**	M**	M**	N**	N**
	56	1.31	0.69	-0.3*	-0.046	-0.08	0.17	0.12	-0.08*	0.15	-0.15
		S	M	N **	N**	N**	N	N	N	N	N
	59	0.79	0.38	0.13	0.08*	0.001	0.019	0.02	0.06	-0.26*	0
		M	W	N**	N	N**	N**	N	N	N**	N**
	60	0.79	0.02	0	0*	0	0	0.19*	0	0.08	0
		M	N**	N**	N**	N	N	N	N	N	N
	62	0.79	0.78	-0.27	0.08*	0.02	0.156	0.3	0.17	-0.31*	0
		M	M	N	N**	N	N**	N**	N	N	N**

* MIC of the phytochemicals, **statistically significant difference between SBF result on thymol and carvacrol ($p<0.05$)

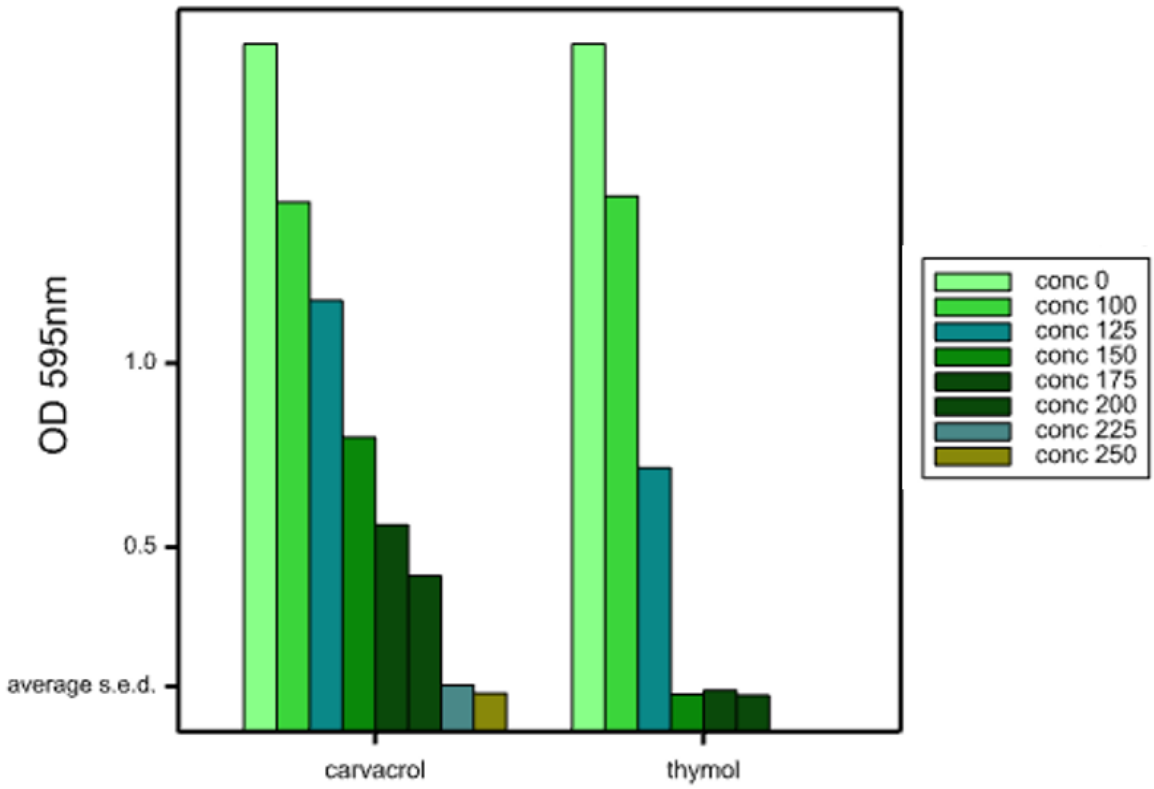

Figure 3. 5 Mean ± OD 595 of stained biofilm attached to *E. coli* control and treated with different concentration of thymol and carvacrol between 0-250 µg l^{-1}. Average of SD bars showed in the graph.

3.3.2.2 The effect on established Biofilm

The efficacy of thymol and carvacrol to reduce established biofilms was tested for nine isolates that were classified as strong biofilm producers **Table 3.5** Statistically significant reductions were noted in biofilms that were treated with thymol and carvacrol at sub-inhibitory to MIC concentrations. The findings indicated that thymol and carvacrol at high concentrations which were near to MICs (100-200 µg l^{-1}) eradicated biofilm with thymol being slightly more effective than carvacrol.

Table 3. 5 Effect of thymol and carvacrol established biofilm formation (mean ± standard deviation).

Isolates		Thymol carvacrol	Control	Phytochemicals concentration µg l^{-1}				
				12.5	25	50	100	200
			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Turkey	88-92	thymol	1.48 ± 0.04	1.58 ± 0.04	1.55 ± 0.08	1.05 ± 0.08	0.53 ± 0.07	0.02 ± 0.02
		carvacrol		1.42 ± 0.02	1.49 ± 0.01	1.31 ± 0.04	1.1 ± 0.005	0.02 ± 0.01
	3208	thymol	1.64 ± 0.01	1.44 ± 0.07	1.2 ± 0.04	0.98 ± 0.07	0.42 ± 0.07	0.00 ± 0.01
		carvacrol		1.44 ± 0.01	1.37 ± 0.04	1.04 ± 0.02	0.99 ± 0.01	0.02 ± 0.01
	3389	thymol	1.64 ± 0.01	1.35 ± 0.07	1.07 ± 0.03	0.99 ± 0.04	0.24 ± 0.09	0.00 ± 0.02
		carvacrol		1.52 ± 0.01	1.49 ± 0.06	1.19 ± 0.08	0.21 ± 0.01	0.04 ± 0.02
Chick	G286	thymol	0.99 ± 0.03	0.75 ± 0.01	0.66 ± 0.01	0.47 ± 0.03	0.26 ± 0.05	0.00 ± 0.02
		carvacrol		0.84 ± 0.01	0.73 ± 0.05	0.72 ± 0.05	0.42 ± 0.03	0.17 ± 0.06
	R3315	thymol	0.98 ± 0.07	0.96 ± 0.04	0.9 ± 0.03	0.71 ± 0.04	0.52 ± 0.02	0.25 ± 0.04
		carvacrol		0.96 ± 0.01	0.92 ± 0.01	0.84 ± 0.06	0.65 ± 0.05	0.43 ± 0.03
	Y173	thymol	0.93 ± 0.03	0.81 ± 0.03	0.73 ± 0.03	0.64 ± 0.01	0.63 ± 0.06	0.01 ± 0.01
		carvacrol		0.9 ± 0.02	0.8 ± 0.03	0.76 ± 0.03	0.62 ± 0.03	0.52 ± 0.04
APEC	58	thymol	0.81 ± 0.03	0.68 ± 0.02	0.58 ± 0.03	0.46 ± 0.02	0.26 ± 0.05	0.00 ± 0.02
		carvacrol		0.98 ± 0.01	0.72 ± 0.06	0.58 ± 0.02	0.48 ± 0.03	0.05 ± 0.07
	62	thymol	0.9 ± 0.06	0.87 ± 0.07	0.73 ± 0.07	0.54 ± 0.08	0.2 ± 0.02	0 ± 0.01
		carvacrol		0.88 ± 0.01	0.74 ± 0.08	0.71 ± 0.06	0.52 ± 0.07	0.12 ± 0.01
	A9	thymol	0.97 ± 0.06	0.64 ± 0.01	0.54 ± 0.06	0.44 ± 0.03	0.26 ± 0.02	0 ± 0.04
		carvacrol		0.75 ± 0.01	0.62 ± 0.01	0.55 ± 0.01	0.37 ± 0.03	0.25 ± 0.02

*Mean for thymol and carvacrol are statistically significant difference ($p<0.05$).

3.3.3 Motility

Swimming motility is a type of bacterial movement which is powered by rotating flagella enabling movement as separate cells through liquid environments. **Figure 3.6 A, B** show that thymol and carvacrol both decreased the swimming motility in a concentration dependent manner ($p= 0.036$). **Figure 3.6 C**, shows representative images of APEC isolate (strain 59) swimming under control, 50, 100, and 150 µg $l^{-1}$of thymol and carvacrol, respectively. It was noteworthy that thymol and carvacrol in high concentrations inhibited the swimming motility.

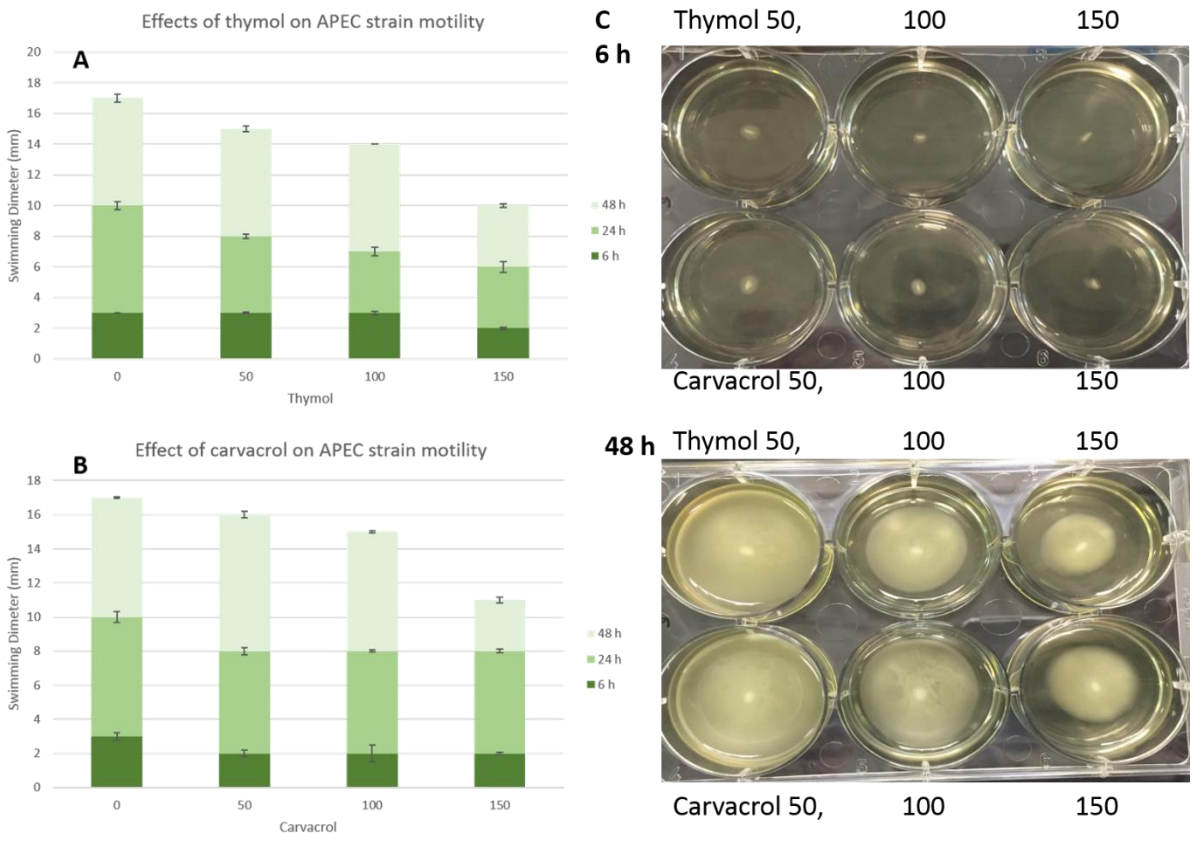

Figure 3. 6 Effect of thymol and carvacrol swimming motility of APEC isolate strain 18. (A) characterisation of the effects of different concentration of thymol on the swimming motility in soft- agar plates. Error bars represent the SD of triplicate samples. significant differences in motility between the control and samples supplemented with thymol 150 µg/ml using paired student's t-test *p= 0.03. (B) with carvacrol, (C) representative images of swimming motility plates for thymol and carvacrol after 6 and 48 hours.

3.3.4 Conjugation

To study the influence of thymol on the ability of bacteria to transfer genetic material by conjugation, mating experiments were performed using as donor strain a wildtype APEC isolate strain 60, and an *E. coli* K12 laboratory strain as recipient, strain DH5α. The donor strain was resistant to ampicillin and the recipient strain has resistant to nalidixc acid. To calculate the efficiency of conjugation the number of surviving donors and recipients were counted after plating dilutions of the mating mixture of on LB amp plates and LB nal plates respectively and the numbers of transconjugants were counted after plating on LB nal amp plates. The frequency of conjugation was expressed as the number of transconjugants divided

by the number of donors. After exposing the mixed mating culture to thymol, the conjugation efficiency was reduced: The conjugation efficiency of *E. coli* in presence of the thymol at sub MICs concentrations 50 µg l^{-1} ($1.78X10^{-5}$ cfu; P value 0.007) and 100 µg l^{-1} ($0.061X10^{-5}$ cfu; p= 0.0008) decrease compared to in the absence of thymol ($3.6X10^{-5}$ cfu) **Figure 3.7.** These results show that exposure to thymol decreased the ability of these *E. coli* to perform plasmid conjugation. A similar number of donors and recipients were recovered after 2 h in the presence and absence of thymol indicating that both donors and recipients were surviving the stress of this level of thymol. Therefore, it can be concluded that the reduced frequency of conjugation is a product of some interference on the mechanics of conjugation by thymol.

This observation indicated that the decrease in *E. coli* conjugation was not due to *E. coli* killing by thymol, but the effect of thymol on *E. coli* conjugation. Conjugation efficiencies were significantly lower in two concentrations of thymol 50 µg l^{-1} (p= 0.004) and 100 µg l^{-1} (p= 0.02) when compared to without thymol **Figure 3.7**. The results suggest that thymol might decrease the ability of *E. coli* to perform conjugation.

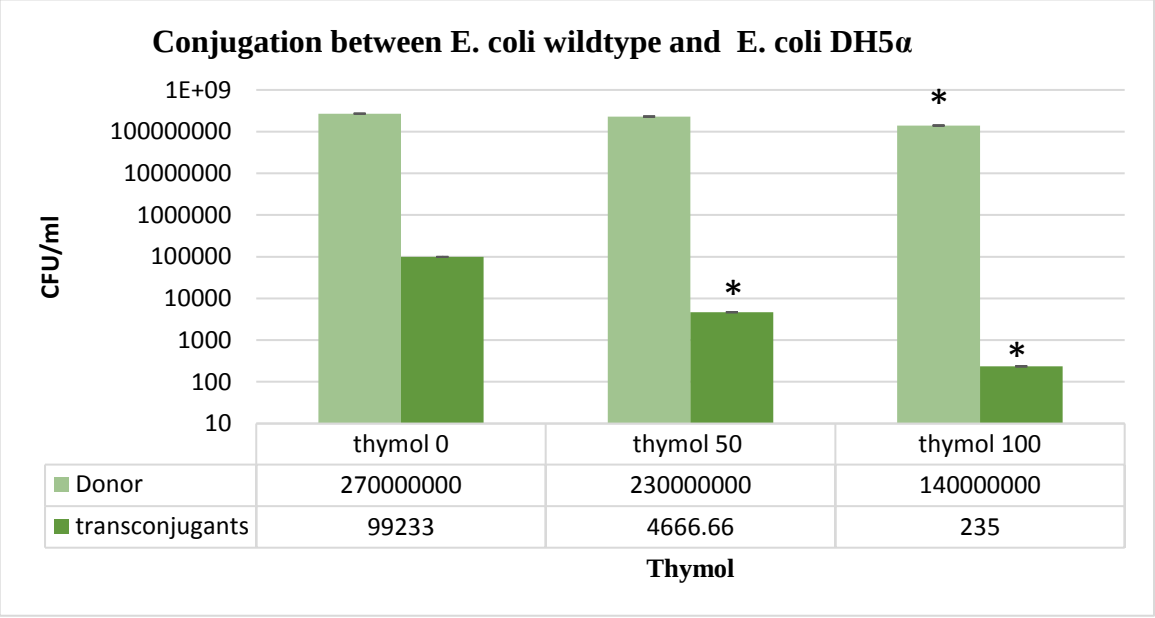

Figure 3. 7 Conjugation in the presence and absence of thymol. * Significant difference.

3.3.5 SEM

SEM observation of the changes to the morphology of *E. coli* cells treated for 2 hours with thymol at a sub-lethal concentration (50-100 µg l^{-1}) are shown in **Figure 3.8**. Compared with the untreated *E. coli* cells (control), SEM evidence indicates that treatment with thymol results in changes in the appearance of the cells, most probably exerted via an effect on the outer cell envelope. Untreated *E. coli* cells are shown in **Figure 3.8 A**, displays a smooth and intact surface. The surface of the cells after treatment with low concentration of thymol looked corrugated with membrane wrinkling and collapse of the cell surface as shown in **Figure 3.8 B**. High concentration of thymol shown in **Figure 3.8 C** shows disintegration of the cell wall and an apparent increase in their compactness. These observations seem to suggest that thymol is able to disrupt the membrane, potentially allowing the leakage of intracellular constituents as well as causing structural alteration of the outer envelope. Moreover, the strain treated with thymol showed more elongated cells than the control cells.

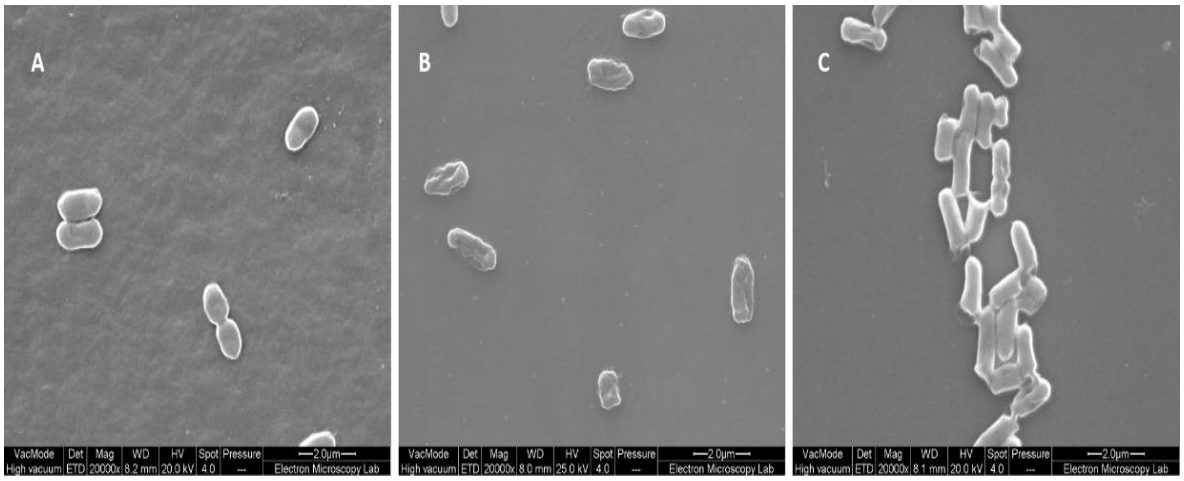

Figure 3. 8 SEM images of *E. coli* cells. (A) Untreated cells, cells after treated with thymol (B) 50 µg l^{-1} and (C) 100 µg l^{-1}.

3.4 Discussion

E. coli infections resulting from consumption of contaminated food has been widely reported and constitutes an enormous public health problem. Also, cells within the biofilm community can become more resistant to biocides than their planktonic counterparts (Das *et al.*, 1998). To reduce health hazard due to *E. coli*, natural products from plants which are mixtures of numerous organic chemicals, have gained importance as antibacterial compounds (Burt, 2004; Niu and Gilbert, 2004). The observations of the antimicrobial activities of thymol and carvacrol against 50 isolates tested from poultry were active against all *E. coli* isolates which is consistent with several earlier studies. MIC and MBC values show that thymol was more activity than carvacrol against *E. coli*, a conclusion that coincides with results of others (Olasupo *et al.*, 2003; Helander *et al.*, 1998). Other reports, however, suggest that the carvacrol was more effective than thymol (Du *et al.*, 2015; Friedman *et al.*, 2002) whilst in other report thymol and carvacrol were equally effective (Xu *et al.*, 2008; Burt *et al.*, 2005; Cosentino *et al.*, 1999). The differences between the concentration and discrepancy of antimicrobial activity may be derived from the methods used to examine antibacterial activity such as dilution or diffusion methods (Xu *et al.*, 2008). According to Guarda and others, the antimicrobial activity of thymol and carvacrol was determined against many microorganisms including foodborne pathogens *E. coli* O157:H7, *S. aureus*, and *L. innocua* were in the range of 125–375 μg l^{-1} (Guarda *et al.*, 2011). In another study, results showed that *E. coli* was inhibited by thymol and carvacrol at concentration of ≥ 500 μg l^{-1} (Rivas *et al.*, 2010) and thymol at 640 μg l^{-1} (Ivanovic *et al.*, 2012). While, Xu and others reported that thymol and carvacrol inhibit the growth of *E. coli* at 200 mg l^{-1} and 100 mg l^{-1}, respectively (Xu *et al.*, 2008; Pei *et al.*, 2009) which was more in keeping with the observations of MIC values of thymol and carvacrol made in this study (range of 125-175 μg l^{-1} and 175-200 μg l^{-1} respectively). Moreover, inhibition profiles of growth curves for thymol and carvacrol were obtained for different levels against *E. coli*.

Normally, at the sub-MIC levels, the lag phase of growth and exponential phase were extended, and both the growth rate and final cell density were reduced with increasing concentrations of both thymol and carvacrol. In a previous study (Skandamis *et al.*, 2001) the presence of oregano essential oils caused an increase in the lag phase and a decrease in the growth rate of *E. coli* O157:H7. In addition, Eucalyptol which is a compound obtained from EOs extended the lag phase of *E. coli* O157:H7 (Zengin and Baysal, 2014). It is known that the lag phase depends on the new environment and the medium containing sub-lethal concentrations of thymol and carvacrol or other EOs which impact on the time required to adjust before growth commences. Nevertheless, the exponential phase was extended with sub-lethal concentrations of thymol and carvacrol. These observations show that *E. coli* can survive and grow, albeit poorly, in sub-lethal concentrations of thymol and carvacrol. This finding was exploited for use sub-lethal for forward examination in this chapter and next two chapters as well.

Thymol and carvacrol stock solutions were prepared in 50% (v/v) ethanol. Ethanol is regularly used as a solvent to prepare stock solutions of EOs before dilution to the required concentrations for microbiological experiments, the solubility of EOs were increased by low concentrations of ethanol and the increased solubility lowered the MIC and MBC of EOs (Canillac and Mourey, 2004; Rivas *et al.*, 2010). High concentrations of ethanol (60%-75%) are considered as bactericidal (Sofos *et al.*, 1998) but other studies suggest growth was inhibited in the presence of as little as 5% ethanol (Oh and Marshall, 1993). In this study, the presence of ethanol in MICs of thymol and carvacrol was 2.8-3.9%, 3.9- 4.5% respectively. Thus, growth curve studies were performed with ethanol added to the basal medium in a range of 0.0-5%. There was no evidence of inhibition. In addition, when a stock preparation of thymol and carvacrol was made by dissolution directly in distilled water or broth rather than ethanol then mixed with broth to achieve the desired test concentration, the antibacterial activity was greatly reduced (data not shown) but improved when dissolved as an ethanol stock preparation.

This finding has been previous confirmed by others (Burt *et al.*, 2005; Periago *et al.*, 2004; Ultee *et al.*, 2000; Walsh *et al.*, 2003).

Results showed variable biofilm formation between isolates groups. Most of the *E. coli* isolates formed weak or no biofilm (56%). Interestingly, the 50 isolates were probed by PCR tests for virulence genes, including adhesion genes (*fimH, papC, csg, crl* and *tsh*). Thus, the correlation between adherence virulence factors and biofilm formation of *E. coli* isolates was not found in the current stage but also reported by others (Reisner *et al.*, 2006; Pavlickova *et al.*, 2017). This observation was somewhat surprising as curli fimbriae that are ubiquitous in *E. coli* are associated with biofilm formation (Allen-Vercoe *et al.*, 1999; Lapidot *et al.*, 2006). The process of biofilm formation by *E. coli* is dependent on different factors such as the growth medium used: minimal or nutrient depleted media gave less observed biofilm less than rich media (Reisner *et al.*, 2006; Skyberg *et al.*, 2007). In this study LB low salt (salt reduction) media was used to determine biofilm formation with phytochemicals as used in other studies (Romling *et al.*, 2014).

Having more-effective antimicrobial agents that are also active against biofilm formation would be a considerable achievement. According to the findings, thymol and carvacrol were effective at reducing biofilm formation by the test isolates. Doses of less than MIC showed a greater influence on biofilm formation of both thymol and carvacrol, so this may suggest that the effects were not just related to reduction in growth rate but also by some direct effect on the biofilm itself. Staining with crystal violet (CV) technique was used as the approach to test the anti-biofilm properties of plant essential oils with success. Its greatest features are that it is inexpensive, relatively quick, and adaptable for use in high-throughput screening with microtiter plates (Djordjevic *et al.*, 2002; Pitts *et al.*, 2003). Whether totally applicable to the aims of this study is open to discussion but a study conducted by Niu and

Gilbert reported that Cinnamomum cassia essential oil reduced the extent of biofilm formation by *E. coli*. (Niu and Gilbert, 2004). This lends support to the validity of the approach used.

The antimicrobial and possibly the anti-biofilm activity of thymol and carvacrol may be attributed to the action of its principal phenolic components, which exhibit significant bactericidal activity (Lambert *et al.*, 2001) interacting with the lipid bilayer of cytoplasmic membranes causing loss of integrity and leakage of cellular material such as ions, ATP and nucleic acid (Helander *et al.*, 1998; Trombetta *et al.*, 2005). Moreover, Tombertta and others have reported thymol possesses a relative hydrophilicity (Trombetta *et al.*, 2005) and this property may enable diffusion through the polysaccharide matrix of the biofilm to destabilise the biofilm and outer membranes. Exposure of *S. aureus* to carvacrol during the early stages of biofilm development resulted in inhibition of matrix formation, with shedding of proteinaceous mass after each antimicrobial pulse (Knowles *et al.*, 2005). It is likely similar processes are at work on *E. coli* also.

Swimming motility has been linked to biofilm formation in several kinds of bacteria (Soutourina and Bertin, 2003), is mediated by flagella (Harshey, 2003), and initiates cell-to-surface contact. In some cases, flagellar motility has been found to be essential for normal biofilm formation (Pratt and Kolter, 1998). However, flagellar motility was not required for initial adhesion and biofilm formation by curli-producing strains of *E. coli* (Prigent-Combaret *et al.*, 2000) or for biofilm formation by *E. coli* strains carrying conjugative plasmids (Reisner *et al.*, 2003). In this work, thymol and carvacrol reduced the swimming motility of *E. coli* at high levels of 150 µg l^{-1} and this could be a reason for biofilm formation reduction as well as slow or reduced *E. coli* growth.

Conjugation is a major process in horizontal gene transfer including multiple antibiotic resistance determinants among pathogenic bacteria and also exchanges of chromosomal genes that has been noted at high frequencies in the gut of the chicken (Lafont *et al.*, 1981; Lafont *et*

al., 1984). EOs compounds may be able to interfere with this process and may potentially prove useful in controlling the spread of antibiotic resistance. To our knowledge this is the first report that EOs or any of their active compounds reduce the transfer of antibiotic resistance among the bacteria. To test this, an *in vitro* model was developed. Sub-lethal thymol (50 and 100 µg l^{-1}) was used to test reduced conjugation without killing *E. coli*. Thymol treatment of donor (APEC isolate) and recipient (*E. coli* K12 laboratory DH5α) (Perez-Mendoza and de la Cruz, 2009) and a mixture of both in broth cultures produced significant reductions in the number of transconjugants observed with 2 h conjugation incubation time as previously described (George and Fagerberg, 1984; Phornphisutthimas *et al.*, 2007). The transconjugants isolated conformed phenotypically similar to recipient cells with acquired resistance to ampicillin suggesting the plasmid had been transferred. Fernandez-Lopez and others reported that unsaturated fatty acids, or other organic acid compounds are conjugation inhibitors (Fernandez-Lopez *et al.*, 2005), which could effect the function of some proteins associated with the bacterial membrane. Another possibility is that the presence of thymol in culture medium may cause a perturbation in the general physiology of *E. coli* such as in osmotic pressure control, membrane potential or energetic balance that impairs conjugation as a pleiotropic effect. Future studies are needed in order test its relevance *in vivo* as well. Moreover, there is a need to determine the influence thymol on the gut population.

Sub-MICs thymol exposure of *E. coli* for 2 h showed shrunken and collapsed surfaces as observed by SEM that provide further evidence of the membrane dependent bacterial disinfection mechanisms. These observations suggest that thymol is able to disrupt the membrane, potentially allowing the leakage of intracellular constituents similar to (Di Pasqua *et al.*, 2007; Lambert *et al.*, 2001). Others treated *E. coli* O157:H7cells with 625µg l^{-1} oregano oil (aqueous solution) which is rich in thymol and carvacrol and found a collapse of cells after loss of their contents (Burt and Reinders, 2003).

This study shows that thymol and carvacrol may contribute to the control and management of *E. coli*. The data suggest these phytochemicals reduce biofilm formation, reduce motility, alter morphology and reduce conjugation between *E. coli* cells. A question arises as to what is their mechanism of action. To investigate this, clues may be gained by training bacteria to tolerance as it may be hypothesised that mutation and/or physiological adaptation arises. If this is the case, then analysis of mutants/adaptants will yield insights into the mechanisms of action of these compounds. This is to be addressed in the next chapter.

CHAPTER FOUR: INVESTIGATIONS INTO THE MECHANISMS OF ACTION OF THYMOL ON *E. COLI*

4.1 Introduction

The antimicrobial activity of many EOs has been widely demonstrated (Burt, 2004; Nazzaro *et al.*, 2013) and have been discussed in detail in the previous chapter (chapter 3). Despite a large body of literature regarding the potential of EOs there are still relatively few applications in real foods due to lack of systematic studies on the antimicrobial activity of the single constituents of EOs and their effects either in model or real systems. In addition, there remains a lack of precise information on the mechanisms of action of bioactive molecules against food-borne microorganisms. More research in this area could substantiate the use of suitable molecule mixtures in relation to food safety requirements (Cosentino *et al.*, 1999). Indeed, a deeper understanding of the microbial targets of EOs and their components as well as of the microbial defence systems may permit a greater use of these antimicrobials in foods and food production. Thymol with carvacrol obtained from labiatae, such as thyme and oregano, are known to possess significant antimicrobial properties and have been the subject of much investigation.

