Phytochemicals (Carvacrol and oregano extract) as possible alternatives to antibiotics in poultry feed

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DECLARATION

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

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Conference papers

- Presented a poster entitled "Effect of carvacrol on *Escherichia coli* (*E. coli*) isolated from infected birds" at Microbiology Research Day, University of Reading on Aug 18, 2016.

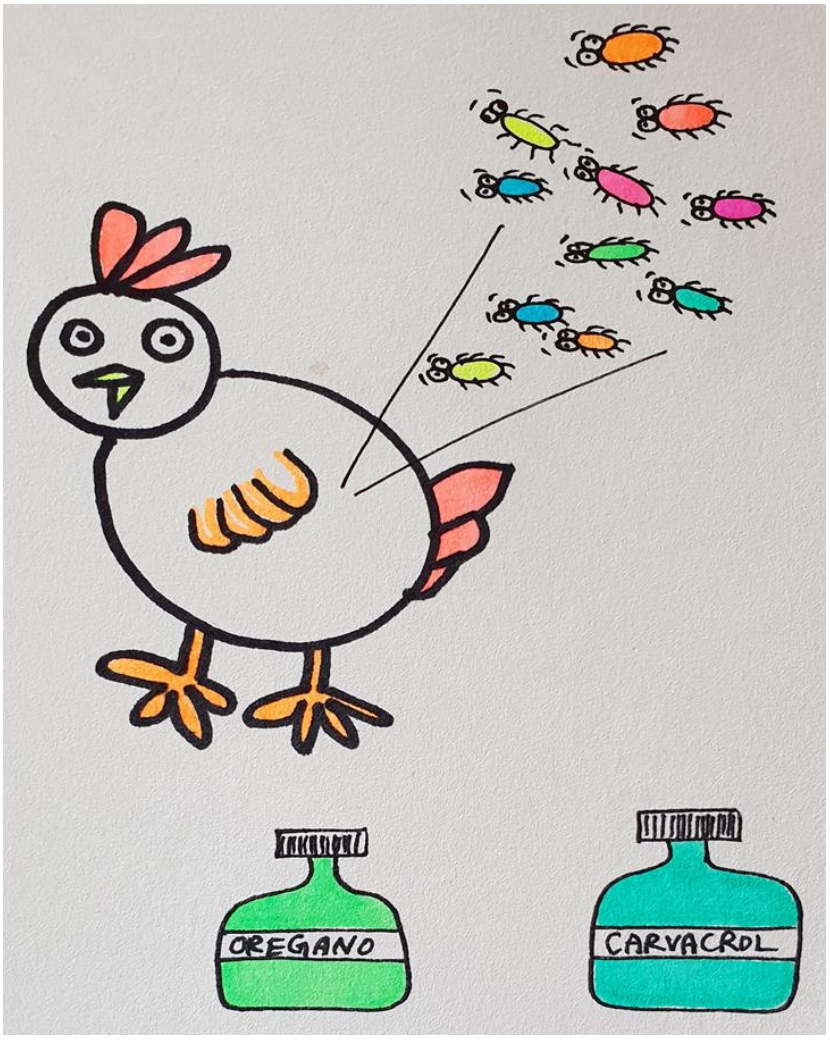
- Presented a poster entitled "Oregano extract as an alternative to antibiotics in poultry feed" at N8 AgriFood International Conference: Food Production for the future at University of Durham, Durham on July 11-13, 2017.

- Presented a poster entitled "Batch culture to study the effect of carvacrol treatment on bacterial population inhabiting the chicken gut" at the 4th Annual European Microbiome Congress Microbiology Research Day, ILEC Conference Centre, London on Nov 14-15, 2018.

ABSTRACT

Poultry provides an important protein source consumed globally by human population. Simultaneously poultry act as a substantial reservoir of antibiotic resistant *Escherichia coli* (*E. coli*) strains including commensal with beneficial roles on poultry health and productivity, and pathogenic not only to poultry but zoonotically to man. This study investigated the role of phytochemicals as possible alternatives to antibiotics and natural anti-bacterial agents to control *E. coli*; carvacrol oil (the active ingredient of oregano) and oregano oil. The possible anti-*E. coli* roles of these phytochemicals were associated with missense mutations in *marR* and *cadC* genes, mutations in *acrA* and *TolC* genes and in *ampC, ampH, pbpC* and *pbpG* genes, which are bacterial strategies to overcome antibiotics. A total of 31 representative *E. coli* strains from 3 different sources of poultry (12 APEC strains, 9 commensal chicken strains, and 10 commensal turkey strains) were shown to be phenotypically and genotypically diverse, and sequentially differentiating between types proved to be difficult. These phytochemicals inhibited the growth and biofilm formation at very low concentrations (MIC values ranging between 0.2 - 0.5μg/ml) in comparison with antibiotics. At sub-MIC levels, these phytochemicals did not induce metabolic changes (SCFAs production) in the *E. coli* strains, but synergistic interactions between carvacrol and ampicillin were observed in controlling the most ampicillin-resistant *E. coli* strains. Though, when this combination therapy was investigated using batch culture with chicken caecal content challenged with an ampicillin-resistant APEC strain, it showed an adverse effect. Carvacrol treatment showed positive impact on the bacterial population by enhancing the growth of probiotic bacteria (*Lactobacillus* sp. and *Lactobacillus salivarius*) and bacteria with anti-Salmonella activity (*Streptococcus* sp. and *Coprococcus* sp.). Thus,

providing evidence that carvacrol as a feed additive might enhance avian gut health by controlling antibiotic-resistant APEC strains, but this needs to be supported by *in vivo* studies.