In recent years, an increasing number of studies have been reported using proteomic, genomic and metabolomic approaches to study the cellular processes and their responses to antibiotic stimuli (Hartman *et al.*, 2014; Tiwari and Tiwari, 2014). These approaches demand a departure from the current drug discovery strategies which typically follow a linear process of identification, evaluation and refinement towards a more integrated parallel process (Bleicher *et al.*, 2003). However, these approaches if used could guide us to identify the mode of action of thymol against *E. coli*. Many antibacterial drug-target interactions of antibiotics that are presently used have been deeply studied, and it is well-known that agents can inhibit various essential cellular functions such as cell wall biosynthesis, transcription, translation, or

DNA supercoiling (Kohanski *et al.*, 2007; Walsh, 2003). Some antimicrobials inhibit the growth of bacteria by targeting protein biosynthesis (Bottger, 1994), such as streptomycin, which interacts directly with the small ribosomal subunit (Carter *et al.*, 2000). The ribosome centre (e.g. t-RNA binding sites) is a highly conserved component of the translational apparatus (Alksne *et al.*, 1993), comprising a rRNA domain and several polypeptides of the small subunit, including the ribosomal protein S12. Lysine-42 mutation in S12 was originally isolated as the cause of streptomycin resistance (Alksne *et al.*, 1993; Noller, 1991). Furthermore, a mutation in the *Euglena* chloroplast 16S rRNA resulted in streptomycin resistance (Montandon *et al.*, 1985), and mutations in different regions of *E. coli* 16S rRNA change the ribosomal response to streptomycin (Frattali *et al.*, 1990). Another example is the quinolone group of antibiotics, which are one of the most commonly prescribed class of antibacterials in the world (Aldred *et al.*, 2014). Quinolones trap DNA topoisomerase II (gyrase) (Gellert *et al.*, 1977; Charron and Hancock, 1990), and DNA topoisomerase IV (Kato *et al.*, 1990), into complexes in which the DNA is broken but constrained by proteins resulting in bacterial chromosome fragmentation (Drlica *et al.*, 2009). The main quinolone resistance mechanism consists of one or a combination of target-site gene mutations that alter the drug-binding affinity of target enzymes (Correia *et al.*, 2017). Such as, topoisomerase enzyme changes structure cause reduced the affinity to fluoroquinolones are caused by mutations in the quinolone resistance determining regions (QRDR) of *gyrA* and *parC* genes (Moon *et al.*, 2010; Ruiz, 2003). However, other mechanisms such as mutations that lead to reduced intracellular drug concentrations by decreased uptake or increased efflux, and plasmid-encoded resistance genes producing either target protection proteins, drug-modifying enzymes or multidrug efflux pumps are known to contribute additively to quinolone resistance (Correia *et al.*, 2017).

Metabolomics in particular offer a unique strategy to detect metabolic changes that occur in an organism in response to drugs and the outcome of such studies can provide insights

into their corresponding mode of action (Dorries *et al.*, 2014). Metabolomics allow comprehensive and quantitative analysis of all the metabolites in a biological system (Khoo *et al.*, 2015). By monitoring the global metabolite profile, metabolomics provides a precise snapshot of the physicochemical state of the cell. In addition, different physiochemical approaches such as mass spectrometry (MS) (Han *et al.*, 2014; Su *et al.*, 2015), Fourier-transform infrared (FT-IR) spectroscopy (Wharfe *et al.*, 2010) and nuclear magnetic resonance (NMR) (Dorries *et al.*, 2014; Halouska *et al.*, 2012) have been used in metabolic or whole-organism profiling of the microbial response to antibiotics. In metabolomics, the investigations carried out so far have focused on studying changes in the intracellular metabolism in response to antibacterial compounds describing the cellular chemical fingerprint of bacteria. Changes in the metabolite composition of microorganisms in response to external stimuli can be measured easily using analytical methods coupled with multivariate statistical analysis (Park *et al.*, 2016). Nowadays, NMR analysis can detect a wide range of low molecular weight metabolites in a single test. NMR is the most frequently used method for the determination and quantitation of microbial metabolites. For this reason, the NMR spectrum of a cell extract is normally regarded as the metabolic profile of an organism, and from this premise a science called metabolomics has been developed based on the chemometric analysis of NMR spectra of biological fluids, including cell extracts (Nicholson *et al.*, 1999). This method allows by measuring against a control population the changes induced by a treatment, through a holistic view of the cellular composition (Ye *et al.*, 2012). In addition, this technique is increasingly used in the investigation on the metabolic effects of natural molecules with bacteriostatic and/or bactericidal action on bacterial growth (Drazic *et al.*, 2015; Grivet and Delort, 2009; Hoerr *et al.*, 2016). Currently, the analysis of the mechanisms of action of EOs' has been improved by applying these various techniques: NMR (Picone *et al.*, 2013) and Mass Spectrometry (Coulier *et al.*, 2006) are regularly used to characterise the metabolomic profile in bacteria.

Several studies have shown the EOs especially thymol can efficiently inactivate pathogens (Cosentino *et al.*, 1999; Burt and Reinders, 2003; Nazzaro *et al.*, 2013; Borges *et al.*, 2015; Du *et al.*, 2015). However, there are only a few reports on the mechanism of action of EOs although Burt and Reinders showed morphological changes in *E. coli* O157 caused by thymol (Burt and Reinders, 2003). Specifically, a comprehensive analysis of changes in bacterial metabolites under treatment with thymol has not been performed. Therefore, the primary purpose of this chapter was to investigate the mechanism of action of thymol in *E. coli*. To do this, two approaches were used. First, exposure to gradually increasing thymol concentrations and selection of resistant *E. coli*, should such mutants arise, genome sequence analysis may identify genetic changes that have arisen and identify natural mechanisms of 'resistance'. An interesting issue arising from this approach is whether or not resistance emerges and if so does this render the EO a non-viable option in pathogen control and might EOs be a public health issue if used. The second approach was to use NMR metabolomics approach to understand how bacterial cells respond to the assault of sub-inhibitory concentrations of the EO. Adapted or trained *E. coli* strains and the wild type strain were to be examined. We hypothesised that a combination of extracellular foot-printing metabolomics, morphological changes, and whole genome sequencing approaches of mutant and original strains would give rise to a more complete mechanistic insight. To evaluate this concept, SEM was used to determine the cells morphology. ^{1}H NMR metabolic analysis was used to profile the metabolic changes in *E. coli* extracellular culture medium of wild-type and mutant following treatment with thymol. Finally, whole genome sequencing of mutant and original strains will be performed to find mutation(s) that might explain how thymol affects *E. coli*.

4.2 Materials and methods

4.2.1 E. coli strains

E. coli isolates used for the adaptation experiment were APEC strains 18, 19 and *E .coli* K12 strain JM109.

4.2.2 Phytochemical stock solutions

Thymol was dissolved in ethanol 50% (v/v) to give a working stock solution of 5mgl^{-1}. The stocks were stored at 4˚C and used within 48hours of making. The stock was diluted with same media (LB broth) used for bacterial culture to achieve concentration ranges required.

4.2.3 Adaptation to increasing concentrations of thymol.

The test was performed after determining the MIC for three strains of *E. coli*, JM109 as a control and two isolates of APEC strains 18 and 19. The MICs for those three strains were 175 µg l^{-1} for JM109 and 125 µg l^{-1} for both APEC isolates. Working Stock solution of thymol at concentration of 5mg l^{-1} was serially diluted using LB broth to give a final concentration from 100µg l^{-1} for the first exposure and thereafter increased by an additional 25 µg l^{-1} so that the test strains would be grown in a rising series of thymol concentrations (100, 125, 150, 175 etc). For each cycle of growth, 4.5 ml of each thymol concentration was added to Greiner CELLATAR® multiwell culture plates 6 wells plates (TC treated with lid). Five colonies of *E. coli* were taken from LB plates, inoculated into 10 ml of LB broth that was incubated aerobically shaking at 150-200 rpm at 37°C for 16-18 hours. When growth was observed, 500µl of the suspension adjusted to an OD 600 = 0.02 (about 1x10^{7} CFU ml^{-1}) were added to each well for the first exposure in LB broth with 100µg l^{-1} thymol. The inoculated plate was incubated at 37ºC with shaking for 48h after which a sample was streaked on to an LB agar plate and a 500 µl sample transferred to a fresh Greiner CELLATAR® multiwell culture plate

containing a concentration of thymol 25 μg l^{-1} stronger than in the previous well. This procedure was continued until minimal growth was observed after 48 h of incubation at 37°C. Adapted strains were stored in -80ºC for subsequent experimentation.

4.2.4 Growth rates of adapted strains.

To investigate the effect of high concentration of thymol on trained and original *E. coli* strain growth, strains were inoculated as described above. From the working stock solution of thymol at concentration of 5 mg l^{-1}, serial dilutions were made fresh in LB broth. Thereafter, 200μl of different thymol concentrations were added to wells of the Greiner CELLSTAR® 96-well plates (sterile, F-bottom with lid), according to the CLSI M31-A3 guidance. The inoculum (25 μl) adjusted to an OD 600 = 0.02 (about $1x10^7$ CFU l^{-1}) of this inoculum were added to each well of the microdilution plates, 3 replicas per strain. To control for experimental conditions, the last column of wells was inoculated without thymol as a negative control. The 96-well plate was covered with a lid and placed in the FLUOstar Omega system (atmospheric control unit for microplate readers-BMG LABTECH, Germany) at 37˚C with orbital shaking (200 rpm) and run for 24 hours with spectrophotometric measurement (at 600 nm) every hour to determine bacterial growth. Immediately after 24 hours incubation 5 μl from each well was transferred to LB agar plates to determine the lowest concentration of thymol at which no growth could be observed after 24 hours of incubation at 37°C. The experiment was performed in triplicate with three repeats on separate days.

4.2.5 Determination of bacterial morphology

The thymol adapted, and non-adapted bacterial strains were observed by scanning electron microscopy. After overnight incubation in LB broth at 37˚C, bacterial cells were suspended to OD 600 = 0.5 in LB broth and divided into two sterile Eppendorf tubes to which thymol was added to one tube at a concentration of 100 μg l^{-1}, whilst the other was left untreated

as a control. Samples were incubated in a rotary shaker set at 200 rpm and 37°C. After 2 hours, the cells were harvested by centrifugation at 14,000x g for 2 minutes, washed twice and resuspended in phosphate buffer saline (PBS). Each suspension (200 µl) was placed on poly-L-lysine-coated glass cover slips for 15 min on both sides. Adhered bacteria were fixed with a solution of 2.5% glutaraldehyde pH7 for 15 min. After fixation, samples were washed with water for 15 min, dehydrated by increasing serial dilution of ethanol (30%, 50%, 70%, 80%, 90%) immersions for 10 minutes each and for 1 h in 100%. Samples were dried in a Balzers critical point dryer (CPD 030), and metal coated in an Edwards sputter coater (S15OB). All samples were observed with a field emission SEM equipped with a cold stage and a cryo-preparation chamber (Quanta 600F). The experiment was performed in triplicate.

4.2.6 ^{1}H NMR

4.2.6.1 Cultivation

Prior to analysis, frozen stock suspensions of wild-type *E. coli* JM109 and its adapted mutant were cultured overnight in 5mL of LB medium at 37°C with shaking at 200 rpm. For the NMR metabolomics analysis, 200 µl of the overnight culture was re-inoculated in 10 ml of M9 defined medium (**Table 4.1**) that had been prepared, filter sterilised and stored in the dark at 4°C a day before the experiment. The salt solution for addition to M9 (**Table 4.2**) was prepared by dissolving individual salts in sterile double distilled water, autoclaved for 15 minutes at 121°C. On the day of experiment, 2mL x 1 mM FeSO4 and 10 ml of trace metal mix solution (Sigma Aldrich, UK) (**Table 4.3**) were added to 1L of filtered M9 solution which was then pre-warmed to 37^{o}C prior to inoculation as described above. Subsequently the culture was incubated at 37^{o}C with shaking to an OD 600 of 0.6 and was used for thymol treatment.

Table 4. 1 NMR M9 medium for 1L of solution

Compounds	Weigh/Volume
H2O autoclaved	900 ml
salt stock solution	100 ml 10 x
glucose 20%	3g
NH4Cl	1g
vitamin B1 (Thiamine)	1ml
1 M CaCl2	100µl
1 M MgSO4	4 ml

Table 4. 2 1L of 10X Salt solution

Compounds	Weight/Volume
H2O	1L
Na2HPO4	60g
KH2PO4	30g
NaCl	5g

Table 4. 3 Trace metal mix

Compounds	Weight/Volume
H_3BO_3	2860 mg
$MnCl_2 \cdot 4H_2O$	1810 mg
$ZnSO_4 \cdot 7H_2O$	222 mg
$Na_2MoO_4 \cdot 2H_2O$	390 mg
$CuSO_4 \cdot 5H_2O$	79 mg
$Co(NO_3)_2 \cdot 6H_2O$	49 mg

4.2.6.2 Bacterial culture supernatant (metabolic profile footprinting)

After incubating multiple 10 ml *E. coli* cultures at an OD 600 of 0.6 in M9 medium, cultures were exposed to a sub-lethal concentration of thymol (50 µg l^{-1}). Positive controls were cultures without thymol. Negative controls were un-inoculated M9 media with or without thymol. There were 6 replicates for each of the treatments and incubation was for 24 h at 37°C. Each 10 ml culture or control was centrifuged at 1000xg for 20 minutes at room temperature

and 1 ml of supernatant were collected immediately afterwards and stored at-80°C until ^{1}H NMR measurement.

4.2.6.3 Preparing culture supernatants

Supernatants were defrosted from -80°C and vortexed. A volume of 400 µl was transferred to a clean microfuge tube. Each sample was buffered with 200µl phosphate buffer (**Table 4.4**), vortexed and centrifuged at 14,000xg for 10 minutes, after which 550 µl of supernatant was transferred into 5 mm internal diameter NMR tube on the day of analysis.

Table 4. 4 Phosphate buffer composition for 500 ml of solution

Compounds	Weight/Volume	Utility
1 mM TSP	0.172 g	Standard
Na_2HPO_4	28.86 g	Buffer
NaH_2PO_4	5.25 g	Buffer
NaN_3.	0.193 g	Antimicrobial
D_2O	1L	NMR lock

4.2.6.4 NMR acquisition and processing

^{1}H NMR spectra were acquired on a Bruker (Bruker Avance III HD, UK) 700 MHz, an automatic tuning-matching unit at 298 K, and an automatic sample changer. To facilitate compound identification, 1D spectra were acquired using standard Bruker 1D nuclear over Hauser enhancement spectroscopy (NOESY) pre-saturation pulse sequence on selected samples (Kumar *et al.*, 1980; Nicholson *et al.*, 1995). After acquisition, spectra were manually phased, processed in order to realign spectrum phasing calibration on TSP at δ 0.00 ppm and baseline correction using MestReNova® software. Stacked spectra were imported into MATLAB (R2015b) MathWork® software where spectra were digitised between δ 0.5-10 ppm in order to delete useless information and avoid data bias; the region containing the water peak was deleted between δ 4.8 and 5.1. Peak assignment was done using online open access

databases (chenomx® and HMDB) and 1D Spectra (for spectroscopy correlation) for molecule identification.

4.2.6.5 Statistical analysis

For footprinting analysis, 6 samples were prepared respectively using 6 biological replicates. Multivariate statistical analysis was done using PCA plots, and analysis of the principal components was done to evaluate the metabolic variations existing between groups. This was done to evaluate the metabolic activity of *E. coli* in M9 media over time and evaluate thymol's potential to modify this metabolic trajectory. Orthogonal projection to latent structure (OPLS) regression was performed on a minimum of 6 replicates per group. The effect of thymol on both wild-type and adapted derivative of *E. coli* was evaluated using this statistical method. PLS regressions were run between each group. OPLS R2Y values around 0.8 were indicative of a good model, with Q2 values of around 0.5 indicate good predictive ability. PCA score plots and OPLS correlation plots were also produced to visualise differences in the metabolome between treatment groups. Loading and contribution plots were extracted to reveal the variables that bear class discriminating power. Moreover, to improve model visualisation and interpretation, S-line plots were extracted to detect metabolites that influence variable selection as they display the overall importance of each variable (X) on all responses (Y) cumulatively over all components.

4.2.7 DNA isolation and sequencing

4.2.7.1 Extraction of Genomic DNA

Trained and original *E. coli* strain JM109 cultures grown for 18-24 h in LB were used for DNA extraction. Genomic DNA was extracted using QIAGEN yeast/bact kit according to manufacturer protocol from fresh samples of bacterial cultures. In summary, overnight culture

(1 ml) was pelleted by centrifugation at 14,000xg for 2 min in a bench top microfuge and incubated at 80˚C for 5 min after adding 300 µl of cell lysis solution to the pellet. RNase A solution (1.5 µl) was added and mixed by gently inverting the tube 25 times then incubated for 15-60 min at 37˚C, followed by 1 min on ice. The tube content was vortexed vigorously for 20 seconds at high speed after addition of 100 µl protein precipitation solution, the mixture was centrifuged for 3 min. The supernatant was transferred to a clean 1.5 ml microfuge containing 300 µl of isopropanol (Sigma-Aldrich, UK), and mixed by gentle inversion 50 times. The mixture was centrifuged at 14,000xg, and the supernatant discarded. The DNA pellet was washed with 300 µl of 70% ethanol and inverted several times. The mixture was centrifuged at 14,000xg for 1 min, ethanol carefully decanted and the DNA pellet was allowed to air dry for 5 min. DNA hydration solution (100 µl) was added to the DNA pellet and vortexed for 5s. The DNA suspension was allowed to dissolve at 65˚C for 1 h, followed by further incubation overnight at room temperature with gentle shaking. The DNA concentration was determined with a ND-1000 Nanodrop spectrophotometer (NanoDrop technologies, USA). DNA concentration was recorded in ng/µl, 260:280 nm and 260:230 nm ratio indicative of DNA quality and purity were recorded (optimal ratio 1.8 ± 0.15). DNA stocks were adjusted to 100ng/µl and stored at -20˚C for sequencing.

4.2.7.2 Sequencing and genome analysis

The commercial, transformation competent *E. coli* strain JM109 were sequenced (microbes NG) according to manufacturer's protocols at 2x250-bp paired–end reads platform following Illumina library preparation. Raw sequence data were processed by an automated analysis pipeline, and reads were trimmed using Trimmomatic tool and the quality was assessed using in-house scripts combined with SAM tools, Bed Tools and BWA-mem. Comparison of the JM109 wild-type strain genome with the JM109 mutant genome was performed using Mauve multiple alignment program (Darling *et al.*, 2011) and annotation with

Prokka (Seemann, 2014). Results refer to positions on a reference *E. coli* genome as ”universal” coordinates using the first published K-12 genome the *E. coli* MG1655 strain. MG1655 sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov/nuccore/NC_000913.3) with accession number NC_000913. The *E. coli* MG1655 genome has been completely sequenced and the annotated sequence, biochemical information, and other available information were used to reconstruct the *E. coli* metabolic map (Edwards and Palsson, 2000).

4.3 Results

4.3.1 Adaptation of E. coli to thymol

MICs of thymol for three strains of *E. coli*, JM109 as an *E. coli* K12 control and two representative isolates of APEC (strain 18 and 19) were established prior to exposure to sub-inhibitory concentrations of thymol and were 175 μg l^{-1} for JM109 and 125 μg l^{-1} for both APEC isolates. As described in materials and methods, each of these three strains was exposed to repeated sub-culture in sub-inhibitory concentrations of thymol. The MIC of thymol adapted strains were determined to be 300 μg l^{-1} (strain 18), 260 μg l^{-1} (strain 19) and 400 μg l^{-1} for JM109 after 16 passages through gradually increasing concentrations of thymol. Tolerance to thymol was shown to be stable as demonstrated by repeated MIC tests after repeated subculture (n = 7) in LB broth without thymol (the strains were passed through every 24h for 7days). After testing for stability, each culture was plated onto NA plates and isolated colonies were used for subsequent analysis. A JM109 adapted derivative was used in subsequent experiments to assess tolerance to high concentrations of thymol.

4.3.2 Growth rate for adapted strain JM109.

Results are displayed in **Figure 4.1** which show the different responses between *E. coli* K12 laboratory strain JM109 and its thymol adapted strain (mutant) that can tolerate and

survive in high concentrations of thymol (p=0.001). The original strain grew well in 100µg l^{-1} but with a shorter exponential phase and an extended stationary phase compared to the control (**Figure 4.1 A**)**.** By contrast, the adapted JM109 derivative was able to grow and tolerate 400 µg l^{-1} although the lag phase was much extended compared to the original strain of JM109. Furthermore, and perhaps more significantly, the adapted JM109 derivative when grown in LB without thymol showed a reduced growth rate and yield compared to the original JM109 strain (**Figure 4.1 B**). In addition, the log or exponential phase was also extended in high thymol concentrations to more than 20 h and in most of the thymol concentrations tested it did not reach a stationary phase.

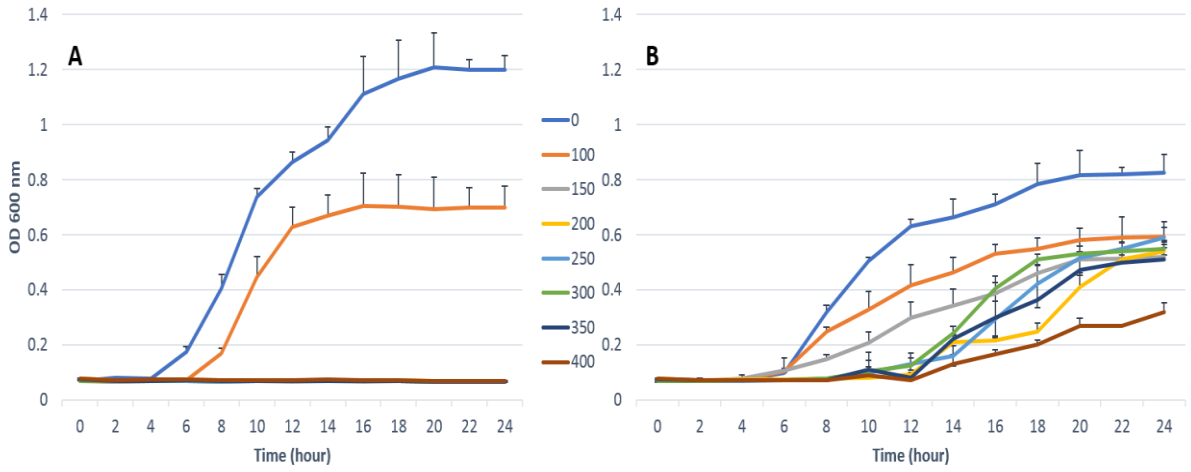

Figure 4. 1 The effects of increasing concentrations of thymol on the growth of the original JM109 *E. coli* (**A**), and its adapted derivative (**B**)

4.3.3 Determination of the morphology of E. coli in the presence of thymol

The morphological changes in *E. coli* cells associated with adaptation and/or exposure to thymol were investigated. SEM analysis revealed that after adaptation to thymol (**Figure 4.2 B**), the cells displayed few morphological changes relative to wild-type (non-adapted) cells (**Figure 4.2 A**) but with a slightly corrugated appearance on the outer surfaces of the cells and appearing thinner and longer. After exposure to sub-lethal concentrations of thymol at 50 µg l^{-}

[1], both adapted and non-adapted cells (**Figure 4.2 C, D**) show considerable morphological alterations in comparison to non-exposed cells (**Figure 4.2 A, B**). The wild-type JM109 had a uniform cylindrical shape and long cells with little evidence of septum formation of 23 whole cells analysed only two showed indications of septum formation. Besides these observations, the overall cell size of wild-type JM109 in the presence of thymol appeared larger than cells without thymol and larger than the adapted derivative of JM109 whether in the presence or absence of thymol. Measurements were made and were as follows [with thymol the average length of the wild-type strain was 1.57 µm whilst the average length of the adapted (mutant) strain was 1.3µm], and these differences were statistically significant (p=0.01). In addition, the adapted (mutant) cells displayed more morphological changes after thymol challenge (**Figure 4.2 D**), the surface appeared to be 'rough' and showed irregularly shaped spots dotted along the cell body.

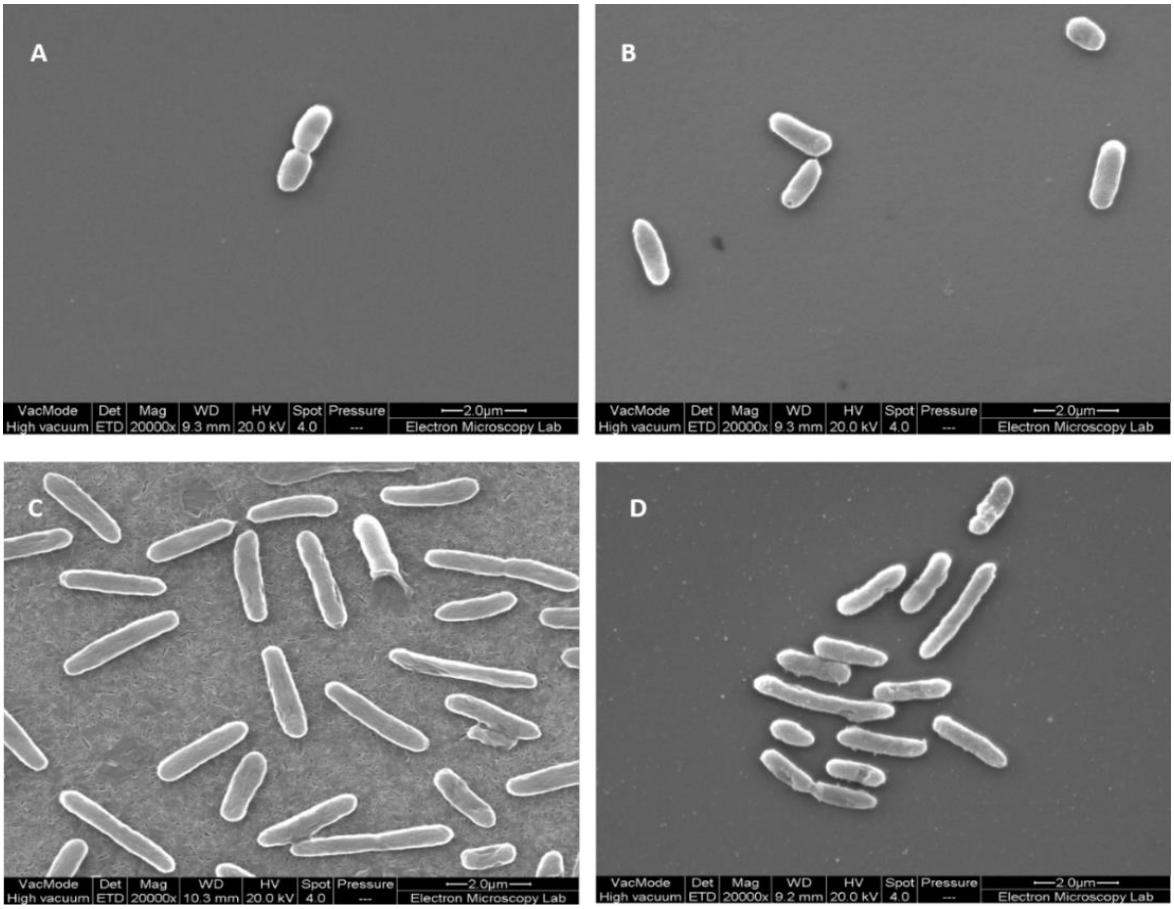

Figure 4. 2 Scanning electron microscope of *E. coli* JM109 cells. (A) non-adapted untreated cells; (B) adapted (mutant) untreated cells; (C) non-adapted treated cells; (D) adapted (mutant) treated cells. For adaptation, cultures were previously exposed to high concentrations of thymol. Treated cells were exposed to thymol sub-lethal concentration that allowed *E. coli* growth after adaptation.

4.3.4 ^{1}HNMR Metabolic footprinting

NMR results consist of a set of 24 ^{1}H-NMR spectra recorded from 6 replicates of each of 4 samples comprising *E. coli* JM109 and the adapted derivative of *E. coli* JM109 wild-type with and without thymol. In addition, 6 replicates of both M9 and M9 with thymol but without *E. coli* were also analysed to allow subtraction of medium only effects. Captured data were analysed using whole metabolome pattern analysis tools which assign the metabolite description and relative concentration (location and height of peaks respectively). To analyse these complex data sets Principle Component Analysis (PCA) was used (**Figure 4.3**) which in this case summarises the original 65536 variables detected. Thus, the direction and distance

covered by the samples can be considered respective indicators of the differences between the metabolic profiles of each strain under the two test conditions, with and without thymol. The metabolic profile of JM109 grown in M9 medium (n = six replicates) were tightly clustered indicating minimal sample to sample variation. However, the metabolic profile of the six replicates of the adapted JM109 grown in M9 medium were more dispersed but significantly discrete from JM109. It is clear that the metabolic profile of the adapted (mutant) strain was different from the wild-type which, given the trajectory; suggests the presence of fewer small metabolites than the wild-type. However, in the presence of thymol both wild-type and its adapted mutant were both very comparable in metabolic profile which may reflect not only more but also very similar small metabolites.

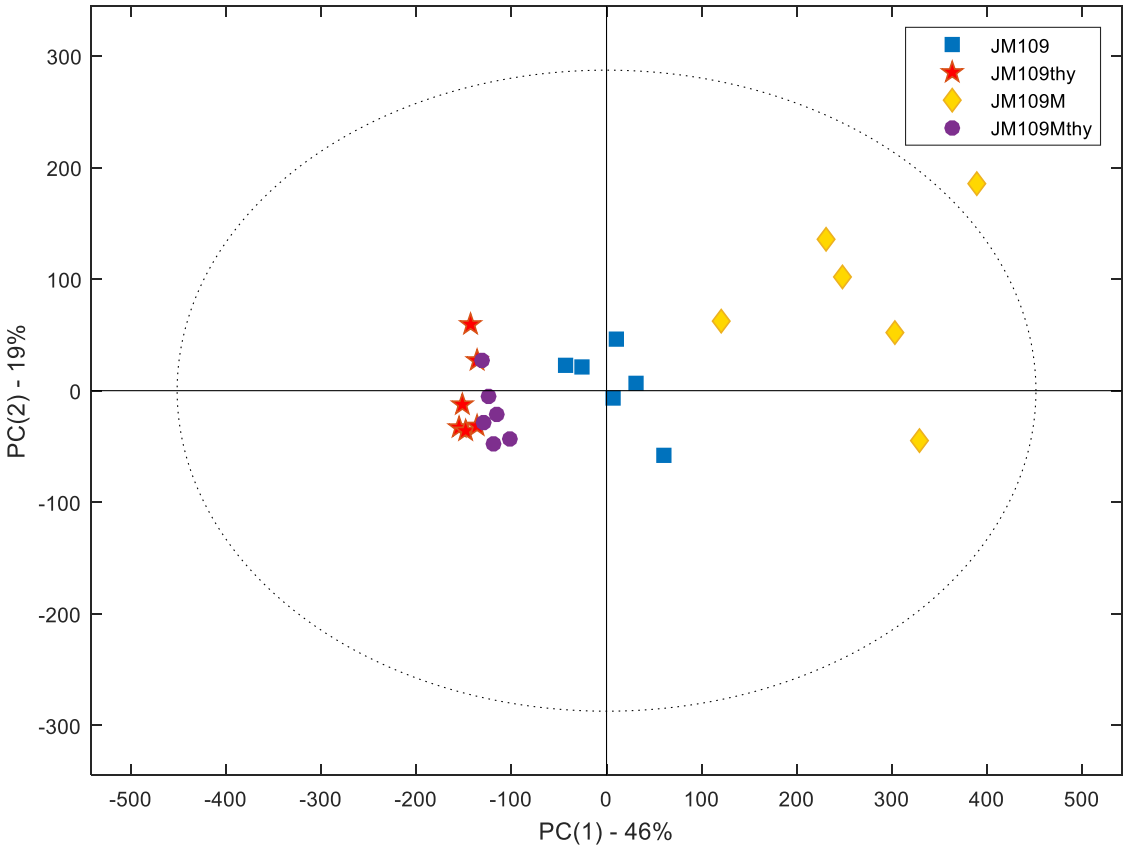

Figure 4. 3 PCA- score plot for effect of different solvent on metabolic footprints derived from *E. coli* MJ109 wild-type and its adapted mutant untreated and treated with a sub-lethal concentration of thymol (50ug l^{-1}). N = 6 for each sample. (JM109; blue squares – JM109 with thymol; orange stars – JM109 adapted mutant; yellow diamonds – JM109 adapted mutant with thymol; purple circles)

PCA score plots indicated differences in metabolic profiles of JM109 and its adapted mutant. The next step in the analysis was to identify the chemical shifts based on correlation coefficient values responsible for the differences. A colour code indicating the weights of the discriminatory variables and the S-line plots are shown in **Figure 4.4 (A)**. Specifically, as the peak colour gradually changes from blue to red, the absolute value of correlation coefficient increases from 0 to 1, indicating the resonances were important for discriminating the metabolite profiles of pairwise analyses, such as wild-type JM109 and its adapted mutant and wild-type JM109 with and without thymol. According to the S-line plot, the spectrum of each extract shows a preponderance of signals related to organic acids, amino acids and sugars in the range between 0 and 4 ppm. The range between 6 and 9 ppm shows the aromatic region such as phenylalanine.

The comparison of wild-type and adapted mutant strain JM109 grown in M9 without thymol (**Figures 4.4 A, B)** shows several peaks that correlate with end products of energy metabolism (ethanol, formate, succinate and acetate) that were significantly higher in the wild type JM109 than its adapted mutant. Succinate is the intermediary synthetic product of Tricarboxylic acid (TCA) cycle, whilst formate and acetate are the end products of the TCA cycle. These findings suggest JM109 wild-type respired aerobically to produce the anticipated end products of energy metabolism. By contrast lactate was significantly higher in the mutant than wild-type. Lactate is one of the main sugar fermentation products of *E. coli*, produced by hydrogenation of pyruvate. Moreover, the aromatic amino acid phenylalanine and other amino acids, such as leucine, valine and alanine that are all the pyruvate family of amino acids were produced more by the adapted mutant than by the wild-type (**Figure 4.4 B**). Therefore, the evidence of increased production of lactate with concomitant increase of the pyruvate family of amino acids in the adapted mutant strain JM109 indicated a possible metabolic shift towards

fermentation. If this is the case, this may well explain why the adapted mutant strain grew slower than the wild-type even without thymol.

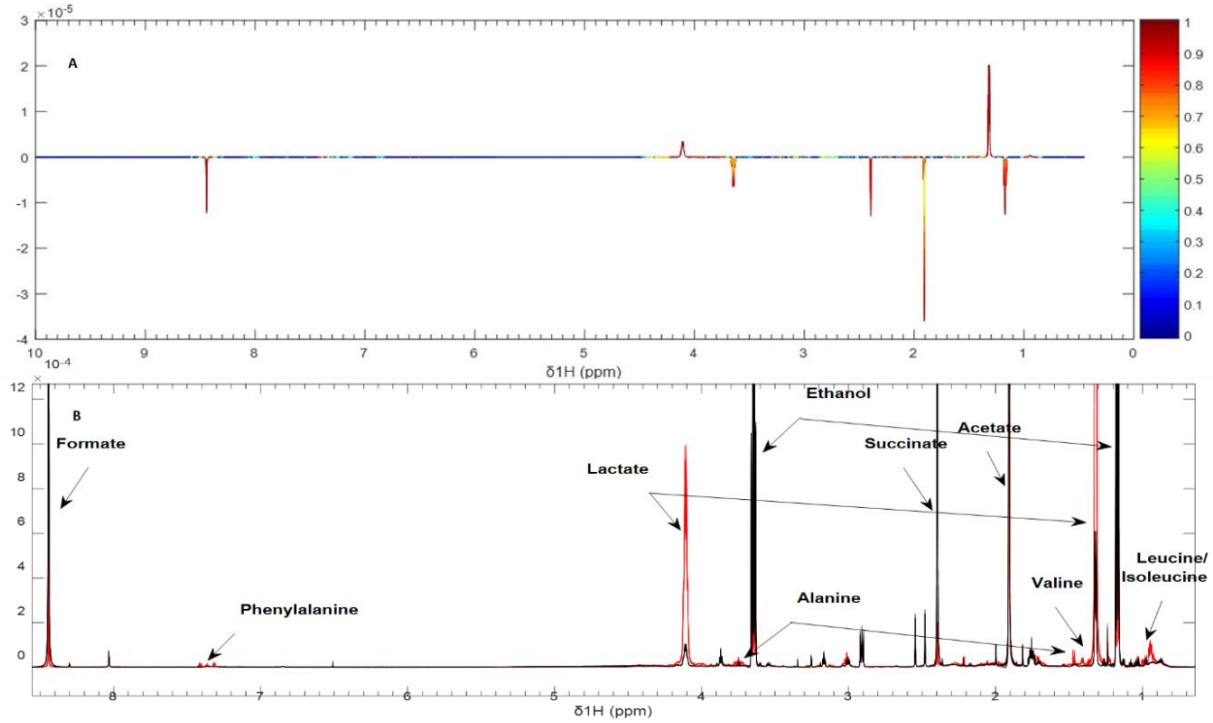

Figure 4. 4 Wild-type and adapted mutant of strain JM109, (A) S-line plot wild-type (bottom) and adapted mutant strain (top). (B) partially assigned 700 MHz 1D spectra of wild-type (black) and adapted mutant strain (red)

Having identified metabolic differences between JM109 and its adapted mutant grown in M9 without thymol, we next examined the metabolic effect of thymol on both strains. A potential confounder of the data was the presence of high levels of ethanol in both experiments as thymol was dissolved in ethanol and this molecule was therefore detected as a common feature in both strains. Thus, the production of ethanol by either strain would be masked by the excess already in the medium. In *E. coli* wild-type (**Figure 4.5 A**), the end products of glucose metabolism featured again (**Figure 4.5 A, B)** but interestingly fumarate and lactate were also observed. In contrast, lactate was observed but at reduced concentrations along with acetate in thymol treated adapted mutant (**Figure 4.6**) suggesting slower growth in thymol possibly due to a shift from aerobic respiration to fermentation.

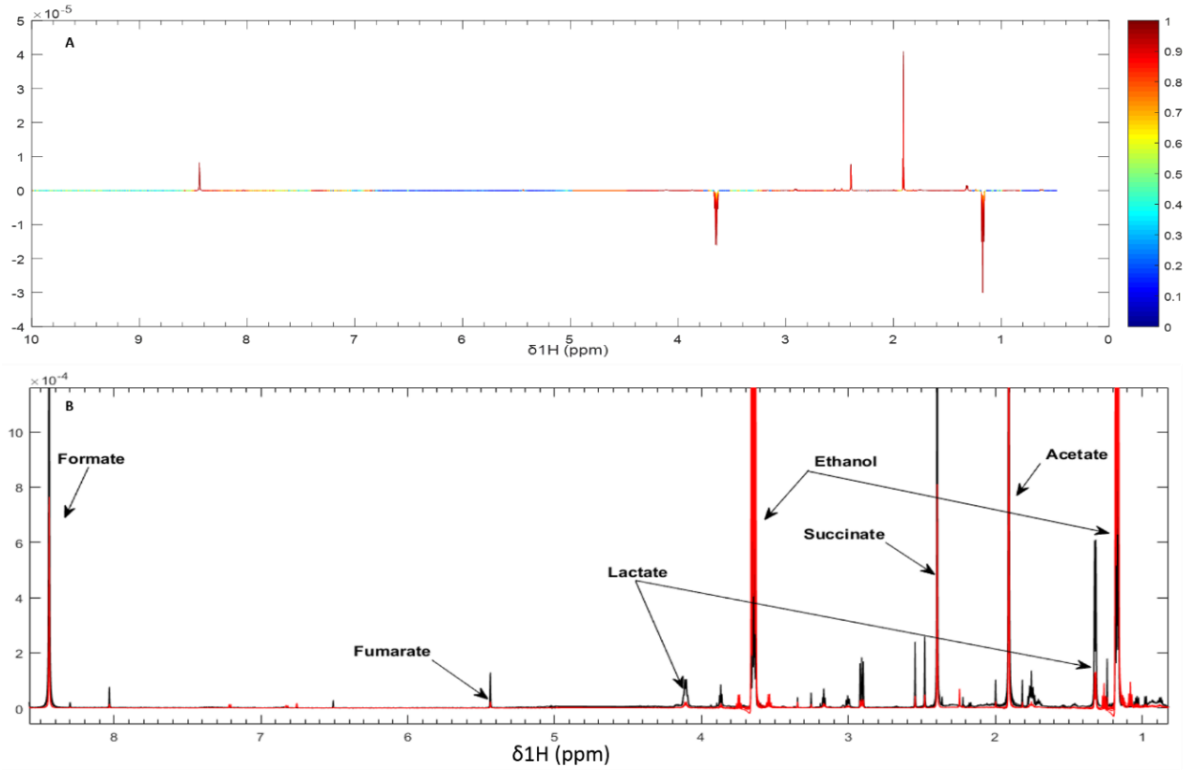

Figure 4. 5 Wild-type JM109 control and treated with thymol. (A) S-line plot wild-type (top) and treated bottom). (B) partially assigned 700 MHz 1D spectra of wild-type (black) and treated (red).

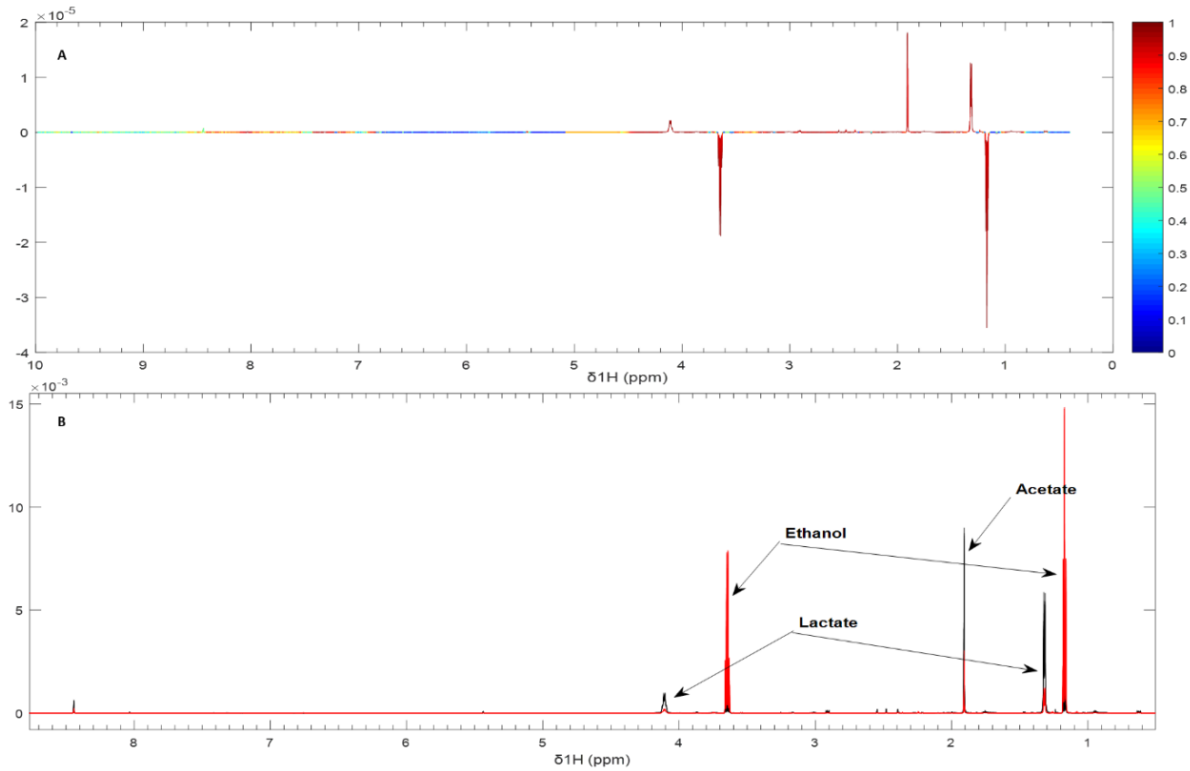

Figure 4. 6 Adapted mutant JM109 control and treated with thymol. (A) S-line plot of adapted mutant JM109 (top) and treated (bottom). (B) partially assigned 700 MHz 1D spectra of adapted mutant JM109 (black) and treated (red).

4.3.5 Sequencing

Having established a non-reverting and therefore stable thymol tolerant derivative of JM109, we sequenced its genome to compare it to its parental JM109 in an attempt to identify mutations that may contribute to thymol tolerance. Results (**Figure 4.7**) show that the parent and derivative tolerant strains both aligned to JM109. There were some major differences that could be ascribed to contig assembly and some regional inversions. The mutant strain harboured a JM109 backbone and was therefore a true derivative. Therefore, any mutations in specific genes are likely to be those that generate the phenotype observed. A mutation was identified in the *acrR* gene that encodes a repressor of AcrAB, which is a multidrug efflux pump. The mutation was a nonsense mutation converting an arginine residue at position 107 to a stop codon in the 215 amino acids long AcrR protein. The location of the mutation in *acrR* was a C to T transition at position 486079 bases (gene size 485761-486408, locus tag="b0464") and abolishes a conserved amino acid residue in the TetR domain C-terminal region. The other possible significant change was an Arginine to Cysteine amino acid change (R to C) at residue 118 in the ribonuclease G protein. The position of this mutation in the *rng* gene is -3397444: *rng* (gene location 3396326-3397795 [reverse orientation], locus tag="b3247"). A visualisation of the mutation on the nucleotide level in an alignment of MG1655, JM109 wild-type and the adapted mutant of JM109 with the effect it has on protein translation is shown in **appendix 6**. Furthermore, an IS5 transposase gene had multiple point mutations not affecting amino acid sequence, and the F-plasmid was missing in the mutant strain.

The data present a tantalising hypothesis that efflux is a mechanism associated with tolerance to thymol. In many ways, this is an entirely plausible interpretation as the AcrAB efflux pump is known to pump out many toxic chemicals from the bacterial cell including antibiotics (Sun *et al.*, 2014; Venter *et al.*, 2015). Therefore, to test this hypothesis, different concentrations of thymol were used in growth experiments with wild-type MG1655 strain and

MG1655 *acrB* (both were provided by colleagues at Birmingham University Prof Laura Piddock and Dr Mark Webber). The hypothesis was that if the AcrAB efflux pump is associated with thymol tolerance by pumping thymol out of the cell, a knock out mutant incapable of synthesising the pump would be 'super-sensitive' to thymol. After exposure to different concentrations of thymol in culture (same protocol used to compare JM109 wild-type and its thymol tolerant derivative strains), growth curves show strain MG1655 was relatively more sensitive to thymol than JM109 but more significantly, that its *acrB* mutant was highly sensitive to thymol as hypothesized (**Figure 4.8 A, B**).

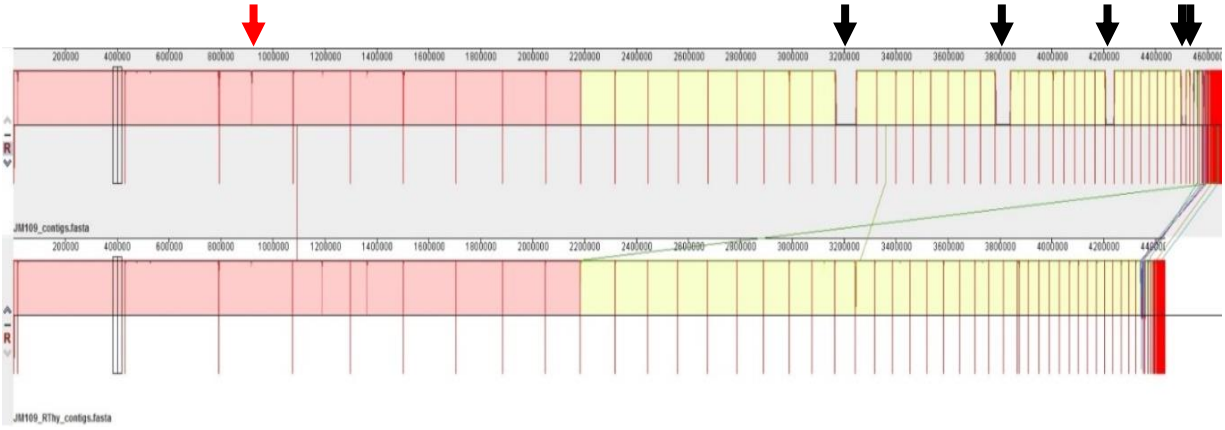

Figure 4. 7 Comparison of the JM109 wildtype strain genome with the JM109 mutant genome, using Mauve multiple alignment program. The black arrows indicate contigs present in the wildtype strain, absent in the mutant. The red lines indicate the contig boundaries, and the red arrow indicates a difference within a contig.

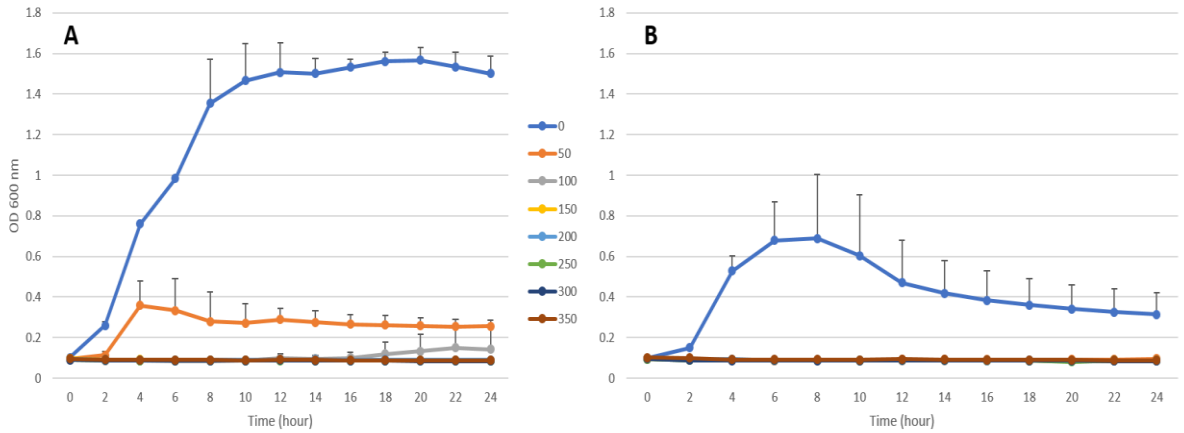

Figure 4. 8 Compares the effect of high concentrations of thymol between the *E. coli* MG1655 (**A**), and mutant *acrB* (**B**).

4.4 Discussion

E. coli JM109 exhibited acquired tolerance to thymol after exposure to increasing sub-inhibitory concentrations of thymol. The increased tolerance to thymol remained after several successive passages under non-selective conditions, suggesting that in *E. coli* this tolerance to thymol could be the result of genetic mutation(s). These conclusions are in agreement with previous studies reported by others (Chung *et al.*, 2006). It was anticipated that if such tolerant mutants arose, genome sequencing would identify at least in part the mechanisms of thymol tolerance and perhaps more importantly insights into the biochemical management of thymol assault. In this study, many cycles of exposure to increasing concentrations of thymol was required to select stable mutants. However, a point should be made that 'high' tolerance was only slightly more than a doubling of the MIC.

The wild-type and adapted mutant *E. coli* were grown in the presence and absence of thymol for comparison. It was noted that the adapted mutant *E. coli* JM109 had extended lag and exponential phases and significantly reduced yield without thymol. This indicated that the adapted mutant strain was slower to grow even in the absence of thymol. It was surmised then that accumulation of mutations that gave increased tolerance to thymol were however, detrimental to growth. Whether this would be true of other tolerance associated mutations would need investigation.

Another observation was that wild-type non-adapted *E. coli* had an extended lag phase in the presence of sub-lethal concentrations of thymol. It is understood that the individual bacteria within a population adjust to a new environment before starting exponential growth. Several factors effect during this phase such as physiological history of the cells, the precise physiochemical status of cells and the growth medium (Swinnen *et al.*, 2004). In a previous study (Skandamis *et al.*, 2001) determined that the presence of oregano essential oils caused an increase in the lag phase and a decrease in the maximum growth rate of *E. coli* O157:H7. In

addition, Eucalyptol which is a compound obtained from EOs extended the lag phase of *E. coli* O157:H7 (Zengin and Baysal, 2014). It is known that the lag phase depends on the new environment, and in these studies and those cited above it is most evident that cells inoculated into media containing sub-lethal concentrations of thymol or other EOs require much time to adjust before growth commences. These findings strongly suggest that gene regulatory processes may be coming into play perhaps to upregulate systems that detoxify thymol or prevent entry, or increase fatty acid synthesis for repair of cell membranes and so forth. Equally some genes encoding such functions as porins may be switched off. The exponential phase of the wild-type *E. coli* was extended with sub-lethal concentrations of thymol and final yields reduced. Moreover, the adapted mutant *E. coli* did not reach stationary phase after 20h which meant the cells were still in exponential phase after more than 20 h. These observations raise important points. First, wild-type *E. coli* can survive and grow, albeit poorly in sub-lethal concentrations of thymol which may indicate that in more complex environments such as the chicken gut, they will not be so competitive. This however, depends upon the impact of thymol on the rest of the gut population: an area for further study for which introductory studies were undertaken in Chapter 5. The second point is that the adapted mutant would appear to be equally disadvantaged even though better able to withstand the stress of thymol. Is it possible that mutations that reduce growth rate are protective toward thymol? Also, it is known that the fraction of a genetically uniform sensitive bacterial population survives exposure to antibiotics or phytochemicals do not all die at the same rate (Balaban *et al.*, 2004). It is possible then that the sensitive un-adapted cells are growing quickly but dying more quickly whereas the adapted mutant is just growing more slowly and not dying so quickly. It may be posited that wild-type cells grow quickly and die quickly when exposed to thymol whereas the mutant grows slowly and, if this were to be the case, microscopic analysis of growing cultures may show this.

Exposure to thymol was inhibitory conferring severe morphological changes in the cell wall and membrane of wild-type *E. coli*. However, SEM analysis revealed that the adapted mutant when exposed to thymol displayed few morphological changes relative to the non-adapted wild-type cells. Exposure of non-adapted wild-type *E. coli* cells to sub-lethal thymol 50µg l^{-1} resulted in the appearance of larger than normal uniform cylindrical shaped cells with corrugated surfaces, with evidence of collapsed cells and perhaps interestingly poor septum formation. These data suggest that thymol render bacteria cell membranes permeable, which was similar to other studies that used EOs (Burt and Reinders, 2003; Zengin and Baysal, 2014). The effect of thymol and carvacrol which are major constituents of oregano EO is to damage the cell membrane (Lambert *et al.*, 2001). Similar studies done by Skandamis and colleagues observed that the minimum concentration of oregano essential oil on *E. coli* O157:H7 cells coagulated the cytoplasmic constituents, while the membrane structure tended to be irregular and rough (Skandamis *et al.*, 2001). Given these findings it may be postulated that thymol disrupts cell membrane structure and function including septum formation which is essential for cellular division and population growth. It maybe surmised also that the cellular response maybe to subvert metabolism away from other processes toward membrane repair and fatty acid synthesis. Studies by Karatzas and others and Randall and others (Karatzas *et al.*, 2007; Randall *et al.*, 2004) showed that strains exposed to and selected as resistant to various disinfectants possessed *fabI* mutations: a gene involved in the regulation of fatty acid biosynthesis, a major component of cell membranes. Also, these studies showed other mutations arose that generated multiple antibiotic resistance, notably upregulation of efflux, as shown in the studies here.

Although the study of single identified metabolites gives a first clue as to the perturbation induced by thymol on the cell metabolism, it was clear that the overall picture of the cellular processes affected by exposure and training to adaptation to thymol was captured

by analysing the metabolome with pattern analysis tools. However, for these studies the focus was on products that were extracellular, rather than the entire cytoplasm for which the reasoning was twofold. First, this approach would capture end products of metabolism that may be perturbed by thymol stress and second gain evidence for general 'leakage' of the bacterial cells as thymol is considered to damage cell membranes (Burt, 2004) and therefore cell integrity. The metabolomic approaches, used here, have been shown in the work of others to be a valuable tool to describe the mode of action of different antibiotics thereby driving drug discovery strategies of identification, modification and evaluation (Bleicher *et al.*, 2003; Halouska *et al.*, 2012). Recently, a study was done to evaluate the metabolic profiles of *E. coli* treated with nine antibiotics with intracellular and extracellular small metabolites separated in fingerprint and footprint analyses to provide complementary information (Hoerr *et al.*, 2016). In order to understand the main sources responsible for the changes associated to each sample, the careful inspection of the loading of first components were performed as shown in **Figure 4.5**. Those results indicated the importance of each peak in the loading plot was influenced by the average amount of the corresponding metabolite in all the replicate samples. Those found to be of particular importance along the plot of wild-type were formate, succinate and acetate that are organic acids present in or at the end of the TCA cycle respiratory pathway. However, the adapted mutant had decreased levels of these metabolites and significantly increased lactate. The hypothesis arising is that increased tolerance to thymol is associated with of a shift away from respiration to fermentation in the adapted mutant strain. A previous study by (Cox *et al.*, 1998) observed the inhibition of respiration in *E. coli* and stimulation of the leakage of intracellular K^+ by tea tree oil. In another study that used vanillin, which is a phenylpropene phenolic aldehyde, it was observed that the mechanism of antibacterial action of vanillin was associated with inhibition of respiration in *E. coli* and *L. innocua,* whilst in some lactic acid bacteria it disrupted K^+ and pH homeostasis (Fitzgerald *et al.*, 2004). Collectively the findings

of these authors and from the study reported in this thesis, the response of bacteria exposed to phenolic compounds is a slowing of growth associated with a shift from respiration to fermentation as indicated by detecting increasing lactate concentrations. Lactate was already present in the all samples tested, suggesting some fermentation, possibly through hypoxia, occurred perhaps during growth or between harvesting and extraction. The adapted mutant strain had high levels of both lactate and the pyruvate family of amino acids. This is compelling evidence of a switch from respiration to fermentation as part of the strategy of *E. coli* to survive assault with polyphenols. It would have been worthwhile to assess if K^+ ions leaked from the bacteria exposed to thymol and will considered in any future work. There was little evidence of other small metabolites leaking suggesting at the concentration of thymol used, a modest 50ug l^{-1}, cell membrane damage was possibly insignificant. **Figure 4.9** shows the overall picture of the respiration shifting to fermentation metabolic leading to increasing amount of lactate instead of decreasing amount of respiration end products.

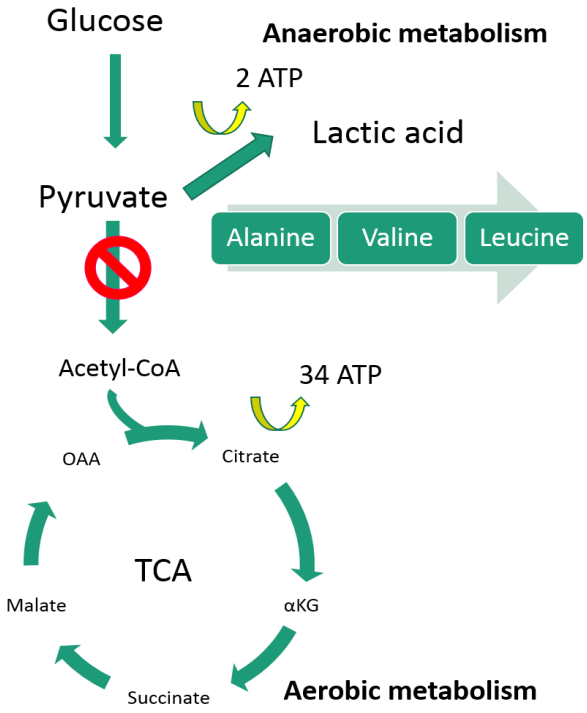

Figure 4. 9 The proposed overall picture of the shift of respiration to fermentation leading to increasing amount of lactate and the pyruvate family of amino acids and decreased end products of respiration.

Whilst strains treated with thymol did not show the same reaction as the adapted mutant *E. coli*, exposure to thymol gave evidence that suggested *E. coli* slowly shift to ferment sugar as carbon source rather than respire. Recently, a study done by (Picone *et al.*, 2013) measured the metabolome of *E. coli* 555 by ^{1}H NMR spectroscopy at different concentrations of carvacrol and they found that, although the adaptation to carvacrol occurring at sub-lethal doses was different from that occurring at higher doses, they showed that towards the higher concentrations of carvacrol there was shift from respiration to fermentation.

The body of data from this study and of others suggest that phenolic compounds disrupt respiration in *E. coli*. Whilst there is no direct evidence for the mechanism of action, it is known that phenolic compounds are highly lipophilic and integrate in the cell membranes, the site of electron transfer in aerobic respiration. It is a tantalising possibility that integration in the cell membrane of phenolic compounds disrupts electron transfer that is essential for respiration with many proteins associated with electron transport are embedded in the cytoplasmic membrane. For example, Hexachlorophane, a phenolic biocide, inhibits the membrane-bound part of the electron transport chain at low concentrations and at higher concentrations, it induces leakage of intracellular contents from *Bacillus megaterium* (Frederick *et al.*, 1974; Joswick *et al.*, 1971). The change in membrane fluidity may disturb the respiratory chain that was observed with the antibacterial activity of flavonoids (Mishra *et al.*, 2009; Haraguchi *et al.*, 1998). In addition, it was reported that the permeability of bacterial cell wall and cell membranes are affected by myrtle extract and EOs, by disruption in the membrane function such as electron transfer, enzyme activity or nutrient absorption (Amensour *et al.*, 2010). This is an important hypothesis that needs investigation in future work.

The result of sequence analysis of the adapted mutant showed a mutation in the *acrR* gene encoding a repressor of the AcrAB efflux pump. The acriflavine resistance regulator (AcrR) is a local transcription factor that regulates the expression of the AcrAB-TolC multidrug

efflux pump. AcrR modulates the expression of *acrRAB* genes (Ma *et al.*, 1996) and the associated AcrAB-TolC multidrug efflux pump (Lee *et al.*, 2014). The multidrug efflux pump AcrAB-TolC gene is part of over 60 genes activated by the global transcriptional regulator MarA (Ruiz and Levy, 2010). The *acrR* gene is divergently located 141bp upstream of the *acrAB* operon (Ma *et al.*, 1996) and encodes a 215-amino acid transcriptional repressor of the TetR family. The N-terminal domain of AcrR contains a DNA-binding helix-turn-helix (HTH) motif, and the C-terminal domain has a unique sequence that is predicted to bind ligands (Ramos *et al.*, 2005). The binding of drugs to the C-terminal domain of AcrR triggers a conformational change in the N-terminal DNA-binding region, resulting in the release of AcrR from DNA and allowing transcription from its cognate promoter (Su *et al.*, 2007). In the AcrR deleted strains (Δ*acrR*) the intracellular accumulation was lowered by enhanced action of the AcrAB-TolC efflux pump, which is involved in exporting a wide range of toxic compounds: these compounds might be antibiotics, disinfectants, organic solvents and phytochemicals (Ma *et al.*, 1995; Tsukagoshi and Aono, 2000). In this study, we found that the *acrR* gene was inactivated, astop codon creating a truncated product, that resulted in the increased function of AcrAB-TolC efflux pump clearance of thymol highlighted by sustained growth of JM109 mutant in higher concentrations of thymol (**Figure 4.10**). The AcrAB is a drug efflux pump that is homologous to other efflux systems (Ma *et al.*, 1993) and its expression is regulated to some extent by the repressor *AcrR* (Ma *et al.*, 1996). *AcrB* is an efflux transporter belonging to the resistance-nodulation-cell division family (Saier *et al.*, 1994) and *AcrA* belongs to the membrane fusion protein family (Dinh *et al.*, 1994) that is thought to connect the transporter protein physically to an outer membrane channel so that the drugs can be exported directly into the external medium by passing the outer membrane barrier, modified figure by (Venter *et al.*, 2015) **Figure 4.11**.