They say “A picture is worth a thousand words”, so I tried to summarise my research into a cartoon/doodle.

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LIST OF ABBREVIATIONS

+	Positive
-	Negative
µg	Microgram
µl	Microlitre
µg/ml	Microgram/milliliter
A	Ampicillin
APEC	Avian Pathogenic *E. coli*
APHA	Animal and Plant Health Agency
API	Analytical Profile Index
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant genes
AST	Antibiotic susceptibility testing
Bp	Base pair
C	Carvacrol
C	Chloramphenicol
CAT	Catalase
CFU	Colony forming unit
CIT	Citrate utilization
CLSI	Clinical and Laboratory Standards Institute
CT	Colistin
CTX	Cefotaxime
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DUL	Dulcitol
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMB	Eosin-methylene blue agar
EPEC	Enteropathogenic *E. coli*
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus-PCR
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
EU	European Union
ExPEC	Extraintestinal pathogenic *E. coli*
F	Forward

FAO	Food and Agriculture Organisation
FCR	Feed conversion ratio
FISH	Fluorescent *in situ* hybridisation
FOS	Fructo-oligosaccharides
G	Gram
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GIT	Gastrointestinal tract
GOS	Galacto-oligosaccharides
GRAS	Generally recognised as safe
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
hr	Hour
I	Intermediate
ICU	Intensive care unit
IS	Internal standard
Kb	Kilobase
L	Litre
LAB	Lactic acid bacteria
LB	Luria-Bertani
mM	Millimolar
MAC	MacConkey agar
Max.	Maximum
MDR	Multi-drug resistant
MDS	Multi-dimensional scaling
MIC	Minimum inhibitory concentration
MID	Merchant Identification Number
ml	Millilitre
MLST	Multi-locus sequence typing
MOS	Mannan-oligosaccharides
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTBSTFA	N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide
NA	Nalidixic acid
NA	Nutrient agar
NB	Nutrient broth
NGS	Next generation sequencing
nm	Nanometer

O	Oregano
OD	Optical density
OTUs	Operational taxonomic units
OXI	Oxidase
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PCoA	Principal coordinates analysis
PERMANOVA	Permutational multivariate analysis of variance
ppm	Parts per million
QIIME	Quantitative Insights Into Microbial Ecology
R	Resistant
R	Reverse
S	Sensitive
S	Streptomycin
SAC	Sucrose
SAM	Ampicillin
SCFAs	Short-chain fatty acids
SOR	Sorbose
STD	Standard deviation
TBE	Tris-borate EDTA
TCA	Tricarboxylic acid
TE	Tetracycline
TOS	Transgalacto-oligosaccharides
UPEC	Urinary pathogenic *E. coli*
UPGMA	Unweighted Pair Group Method with Arithmetic Averages
UTI	Urinary tract infection
V	Vessel
V	Voltage
VL	Viande-Leuvre
VNC	Viable but non-culturable
WGS	Whole genome sequencing
WHO	World Health Organisation
WT	Wild-type
wt/vol	Unit of measure (weight/volume)

CHAPTER 1: General introduction

1.1 Poultry production

The UK is one of the leading countries in poultry production within the European Union (EU) (Kools et al., 2008). Chicken is the most UK's consumed meat, marking 40% - 50% of the total meat consumption (Valceschini, 2006). To meet the continuous increase in local and global demand for white meat and feed the growing population, improvements in white meat productivity have increased significantly in the recent years (Delgado, 2005). As a result, huge efforts have been put in order to achieve higher level of effectiveness in poultry production such as improving diet and husbandry practices (Thornton, 2010) which collectively led to improving feed conversion ratio (FCR) to 1.4 (Science, 1999). Though the use of in-feed antibiotic medication also made a contribution to an improved productivity (Bunyan et al., 1977), but this resulted in negative consequences of selecting highly resistant bacteria. As a result, the World Health Organization (WHO) set guidelines and recommendations to stop the use of antibiotics as growth promoters in 1997 (Caron et al., 2009). After that, the EU imposed a ban on the use of prophylactic antibiotics in animal feed in 2006 (Millet and Maertens, 2011). These precautionary measurements are being taken into consideration as they are important for the poultry welfare and its sustainability, and moreover for human (Casewell et al., 2003) as they are at the top of the food chain hierarchy. One aspect of current research focuses on the effect of non-medicated diet on the intestinal microbiota since diet has a direct impact on productivity and animal health (Borda-Molina et al., 2018), so there is a need to understand the impact of this change through the food chain.