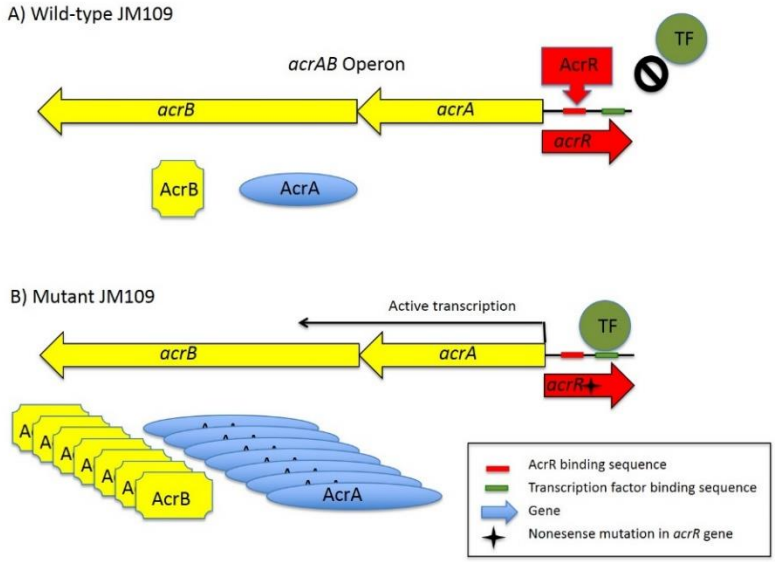

Figure 4. 10 Illustrates a graphic representation of wild type thymol sensitive JM109 (A) due to the active repression of *acrAB* gene transcription by the action of active AcrR inhibiting the binding of transcription factors (TF). Whereas in mutant (B), thymol resistant JM109 the *acrR* gene has a nonesense mutation resulting in a defective AcrR protein of 107 amino acids long that can no longer suppress *acrAB* transcription allowing the binding of potent transcription factors to produce more AcrAB proteins to construct efflux pumps.

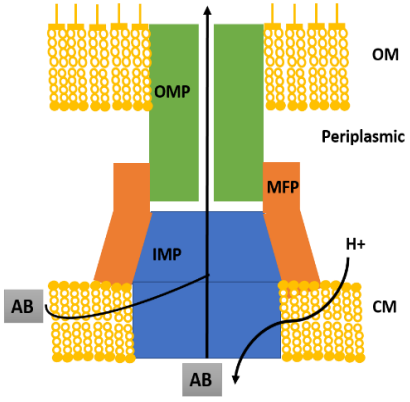

Figure 4. 11 A hypothetical mechanism for AcrAB pumps. The complex consists of three proteins which span the cytoplasmic membrane(CM), the outer membrane(OM), and the periplasmic space. The inner-membrane protein(IMP) *AcrB*, is responsible for substrate specificity and catalyses pH dependent drug transport. Examples for outer membrane protein(OMP) is *TolC* and the periplasmic membrane fusion protein (MFP) is *AcrA* connects the IMP and the OMP.

Furthermore, efflux pumps AcrAB are thought to produce a multiprotein complex traversing the cytoplasmic as well as the outer membrane (Ma *et al.*, 1994; Nikaido, 1994). The absence of one or two of the component proteins such as in our MG1655 *acrB* mutant strain will result in the formation of a defective complex resulting sustained accumulation of thymol in the cell. In addition, thymol has been shown to increase the permeability of bacterial membranes due to its lipophilic nature and result in macromolecules leakage, and possibly allowing leakage of drugs into the cells (Trombetta *et al.*, 2005). Therefore, sensitivity to low

concentrations of thymol might be the result of a defective efflux process. Moreover, it has been shown that thymol inhibits the ATP synthase and cell growth of *E. coli* resulting in slowed growth or bacterial senescence (Liu *et al.*, 2017). In summary, our hypothesis was inactivation of *acrR* is effective in increasing the MICs of thymol. These results indicate that AcrAB plays an important role in survival against thymol. Therefore, the AcrAB efflux pump is involved in cellular responses to thymol. Most probably this mechanism in the comparative 'resistance' to thymol is the same mechanism created in response to the presence of antibiotics. Therefore, AcrAB efflux pump inactivation is a primary candidate to increase bacterial sensitivity to antibiotics/ phytochemicals. It would be interesting to test this hypothesis by using specific efflux inhibitors such as phenylalanine arginyl *β*-naphthylamide (PA*β*N). It would be anticipated that efflux inhibitors would increase sensitivity to thymol (Askoura *et al.*, 2011).

The other mutation was in the *rng* gene encoding ribonuclease G (RNase G) that was a non-synonymous amino acid change (R to C). RNAse G functions in mRNA decay, tRNA and rRNA cleavage and maturation in conjunction with other RNase E and G family endoribonucleases (Deutscher, 2006). *E. coli* RNase G was originally identified as an endoribonuclease involved in the maturation of 16S rRNA (Li *et al.*, 1999). RNase G has a high similarity with the N-terminal catalytic domain of RNase E. Both RNase E and G attack substrate RNA in a single stranded AU-rich region (Lin-Chao *et al.*, 1994; Tock *et al.*, 2000) and prefer substrates with mono-phosphorylated 5'-ends to those with tri-phosphorylated termini (Mackie, 1998). *E. coli* RNase G was also involved in the degradation of *adh*E mRNA encoding fermentative alcohol dehydrogenase (Kaga *et al.*, 2002; Umitsuki *et al.*, 2001). The mutation discovered in these studies is located in the S1-like RNA-binding domain possibly have resulted in the noted slowed growth confirming previous studies that mutations in this domain results in slowed growth of *E. coli* cultures (Chung *et al.*, 2010).

A point that needs to be raised here is the fact that at least two mutations were observed in the adapted mutant. Despite the presence of these 'protective' mutations the bacteria were very slow growing, and it could be argued that these bacteria would be uncompetitive in the environments in which these bacteria are found. That two mutations and possibly others that bioinformatics analysis has yet to confirm suggest that exposure to thymol will not readily select resistant mutants in the 'real world': remember, in these studies the bacteria were exposed to thymol under highly artificial circumstances and in mono-culture. It may be argued therefore that thymol and other EOs used in complex environments such as in chicken feed may pose little or even no threat of generating resistance unlike antibiotics. Whilst tempting to speculate EOs could be the new antibiotics of the future, but further work is needed in this area.

CHAPTER 5: THE IMPACT OF THYMOL ON THE GUT MICROBIOME OF THE CHICKEN: AN *IN VITRO* STUDY

5.1 Introduction

The gastrointestinal tract of poultry is densely populated with various microbes, primarily consisting of complex communities of bacteria colonising different parts of the gut: the most densely colonised being the caeca and lower gut (Zhu *et al.*, 2002). It has been long known that the microbiota has a major and some consider predictable impact on host health and nutrition, specifically with regard to having an important role in the growth and health of the host (Brisbin *et al.*, 2008; Stanley *et al.*, 2014) . The gastrointestinal tract consists of different organs; proventricularus, gizzard, small intestine (duodenum, jejunum and ileum), caecum and large intestine. The diversity of the microbiome and the number of anaerobic bacteria has been found to be at the greatest density in the caecum (10^{10} CFU/g intestinal content) of the entire digestive tract (Ranjitkar *et al.*, 2016; Bjerrum *et al.*, 2006). Here the environment is conducive to bacterial fermentation with a relatively low oxygen partial pressure if not anaerobic due to bacterial oxygen scavenging and decreased host derived enzymes and bile salt concentrations that create conditions suitable for a variety of gut adapted bacteria (Gabriel *et al.*, 2006). In addition, the caeca are a pair of blind sacks which fill from passage of luminal content from the large intestine and consequently have a rather slow passage rate: that said the caeca often actively eject their contents once every 24h or so. Of the compartments of the poultry gut, the most studied intestinal microbiome of poultry is indeed from the caecal microbiome (Pan and Yu, 2014; Waite *et al.*, 2012; Mohd Shaufi *et al.*, 2015; Wei *et al.*, 2013).

The caecal microbiome is dominated by strictly anaerobic bacteria, many of which belong to unknown bacterial genera. Earlier culture-based studies have showed that the phylum *Firmicutes* and *Bacteroidetes* dominated the caecal microbiota (Salanitro *et al.*, 1974; Mead,

1989; Park *et al.*, 2017). However, using abundance estimates based on 16S rRNA gene sequences, a culture independent phylogenetic profiling tool, have given enhanced insights into the composition of the chicken caecal microbiome (Zhu *et al.*, 2002; Wei *et al.*, 2013). These recent studies have shown that from 12 bacterial phyla identified in both chicken and turkey, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the most prevalent phyla (Wei *et al.*, 2013). However, more than half of these bacteria belong to phylum *Firmicutes* especially the order *Clostridiales* (families *Clostridiaceae, Lachnospiraceae* and *Ruminococcaceae* (Bjerrum *et al.*, 2006; Lu *et al.*, 2003; Danzeisen *et al.*, 2011; Ranjitkar *et al.*, 2016)). The caecal microbiome is composed primarily of commensal bacteria although some pathogenic bacteria may be harboured there also that are controlled by many factors, such as host, age, diet, the existing microbiota (the Nurmi effect), commercial environmental conditions, management practices such as litter management, disinfection regimes between batches of birds, feed additives such as phytochemicals and so forth (Wei *et al.*, 2013; Lu *et al.*, 2003; Nurmi and Rantala, 1973). Frequent efforts, especially dietary intervention and litter management, have been attempted to modulate the intestinal microbiome to enhance feed conversion and gut health (Ruiz *et al.*, 2008; Wang *et al.*, 2016). However, few of these interventions have achieved consistent or sustainable improvement.

The search for alternative products for pathogen control has emerged because of the continued use of antibiotics in poultry production. An increase in consumer awareness and interest in these issues has stimulated research to prudent antibiotic-use practices seeking acceptable replacements (Apajalahti, 2005). Non-antibiotic products include: probiotics, prebiotics, synbiotics (Gaggia *et al.*, 2010), enzymes, phytobiotics (Yang *et al.*, 2009) and EOs (Brenes and Roura, 2010), which may be useful in alternative production systems. An assessment of the changes in the intestinal microbiome community and interactions following treatment with these novel products are an important aspect of comparing the efficacy of

alterative products to antibiotics (Roberts *et al.*, 2015; Janardhana *et al.*, 2009). A few studies were done that demonstrated mixed results concerning the performance of EOs, such as oregano and thymol, with several of these studies suggesting little or no effect on chicken broiler performance (Brenes and Roura, 2010). Conversely, by investigating the diversity of the caecal microbiome community using DGGE of 16S rRNA gene PCR amplicons, Hume and others found coccidia species, and that the essential oils they used modulated the intestinal microbiome communities reducing coccidia. The birds were treated with anticoccidials and the antibiotic bacitracin, in addition to avoiding drastic shifts after a mixed challenge (Hume *et al.*, 2006).

If the poultry industry is to transition from antibiotic use to alternatives it is recognized that a better understanding of the interactions of the intestinal microbiome with the host and with non-antibiotic products is required to further enhance poultry nutrition and gut health. Currently, the lack of satisfactory knowledge on the bacterial diversity in poultry intestines is considered one of the major knowledge gaps that hinder this transition. The knowledge of the microbiota host interaction with non-antibiotic products such as the use of prebiotics, probiotics, and phytochemicals could lead to an understanding of the effects on growth and health status. The investigation and characterization of the changes in the caecal microbiome that occur in chicken by using in-feed phytochemicals, for example thymol, may be done by a combination of *in vitro* experiments and *in vivo* trials. *In vitro* surrogate gut model experiments will provide information regarding the microbiota and may help explain and differentiate the extent of bioconversion processes mediated by the host itself (Jacobs *et al.*, 2009; Bolca *et al.*, 2007a). From an ethical perspective, these in *vitro* studies should be performed prior to *in vivo* trials but one complication of *in vitro* gut models is their diversity, ranging from simple static models (batch culture fermentation) to advanced continuous models (gastrointestinal simulators).

Batch culture fermentation (static gut models) is basically a closed system using sealed bottles containing a slurry of caecal material that are maintained under anaerobic conditions. This system is relatively easy to operate and is cost effective, allowing for parallel screening. This model may be used primarily to evaluate the effects of thymol on chicken derived gut microbiota and to assess the environmental conditions and thymol concentration that favours or limits the caecal microbiome. Nevertheless, these closed systems are only adequate for simulating short-term conditions in the gut; long-term adaptations need more complex dynamic models such as whole gut model continuous flow models (Rowland *et al.*, 2017). Given that little is known regarding the impact of any EO on the gut microbiota and because ethically it is essential to undertake *in vitro* studies prior to animal trials, it was considered appropriate to use simple static batch cultures to assess the impact of thymol on caecal gut microbiota and its metabolism. To do this, 16S rRNA population analysis and NMR metabolic analysis of static gut models collected at 5-time points over a 48h incubation period were performed. Although preliminary and far removed from the actual caecum of a live bird the data generated should provide some clues to possible outcomes that may be generated *in vivo*.

5.2 Materials and Methods

5.2.1 Chicken caecal batch fermentation model

5.2.1.1 Thymol stock.

Thymol was dissolved in ethanol 50% (v/v) to give a working stock solution of 5 mgl^{-1}. The stock was stored at 4˚C and used within 48 hours of making. Serial dilutions were made in fresh sterile VL medium to give 50 and 100 $\mu g\ l^{-1}$ concentrations.

5.2.1.2 E. coli strains

E. coli strain (APEC 18) stored at -80°C was grown on LB agar plates at 37°C for 16-18 hours and colonies were picked into LB broth cultures and incubated aerobically with shaking at 150- 200 rpm at 37 °C for 16-18 hours.

5.2.1.3 VL medium

Viande-Leuvre medium (VL) was prepared in 1L. All solid ingredients (**Table 5.1**) were weighed in except hemin which was made into a solution by adding it to water and dissolving it with a couple of drops of NaOH (1M). The ingredients were dissolved in 900 ml of distilled water and after dissolution of all substrates the volume was made up to a final volume of 1L and sterilised by an autoclave (121^{o}C 15min). Fresh stock solutions of Vitamin K and Resazurin were prepared separately by dissolving the reagents in water, and were filtered sterilised prior to adding them to the VL medium.

Table 5. 1 VL medium for 1L

No.	Compounds	Weigh/Volume
1	Meat extract	2.5 g
2	Glucose	2.5 g
3	Tryptose	10 g
4	Yeast Extract	5 g
5	NaCl	5 g
6	Haemin	0.05 g
7	Vitamin K	10 ul
8	L-Cysteine HCl	0.6 g
9	Resazurin solution (0.025g/100ml)	4 ml

5.2.1.4 Collection of caecal content and setting up the closed fermentation model

Caecal samples were collected from 6 Ross 308 male broilers at 15 days of age that had been housed, fed and managed indoors under standard commercial conditions at CEDAR (The University of Reading Veterinary Officer). The broilers had been fed on *ad libitum,* a basal starter diet from hatching to ten days of age prior to their transfer to a grower diet from day 10 onwards. Six birds were selected at random and killed by cervical dislocation. The birds were opened and the gut exteriorised and the caeca removed. Each caecum was squeezed out between the thumb and forefinger of gloved hands, so its content was placed into a Duran bottle. All samples were collected together. Ten grams of caecal contents were weighed and added to 30 ml of VL medium (25% dilution), and transported to the laboratory in an anaerobic jar. These procedures were performed as quickly as possible using VL medium purged of oxygen in order to reduce loss of strictly anaerobic bacteria. Inside an anaerobic chamber (Whitly MG1000 anaerobic workstation) operating on a cylinder of conventional anaerobic gas mixture (80% N_2, H 10% and 10% CO_2), the caecal slurry was divided into 4 separate vessels so that each one contained 10 ml of the caecal slurry. The pH was adjusted to between 5.8 and 6.0 using 1M NaOH/HCl solution for each vessel. Four treatments were prepared (**Table 5.2**). The model was run as a closed-fermentation system for 48 hours within the anaerobic cabinet and samples (1.5 ml) were collected after (0, 4, 8, 24, and 48 hours) into a sterile 1oz screw cap bottles that were maintained in the cabinet briefly prior to storage at -80˚C. In addition, the pH of the slurry was checked when samples were collected at every time point by pH indicator strips.

Table 5. 2 caecal slurry cultures

No.	Culture sample	Thymol final concentration	E. coli culture strain 18
1	Control (negative)	0	0
2	Control (positive)	0	$5.5x10^5$
3	Low thymol	50 µg l^{-1}	$5.5x10^5$
4	High thymol	100 µg l^{-1}	$5.5x10^5$

5.2.2 16S rRNA sequence of the gut population

5.2.2.1 DNA extraction

DNA was isolated from each caecal culture sample using a PowerSoil® DNA Isolation kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, 1 ml of caecal culture sample was added to a Power Bead tube and gently vortexed to mix. To the homogenised solution, 60 µl of solution C1 was added and vortexed for 30 seconds. Solution C1 is a lysis buffer and contains SDS which breaks down lipids and fatty acids in the cell membrane. The tubes were then placed in a multi-tube vortex (VWR DNX-2500) at maximum speed for 10 minutes, before centrifuging for 30 second at 14,000xg. The supernatant was transferred to a clean 2 ml microfuge tube and 250 µl of Solution C2 was added and vortexed for 5 seconds followed by incubating at 4°C for 5 minutes. Solution C2 removes inhibitors from the sample. The sample was centrifuged at 14,000xg for one minute at room temperature and 600 µl of supernatant was transferred to a new 2 ml microfuge tube. To the supernatant, 200 µl of solution C3 was added, inverted twice and incubated at 4°C for 5 minutes followed by a one-minute centrifugation at 14,000xg. Solution C3 is another inhibitor removal reagent. The 750 µl of supernatant was added to a 2 ml tube containing 1200 µl of solution C4 and the tube was vortexed for 5 seconds. Solution C4 contains a high concentration of salt which allows the DNA to bind to the column. To a spin filter membrane, 675 µl of the solution was applied and centrifuged for one minute at 14,000xg. This step was repeated until the full 1.2 ml and the

supernatant had passed through the column. Subsequently, 500 µl of solution C5 was applied to the membrane and centrifuged for 30 seconds at 14,000xg. Solution C5 is an ethanol wash that reduces the salt concentration and removes other contaminants. The flow-through was discarded before centrifuging for a further one minute at 14,000xg to ensure all residual solution C5 was removed. The collection tube was replaced with a sterile 1.5 ml microfuge tube before 100 µl of solution C6 was pipetted onto the centre of the spin filter and incubated for two-five minutes at room temperature followed by centrifugation for 30 seconds at 14,000xg. Solution C6 is a low salt elution buffer and therefore the DNA is removed from the membrane. Eluted DNA was stored at -80˚C until required. The DNA samples recovered from the extraction process were quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop technologies, USA). The Nanodrop pedestal was cleaned by pipetting 2 µl of dH20 onto it and wiping with Whatman filter paper. The Nanodrop was blanked with 1µl of appropriate solution C6 before pipetting 1 µl of sample and taking a reading. The concentration (OD 260 nm), 260:280 nm ratio and 260:230 nm was recorded and considered acceptable for downstream applications if ratio values were 1.8 ± 0.15 at a minimum concentration of 10 ng/µl^{-1}. All DNA samples were normalised to standard concentration of 10 ng/ µl^{-1}, aliquoted and loaded into a 96-well plate.

5.2.2.2 Next Generation Sequencing (NGS)

DNA samples for 4 treatments collected at 5-time points were submitted to the Animal and Plant Health Association (APHA, Surrey, UK) Genomic Services and Development Unit (GSDU) for next generation sequencing (NGS) using Illumina sequencing technology to sequence genomic DNA or PCR amplicons. Aliquots of extracted DNA were amplified with universal primers for the V4 and V5 regions of the 16S rRNA gene using primers U515F (5'-GTGYCAGCMGCCGCGGTA-3') and U927R (5'-CCCGYCAATTCMTTTRAGT-3'), which were designed to permit amplification of both bacterial and archaeal ribosomal RNA

gene regions, whilst providing the best possible taxonomic resolution based on published information (Wang and Qian, 2009). Forward and reverse fusion primers consisted of the Illumina overhang forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and reverse adapter (5'-GTCTCGTGGGCTCGGAGATGTGTAATAAGAGACAG-3') respectively.

Amplification was performed with FastStart HiFi DNA Polymerase (Roche Diagnostics Ltd, UK) using the following cycling conditions: 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 35 s, 72 °C for 1 min; followed by 72 °C for 8 min. Amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). Each sample was then tagged with a unique pair of indices and sequencing primers, using Nextera XT v2 Index kits, and 2x KAPA HiFi HotStart ReadyMix using the following cycling conditions: 95 °C for 3 min; 12 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; followed by 72 °C for 5 min. Index-tagged amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). The concentration of each sample was measured using the fluorescence-based Picogreen assay (Invitrogen). Concentrations were normalized before pooling all samples, each of which would be subsequently identified by its unique MID. Sequencing was performed on an Illumina MiSeq with 2 x 300 base reads according to the manufacturer's instructions (Illumina Cambridge UK).

5.2.2.3 Data Analysis

Sequence reads were processed according to the microbiome-helper pipeline (https://github.com/mlangill/microbiome_helper/wiki/16S-standard-operating-procedure). Paired-end reads were merged based on overlapping ends using PEAR (http://sco.h-its.org/exelixis/web/software/pear/), before filtering the data for base-calling quality and amplicon length with a cut-off of 97%. The processed sequences were then classified using

the pick open reference and were assigned to operational taxonomic units (OTUs) implemented in QIIME v1.9.1. Representative sequences for each OTU were assigned to different bacterial taxonomy levels-phylum, class, order, family and genus by using Greengenes 16S rRNA gene database (http://greengenes.secondgenome.com/). The resultant distribution of OTUs across the multiple samples was further analysed using QIIME v1.9.1 to summarise their distribution and explore alpha and beta diversity. Alpha diversity was explored by plotting the number of sequences per sample against *Chao1* values, and beta diversity was explored through principal component analysis (PCA) in MATLAB R2015b.

5.2.3 ^{1}H NMR

5.2.3.1 Preparing culture supernatants (footprinting)

The supernatants of 48 samples from the caecal fermentation model experiment collected from 3 technical replicates of 4 treatments at 4 different times points (0, 8, 24 and 48 h) were defrosted from -80 °C and vortexed (time point 4 was removed for convenience and as we found in the NSG result not much change in this time point). Samples (400 µl) were transferred to clean microfuge tubes and each sample was buffered with a 200µl phosphate buffer **Table 5.3**, vortexed and centrifuged at 14,000xg for 10 minutes. A total of 550 µl of each supernatant was transferred into 5mm internal diameter NMR tubes on the same day for analysis.

Table 5. 3 Phosphate buffer composition for 500 ml of solution

Compounds	Weigh/Volume	Utility
1 mM TSP	0.172 g	Standard
Na_2HPO_4	28.86 g	Buffer
NaH_2PO_4	5.25 g	Buffer
NaN_3.	0.193 g	Antimicrobial
D_2O	1L	NMR lock

5.2.3.2 NMR acquisition and processing

^{1}H NMR spectra were acquired on a Bruker 700 MHz (Bruker Avance III HD, UK), an automatic tuning-matching unit at 298 K, and an automatic sample changer. To facilitate compound identification, 1D spectra were acquired using standard Burker 1D nuclear over Hauser enhancement spectroscopy (NOESY) presaturation pulse sequence on selected samples (Kumar *et al.*, 1980; Nicholson *et al.*, 1995). After acquisition, spectra were manually phased, processed in order to affine spectrum phasing calibration on TSP at δ 0.00 ppm and baseline correction using MestReNova® software. Stacked spectra were imported into MATLAB (R2015b) MathWork ®software where spectra were digitalised between δ 0.5-10 ppm, in order to delete useless information and avoid bias of the data; the region containing the water peak was deleted between δ 4.8 and 5.1. Peak assignment was done using online open access databases (chenomx® and HMDB) and 1D Spectra (for spectroscopy correlation) for molecule identifications. Chenomx® Software was also used for quantification.

5.2.3.3 Data analysis

For footprinting analysis, 48 samples were prepared respectively using 3 technical replicates as described in section 5.2.3.1. Multivariate statistical analysis was evaluated using principal coordinate analysis (PCA) plots and analyses of the loading of the principal components were done to evaluate the metabolic variations existing between the treatments. Orthogonal projections to latent structure (OPLS) regression performed a minimum of 3 replicates per treatments were used to analysis the data. PLS regressions were run in between each group for each time. OPLS R2Y values around 0.8 were indicative of a good model, with Q2 values of around 0.5 indicating good predictive ability. PCA score plots and OPLS correlation plots were also produced to visualise differences in the metabolome between treatment groups. Loading and contribution plots were extracted to reveal the variables that

bear class discriminating power. Moreover, to improve model visualization and interpretation, S-line plots were extracted to detect the metabolites that influence variable selection as they display the overall importance of each variable (X) on all responses (Y) cumulatively over all components.

5.3 Results

5.3.1 Next Generation Sequencing (NGS)

5.3.1.1 Change in caecal Bacterial Alpha and Beta Diversity

Genomic sequences from all 20 samples were assigned to 92,572 operational taxonomic units (OTU) using a 97% similarity cut off. The diversity of the gut microbiota within a community was measured with alpha diversity indices, in particular the number of OTUs. Rarefaction curves of alpha and chao1 diversity of the observed OTUs are shown in **Figure 5.1 (A and B)** respectively, but Shannon diversity indices are not shown.

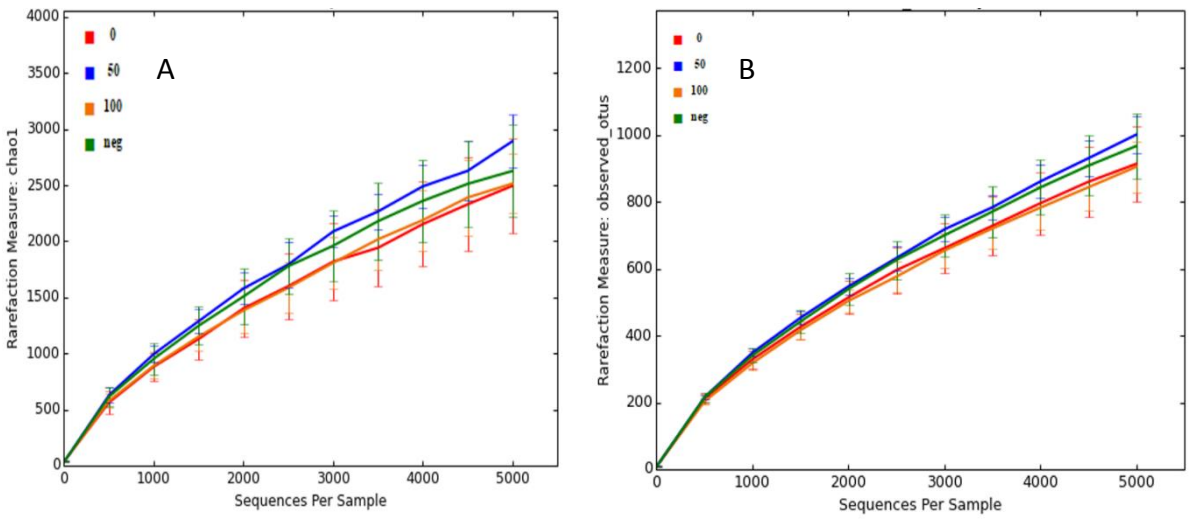

Figure 5. 1 Alpha diversity rarefaction curves measuring **(A),** and chao1 **(B)** observed OTUs with respect to sequence accumulation per sample of four treatment segments. Negative (neg), positive control (0), low thymol (50) and high thymol concentration (100). The alpha diversity plot for each segment represents the pooled data for five sampling hours (0,4,8, 24, and 48).

There were no differences between previous plots. Likewise, the bacterial diversity between samples, as demonstrated with PCA based on an unweighted (qualitative) phylogenetic UniFrac-based distance matrices (beta diversity) showed no significant difference in the total microbiota composition between the four treatments as shown in **Figure 5.2 (A)**. However, beta diversity metrics clearly indicated that the difference in bacterial communities observed was related to the time of incubation **Figure 5.2 (B)** and not the treatment. The same result was revealed when weighted (quantitative) phylogenetic UniFrac distance matrix was plotted **Figure 5.3**. There was no treatment effect on beta diversity of the bacterial microbiota **Figure 5.3 (A)**, and the differences observed were related to time **Figure 5.3 (B)**.

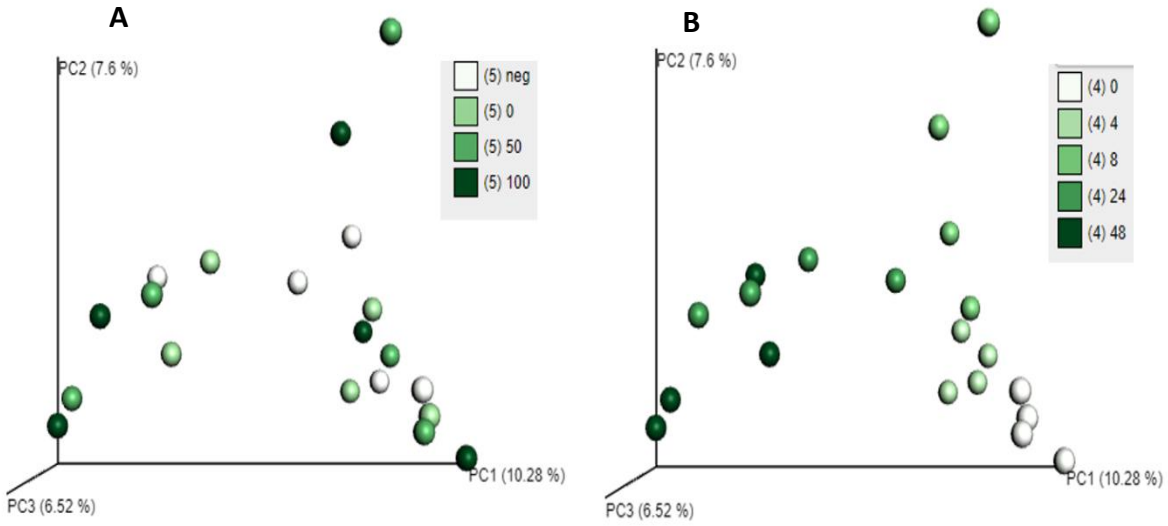

Figure 5. 2 Principal coordinate analysis plot of unweighted pairwise UniFrac distances beta diversity showing clustering of bacterial groups (**A**). Four treatments were performed (white, negative control; light green, positive control; green, low thymol; dark green, high thymol concentration), plots for each treatment represent the pooled data of five sampling time points (0, 4, 8, 24 and 48). (**B**) Five-time points (white, zero time; light green, 4; grass green, 8; green, 24 and dark green 48 hours). The plot for each treatment represents the pooled data for four treatments (negative control, positive control, low thymol and high thymol concentration).

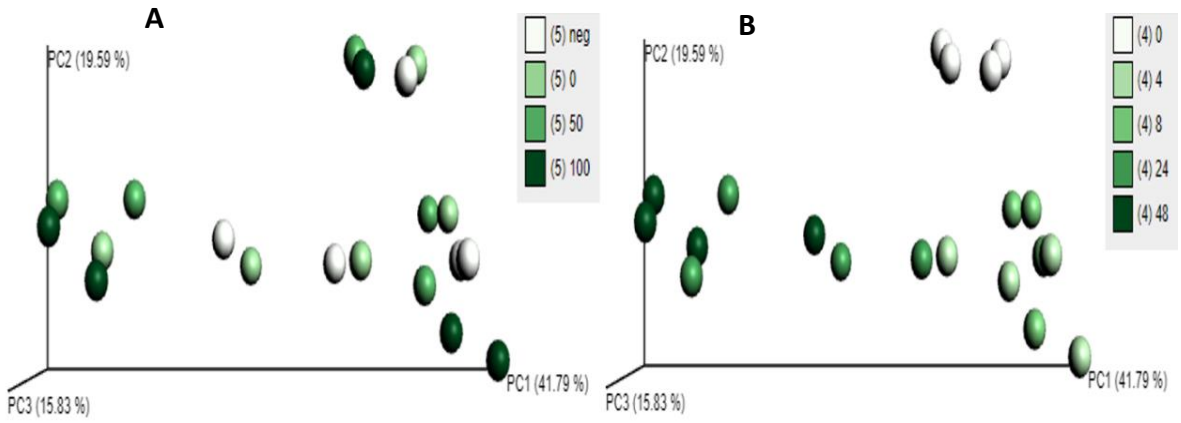

Figure 5. 3 Principal coordinate analysis plot of weighted pair-wise UniFrac distances beta diversity showing clustering of bacterial groups, (**A**) four treatments (white negative control, light green positive control, green low thymol and dark green high thymol concentration), the plot for each treatment represents the pooled data of five sampling time points (0, 4, 8, 24 and 48 hours). (**B**) Five timepoints (white zero time, light green 4, grass green 8, green 24 and dark green 48 hours). The plot for each treatment represents the pooled data for four segments (negative control, positive control, low thymol and high thymol concentration).

5.3.1.2 Phylum level changes of caecal bacteria

The total number of sequence reads defined by taxonomic classification of OTUs for each treatment at each time point was analysed and the composition determined at phylum, class, order, family and genus levels. Relative abundances at the phylum level (**Figure 5.4**) were classified into 4 phyla in all treatment groups, *Firmicutes* were the predominant phylum throughout all cultures regardless of treatment or time, representing ~87% at the start and increasing to 90% in thymol treatment, and 91% in both negative and positive control after 48 hours. The second most abundant phylum was *Tenericutes*, which started with ~11% in all treatments and after 48 hours had decreased in all treatments at differing percentages. The negative control was reduced to 7.3% followed by the positive control 8.2%, thymol 50 µg 8.8% and thymol 100 µg 9% respectively (data not shown). The third phylum was *Proteobacteria* which includes the family *Enterobacteriaceae* and the genus *E. coli*. We added an apparently thymol sensitive *E. coli* to the treatments to test the effects of thymol on selection. However, *Proteobacteria* was the only phylum level that was reduced over time, not only in treated cultures but all cultures declined by time 48 hours at different percentages depending

on the treatment **Figure 5.5A.** In addition, *E. coli* in each treatment was counted at all time point and the results show that the *E. coli* added to the system were detected and secondly that the number of detectable *E. coli* reduced over time, a feature associated with a closed system rather than the presence of thymol **Figure 5.5B**. It was clearly evident that the numbers of *Proteobacteria* declined very rapidly within the first 4 hours of incubation and this drop was statistically significant (p=0.0007) in all four samples at each time point were treated as replicas (n=4) and compared by Student t-test at time values of 4 hours. The base line was 0.8-1% in all cultures at the start of treatment incubation but over time there was a gradual decrease in all treatments. The thymol treated cultures decreased to 0.2% similar to the controls 0.2-0.3%. This indicated that static culture was unfavourable to Proteobacteria. The phylum *Bacteroidetes* showed few changes between treatments over time. At the start *Bacteroidetes* comprised 0.3% of OTUs and later decreased in all treatments over the time to 0.2%.

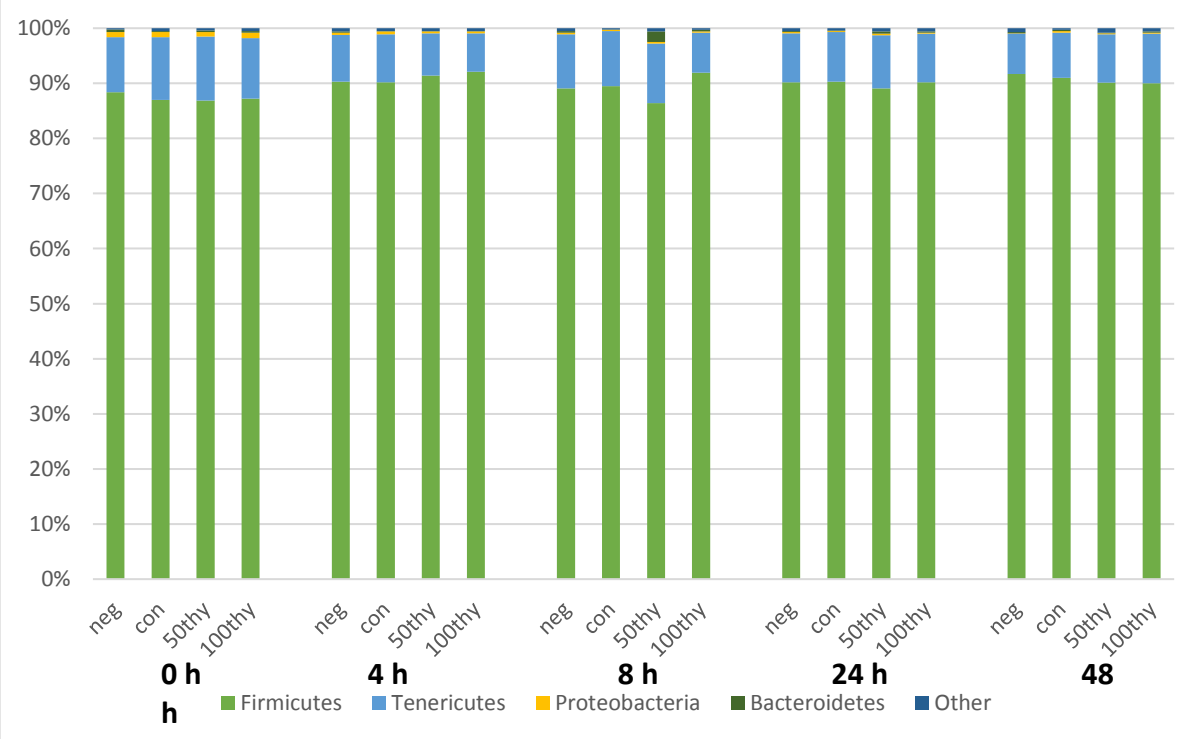

Figure 5. 4 Bacterial phyla distributions (relative abundances (%)) throughout 48-hours of incubation for each treatment in an in *vitro* closed culture fermentation inoculated with caecal isolates. (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) at all timepoint (0,4,8,24, and 48 hours).

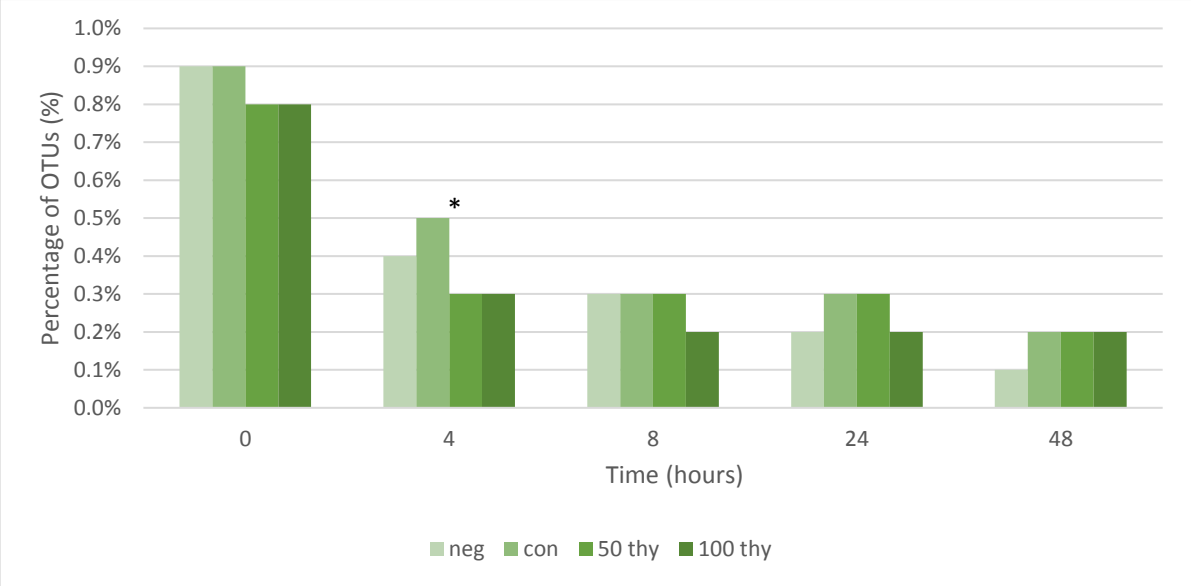

Figure 5. 5A Phylum *Proteobacteria* family *Enterobacteriaceae* relative abundance (%) in all treatments (neg=negative control, con= positive control, 50thy= 50µg l^{-1} thymol and 100thy= 100µg l^{-1} thymol) over times. * after 4 hours of incubation drop off was statistically significant (p=0.0007) in all four samples.

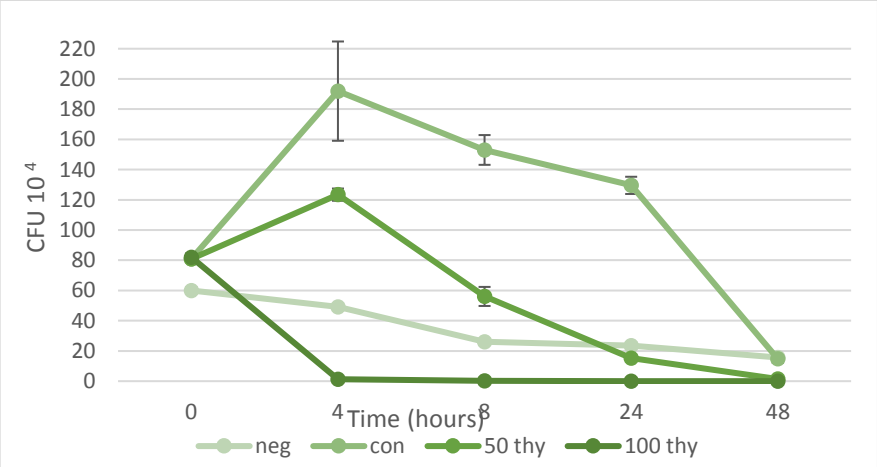

Figure 5.5B *E. coli* count in the different time point (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol).

5.3.1.3 Family level changes of caecal bacteria

The relative abundance at the family level within the phylum *Firmicutes* which was the most abundant group was interrogated. Differences between caecal culture treatments were evident at the family level, specifically for the most abundant phyla, the *Firmicutes* (**Figure 5.6**).

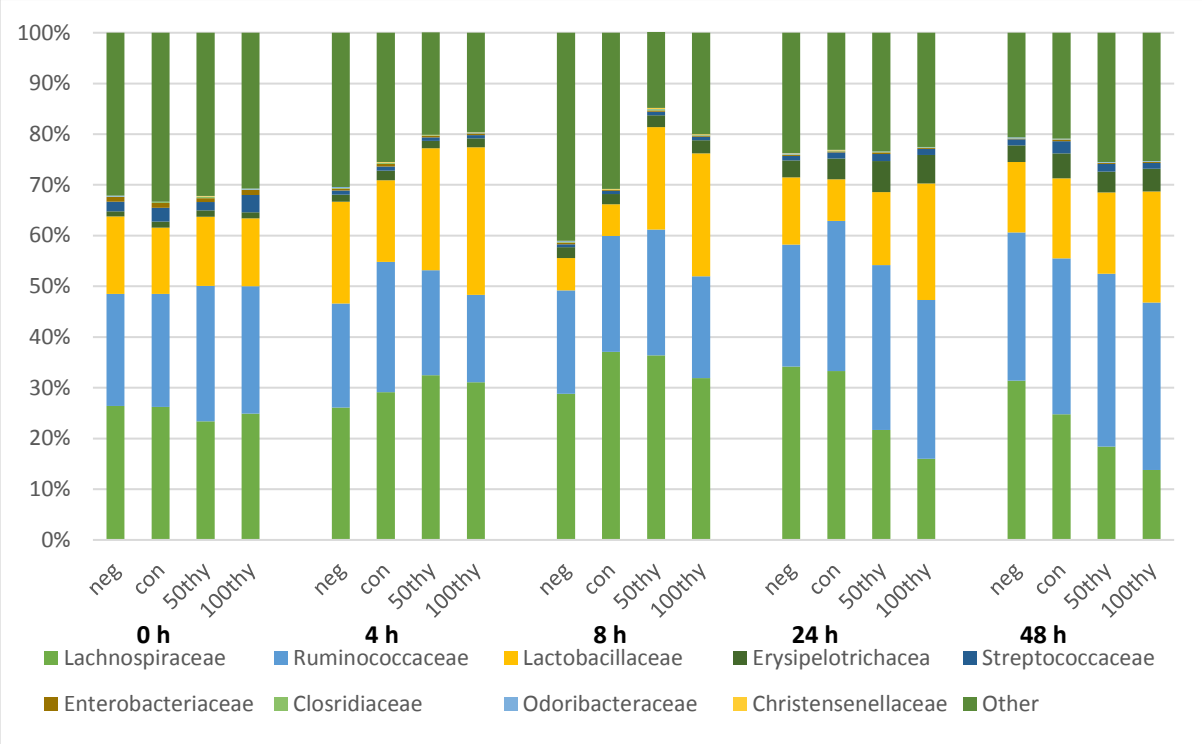

Figure 5. 6 Bacterial family distributions (relative abundances (%)) throughout 48-hours for each treatment in i*n vitro* closed culture fermentations inoculated with caecal isolates using QIIME v1.9.1. (neg=negative control, con= positive control, 50thy= 50µg l^{-1} thymol and 100thy= 100µg l^{-1} thymol) and all timepoint (0,4,8,24, and 48 hours).

The *Lachnospiraceae* was the most abundant family in all treatments at the start (t=0h) comprising 23.4-26% of OTUs and these showed an increase in abundance in all cultures up until 8 hours but thereafter the cultures treated with thymol showed a decline greater than that of either control (**Figure 5.7**). The graph shows a significant difference between the control and cultures treated with thymol at time points 24 and 48 h. By taking time points 24 and 48 hour for both treated groups (n=4), and similarly for the controls (n=4) and performing a Student t-test the resultant differences were significant for controls (p=0.05) and thymol treated (p=0.003).

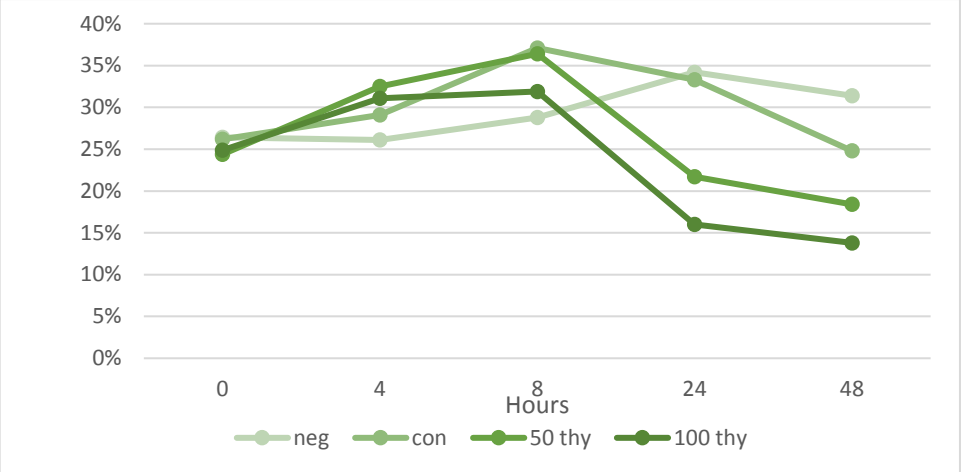

Figure 5. 7 Family *Lachnospiraceae* relative abundances (%), all treatments (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) over all time points.

The *Ruminococcaceae* at the start (time point 0h) were the second most abundant family (22% to 26%) followed by *Lactobacillaceae* (13% to 15%). Both of these families increased over time in all cultures indicating these groups of organisms were at a growth advantage in static anaerobic culture in VL medium irrespective of the presence or absence of thymol. Three families were found within the phylum *Firmicutes* had low abundance these were; *Erysipelotrichacea* (1% to 3.3%), *Streptococcaceae* (1% to 3%) and *Closidiaceae* (0.1% to 0.2%). *Enterobacteriaceae* family members from phylum *Proteobacteria* (0.1% to 1%) were mentioned above (**Figure 5.5**). *Odribacteraceae* member of phylum *Bacteroidetes* was the lowest abundant family (0.1% to 0.2%).

5.3.1.4 Genus level changes of caecal bacteria

The dominant genera were from the phylum *Firmicutes* **Figure 5.8**. The most prevalent genus was *Lactobacillus* of the *Lactobacillaceae* family comprising 13% -15% of the total at time point 0 h and these increased at different ratios between treatments by time 4 h, with the highest increase (29%) associated with high concentration of thymol **Figure 5.9**. By time point 8 h the percentage abundance decreased in all treatments but with distinctions between them: with thymol the abundance decreased by ~8% (p=0.03), but without thymol the abundance

decreased by 16% (p=0.007). However, there was no significant difference between any treatment group by time 48 h (p=0.23). This suggests a short-term impact of thymol that may be limited to the early stages of the fermentation.

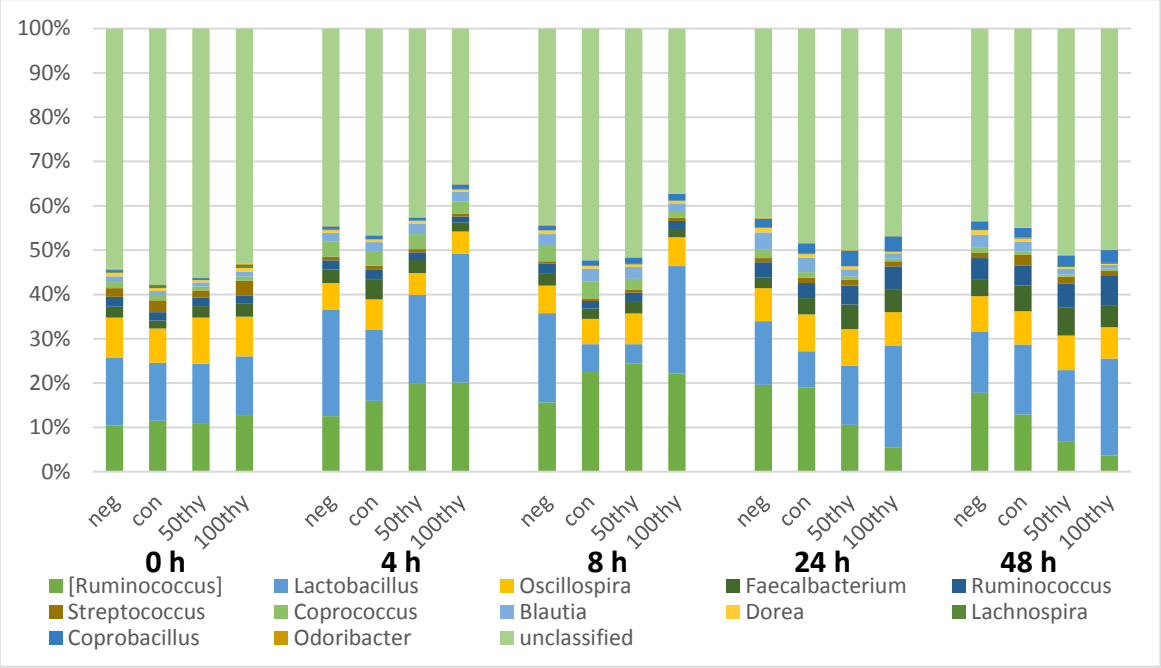

Figure 5. 8 Bacteria genus distributions (relative abundances (%)) throughout 48-hours for each treatment in vitro closed culture fermentations inoculated with cecal using QIIME v1.9.1. (neg=negative control, con=positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) and all timepoint (0,4,8,24, and 48 hours).

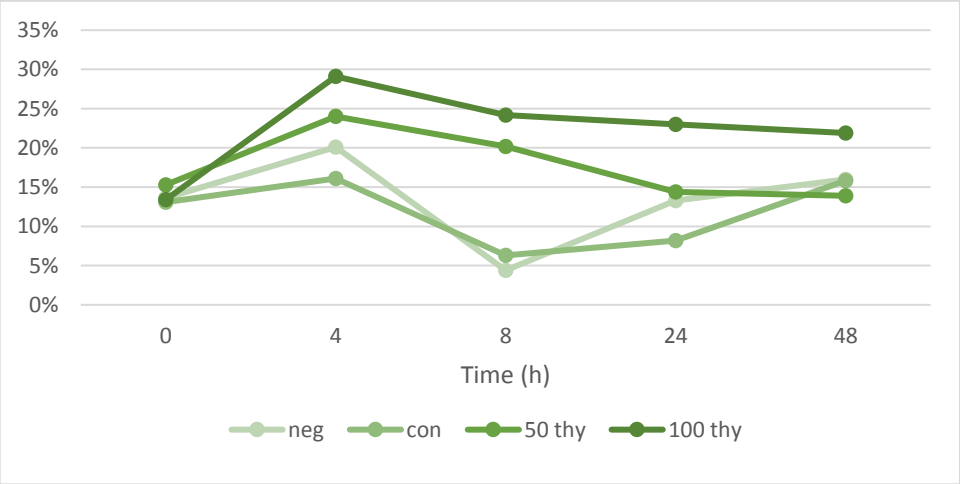

Figure 5. 9 Genus *Lactobacillus* of *Lactobacillaceae* family relative abundances (%), all treatment (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) over times.

Another genus with a high relative abundance (14.7%) was an unknown genus but according to 16S rRNA sequence homology, it was similar to *Ruminococcus* from the *Lachnospiraceae* family **Figure 5.10**. For all treatments, there was an increased abundance at the first 8h but thereafter all decreased. However, cultures treated with thymol declined more rapidly by time 48 h. These results show significant differences between cultures treated with thymol, and both control cultures at time 24 h. By taking the time points 24 and 48 hours for both treated groups (n=4) and similarly for the controls (n=4) and performing a Student t-test the differences shown were significant for controls (p=0.02) and thymol treated (p=0.008).

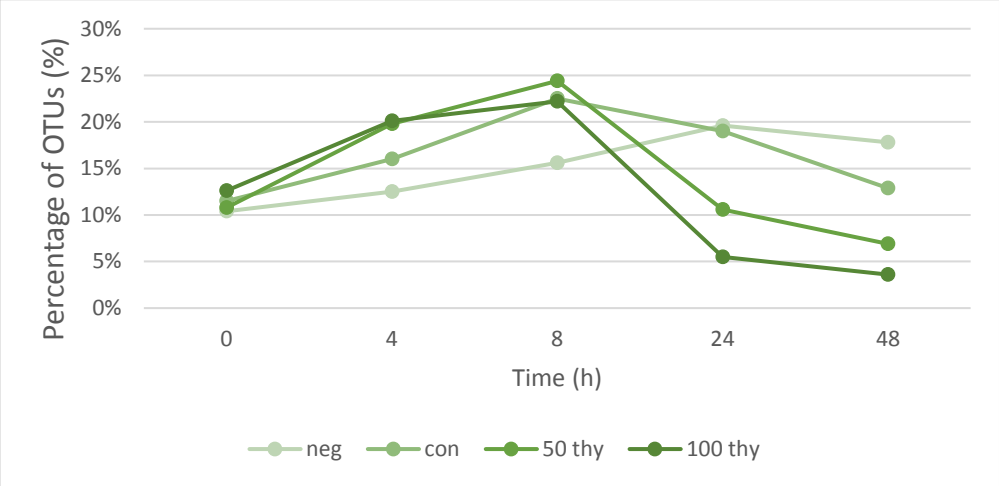

Figure 5. 10 Genus unknown [*Ruminococcus*] of *Lachnospiraceae* family relative abundances (%), all treatment (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) over times.

In addition, *Blautia*, *Dorea* and *Coprococcus* of the *Lachnospiraceae* family were decreased in relative abundances at 48 h in cultures treated with thymol, whereas both controls were increased at different levels over time. In more detail, *Blautia* showed an initial increase in abundance up to 8 h and thereafter decreased compared to controls (**Table 5.4**). The Student t-test was performed as previously described. *Dorea* reacted similarly although declined from time 0 h onwards. In both genera the culture with high concentration of thymol achieved the

lowest percentage abundance compared to controls **Figure 5.11**. However, *Coprococcus* was increased in all cultures at time 4 h but cultures treated with thymol were less abundant by 8 h. All cultures gradually had reduced abundances so that by 48 h there were no differences between the cultures **Figure 5.12**.

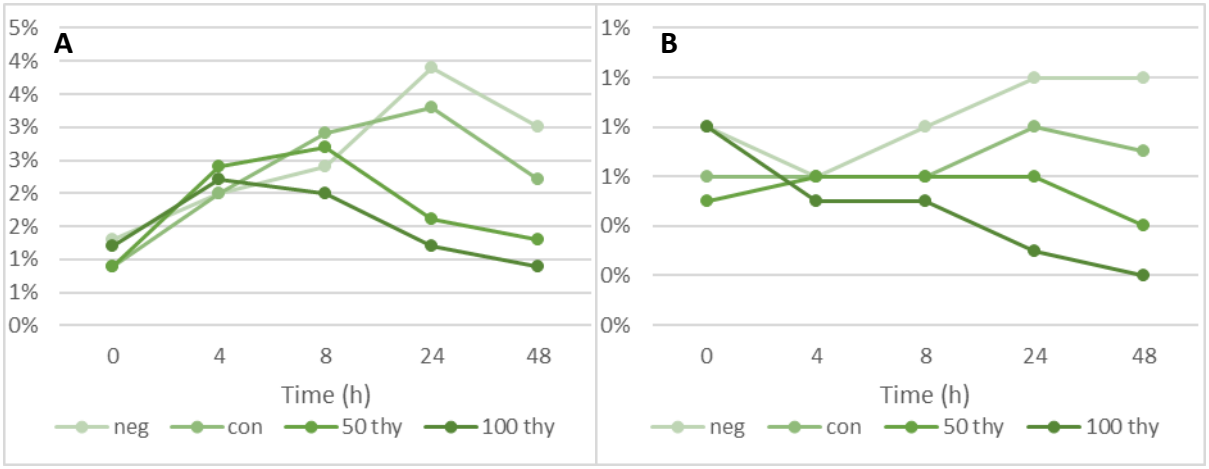

Figure 5. 11 Genus *Blautia* (A) and genus *Dorea* (B) of *Lachnospiraceae* family relative abundances (%), all treatments (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) over time points.

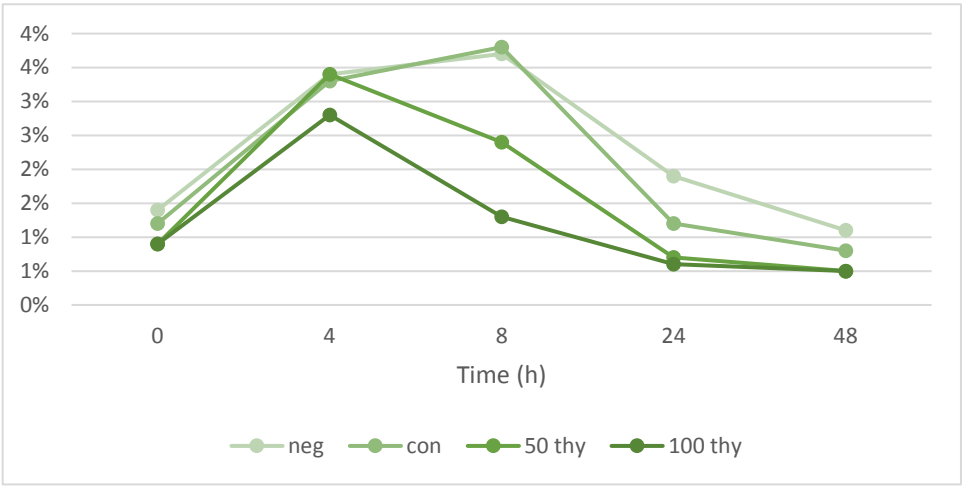

Figure 5. 12 Genus *Coprococcus* of *Lachnospiraceae* family relative abundances (%), all treatments (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) over time points.

Faecalbacterium and *Ruminococcus*, representatives of the *Ruminococcaceae* family, were stably persistent at between 2 and 3% abundance for the first 8 h **Figure 5.13**. Thereafter their abundances increased in all cultures, but it was differences between presence and absence of thymol: those cultures treated with thymol were higher than controls. By taking the time points 24 and 48 hour for both treated groups (n = 4) and similarly for the controls (n = 4) and performing Student t-test the differences shown were significant for controls (p= 0.05) and treated (p= 0.00006) in *Faecalbacterium* **Figure 5.13 (A)**. Whereas, *Ruminococcaceae* **Figure 5.13 (B)** the relative abundances in cultures treated with thymol were higher than controls (p= 0.01, 0.003) respectively.

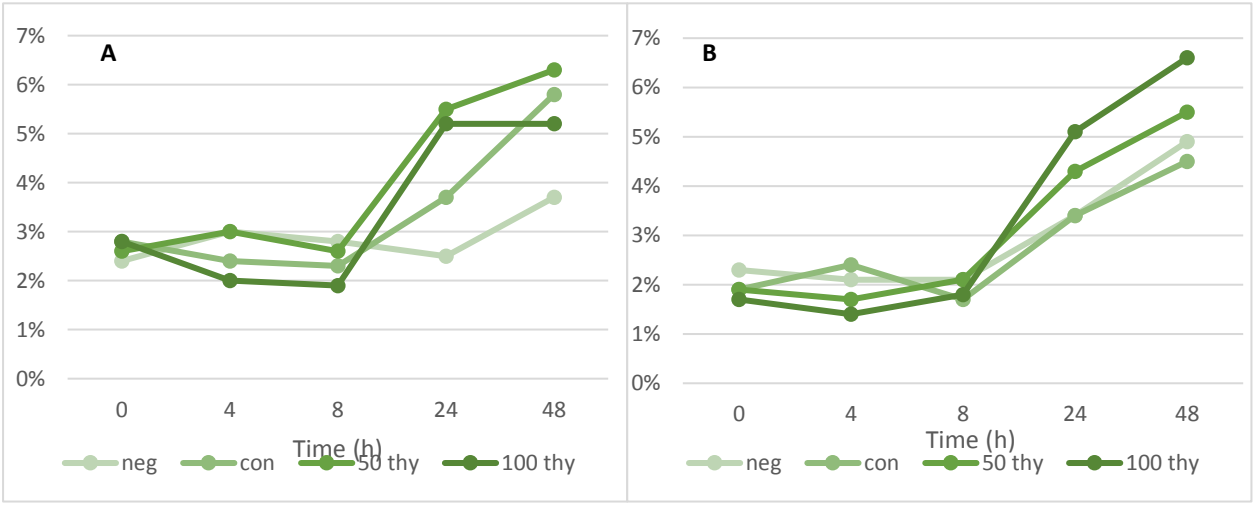

Figure 5. 13 Genus *Faecalbacterium* (A) and genus *Ruminococcus* (B) of *Ruminococcaceae* family relative abundances (%), all treatment (neg=negative control, con= positive control, 50thy= 50 µg l^{-1} thymol and 100thy= 100 µg l^{-1} thymol) over times.

Thymol influenced the abundance of the genus *Coprobacillus* that belongs to the *Erysipelotrichacea* family **Figure 5.14.** At baseline (t=0 h), all cultures were (0.3%) which gradually increased at time points 4 h and 8 h, followed by a significant difference observed after 24 h in which cultures treated with thymol had increased abundance (3.5%), higher than the controls (2%). The abundance in controls continued to increase whilst their abundance in thymol treated cultures decreased at time 48 h. A summary of the findings is presented in **Table 5.4**

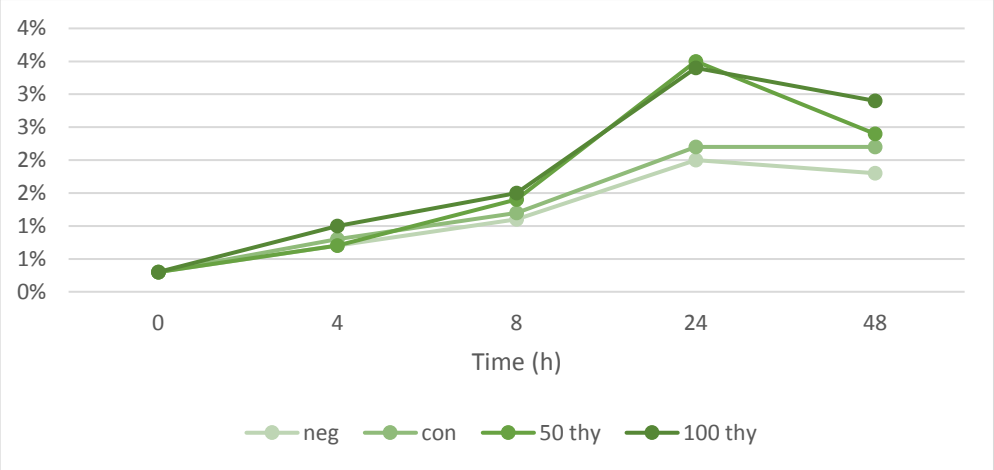

Figure 5. 14 Genus *Coprobacillus* of *Erysipelotrichacea* family family relative abundances (%), all treatments (neg=negative control, con= positive control, 50thy= 50µg l^{-1} thymol and 100thy= 100µg l^{-1} thymol) over time points recorded.

Table 5. 4 Genera that differed in relative abundance (% of total sequences) between the control and the treated with thymol. (Neg=negative control, con= positive control, low= 50 µg l^{-1} thymol and high= 100 µg l^{-1} thymol), * not significant.

Genera	Time point	After treatment	Controls		Thymol treatment	
			Neg- con %	P-values	Low-high %	P-values
Lactobacillus	8 h	↑	4 - 6	0.007	20 – 24	0.03
[Ruminococcus]	24 h	↓	19	0.02	10.6 – 5.5	0.008
Blautia,	24 h	↓	3 – 2.2	0.01	1.3 – 0.9	0.22 *
Dorea	24 h	↓	1 – 0.7	0.023	0.4 – 0.2	0.044
Coprococcus	24 h	↓	1.1 – 0.8	0.44 *	0.5	0.005
Faecalbacterium	24 h	↑	2.5 – 3.7	0.05	5.5 – 5.2	0.00006
Ruminococcaceae	24 h	↑	3.4	0.01	4.3 – 5.1	0.003
Coprobacillus	24 h	↑	1.8 – 2.2	0.0001	2.4 – 2.9	0.0009

5.3.2 Metabolic response to thymol by ^{1}H NMR

Anaerobic batch culture fermenters were inoculated with caecal samples from 6 chickens (15 days old). Four cultures were set up: two for controls (one with added *E. coli* and the other without), and two for a thymol treatment (50 & 100 µg l^{-1}). Metabolic profiles were characterised by ^{1}H-NMR spectroscopy. The experimental design consisted of a set of 16 NMR

spectra recorded from 4 cultures at 4 different time points (0, 8, 24 and 48 h) comprising the influence of sub-lethal concentration of thymol on the metabolic profile of caecal isolates at different time points. Whiles, time point 4h not included because limited of NMR samples. A characteristic NMR spectrum was acquired of the negative control fermented caecal isolate is displayed in **Figure 5.15**, with annotations of the identified metabolites. The assignment of spectral lines was performed using 1D NMR experiments, complemented with results from Chenomx software and Human Metabolome Database (http://www.hmdb.ca/).

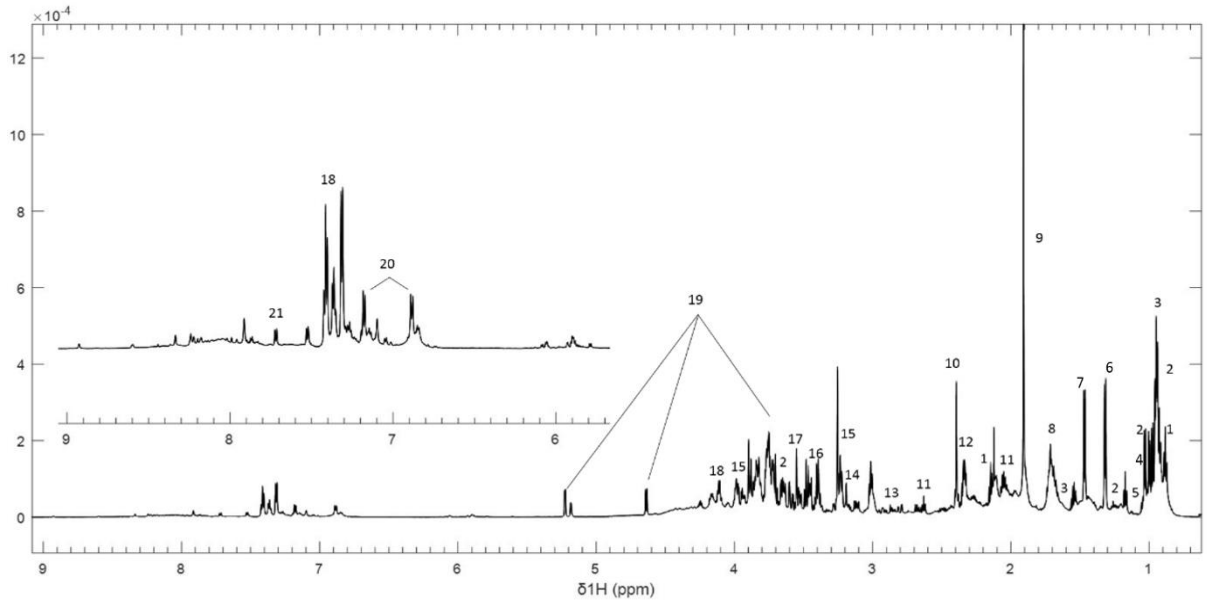

Figure 5. 15 A characteristic NMR spectrum acquired of the negative control fermented caecal, isolate with annotations on the identified metabolites:1. Butyrate, 2. Isoleucine, 3. Leucine, 4. Propionate, 5. Valine, 6. lactate, 7. Alanine, 8. Lysine, 9. Acetate, 10. Succinate, 11. Glutamate, 12. Glutamine, 13. Aspartate, 14. Choline, 15. Betaine, 16. Glycine, 17. Glycerol, 18. Phenylalanine, 19. Glucose, 20. Tyrosine, 21. Uracil

Orthogonal partial least squares (OPLS) class models examined the homogeneity among the samples associated with the first principal component of the metabolic profile resulted in an R2 and Q2 values of 66 % for the first two components **Figure. 5.16**. In these models, a clear separation was evident based on the different concentrations of thymol and controls within the time recorded; therefore, the results could suggest that different metabolic patterns were observed. However, one of the major differences between control and treatment

is the presence not only of thymol but ethanol which the thymol was dissolved. Both of these are small metabolites and would contribute to differences seen. Moreover, across different time points the results also show separation with slight overlapping between 8 h and 24 h especially between controls, so the samples were divided into groups depending on sampling time points.

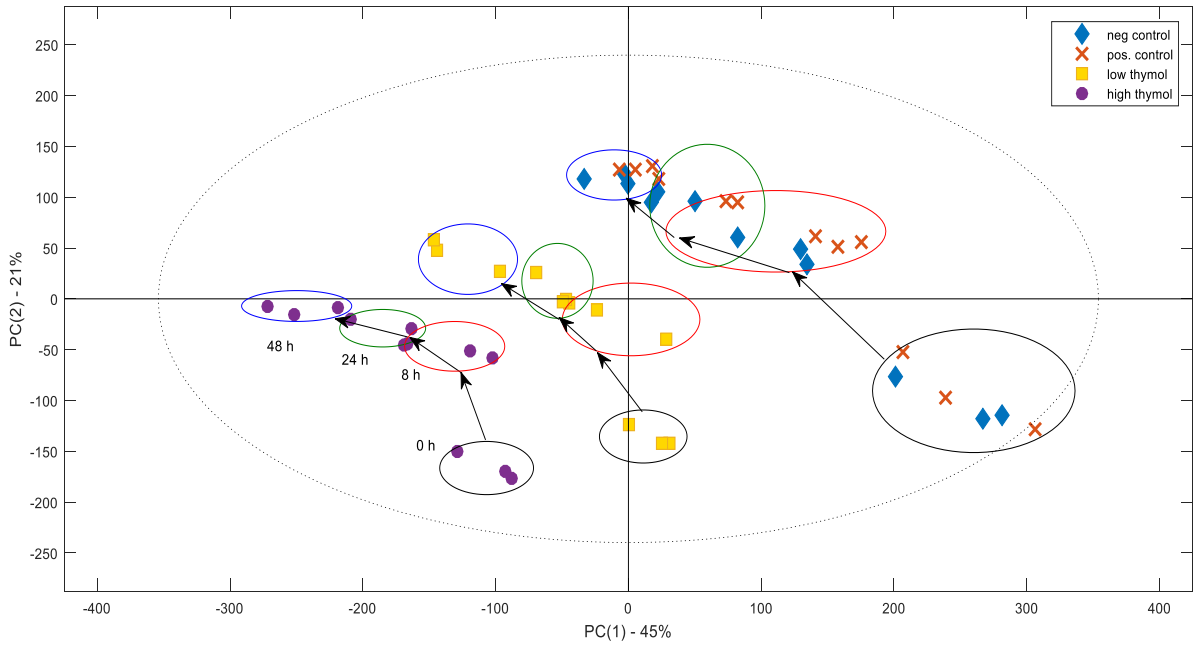

Figure 5. 16 OPLS - score plot for metabolic controls and treated with a two sub-lethal concentration of thymol (50 ug l^{-1} low thymol and 100 ug l^{-1} high thymol). N = 3 for each sample. (negative control; blue diamonds, positive control; orange cross, low thymol; yellow squares, high thymol; purple circles).

To identify metabolic variation based on correlation coefficient values between the samples, S-line plots' assignment of spectral lines over time between the different samples for all four-times points collected was applied to metabolic profiles shown in **Figure 5.17**. A colour code indicating the weights of the discriminatory variables, as the peak colour gradually changes from blue to red, the absolute value of correlation coefficient increases from 0 to 1, indicating the resonances were important for discriminating metabolite profiles of pairwise analyses. The result clearly differentiated the controls' samples from the thymol treated by ethanol peaks and the different component concentrations between them. Moreover, the metabolic variation at time 8 and 24 h showed very close concentrations in metabolites (F**igure 5.17**).

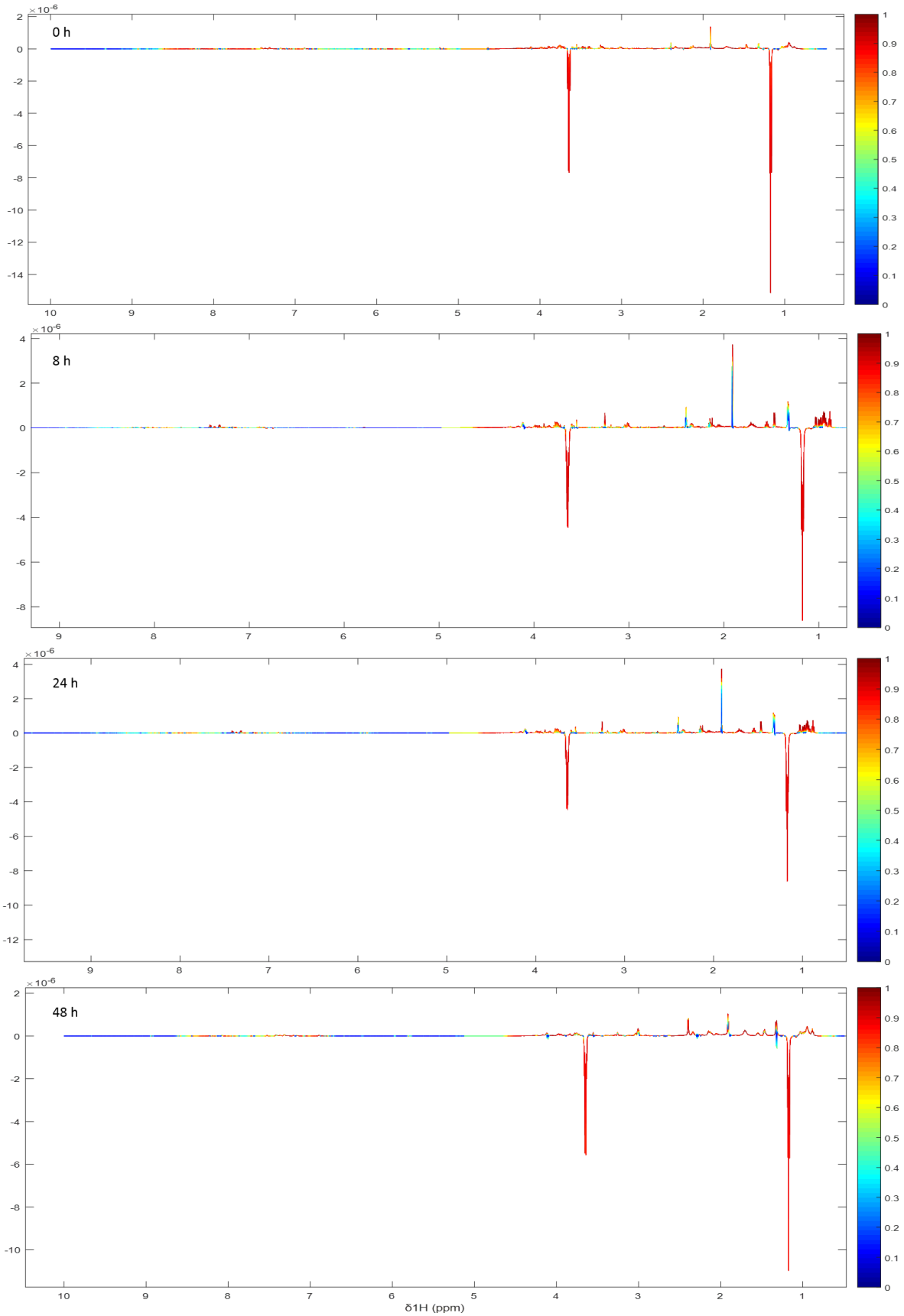

Figure 5. 17 S-line plot show the metabolites differentiation between ^{1}H NMR spectra of controls (top) and treated with thymol (bottom) caecal cultures over time.

An OPLS plot clearly discriminated the control samples from those samples treated with thymol samples along the first principal component at time point 8 h (**Figure 5.18**). Caecal batch cultures contained greater amounts of amino acids, such as leucine, isoleucine, valine, alanine, lysine, tyrosine, betaine and phenylalanine, which were increased in control samples than cultures treated with thymol. Butyrate, propionate and glutamate were also present in control cultures. However, fumarate was the only one detected in cultures treated with thymol. **Figure 5.19** shows S-line plot for time 8h indicating the key metabolites that contributed to this discrimination. However, lactate, acetate and succinate were produced in both cultures with an insignificant increase in control cultures. Fumarate was present in thymol treated cultures alongside ethanol which is explained by the dissolution of thymol in ethanol. All amino acids and short fatty acids metabolites were decreased over time (48 h) in the cultures (**Figure 5.20**), but phenylalanine and propionate were produced more in thymol treated cultures.

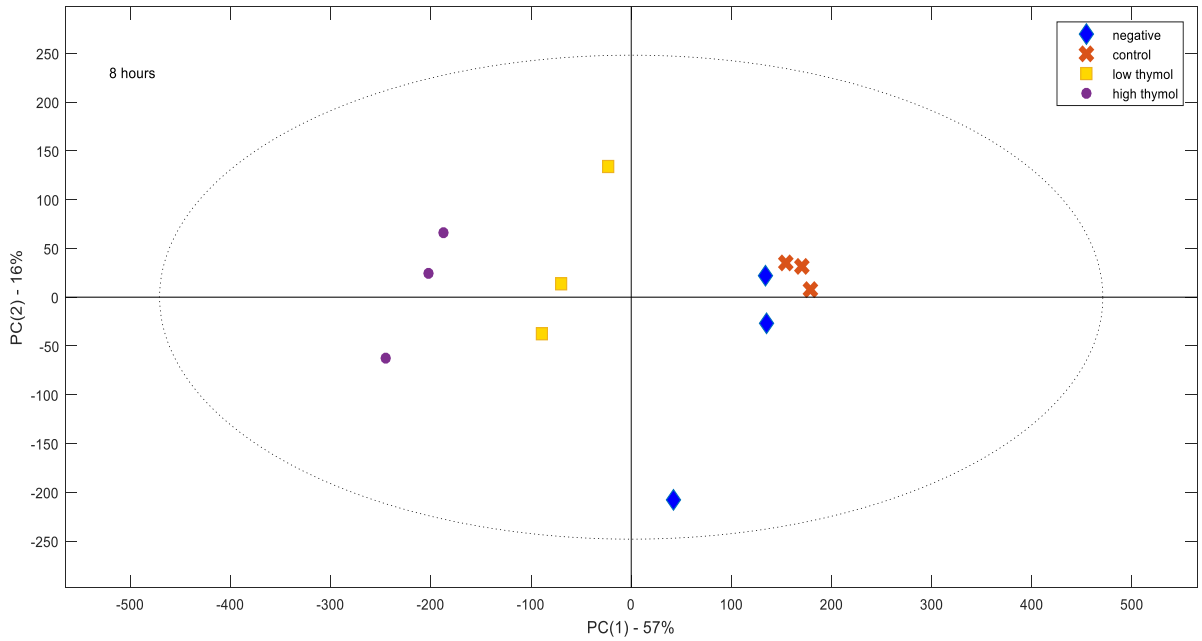

Figure 5. 18 OPLS plot (*n* = 12; R2X= 0.55; R2Y= 0.84; and Q2= 0.77) discriminating the controls samples from the samples treated with thymol.

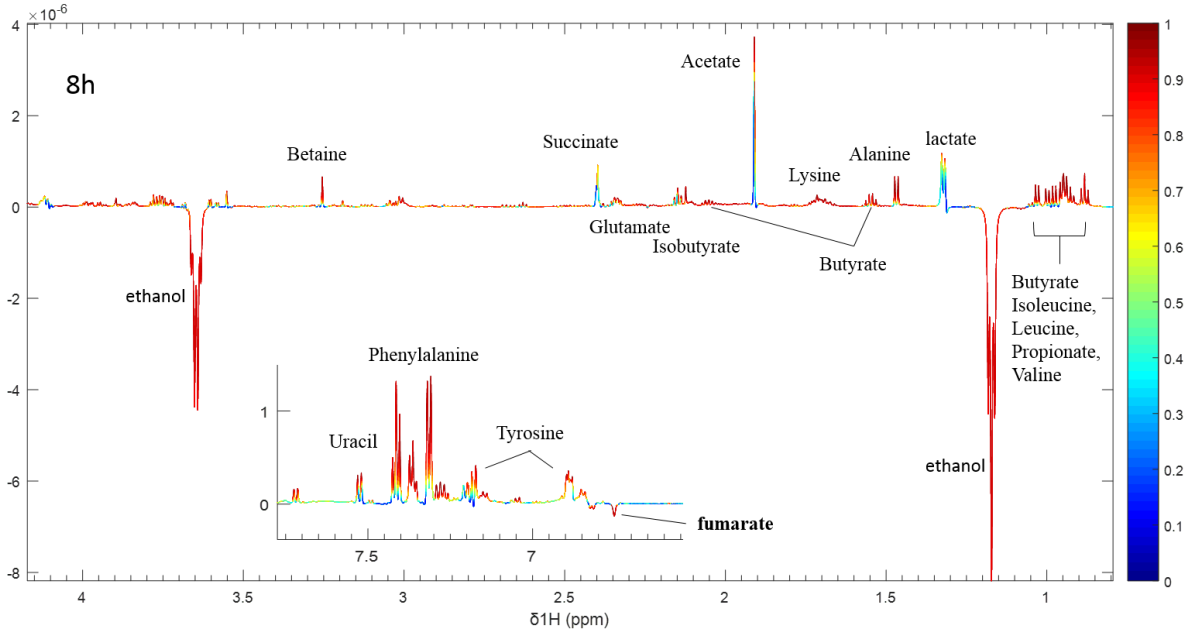

Figure 5. 19 S-line plot highlighting the most important metabolites for the differentiation between ^{1}H NMR spectra of controls (top) and treated with thymol (bottom) at time point 8h.

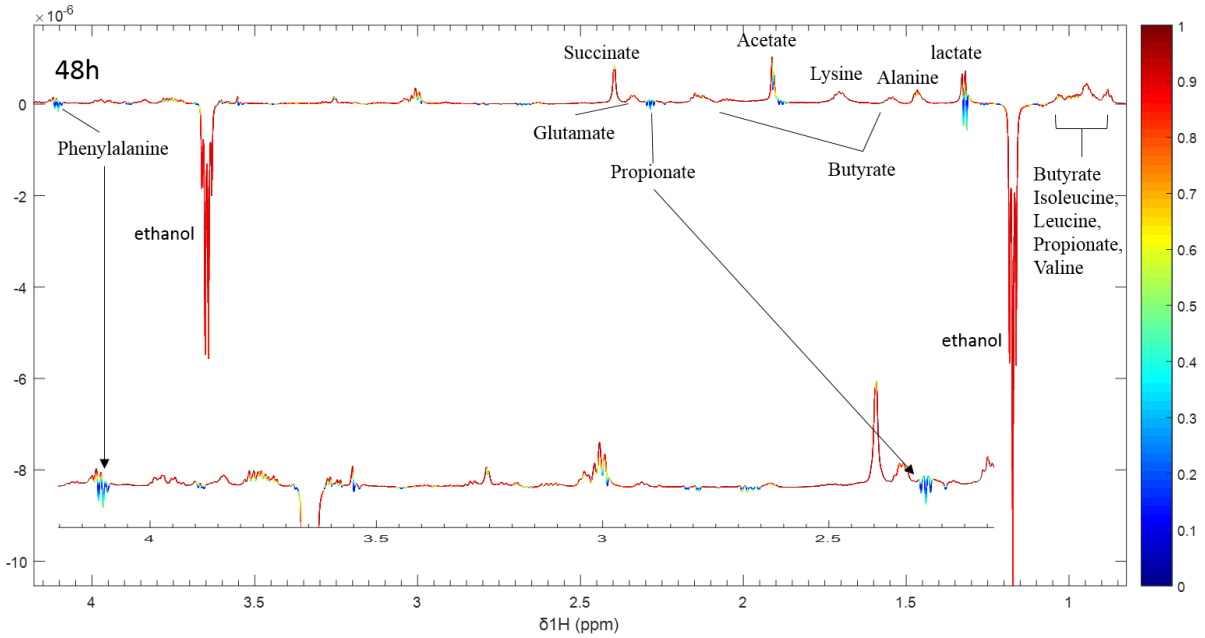

Figure 5. 20 S-line plot highlighting the most important metabolites for the differentiation between ^{1}H NMR spectra of controls (top) and treated with thymol (bottom) at time point 48h

5.4 Discussion

The present *in vitro* study assessed the impact of thymol on the composition and activity of caecal associated gut microbiota. A simple static gut model, an *in vitro* batch culture fermentation assembly was used, which was a closed system maintained under anaerobic conditions. Duenas and others suggested these static gut models are suitable for simulating short-term conditions in the gut microbiota by dietary phenolic compounds (Duenas *et al.*, 2015). However, it is essential that interpretation of the data takes into account a number of factors. Firstly, the inoculum may not represent all the gut microbiota, especially oxygen sensitive anaerobes due to the inevitable exposure to oxygen during manipulations and the time taken to prime the *in vitro* system. Secondly, there was no flow-through of fresh nutrients to represent the flow of the digesta in the gut. Thirdly, there were no host factors, such as antibodies and mucin, other than what was in the digesta used to prime the system. Fourthly, there was no selective removal of metabolites such as butyrate by host epithelial cells for example. Given these limitations it is advised to consider the changes with caution but as suggested by Duenas et al., (2015) the responses observed in the short term are possibly more relevant and potentially representative of what might happen *in vivo*.

Molecular technologies based on the 16S rRNA sequences have developed the field of gut microbial ecology. Next generation DNA sequencing allows culture-independent methods to determine the gut microbiome (Turnbaugh and Gordon, 2009) and can be a powerful tool to monitor gut community shifts induced by polyphenols (Andersson *et al.*, 2008). Here, the effects of thymol on the taxonomic distribution as predicted from the 16S analysis were observed at phylum, family and genus/species level. The first point to make is that the profile of the starting populations in these studies (t=0h) showed a distribution of Phyla/Families/Genera consistent with those reported by many other authors (Wei *et al.*, 2013;

Sergeant *et al.*, 2014; Oakley *et al.*, 2014; Awad *et al.*, 2016). In this study the caecal content was taken from 6 chickens of 15 days of age, and the results showed that the chicken caecal content is dominated by the phylum *Firmicutes*, with lower proportions of *Proteobacteria*, *Bacteroidetes* and *Tenericutes*, in both controls and those treated with thymol at t=0 h.

As a generalisation, in all treatments, *Firmicutes* were the predominant phylum and increased with time, while other phyla (*Proteobacteria*, *Tenericutes* and *Bacterioidetes*) tended to decrease. On the other hand, the composition of the gut microbiota can be affected by several factors, such as diet, environment, treatment, feed additive, type of chicken and age which is perhaps the most significant (Qu *et al.*, 2008; Danzeisen *et al.*, 2011). The reduction of *Bacteroidetes* in these static cultures may not reflect the maturation process in the chicken as it is recognised the abundance of *Bacteroidetes* does in fact increase to become, often, the second most prevalent family with age (Awad *et al.*, 2016; Ranjitkar *et al.*, 2016). In the current study, the phylum *Proteobacteria* which includes the family *Enterobacteriaceae* and the genus *E. coli*, declined over time in all cultures. *E. coli*, a facultative anaerobic bacterium, is a dominant species in the gut in the early stages of chicken development. Therefore, a reduction of *E. coli* during the second week of life could be attributed to the dominance of anaerobes (Zhu and Joerger, 2003). Our results did not show a relative abundance of *E. coli* that may reflect the age of the chicken used in this experiment: the digesta was from 15-day old chicks. Brown and colleagues reported that members of the phylum *Firmicutes* can inhibit the growth of opportunistic pathogens, such as *E. coli*, (Brown *et al.*, 2012). Given the high abundance of *Firmicutes* it would seem logical to assume the rapid decline of *E. coli* and other *Enterobacteriaceae* was due to the pressure of competing with *Firmicutes* in this environment. The question arises as to whether the addition of thymol had any additive effect on the reduction: the data shown in **figure 5.5** were equivocal suggesting this is not the case. This is

entirely plausible as the thymol MIC of the *E. coli* tested (and the strain added to one of the controls) were all well above the level of thymol added to the system.

One of the 'control' systems was primed not only with 15 day old chicken caecal digesta but also a well characterised *E. coli* isolate (strain 18). Even with this additional source of *E. coli* their number reduced rapidly. This finding re-enforces the concept that the system and micro-organisms within it were very effective at competing against this additional *E. coli*, a well-defined pathogenic type. It is argued that this hostile environment would militate against the survival and/or persistence of *E. coli* without host tissue on which to adhere. As for all other findings, it would be useful to study the impact of thymol *in vivo*.

The genera *Dorea*, *Blautia* and *Coprococcus* all decreased with in the presence of thymol by t=48 h although at different rates than other bacteria. A key point here is that little is known of the role of these bacteria in the chicken. *Dorea* found in high abundance is associated with colorectal cancer in humans along with other bacteria, and some of which may be candidate drivers of the colorectal cancer pathway in the proximal colon (Peters *et al.*, 2016). Shen and co-authors showed that colorectal cancer was associated with increased *Dorea* spp. ($p < 0.005$) and *Coprococcus spp.* ($p < 0.05$) in colorectal cancer cases (Shen *et al.*, 2010). Their role in the chicken in unknown but the observation made in this study showed the presence of thymol was associated with a decrease of both. Is this potentially beneficial? *Dorea* and *Blautia*, express spore proteins and *Blautia* preferentially expresses enzymes for propionate production pathways rather than butyrate biosynthesis (Polansky *et al.*, 2015). Butyrate is a known colorectal cancer chemo-preventive and possibly therapeutic short-chain fatty acid in humans (Encarnacao *et al.*, 2015). Moreover, in a study done by (Org *et al.*, 2017) it was shown that fasting serum levels of glycerol, monounsaturated fatty acids, and saturated fatty acids are strongly associated with increased abundance of *Blautia* and *Dorea* and

decreased abundance of *Coprococcus.* Also, an increase in abundance of *Blautia* was shown in individuals with high BMI levels (Org *et al.*, 2017), which supports the previous data from (Kasai *et al.*, 2015) who showed the *Blautia* genus is associated with obesity. Furthermore, a recent study found an association between *Blautia* and human genetic variants in a genomic region that has been associated with obesity and BMI (Bonder *et al.*, 2016). On the other hand, increased abundance of commensal bacteria belonging to the *Blautia* genus is associated with reduced lethal graft-versus-host disease (GVHD) and improved overall survival (Jenq *et al.*, 2015). The questions raised for the chicken relate to the role of these bacteria in the health and nutrition of the chick. Is it possible that manipulation of these bacteria may influence fat metabolism? Whilst highly speculative the drive toward healthy meat products with low fat and low cholesterol is a current focus. Indeed, the studies of Le Roy et al., (2017) showed that chickens treated with an antibiotic (Tiamulin) produced high levels of fat and very low density lipo-proteins (Le Roy *et al.*, 2017). Thus, replacing this antibiotic with EOs may be beneficial not only for reducing antibiotic use but also having a positive nutritional impact if such changes are associated with reduction of the bacterial genera discussed above. Again, *in vivo* studies are required to assess and confirm efficacy.

The *Lachnospiraceae* and *Ruminococcaceae* are two of the most abundant families from the order *Clostridiales* found in the gut environment. One important characteristic of *Lachnospiraceae* and *Ruminococcaceae* families is the ability to produce butyrate by utilising complex plant derived carbohydrates as energy sources (Biddle *et al.*, 2013). Butyrate is a short chain fatty acid (SCFA) that has been associated with the maintenance of gut health (Berni Canani *et al.*, 2012) and is anti-inflammatory (Van Immerseel *et al.*, 2010; Celasco *et al.*, 2014). Butyrate provides energy to colonic mucosa (Roediger, 1980) and thus has important implications on intestinal health (Hamer *et al.*, 2008). *Faecalibacterium* was assigned to the known butyrate-producing genera (Duncan *et al.*, 2002; Louis and Flint, 2009). Also, increased

caecal richness in *Ruminococcus* which produces cellulose degrading enzymes as well (Morrison and Miron, 2000) indicates possible synergy between butyrate producing species and *Ruminococcus* (Pryde *et al.*, 2002). Thymol treatment was associated with the increased relative abundance of butyrate-producing species such as *Faecalibacterium* and *Ruminococcus*. If the link between thymol and those genera can be confirmed *in vivo*, this would be highly useful novel information. In previous studies, the effect on the intestinal microbiota after introducing grape pomace extracts in the diet of broiler chicken, found that the cecum had higher populations of *E. coli*, *Lactobacillus,* and *Enterococcus* species than the birds in any other treatment group (Viveros *et al.*, 2011). They concluded that grape polyphenol-rich products modified the gut morphology and intestinal microbiota and increased the biodiversity degree of intestinal bacteria in broiler chicken. This finding is contrary to the data produced in this study suggesting further work regarding *E. coli* is required *in vivo*. Also, the grape seed polyphenols diet in pig has been shown to shift the microbiome, dramatically increasing *Lachnospiraceae, Clostridales, Lactobacillus* and *Ruminococcacceae*. Moreover, the relationship between dietary and colon health may be attributable to the altered bacterial populations or phenolic compounds in the colon (Choy *et al.*, 2014). Another study used a blend of EOs including thymol (15g/tonne) and assessed its effect on the performance and intestinal microbiota of broilers by quantitive PCR measurements of caecal microbes, and their results showed higher percentage of *Lactobacillus* and *E. coli* in the EOs group compared to the control group at 41 days (Tiihonen *et al.*, 2010). In addition, other studies have shown *Lactobacillus* counts were increased by dietary EO (Du *et al.*, 2015; Jamroz *et al.*, 2005). The findings in this study are thymol was associated with an increase in *Lactobacillus* species by 4 h followed by a modest decrease by 48 h. Further work, and preferably *in vivo* studies, is needed to tease out the impact of EOs on *Lactobacillus* species especially so as they are known

probiotics used to reduce the colonisation of pathogenic bacteria in food animals, and therefore reduce the risk of food borne illness to consumers (Neal-McKinney *et al.*, 2012).

Thymol treatment was associated with the gradual increase in the abundance of *Coprobacillus* (of the *Erysipelotrichacea* family) beyond 24 h. Little is known about *Coprobacillus* but in a previous study in mice, increased amounts of bacteria of the *Erysipelotrichi* family were found in a mucus layer impenetrable to other bacteria (Jakobsson *et al.*, 2015). In another study, this type of bacterium decreased with sessile serrated adenoma (SSA) cases in humans (Peters *et al.*, 2016). From these few reports, these bacteria have been suggested to have beneficial effects possibly enhancing mucus production (Everard *et al.*, 2014). The findings in the study reported here suggest *Coprobacillus* increases in the presence of thymol and if the properties attributed to these bacteria from human and mouse studies are accurate, these bacteria may be beneficial in enhancing gut health through mucus secretion: very speculative and of course needs to be tested *in vivo*.

The antimicrobial effect of thymol treatment appears to be selective for certain microbes, as indicated previously low concentrations of thymol are associated with decreased growth of some bacteria however appear beneficial to bacteria such as *Faecalbacterium* and *Ruminococcus* and *Lactobacillus*. This finding is similar to (Ouwehand *et al.*, 2010) as they reported *in vitro* work using thymol at the level of 50 mg/l decreased the growth of *E. coli*, however the beneficial *Bifidobacterium* and *Lactobacillus* were not affected to the same degree. *Salmonella typhimurium* was also reported to be more sensitive to thymol as well (Mith *et al.*, 2014). Consuming thymol in chicken diets may be an appropriate treatment to improve the chicken gut microbiota and the microbial fermentation profiles of broiler chicken (Helander *et al.*, 1998). The reviews by Rehman and others suggested that the Guanine-Cytosine ratio of the total genomic DNA content of the caecal microbiota was unaffected by EO treatment

(Rehman *et al.*, 2007). This is a relatively crude measure of the population that may disguise some shifts. However, the findings of this work suggest Proteobacteria decline dramatically but this may be due to pressure from the other microbiota and/or the type of environment of the static culture but not necessarily thymol. Many of the effects seen were statistically significant from 24 h onwards but we must return to the valid point made by Duenas et al., (2015) who indicated static cultures may only be relevant for the first few hours or so. It could be argued that the shifts seen at 24 and 48 h are more related to adaptation to the static mixed culture than relevant to the chicken gut where transit times are measured in a few hours. The major exception is the caecum where fermentation is often for 24h or more. The findings in this study generate testable hypotheses to test what population shifts are observed in the caecum of the live animal and whether they correlate with those observed in static mixed culture.

The different components of the metabolic profiles were clearly distinct between the different concentrations of thymol treatments and controls at all time points. At 8 h the result indicates that a significant increase of mostly SCFA and amino acids were in the controls cultures. So, this distinction may be caused by thymol affecting the amount and rate of production of these metabolites. The amount and rate of SCFA production depends on the site of fermentation also the composition and density of the gut microbiota in combination with the type of nutrients available for microbial fermentation (Macfarlane *et al.*, 2006). As mentioned in a previous chapter, the response of *E. coli* exposed to thymol is a slowed growth rate associated with a shift from respiration to fermentation as indicated by detecting increasing lactate concentrations (Fitzgerald *et al.*, 2004). Therefore, thymol may affect the rate of growth of many other members of the gut microbiota. All variations in SCFA and amino acids such as butyrate and alanine concentrations could be indicative of changes in energy production due to thymol present in culture that could also be related to the observed variations in TCA/Krebs cycle intermediates (Liu *et al.*, 2012). Moreover, the presence of ethanol in high levels in

thymol treated cultures can be discounted. Ethanol was used to dissolve the thymol and is therefore an artefact of the system. Sadly, this mask any changes in ethanol production by the microbiota.

Lactate was already present in similar amounts in all cultures, as well succinate and acetate at time 8 h. Both lactate and succinate are intermediates in the global fermentation process and are to varying extents metabolised to SCFA by cross-feeding species (Belenguer *et al.*, 2007; Macfarlane and Macfarlane, 2012). Lactate is the major fermentation product of lactic acid bacteria, such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* (Barcenilla *et al.*, 2000), which was consistent with NGS results showing increased *Lactobacillus* in thymol treatment most notably in the first 4 h of incubation. In addition, lactate tends to reduce residual pH more than SCFA (Rinttila and Apajalathi, 2013). Succinate is an end-product of some *Bacteroidetes* under some environmental conditions (Rautio *et al.*, 2003; Macfarlane *et al.*, 2006). The presence of thymol may induce those environmental conditions necessary for succinate production by *Bacteroidetes*. In addition, acetate was found in similar proportions in both controls and thymol treated cultures despite thymol slowing bacterial growth. Acetate is the most abundant SCFA, the high acetate content could be attributed to exogenous mechanisms such as bacterial fermentation in the digestive tract (Louis *et al.*, 2014). It is assessed that more than 200 bacterial species are contained in the avian gut (Wallace *et al.*, 2010). Acetogens are a group of obligate anaerobic bacteria related to the genera *Ruminococcus* (Drake *et al.*, 2008), which were increased in cultures treated with thymol based on NGS findings. Fumarate was the only SCFA found in thymol treatment cultures, which is an early product of the anaerobic electron transport chain. Anaerobic electron transport chain starts with the carboxylation of phosphoenolpyruvate and the resulting oxaloacetate is reduced to fumarate. When the partial pressure of CO_2 is low, succinate, the product of fumarate reductase,

is converted into methylmalonate, which is cleaved into propionate and CO_2 (Macy *et al.*, 1978; Macy and Probst, 1979). Propionate was increased over the time in our study.

Propionate was found more in control cultures at time 8 h than thymol treated systems although by time 48 h the amount of propionate was increased in the thymol treatment culture. Similar findings were noted for phenylalanine as well. To produce propionate there are different pathways, it is mostly formed through the succinate pathway by *Bacteroidetes* and some *Firmicutes* (Louis *et al.*, 2014; Salonen *et al.*, 2014). *Bacteroides* spp. can also convert lactate to propionate (Flint *et al.*, 2012). Therefore, the relative abundance of propionate was probably associated with *Bacteroidetes*, which can produce propionate from succinate and/or lactate pathways. Propionate has potential health-promoting effects that include anti-lipogenic, anti-inflammatory and anti-carcinogenic actions albeit to lesser extents than Butyrate (Jan *et al.*, 2002; Hosseini *et al.*, 2011). Moreover, propionate inhibits stimuli-induced expression of adhesion molecules (Vinolo *et al.*, 2011). Phenylalanine is produced from bacterial degradation of aromatic amino acids with bacterial metabolism of phenylalanine producing derivatives such as phenylpyruvate and phenylacetate (Windey *et al.*, 2012). Intestinal bacteria that are involved in these processes include *Bacteroides*, *Enterobacteria*, and *Lactobacillius* (Fuchs *et al.*, 2014; Botsford and Demoss, 1972; Hughes *et al.*, 2000).

The current study shows that supplementation with thymol exerts a positive effect on intestinal microbiota with a concomitant enhancement in growth performance, which might be related to changes in intestinal microbiota. More specific studies are required to improve luminal availability of thymol, and clarify how it affects intestinal microbiota *in vivo*. However, it is essential that caution be advised that static gut models are suitable for simulating short-term conditions in the gut microbiota by dietary phenolic compounds (Duenas *et al.*, 2015). The question is how short term? Many effects were observed in the first 8h of incubation and that may more readily reflect the impact in the gut. Changes later between 24h and 48h may be

purely artefacts of extending incubation in an inappropriate system for too long. That said, what has been learnt here is that there are bacteria that thrive in the model in the presence of thymol and many of these may be probiotic in nature.

CHAPTER 6: GENERAL DISCUSSION

6.1 Discussion

There is much debate regarding the definition of commensal, opportunistic and pathogenic *E. coli*. Whilst some types can be defined as pathogens such as *E. coli* O157:H7 carrying the Locus of Enterocyte Effacement (LEE) and the Verocytoxins (*stx1*, *stx2*), the clear distinction between commensals and pathogens in avian hosts remains unclear (Johnson *et al.*, 2012; Nakazato *et al.*, 2009). This study used a range of tests that were selected for being applicable to routine and diagnostic laboratories, as it was reasoned that is where the differentiation is most needed for clinical purposes. Considerable diversity both, phenotypic and genotypic, was observed even though analysis was of a relatively small number of isolates. In terms of genotypic diversity, most *E. coli* were potentially pathogenic as most carried multiple virulence determinants: the Nolan laboratory often quotes the presence of five or more as evidence of being a pathogen (Johnson *et al.*, 2008a). Known APEC tended to encode more virulence determinants than presumptive commensals. The problem for this study was the use of healthy poultry (either chicken or turkey) that may carry both true commensals, if such a type exists, and pathogenic types that were not associated with disease. Thus, the pool of presumptive commensals is likely to be a mixture of both types. However, the genetic analysis based on ERIC-PCR clearly separated APEC from healthy turkey and chicken isolates, which also generally possessed less virulence determinants, whereas the presumptive commensals from chicken seemed to share similarities with turkey isolates and APEC. In a recent study, *E. coli* isolates from chickens showed 100% genetic identity between isolates made from pathological lesions and healthy chicken in the same flocks (Paudel *et al.*, 2016). This may be explained by the transition of a pathogen resident in the gut, perhaps behaving as non-harmful or a commensal, until stress or primary infection permits expression of pathogenic capability. Other reports do not support this finding but rather point to a high heterogeneity of *E. coli*

isolates in chicken (Pasquali *et al.*, 2015; Kemmett *et al.*, 2013). The findings here indicate that isolates associated with healthy chickens represented genuine commensals at the time of isolation without inducing fulminant disease. Some may go on to produce disease if appropriate stresses are applied to the chickens. On the other hand, the similarity between some of the chicken and turkey isolates that generally encoded the fewest virulence factors may suggest that similar conditions and diet in the host may favour these types as commensals. It may be speculated that *E. coli* isolates from turkey lacking many virulence determinants are either commensals or potential pathogens that harbour different virulence traits associated with disease in turkeys and are unrelated to chicken adapted APEC isolates. What can be argued is that the virulence determinants and limited range of tests used were not sufficient to understand the differences between isolates and other methods are required, one method being whole genome sequencing (WGS). It can be concluded the limited tests used in this study may not yield the specific differentiation required by the routine laboratory.

Perhaps it could be argued that the selection of these in the studies were of limited diagnostic value. That said the Nolan laboratory uses them as targets in their analyses (Johnson *et al.*, 2008a). For APEC, understanding genotype-to-phenotype and host-pathogen relationships need to be established, evaluated and deployed in parallel with present methods, which will require a major effort leading to gradual replacement of current day methodologies. An important point to raise here is not just presence of putative virulence determinants but also whether these genes are expressed *in vivo*.

This study focused upon cost effective and easily performed tests that could be undertaken in a non-reference laboratory setting with very limited funding that was not unlimited in this PhD study. A discussion needs to be had here about the number and range of tests. PCR for the forty or so currently defined APEC virulence determinants could have been

exploited. Many of these virulence factors are not yet well characterised and many are in a very small minority of APEC isolates. For example, *fyuA* was present in all colibacillosis-affected isolates, with a significantly lower prevalence in the control isolates (Vandekerchove *et al.*, 2005). Another author found seven virulence genes *iutA, iss, cvaC, tsh, papC, papG* and *felA* were detected significantly more often amongst colibacillosis isolates than healthy birds, which were several of those genes were not detected in many colibacillosis isolates, that suggests variants of those genes and yet undetected virulence factors (Delicato *et al.*, 2003). Those selected were regarded as the most prevalent in APEC (Lisa Nolan personal communication) and potentially the most relevant to detect. Similarly, a limited range of antibiotic resistance was tested when in reference laboratories 18 are tested. A discussion needs to be had about whether or not antibiotic resistance can be considered as virulence determinants. Given poultry are heavily treated with ampicillin and nalidixic acid, resistance gives a selective advantage and a correlation between virulence determinants and antibiotic resistance was shown, as APEC generally harboured more resistance genes than the other isolates. Thus, it could be argued that co-selection of traits adds to the virulence potential even though in many instances resistance is likely to be highly transient being on mobile genetic elements such as plasmids. As for metabolite use, the question arises why test eleven substrates when 2000 metabolites can be tested in some platforms such as Biolog's phenotype microarray. Based on current list prices the cost per organism for full genome sequencing and whole metabolism profiling by Biolog, is in the region of £400-£600, a grand total of £80,000-£120,000 for 200 isolates. In addition, literature search to identify variable characteristics that could be used collectively to gain insight was conducted. This study has certainly indicated some interesting avenues for new work by assessing those differences in a wider population of isolates. It is recognised that a weakness of the current study is the limited representation of isolates. But the aim was to investigate the diversity of *E. coli* populations in poultry using a set of relatively simple and

readily transferable tests that a front-line diagnostic laboratory rather than a specialist reference laboratory can undertake. As already discussed whole genome analysis is readily available albeit expensive, although reductions in cost are anticipated, the time required for a detailed analysis remains the significant bottleneck compounded by the fact that there is no clear definition of APEC and commensal although the Nolan laboratory has suggested approaches to accurately differentiate faecal isolates from pathogens (Johnson *et al.*, 2008a) although not repeated in this study! Thus, until a pipeline for the bioinformatics is developed that calls APEC confidently, there remains some debate surrounding the definition of APEC. Furthermore, the time taken to obtain results from WGS for an 'in-the-field' situation where suspect disease is being investigated, there is a real need for simple, rapid and well-established methods of analysis of which WGS is most certainly not. The data of this study, and those of many others, indicates that we still lack of a satisfactory understanding of the differences between commensals and APEC to be able to effectively define them without recourse to testing Koch' postulates in animal models. That is untenable for routine diagnosis obviously but is a direction that needs pursuing.

This work did generate some interesting differences between isolates such that some turkey isolates were considered commensal and some APEC defined as likely to be highly virulent. Indeed, isolates were selected that were considered statistically diametrical opposite in terms of the variables that were tested for *in vivo* studies. The Galleria model was selected according to the following criteria; the 3Rs principles (**R**eplacement, **R**eduction and **R**efinement), cost limitation, and it has been used for *E. coli* by others in published reports. Despite initial excitement that all the *in vitro* work had identified suitable differential tests, the Galleria data were extremely disappointing. As with other authors (Alghoribi *et al.*, 2014), the model was found wanting and perhaps use of a relevant model, namely poultry, is needed to verify or refute those interesting differences between isolates.

Using EOs for chicken nutritional performance has deserved a lot of attention during the last decade, with special emphasis on their antimicrobial properties. Regardless of an overwhelming amount of *in vitro* data concerning chicken, few *in vivo* trials with pure compounds have been published. In this thesis, 2 pure components (thymol and carvacrol) were selected based on the literature and were investigated for their anti-microbial properties. Here, the work was with pure compounds rather than extracts from plants containing EOs to ensure that the biological activities observed are related to the compound itself. The composition of a plant EO can differ according to many factors such as environmental conditions, geographic origin, and plant age (Sela *et al.*, 2015; Fischer *et al.*, 2011), therefore to eliminate these experimental confounders a pure compound was used.

Based on using EO as an antimicrobial, could bacteria become resistance to EOs or could EOs induce multiple mutations in bacteria? There was limited evidence provided from studies suggesting the spontaneous occurrence of essential oils resistance. It is probable that the multi-component nature of essential oils may reduce their potential to select resistance to occur because numerous targets need to be adapted by the bacteria to hamper the effects of the essential oils. Moreover, if membrane permeabilisation is one of the modes of action of the essential oil, it is unlikely that resistance will develop spontaneously (Langeveld *et al.*, 2014; Hurdle *et al.*, 2011).

Regarding the use of the Galleria model to differentiate between commensals and APEC isolates a question arises as to how the model may correlate with poultry? It is worthy to use this model again to test the mutant strain with the wild strain. We assume that the mutant strain becomes thymol resistant, so if the wild strain was pathogenic, will the mutant remain pathogenic or become less pathogenic *in vivo*? As the advantages of *G. mellonella* as an infection model are well suited to investigate the mutant strain (Champion *et al.*, 2010), and

that it was determined by this study that the mutant strain is a slow grower compared to the wild strain, we expect the Galleria model result to be a significant confirmatory addition of the mutant's alterations *in lieu* of resistance to thymol.

The work in chapter 5 up to our knowledge is the first study that indicates the impact of thymol and its phenolic metabolites on caecal isolates, and the association of the effect of thymol treatment on the gut bacterial population. The purified ingredients of EOs are suitable alternatives to antibiotics, and it is recognized as safe to use in feeding (Cosentino *et al.*, 1999). The original testable hypothesis based on our studies with APEC was that sub-MIC concentrations would impair the growth of *E. coli*. It would seem the static gut model with its complex bacterial population reduced all *Proteobacteria*. So, the hypothesis was therefore untested. The logic would be to take the batch culture to the next stage as continuous culture (Gibson and Wang, 1994; Macfarlane *et al.*, 2005) and then to *in vivo* studies.

Although *in vivo* intervention trials are physiologically the most relevant to study both metabolism and microbial modulation, *in vitro* tools have been considered to simulate intestinal conditions. In combination with *in vivo* trials, *in vitro* experiments may help to elucidate the extent bioconversion processes mediated by the host itself (Jacobs *et al.*, 2009; Bolca *et al.*, 2007b). The complexity of *in vitro* gut models is diverse, ranging from simple static models (batch culture fermentation) to advanced continuous models (gastrointestinal simulators). The system we used could be valid and relevant up to 24 h, as the caeca are filled up after 24 h before they vacate.

The transmission of plasmids bearing antibiotic resistance genes by conjugation between enterobacteria has been demonstrated in the digestive tracts of humans and various animals, such as chicken (Lafont *et al.*, 1981). Also, exchange of chromosomal genes occurs at high frequencies in the gut of the chicken (Lafont *et al.*, 1984). To inhibit this transmission and genetic exchange by conjugation, a model was developed *in vitro* testing the effects thymol

on *E. coli* conjugation. According to MIC results, thymol was inhibited plasmid transfer between *E. coli* isolates, and the data in chapter four suggest that phenolic compounds disrupt respiration in *E. coli*. The inhibited respiration in *E. coli* stimulates the leakage of intracellular K^+ and disrupts pH homeostasis (Cox *et al.*, 1998; Fitzgerald *et al.*, 2004). The study of conjugation was very preliminary and limited to one mating experiment repeated three times; therefore, it is needed to test a wider range of plasmids and resistance types. Of importance, respiration is essential for plasmid gene transfer (Watmough and Frerman, 2010) and integrity of membrane interaction is also essential (Di Pasqua *et al.*, 2007). So, it does seem very logical that thymol is likely to be highly disruptive of plasmid transfer by disrupting these two essential aspects of plasmid transfer.

Based on all findings of this study, it was of great interest to study the effect of thymol on gut bacteria (commensals and other bacteria type) and functionality in the chicken. In addition, determining the impact of thymol on the host, and whether it improves nutritional performance and intestinal health is critical. These investigations remain part of the future work following this preliminary study. As mentioned previously that EOs (thymol and carvacrol) were suitable alternatives to antibiotics, and are recognized as safe to use in feeding, many of its benefits to public health beside antimicrobial agents, as it also has different applications such as an antioxidant, anti-inflammatory, immuno-modulating effects and enhancement of endogenous secretions. Thus, it could be used in food producing animals. However, it is necessary to study the MIC range concentration on the host cells. Using tissue culture and ligated gut models is very relevant here to assess the safety and the efficacy of potential in-feed compounds. There is a need to understand their effect on the host cells. For example, epithelial cell culture models have been widely employed to assess the absorption of drug molecules across intestinal mucosa and three-dimensional culture models improve the opportunity to evaluate drug permeability *in vitro* (Li *et al.*, 2013).

6.2 Conclusion

The differences between commensal and pathogenic *E. coli* cannot be simply defined, and the evaluation of genotypic and phenotypic diversity in a mixture of isolates is complex. The diverse genetic distance of *E. coli* strains isolated from presumptive commensals in turkey and chicken grew in the same environment compared to APEC stains isolated from infected birds. However, the significance of specific phenotypes and the perceived distance between diverse types were less clear.

Essential oils of plant products have been studied as substitutes for antibiotics in combating *E. coli* infections to find new antimicrobial agents. In this work, we show convincing evidence of the antimicrobial effect of thymol and carvacrol. The antimicrobial activity of these phytochemicals and their ability to control biofilm formation is mediated in part by reducing bacterial motility and altering bacterial morphology and probably membrane integrity. In addition, reduction of conjugation between *E. coli* was observed in the presence of thymol, and it is known that conjugation occurs in biofilms. Is it possible that thymol may have added impacts in biofilms reducing gene transfer? However, we also provided evidence of the evolution of thymol resistance by training bacteria to tolerate high concentrations of thymol. Thymol resistant mutants acquired growth-limiting mutations and identification of the genes involved might provide novel targets that may be amenable to pharmaceutical bactericidal if such thymol resistant mutants arise in nature.

The analysis of mutants showed that at least two mutations in the adapted mutant, of which one was a nonsense mutation in the *acrR* gene encoding a repressor of the AcrAB efflux pump, and the second was a missense mutation in the *rng* gene encoding ribonuclease G (RNase G). Therefore, inhibitors of efflux such as Hoescht33323 bisbenzimide may be deployed to enhance the toxicity of thymol (Nikaido *et al.*, 2011). Moreover, this study identified bacterial metabolic profile associated with thymol treatment that indicated disruption

of respiration in *E. coli*, which is not a direct evidence for thymol mechanism of action albeit a plausible hypothesis that integration of thymol in bacterial cell membrane disrupts electron transfer proteins and pathway that is essential for respiration.

The use of thymol in batch culture closed system indicated that supplementation with thymol exerts a positive effect on intestinal microbiota such as increased LAB and the question is whether this will occur *in vivo* and enable a concomitant enhancement in growth performance. More specific studies are required to improve luminal availability of thymol, and clarify how it affects intestinal microbiota *in vivo*. Collectively, the results of this study support the applicability of plant essential oils as an effective alternative to antibiotic use in agriculture and poultry industries to minimize the burden of antibiotic resistant bacteria worldwide. In addition, this study provided insight into possible essential oils' bactericidal mode of action, and the possible evolution of EO resistant bacteria. Further investigations into the effects of essential oils on avian, mammalian and plant bacterial hosts are warranted and should be pursued.

6.3 Future work

- Whole genome sequencing of strains that were isolated from turkey and seem similar phenotypically and genotypically but were classified as highly pathogenic and less pathogenic based on the Galleria model. Comparison of the genetic make-up of these two strains may identify the differences between them.
- Using the Galleria model to understand the differences between commensals and APEC of two strains were isolated from the same host and seem similar in phenotypic and genotypic characteristics. One of the strains is of high pathogenicity and the other is less pathogenic and comparison of the genetic make-up of these two strains may identify the differences between them that impact their host-pathogen interaction

mechanisms in the *in vivo* experiment. This could be an interesting work for the future, but we must be cautious as this approach may identify factors specific for pathogenicity in Galleria and not poultry.

- Using epithelial cell culture models to test the range of MICs to assess the safety and the efficacy of potential in-feed compounds.
- Determining the impact of thymol on the host, and whether it improves nutritional performance and intestinal health *in vivo* is critical.

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Appendix

Appendix 1: The manufacturer/supplier details for all medium used

Tryptophan Broth

Ingredients	Grams/Litre
Casein enzymic hydrolysate	10.0
Sodium chloride	5.0
DL-Tryptophan	1.0

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C. Use before expiry date on the label.
Directions: Dissolve the ingredients in water by heating. Dispense 3 ml per test tube. Close the tubes with cotton plugs, plastic or metal caps. Autoclave for 15 minutes at 121 ± 3°C.

Nutrient Broth (NB)

Ingredients	Grams/Litre
Peptone	15.0
Yeast extract	3.0
Sodium chloride	6.0
D(+)-Glucose	1.0

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.
Directions: Dissolve 25 g in 1 litre distilled water and fill into tubes. Sterilize by autoclaving at 121°C for 15 minutes.

Luria-Bertani broth (LB)

Ingredients	Grams/Litre
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.
Directions: Suspend 20 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Luria-Bertani low salt broth (LB low salt)

Ingredients	Grams/Litre
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	0.5

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.
Directions: Suspend 15.5 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Nutrient Agar NA

Ingredients	**Grams/Litre**
Meat extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.
Directions: Suspend 28 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Luria-Bertani Agar (LB)

Ingredients	Grams/Litre
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	15.0

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.
Directions: Suspend 20 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Pour into sterile petri plates. Dry the surface of the gel before inoculation.

Eosin Methylene Blue (EMB)

Ingredients	**Grams/Litre**
Peptone	10.0
Lactose	10.0
Dipotassiummonohydrogenphosphate	2.0
Methylene Blue	0.065
Eosine Y	0.4
Agar	15.0

Store prepared media below 8°C and protected from direct light. Store dehydrated powder in a dry place, in tightly sealed containers at 2-25°C.
Directions: Suspend 37.5 g in 1 litre of distilled water. Heat to dissolve completely, sterilize by autoclaving at 121°C for 15 minutes. Cool to 60°C and shake the medium in order to oxidize the methylene blue and to suspend the precipitate.

Salmonella Shigella Agar (SS Agar)

Ingredients	Grams/Litre
Meat extract	5.0
Peptone	5.0
Lactose	10.0
Ox bile, dehydrated	8.5
Sodium citrate	10.0
Sodium thiosulfate	8.5
Ferric citrate	1.0
Brillant green	0.0003
Neutral red	0.025
Agar	15.0

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.
Directions: Suspend 63 g in 1 litre of distilled water. Bring to boiling with frequent agitation and allow to simmer gently to dissolve the agar. Do not autoclave. Cool to about 50°C, mix and pour into petri dishes.

MacConkey agar (McC)

Ingredients	Grams/Litre
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.
Directions: Suspend 52 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Pour into sterile petri plates. Dry the surface of the gel before inoculation.

Appendix 2: isolated and storage of *E. coli* strains

A. Chicken isolates

The samples from chickens were made from an experiment that used different levels of dietary fibre. Corn Distiller Dried Grains with soluble (CDDGs) were added to a balanced diet containing normal levels of protein and phosphorus as in poultry diets. Commercial diet starter was fed till day 9 (Corn/soy based diet), after that used CDDGs as dietary fibre source by three treatments 14%, 21%, 28% and control (corn/soy based diet). Samples were collected from day 24 and 35 of chicken age.

NO.	Age (d)	Dietary	Bird ID
1	24	0%	G239
2			Y169
3			Y195
4			Y173
5		14%	B174
6			G291
7			B152
8			R3336
9			Y209
10		21%	B140
11			B154
12		28%	G260
13			G263
14			R3317
15	35	0%	B27
16			R3306
17			B178
18			R3304
19			R3315
20			Y208
21			G268
22		14%	R3347
23			Y183
24			Y175

25			G258
26			R3301
27			R3350
28		21%	B138
29			G267
30			Y186
31			G228
32			B136
33		28%	G286
34			R3309
35			R3323

B. Turkey isolates

The samples from turkeys were made from an experiment that established to evaluate supplementing turkey diets with whole grain wheat on the maintenance of gut health. The birds were fed two different diets during the experiment; two treatment groups are a standard diet (concentrated supplement, CON) and whole grain wheat (WGW). Samples were collected after one week of arrived birds and for a total of eight subsequent weeks.

No.	Date	Pen#	Dietary	Bird ID
1	21/10/2014 week 1	2	WGW	3362
2		3	CON	3222
3		4	WGW	34
4		5	CON	3386
5		6	WGW	3287
6		7	CON	3279
7		9	CON	3372
8		9	CON	3372R
9		10	WGW	3272
10	28/10/2014 week 2	1	CON	3369
11		4	WGW	3254
12		8	WGW	110R
13		10	WGW	3413
14		12	WGW	3251

15	4/11/2014 week 3	1	CON	3383
16		2	WGW	3233
17		3	CON	3298
18		3	CON	3298R
19		4	WGW	108
20		5	CON	3204
21		6	WGW	46
22		7	CON	3280
23		9	CON	3235
24		11	CON	3253
25		12	WGW	3219
26	11/11/2014 week 4	1	CON	3281
27		1	CON	3281R
28		2	WGW	3283
29		3	CON	84
30		3	CON	84R
31		4	WGW	3275
32		4	WGW	3275R
33		5	CON	3212
34		6	WGW	3227
35		6	WGW	3227R
36		7	CON	98
37		7	CON	98 R
38		8	WGW	107
39		8	WGW	107R
40		9	CON	85R
41		10	WGW	3206
42		10	WGW	3206R
43		11	CON	3389
44		12	WGW	3208
45	18/11/2014 week 5	1	CON	113
46		2	WGW	3250
47		3	CON	3276
48		3	CON	3276R

49		2	WGW	88-92
50		2	WGW	88-92R
51		3	CON	99-3217R
52	25/11/2014 week 6	5	CON	111-106
53		5	CON	111-106R
54		6	WGW	3373-3242
55		6	WGW	3373-3242R
56		1	CON	3380
57		1	CON	3380R
58		2	WGW	3271
59		2	WGW	3271R
60		3	CON	105
61		3	CON	105R
62		5	CON	3397
63		5	CON	3397R
64		6	WGW	104R
65		7	CON	35R
66		8	WGW	3270
67	2/12/2014 week 7	8	WGW	3270R
68		11	CON	32
69		12	WGW	3287
70		3	CON	3396
71		4	WGW	3215
72		4	WGW	3215R
73		5	CON	3265
74		6	WGW	3398
75		9	CON	93
76		9	CON	93R
77		10	WGW	3268
78		11	CON	30
79		12	WGW	3240
80		1	CON	3262
81	9/12/2014 week 8	1	CON	3262R
82		3	CON	3234

83		3	CON	3234R
84		4	WGW	3376
85		4	WGW	3376R
86		5	CON	3216
87		5	CON	3216R
88		6	WGW	3220
89		7	CON	3211
90		8	WGW	44
91		9	CON	3257
92		9	CON	3257R
93		10	WGW	3366R
94		11	CON	3364
95		11	CON	3364R
96		12	WGW	42
97		4	WGW	40
98		7	CON	91R
99		9	CON	3210R
100		10	WGW	3288R

C. APEC isolates

Other *E. coli* 65 samples were provided by University of Surrey (UoS) collected from infected chicken and confirmed as avian pathogenic *E. coli* (APEC).

Appendix 3: Basic statistical correlation between virulence genes, utilise carbon sources and antimicrobial resistant

	fim1	*papC*	*csg*	*crl*
papC	0.036 0.614			
csg	0.103 0.146	0.044 0.533		
crl	0.403 0.000	0.014 0.839	-0.015 0.829	
tsh	0.171 0.016	-0.045 0.528	-0.031 0.667	0.069 0.333
iucD	0.042 0.559	0.004 0.953	0.076 0.284	0.090 0.207
irp2	0.028 0.689	-0.086 0.224	-0.078 0.271	-0.026 0.719
iss	0.029 0.679	0.063 0.377	0.036 0.610	0.120 0.092
astA	0.027 0.700	0.037 0.600	-0.015 0.831	0.059 0.406
cva	0.060 0.396	0.049 0.491	-0.070 0.323	0.072 0.314
K1	0.075 0.289	0.124 0.080	-0.174 0.014	0.030 0.670
Sucrose	0.047 0.511	0.002 0.981	-0.061 0.389	0.058 0.417
Raffinose	-0.008 0.913	0.033 0.645	-0.077 0.278	0.063 0.376
Inositol	-0.073 0.303	-0.042 0.556	-0.045 0.523	0.015 0.838
Adonitol	-0.046 0.521	-0.110 0.121	0.001 0.984	-0.132 0.063
Arabitol	-0.040 0.577	-0.110 0.121	0.062 0.385	-0.124 0.079
Dulcitol	0.090 0.203	0.116 0.103	-0.119 0.093	0.069 0.333
Allantoin	0.020 0.774	-0.024 0.739	-0.115 0.105	0.008 0.908
Proline	-0.014 0.844	0.016 0.819	-0.162 0.022	0.041 0.560
Sorbose	0.014 0.841	0.000 1.000	-0.170 0.016	0.052 0.467
Metzitose	-0.153 0.030	-0.035 0.621	-0.189 0.007	0.012 0.864
Salicin	0.032	-0.037	-0.199	0.013

	0.654	0.603	0.005	0.857
Nalidixic acid	0.031	-0.020	0.125	-0.073
	0.665	0.775	0.077	0.303
Amikacin	0.050	0.081	0.062	0.020
	0.478	0.255	0.381	0.775
Ampicillin	-0.104	-0.131	0.019	-0.062
	0.144	0.063	0.788	0.381
Chloramphenicol	0.049	0.057	-0.209	0.020
	0.489	0.422	0.003	0.780
Colistin	0.044	-0.051	-0.145	0.018
	0.540	0.477	0.040	0.805
Streptomycin	-0.110	-0.049	-0.136	-0.196
	0.120	0.490	0.054	0.005
Trimethoprim	-0.098	-0.006	-0.189	0.027
	0.168	0.936	0.007	0.706

	tsh	*iucD*	*irp2*	*iss*
iucD	0.459			
	0.000			
irp2	0.099	0.037		
	0.163	0.604		
iss	0.142	0.304	0.072	
	0.045	0.000	0.310	
astA	-0.016	-0.113	0.141	-0.224
	0.826	0.110	0.046	0.001
cva	0.240	0.347	-0.012	0.280
	0.001	0.000	0.865	0.000
K1	0.054	0.169	-0.149	0.128
	0.445	0.017	0.036	0.071
Sucrose	0.049	0.154	-0.145	0.257
	0.490	0.029	0.041	0.000
Raffinose	0.159	0.300	-0.031	0.319
	0.024	0.000	0.667	0.000
Inositol	-0.012	0.162	-0.101	0.079
	0.861	0.022	0.153	0.266
Adonitol	-0.068	-0.263	0.082	-0.363
	0.341	0.000	0.250	0.000
Arabitol	-0.064	-0.234	0.051	-0.308
	0.367	0.001	0.477	0.000
Dulcitol	0.138	0.332	-0.014	0.232
	0.051	0.000	0.840	0.001
Allantoin	-0.055	0.092	-0.049	0.003
	0.442	0.195	0.489	0.970
Proline	0.164	0.258	-0.206	0.195

	0.020	0.000	0.003	0.006
Sorbose	0.168	0.257	0.080	0.255
	0.018	0.000	0.260	0.000
Metzitose	0.052	0.072	-0.134	0.066
	0.463	0.312	0.058	0.350
Salicin	0.055	0.143	-0.038	0.070
	0.440	0.043	0.593	0.325
Nalidixic acid	0.078	0.132	-0.003	-0.054
	0.274	0.062	0.965	0.444
Amikacin	0.214	0.227	-0.196	0.108
	0.002	0.001	0.005	0.129
Ampicillin	0.069	0.038	-0.017	0.015
	0.329	0.595	0.814	0.836
Chloramphenicol	0.132	0.037	-0.004	0.013
	0.063	0.599	0.951	0.858
Colistin	-0.020	0.026	-0.213	0.053
	0.777	0.711	0.002	0.458
Streptomycin	-0.027	0.184	-0.092	0.170
	0.706	0.009	0.194	0.016
Trimethoprim	0.054	0.069	-0.127	0.129
	0.452	0.331	0.072	0.069

	astA	*cva*	K1	Sucrose
cva	-0.313			
	0.000			
K1	-0.160	0.231		
	0.023	0.001		
Sucrose	-0.060	0.049	0.174	
	0.399	0.487	0.014	
Raffinose	-0.172	0.180	0.257	0.484
	0.015	0.011	0.000	0.000
Inositol	0.132	0.091	0.067	-0.044
	0.062	0.199	0.348	0.534
Adonitol	0.013	-0.209	-0.231	-0.399
	0.851	0.003	0.001	0.000
Arabitol	0.017	-0.201	-0.231	-0.420
	0.811	0.004	0.001	0.000
Dulcitol	-0.170	0.305	0.264	0.334
	0.016	0.000	0.000	0.000
Allantoin	0.080	0.057	-0.050	0.049
	0.258	0.423	0.484	0.493
Proline	-0.082	0.260	0.392	0.343
	0.248	0.000	0.000	0.000
Sorbose	-0.173	0.236	0.171	0.238

	0.014	0.001	0.015	0.001
Metzitose	0.079	0.077	-0.074	-0.011
	0.265	0.281	0.299	0.872
Salicin	0.016	0.114	0.105	0.015
	0.817	0.109	0.141	0.833
Nalidixic acid	-0.005	0.065	-0.108	-0.142
	0.946	0.363	0.127	0.044
Amikacin	-0.184	0.257	0.217	0.014
	0.009	0.000	0.002	0.849
Ampicillin	0.007	0.000	-0.056	-0.229
	0.919	0.999	0.428	0.001
Chloramphenicol	-0.097	0.098	0.128	-0.025
	0.173	0.167	0.072	0.722
Colistin	-0.123	0.135	0.274	0.138
	0.084	0.056	0.000	0.052
Streptomycin	-0.156	0.148	0.121	0.102
	0.028	0.037	0.087	0.149
Trimethoprim	-0.088	0.151	0.148	0.107
	0.217	0.033	0.036	0.132

	Raffinose	Inositol	Adonitol	Arabitol
Inositol	0.105			
	0.140			
Adonitol	-0.445	-0.066		
	0.000	0.354		
Arabitol	-0.367	-0.025	0.919	
	0.000	0.725	0.000	
Dulcitol	0.418	0.056	-0.490	-0.492
	0.000	0.433	0.000	0.000
Allantoin	0.019	0.301	0.007	0.004
	0.793	0.000	0.922	0.955
Proline	0.335	0.029	-0.299	-0.239
	0.000	0.686	0.000	0.001
Sorbose	0.440	-0.005	-0.370	-0.371
	0.000	0.949	0.000	0.000
Metzitose	0.263	0.373	-0.055	0.123
	0.000	0.000	0.437	0.083
Salicin	0.139	0.270	-0.058	0.054
	0.050	0.000	0.413	0.451
Nalidixic acid	-0.084	-0.126	0.296	0.307
	0.239	0.076	0.000	0.000
Amikacin	0.223	-0.059	-0.155	-0.155
	0.002	0.408	0.029	0.029
Ampicillin	-0.157	-0.014	0.182	0.195
	0.027	0.839	0.010	0.006
Chloramphenicol	0.190	-0.057	-0.151	-0.151

	0.007	0.420	0.033	0.033
Colistin	0.126	-0.051	-0.068	-0.070
	0.074	0.475	0.342	0.321
Streptomycin	0.169	-0.031	-0.180	-0.183
	0.017	0.667	0.011	0.010
Trimethoprim	0.252	-0.025	-0.204	-0.205
	0.000	0.721	0.004	0.004

	Dulcitol	Allantoin	Proline	Sorbose
Allantoin	-0.033			
	0.639			
Proline	0.295	0.009		
	0.000	0.896		
Sorbose	0.342	-0.085	0.284	
	0.000	0.233	0.000	
Metzitose	-0.060	0.253	0.170	-0.004
	0.398	0.000	0.016	0.957
Salicin	0.027	0.266	0.179	-0.004
	0.706	0.000	0.011	0.955
Nalidixic acid	-0.063	-0.090	-0.191	-0.040
	0.379	0.203	0.007	0.578
Amikacin	0.076	-0.033	0.268	0.189
	0.283	0.639	0.000	0.007
Ampicillin	-0.229	0.001	0.028	0.099
	0.001	0.989	0.692	0.161
Chloramphenicol	0.157	-0.033	0.179	0.188
	0.027	0.647	0.011	0.008
Colistin	0.042	-0.029	0.367	0.167
	0.557	0.686	0.000	0.018
Streptomycin	0.207	-0.051	0.232	0.255
	0.003	0.471	0.001	0.000
Trimethoprim	0.073	-0.044	0.263	0.188
	0.301	0.535	0.000	0.008

	Metzitose	Salicin	Nalidixic acid	Amikacin
Salicin	0.433			
	0.000			
Nalidixic acid	0.036	0.038		
	0.613	0.594		
Amikacin	-0.049	-0.052	0.098	
	0.487	0.464	0.167	
Ampicillin	0.070	-0.013	0.290	0.133
	0.327	0.858	0.000	0.060
Chloramphenicol	0.092	0.097	0.005	0.164
	0.195	0.172	0.943	0.020
Colistin	0.044	-0.045	0.006	0.192

	0.540	0.527	0.927	0.006
Streptomycin	-0.026	0.079	0.049	0.290
	0.718	0.269	0.494	0.000
Trimethoprim	0.199	0.163	0.035	0.237
	0.005	0.021	0.619	0.001

	Ampicillin	Chloramphenicol	Colistin	Streptomycin
Chloramphenicol	0.129			
	0.068			
Colistin	0.098	0.023		
	0.167	0.745		
Streptomycin	0.262	0.164	0.133	
	0.000	0.021	0.060	
Trimethoprim	0.187	0.240	0.293	0.351
	0.008	0.001	0.000	0.000

Cell Contents: Pearson correlation
P-Value

Appendix 4: *E. coli* strains isolated from a poultry,15 chicken, 15 turkey, 20 APEC and K12 (JM109 and DH5α) for control to determine the susceptibility of thymol and carvacrol.

no	Isolated strains	Thymol (µg/mL)		Carvacrol (µg/mL)		Antimicrobial resistance	Virulence genes	Carbon sources utilised
		MIC	MBC	MIC	MBC			
1	turkey	125	150	175	225	0	6	5
2		125	150	175	200	0	7	1
3		175	200	175	200	1	8	0
4		175	200	200	225	0	7	3
5		175	200	200	225	0	5	2
6		175	200	200	225	2	4	0
7		125	200	175	200	1	3	2
8		175	200	175	200	0	6	3
9		125	150	175	200	2	2	2
10		175	200	175	200	0	8	1
11		125	150	175	200	1	5	4
12		175	200	175	200	0	5	3
13		175	200	175	200	0	5	2
14		125	150	175	200	0	6	1
15		125	150	175	200	1	6	3
16	chicken	150	175	175	200	2	7	0
17		150	175	175	200	2	8	0
18		150	175	200	225	2	7	1
19		150	175	175	200	1	8	1
20		125	175	200	225	1	8	2
21		150	175	200	225	1	8	1
22		125	150	150	175	0	8	4
23		125	150	150	175	0	8	4
24		175	200	175	200	1	9	1
25		150	175	200	225	3	6	1
26		125	150	175	200	0	8	1
27		125	150	175	200	0	9	1
28		125	150	150	175	0	7	0
29		125	150	175	200	0	6	1
30		125	150	150	175	3	10	1
31	APEC	125	200	200	225	1	11	3
32		125	150	200	225	1	9	2
33		125	150	175	200	2	5	1
34		125	150	200	225	1	7	3
35		125	200	200	225	0	8	0
36		175	150	175	200	0	7	2
37		125	150	200	225	0	7	3

38		125	150	200	225	4	8	3
39		125	200	225	250	3	10	2
40		125	150	200	225	1	6	2
41		125	150	100	150	2	5	1
42		125	125	150	175	0	9	0
43		125	125	150	150	0	8	2
44		150	175	175	200	0	7	3
45		125	200	100	150	1	7	4
46		125	150	125	150	2	5	4
47		125	150	150	200	3	6	1
48		150	175	175	200	1	7	2
49		125	125	150	175	4	7	3
50		150	175	200	225	1	6	2
51	JM109	175	200	175	200		3	
52	DH5α	150	200	175	200		2	

Appendix 5: Biofilm formation and Motility

no	Bird ID	SBF	result	SBF
1	3222	0.145562815	negative	0
2	84	2.046429964	strong	1
3	98	0.048742862	negative	0
4	3389	1.509690033	strong	1
5	3208	2.447249879	Strong	1
6	88-92	0.707424305	moderate	-1
7	105	0.151195401	negative	0
8	3397	0.455205204	weak	0
9	35R	0.082697643	negative	0
10	3270	0.07369005	negative	0
11	3287	0.127284439	negative	0
12	3376	0.051647945	negative	0
13	3216	0.379334833	weak	0
14	3257	0.105873134	negative	0
15	3364	0.108486223	negative	0
16	Y195	1.25741782	strong	1
17	Y173	0.883354236	moderat	-1
18	B152	0.151456086	negative	0
19	R3315	1.341174901	strong	1
20	G260	0.032129068	negative	0
21	B27	0.009001619	negative	0
22	R3304	0.041931467	negative	0
23	Y208	0.049217367	negative	0
24	Y175	1.492918264	strong	1
25	B138	0.13565811	negative	0
26	G286	1.983463784	strong	1
27	R3309	0.15930652	negative	0
28	G228	0.726703286	moderat	-1
29	R3323	0.125051247	negative	0
30	R3314	0.046853511	negative	0
31	12	0.880372453	moderate	-1
32	14	0.020498213	negative	0
33	18	0.206787619	negative	0
34	19	1.946100323	strong	1
35	22	0.09780344	negative	0
36	33	0.069196712	negative	0
37	43	0.069789633	negative	0
38	48	0.005391577	negative	0
39	54	0.010888073	negative	0
40	55	0.602282149	moderate	-1

41	56	1.620107968	strong	1
42	57	0.171846166	negative	0
43	58	0.55214451	moderate	-1
44	59	0.68491638	moderate	-1
45	60	0.805213267	moderate	-1
46	61	0.285297567	negative	0
47	62	0.883644121	moderate	-1
48	63	0.547376088	moderate	-1
49	64	0.250122393	negative	0
50	65	0.727697354	moderate	-1
51	JM109	0.042243094	negative	0
52	DH5α	0.111516538	negative	0

Appendix 6: A visualisation of the mutation on the nucleotide level in an alignment of the MG1655, JM109 wildtype and mutant genes with the effect it had on protein translation

acrR (gene location 485761-486408, locus tag="b0464")

```
MG1655_b0464_acrR      ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCT
JM109_WT_acrR          ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCT
JM109_RThy_acrR        ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCT
                       ************************************************************

MG1655_b0464_acrR      CTACGTCTTTTCTCACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCA
JM109_WT_acrR          CTACGTCTTTTCTCACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCA
JM109_RThy_acrR        CTACGTCTTTTCTCACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCA
                       ************************************************************

MG1655_b0464_acrR      GCTGGCGTTACGCGCGGTGCAATCTACTGGCATTTTAAAGACAAGTCGGATTTGTTCAGT
JM109_WT_acrR          GCTGGCGTTACGCGCGGTGCAATCTACTGGCATTTTAAAGACAAGTCGGATTTGTTCAGT
JM109_RThy_acrR        GCTGGCGTTACGCGCGGTGCAATCTACTGGCATTTTAAAGACAAGTCGGATTTGTTCAGT
                       ************************************************************

MG1655_b0464_acrR      GAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAGCTTGAGTATCAGGCAAAA
JM109_WT_acrR          GAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAGCTTGAGTATCAGGCAAAA
JM109_RThy_acrR        GAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAGCTTGAGTATCAGGCAAAA
                       ************************************************************

MG1655_b0464_acrR      TTCCCTGGCGATCCACTCTCAGTATTAAGAGAGATATTAATTCATGTTCTTGAATCCACG
JM109_WT_acrR          TTCCCTGGCGATCCACTCTCAGTATTAAGAGAGATATTAATTCATGTTCTTGAATCCACG
JM109_RThy_acrR        TTCCCTGGCGATCCACTCTCAGTATTAAGAGAGATATTAATTCATGTTCTTGAATCCACG
                       ************************************************************

MG1655                              486079
                                    |
MG1655_b0464_acrR      GTGACAGAAGAACGGCGTCGATTATTGATGGAGATTATATTCCACAAATGCGAATTTGTC
JM109_WT_acrR          GTGACAGAAGAACGGCGTCGATTATTGATGGAGATTATATTCCACAAATGCGAATTTGTC
JM109_RThy_acrR        GTGACAGAAGAACGGCGTTGATTATTGATGGAGATTATATTCCACAAATGCGAATTTGTC
                       ***************** ****************************************

MG1655_b0464_acrR      GGAGAAATGGCTGTTGTGCAACAGGCACAACGTAATCTCTGTCTGGAAAGTTATGACCGT
JM109_WT_acrR          GGAGAAATGGCTGTTGTGCAACAGGCACAACGTAATCTCTGTCTGGAAAGTTATGACCGT
JM109_RThy_acrR        GGAGAAATGGCTGTTGTGCAACAGGCACAACGTAATCTCTGTCTGGAAAGTTATGACCGT
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                    ************************************************************

MG1655_b0464_acrR   ATAGAACAAACGTTAAAACATTGTATTGAAGCGAAAATGTTGCCTGCGGATTTAATGACG
JM109_WT_acrR       ATAGAACAAACGTTAAAACATTGTATTGAAGCGAAAATGTTGCCTGCGGATTTAATGACG
JM109_RThy_acrR     ATAGAACAAACGTTAAAACATTGTATTGAAGCGAAAATGTTGCCTGCGGATTTAATGACG
                    ************************************************************

MG1655_b0464_acrR   CGTCGCGCAGCAATTATTATGCGCGGCTATATTTCCGGCCTGATGGAAAACTGGCTCTTT
JM109_WT_acrR       CGTCGCGCAGCAATTATTATGCGCGGCTATATTTCCGGCCTGATGGAAAACTGGCTCTTT
JM109_RThy_acrR     CGTCGCGCAGCAATTATTATGCGCGGCTATATTTCCGGCCTGATGGAAAACTGGCTCTTT
                    ************************************************************

MG1655_b0464_acrR   GCCCCGCAATCTTTTGATCTTAAAAAAGAAGCCCGCGATTACGTTGCCATCTTACTGGAG
JM109_WT_acrR       GCCCCGCAATCTTTTGATCTTAAAAAAGAAGCCCGCGATTACGTTGCCATCTTACTGGAG
JM109_RThy_acrR     GCCCCGCAATCTTTTGATCTTAAAAAAGAAGCCCGCGATTACGTTGCCATCTTACTGGAG
                    ************************************************************

MG1655_b0464_acrR   ATGTATCTCCTGTGCCCCACGCTTCGTAATCCTGCCACTAACGAATAA
JM109_WT_acrR       ATGTATCTCCTGTGCCCCACGCTTCGTAATCCTGCCACTAACGAATAA
JM109_RThy_acrR     ATGTATCTCCTGTGCCCCACGCTTCGTAATCCTGCCACTAACGAATAA
                    ***********************************************

                    Val Tyr Glu Glu Arg Arg Arg Leu Leu Met Glu
JM109_WT_acrR       GTG ACA GAA GAA CGG CGT CGA TTA TTG ATG GAG
JM109_RThy_acrR     GTG ACA GAA GAA CGG CGT TGA TTA TTG ATG GAG
                    Val Tyr Glu Glu Arg Arg StopLeu Leu Met Glu
```

rng (gene location 3396326-3397795 [reverse orientation], locus tag="b3247")

```
MG1655_b3247_rng      ATGACGGCTGAATTGTTAGTAAACGTAACGCCTTCGGAAACGCGAGTGGCGTATATTGAT
JM109_WT_rng          ATGACGGCTGAATTGTTAGTAAACGTAACGCCTTCGGAAACGCGAGTGGCGTATATTGAT
JM109_RThy_rng        ATGACGGCTGAATTGTTAGTAAACGTAACGCCTTCGGAAACGCGAGTGGCGTATATTGAT
                      ************************************************************

MG1655_b3247_rng      GGCGGTATTCTGCAGGAAATTCATATTGAACGTGAGGCGCGACGCGGAATAGTAGGCAAT
JM109_WT_rng          GGCGGTATTCTGCAGGAAATTCATATTGAACGTGAGGCGCGACGCGGAATAGTAGGCAAT
JM109_RThy_rng        GGCGGTATTCTGCAGGAAATTCATATTGAACGTGAGGCGCGACGCGGAATAGTAGGCAAT
                      ************************************************************

MG1655_b3247_rng      ATCTACAAGGGTCGTGTAAGTCGTGTACTTCCGGGTATGCAGGCGGCTTTTGTAGATATT
JM109_WT_rng          ATCTACAAGGGTCGTGTAAGTCGTGTACTTCCGGGTATGCAGGCGGCTTTTGTAGATATT
JM109_RThy_rng        ATCTACAAGGGTCGTGTAAGTCGTGTACTTCCGGGTATGCAGGCGGCTTTTGTAGATATT
                      ************************************************************

MG1655_b3247_rng      GGGCTGGATAAAGCCGCGTTTCTTCATGCATCCGACATCATGCCGCACACCGAATGTGTG
JM109_WT_rng          GGGCTGGATAAAGCCGCGTTTCTTCATGCATCCGACATCATGCCGCACACCGAATGTGTG
JM109_RThy_rng        GGGCTGGATAAAGCCGCGTTTCTTCATGCATCCGACATCATGCCGCACACCGAATGTGTG
                      ************************************************************

MG1655_b3247_rng      GCGGGTGAAGAACAAAAGCAATTCACGGTGCGCGACATCTCGGAACTGGTTCGTCAGGGG
JM109_WT_rng          GCGGGTGAAGAACAAAAGCAATTCACGGTGCGCGACATCTCGGAACTGGTTCGTCAGGGG
JM109_RThy_rng        GCGGGTGAAGAACAAAAGCAATTCACGGTGCGCGACATCTCGGAACTGGTTCGTCAGGGG
                      ************************************************************

MG1655                                                          3397444
                                                                      |
MG1655_b3247_rng      CAAGATCTGATGGTGCAGGTGGTGAAAGATCCGCTTGGCACTAAAGGTGCGCGCCTGACC
JM109_WT_rng          CAAGATCTGATGGTGCAGGTGGTGAAAGATCCGCTTGGCACTAAAGGTGCGCGCCTGACC
JM109_RThy_rng        CAAGATCTGATGGTGCAGGTGGTGAAAGATCCGCTTGGCACTAAAGGTGCGTGCCTGACC
                      ************************************************** ********

MG1655_b3247_rng      ACCGATATCACGCTCCCTTCTCGCTATCTGGTGTTTATGCCAGGGGCTTCTCACGTTGGG
JM109_WT_rng          ACCGATATCACGCTCCCTTCTCGCTATCTGGTGTTTATGCCAGGGGCTTCTCACGTTGGG
JM109_RThy_rng        ACCGATATCACGCTCCCTTCTCGCTATCTGGTGTTTATGCCAGGGGCTTCTCACGTTGGG
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MG1655_b3247_rng      GTTTCCCAACGTATTGAAAGCGAATCAGAACGTGAACGCCTGAAAAAAGTGGTCGCAGAG
JM109_WT_rng          GTTTCCCAACGTATTGAAAGCGAATCAGAACGTGAACGCCTGAAAAAAGTGGTCGCAGAG
JM109_RThy_rng        GTTTCCCAACGTATTGAAAGCGAATCAGAACGTGAACGCCTGAAAAAAGTGGTCGCAGAG
                      ************************************************************

MG1655_b3247_rng      TATTGCGACGAGCAGGGCGGGTTTATCATCCGTACCGCAGCGGAAGGGGTTGGCGAGGCT
JM109_WT_rng          TATTGCGACGAGCAGGGCGGGTTTATCATCCGTACCGCAGCGGAAGGGGTTGGCGAGGCT
JM109_RThy_rng        TATTGCGACGAGCAGGGCGGGTTTATCATCCGTACCGCAGCGGAAGGGGTTGGCGAGGCT
                      ************************************************************

MG1655_b3247_rng      GAACTGGCCTCCGATGCCGCTTATCTGAAACGCGTCTGGACCAAAGTTATGGAGCGTAAA
JM109_WT_rng          GAACTGGCCTCCGATGCCGCTTATCTGAAACGCGTCTGGACCAAAGTTATGGAGCGTAAA
JM109_RThy_rng        GAACTGGCCTCCGATGCCGCTTATCTGAAACGCGTCTGGACCAAAGTTATGGAGCGTAAA
                      ************************************************************

MG1655_b3247_rng      AAACGCCCGCAGACCCGTTATCAGCTGTACGGCGAACTGGCGCTGGCGCAGCGTGTTCTG
JM109_WT_rng          AAACGCCCGCAGACCCGTTATCAGCTGTACGGCGAACTGGCGCTGGCGCAGCGTGTTCTG
JM109_RThy_rng        AAACGCCCGCAGACCCGTTATCAGCTGTACGGCGAACTGGCGCTGGCGCAGCGTGTTCTG
                      ************************************************************

MG1655_b3247_rng      CGTGATTTCGCCGATGCCGAACTGGACCGCATTCGCGTTGACTCACGCCTGACTTACGAA
JM109_WT_rng          CGTGATTTCGCCGATGCCGAACTGGACCGCATTCGCGTTGACTCACGCCTGACTTACGAA
JM109_RThy_rng        CGTGATTTCGCCGATGCCGAACTGGACCGCATTCGCGTTGACTCACGCCTGACTTACGAA
                      ************************************************************

MG1655_b3247_rng      GCGTTACTTGAGTTCACCTCGGAGTACATTCCCGAGATGACAAGCAAGCTGGAGCATTAC
JM109_WT_rng          GCGTTACTTGAGTTCACCTCGGAGTACATTCCCGAGATGACAAGCAAGCTGGAGCATTAC
JM109_RThy_rng        GCGTTACTTGAGTTCACCTCGGAGTACATTCCCGAGATGACAAGCAAGCTGGAGCATTAC
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MG1655_b3247_rng      ACAGGACGCCAGCCGATTTTCGATCTCTTTGATGTCGAAAACGAAATCCAGCGAGCGCTG
JM109_WT_rng          ACAGGACGCCAGCCGATTTTCGATCTCTTTGATGTCGAAAACGAAATCCAGCGAGCGCTG
JM109_RThy_rng        ACAGGACGCCAGCCGATTTTCGATCTCTTTGATGTCGAAAACGAAATCCAGCGAGCGCTG
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MG1655_b3247_rng      GAACGCAAAGTAGAACTGAAATCCGGTGGTTATCTCATTATCGACCAGACCGAAGCGATG
JM109_WT_rng          GAACGCAAAGTAGAACTGAAATCCGGTGGTTATCTCATTATCGACCAGACCGAAGCGATG
JM109_RThy_rng        GAACGCAAAGTAGAACTGAAATCCGGTGGTTATCTCATTATCGACCAGACCGAAGCGATG
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MG1655_b3247_rng      ACCACCGTGGACATCAATACCGGAGCGTTTGTCGGTCATCGCAATCTGGACGACACCATT
JM109_WT_rng          ACCACCGTGGACATCAATACCGGAGCGTTTGTCGGTCATCGCAATCTGGACGACACCATT
JM109_RThy_rng        ACCACCGTGGACATCAATACCGGAGCGTTTGTCGGTCATCGCAATCTGGACGACACCATT
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MG1655_b3247_rng      TTCAATACCAATATTGAAGCGACGCAGGCTATCGCTCGCCAGTTACGGTTGCGTAATCTG
JM109_WT_rng          TTCAATACCAATATTGAAGCGACGCAGGCTATCGCTCGCCAGTTACGGTTGCGTAATCTG
JM109_RThy_rng        TTCAATACCAATATTGAAGCGACGCAGGCTATCGCTCGCCAGTTACGGTTGCGTAATCTG
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MG1655_b3247_rng      GGCGGGATTATCATTATTGATTTCATCGATATGAATAATGAAGATCACCGCCGCCGAGTG
JM109_WT_rng          GGCGGGATTATCATTATTGATTTCATCGATATGAATAATGAAGATCACCGCCGCCGAGTG
JM109_RThy_rng        GGCGGGATTATCATTATTGATTTCATCGATATGAATAATGAAGATCACCGCCGCCGAGTG
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MG1655_b3247_rng      CTGCACTCGCTGGAGCAGGCGTTGAGCAAAGACCGGGTGAAAACCAGCGTTAATGGTTTT
JM109_WT_rng          CTGCACTCGCTGGAGCAGGCGTTGAGCAAAGACCGGGTGAAAACCAGCGTTAATGGTTTT
JM109_RThy_rng        CTGCACTCGCTGGAGCAGGCGTTGAGCAAAGACCGGGTGAAAACCAGCGTTAATGGTTTT
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MG1655_b3247_rng      TCGGCGCTGGGGCTGGTGGAGATGACGCGTAAACGCACCCGCGAAAGCATTGAGCACGTA
JM109_WT_rng          TCGGCGCTGGGGCTGGTGGAGATGACGCGTAAACGCACCCGCGAAAGCATTGAGCACGTA
JM109_RThy_rng        TCGGCGCTGGGGCTGGTGGAGATGACGCGTAAACGCACCCGCGAAAGCATTGAGCACGTA
                      ************************************************************

MG1655_b3247_rng      CTGTGTAACGAATGCCCAACCTGCCACGGTCGCGGAACGGTGAAAACCGTGGAAACGGTA
JM109_WT_rng          CTGTGTAACGAATGCCCAACCTGCCACGGTCGCGGAACGGTGAAAACCGTGGAAACGGTA
JM109_RThy_rng        CTGTGTAACGAATGCCCAACCTGCCACGGTCGCGGAACGGTGAAAACCGTGGAAACGGTA
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MG1655_b3247_rng      TGCTATGAAATCATGCGCGAGATTGTTCGTGTCCACCATGCTTACGACTCCGACCGTTTC
JM109_WT_rng          TGCTATGAAATCATGCGCGAGATTGTTCGTGTCCACCATGCTTACGACTCCGACCGTTTC
JM109_RThy_rng        TGCTATGAAATCATGCGCGAGATTGTTCGTGTCCACCATGCTTACGACTCCGACCGTTTC
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MG1655_b3247_rng      CTGGTCTATGCTTCTCCGGCAGTAGCTGAAGCCTTGAAAGGCGAAGAGTCACACTCGCTG
JM109_WT_rng          CTGGTCTATGCTTCTCCGGCAGTAGCTGAAGCCTTGAAAGGCGAAGAGTCACACTCGCTG
JM109_RThy_rng        CTGGTCTATGCTTCTCCGGCAGTAGCTGAAGCCTTGAAAGGCGAAGAGTCACACTCGCTG
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MG1655_b3247_rng    GCGGAAGTGGAAATTTTCGTTGGCAAACAGGTTAAAGTACAAATTGAACCGCTCTATAAC
JM109_WT_rng        GCGGAAGTGGAAATTTTCGTTGGCAAACAGGTTAAAGTACAAATTGAACCGCTCTATAAC
JM109_RThy_rng      GCGGAAGTGGAAATTTTCGTTGGCAAACAGGTTAAAGTACAAATTGAACCGCTCTATAAC
                    ************************************************************

MG1655_b3247_rng    CAGGAGCAGTTTGACGTCGTAATGATGTAA
JM109_WT_rng        CAGGAGCAGTTTGACGTCGTAATGATGTAA
JM109_RThy_rng      CAGGAGCAGTTTGACGTCGTAATGATGTAA
                    ****************************


                     Thr Lys Gly Ala Arg Leu Thr
JM109_WT_rng         ACT AAA GGT GCG CGC CTG ACC
JM109_RThy_rng       ACT AAA GGT GCG TGC CTG ACC
                     Thr Lys Gly Ala Cys Leu Thr
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