1.2 Feed additives in poultry

1.2.1 Antibiotics

Antibiotics are chemical compounds that kill or inhibit bacterial growth. They were discovered in the early 20th century as a cure to certain diseases (Davies and Davies, 2010). Since the discovery of penicillin by Alexander Fleming, many other antibiotics belonging to many different classes and with differing anti-bacterial properties have been discovered and chemically synthesised or manufactured *de novo* (Macfarlane, 1984). The growth promoting effect of antibiotics was discovered in the 1940s (Hughes and Datta, 1983), and later in the 1950s and 1960s, they were authorised with set guidelines by the EU to be used in animal feeds (Castanon, 2007). However, the 1980s marked the emergence of global antibiotic resistant bacteria (ARB) carrying antibiotic resistant genes (ARG) (Aarestrup, 2003). This raised worries as these ARG would be transferred through the food chain from animals to man (Greko, 2001). A European surveillance study conducted in 2005 proved the presence of ARB of animal origin among patients admitted to intensive care units (ICU) (Hanberger et al., 2009). ARB were the reason behind high numbers of medical illnesses and even death (Cosgrove, 2006).

Antibiotics used as a prophylactic agent have shown to have positive effects on the growth performance of chicken as a presumed result of reduced pathogen load, and reduction in competition for nutrients in the small intestine, reduction of inflammation, and improvement of digestion (Thomke and Elwinger, 1998). Also, they were used to fight bacterial infections as a therapeutic drug and at sub-therapeutic levels as feed ingredients, because they were shown to enhance growth, but unfortunately this led to the rise of the

first incident of resistant *Salmonella enterica* ser. Typhimurium in 1963 (Dewey et al., 1997).

The usage of antibiotics results in disturbance of homeostasis of the human microbiota (Dethlefsen et al., 2008). In human, antibiotic induced diarrhea is a common phenomenon associated with bacterial dysbiosis (Pham and Lawley, 2014). Antibiotics affect the colonisation of the native microbiota, competition for food and space, and increases availability of ARG in pathogens (Sommer et al., 2009). This factor causes major shifts in the composition of microbiota in comparison with the other factors (Dave et al., 2012). For example, the decrease in *Lactobacillus* growth level gives space for *Candida* species to flourish in the caecum, due to the production of prostaglandin E_2 (Kim et al., 2014). This is due to the fast effect and potency of antibiotics in killing or inhibiting the growth of bacterial communities. However, the gut microbiota can re-gain its composition after a short period of time from finishing the antibiotics course, due to its elasticity and flexibility (Dethlefsen et al., 2008). Unfortunately, long-term usage of antibiotics causes incomplete recovery (Dethlefsen and Relman, 2011).

Bacteria are evolving continuously and developing antibiotic resistance at a high level, which most professionals in the field of disease control agree is due to ‘abuse’ of these antibiotics that led to the prevalence of ARB and ARG (Levy and Marshall, 2004). There are many factors that contributed to this issue with probably the over and inappropriate use of antibiotics in agriculture, and human and animal medication (Alekshun and Levy, 2007) being considered one of the primary causes. There is a continuous pressure being applied to reduce antibiotic usage in many areas, including general practice and hospitals

(Goossens et al., 2005), as the development of new antibiotics is a long procedure hindered by a very high resistance against existing antibiotics (Spellberg et al., 2004). There is an urgent need to find alternatives to antibiotics with a direct beneficial effect on the animal gut microbiota as their principal mode of action.

1.2.1.1 Antibiotic resistance

ARB carrying ARG increases the reservoir of resistance genes in the human microbiome (Sommer et al., 2010). In fact, many nosocomial infections these days are due to the dissemination of multi-drug resistant (MDR) Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriacea* producing extended spectrum β-lactamases (ESBL) (Tacconelli et al., 2014). While, 40-60% of the nosocomial infections are MDR of Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Clancy et al., 2006). The prolonged use of antibiotics selects for resistant bacteria (Levy, 1985), but on the other hand, the selected bacteria and resistance genes reservoir will decrease in numbers once the treatment stops (Alekshun and Levy, 1997). Therefore, the usage of antibiotics should be strictly controlled and limited in the medical and veterinary areas (Khachatourians, 1998).

There are mechanisms which lead to the MDR phenomenon, which are encoded by plasmids and transposons (DeFlaun and Levy, 1989), bacteriophages (Levy and Marshall, 2004), and through gene mutations, which can be transferred from one bacterium to another through horizontal gene transfer (HGT) (Chadwick and Goode, 1997). Antibiotic resistance plasmids have received a lot of interest, due to their medical and practical importance (Norman et al., 2009). Plasmids are covalently-closed circular DNA, extra-

chromosomal elements possessing the ability to replicate independently inside most bacterial cells, and some archaea and eukaryotic cells. They differ in size, which is measured by kilobase (kb) pairs, ranging from a small number of kb to more than hundreds of kb (Wegrzyn, 2005). They also differ in the genes they carry, such as antimicrobial resistance or virulence gene, which will give an extra advantage to the bacterial cell under the appropriate conditions (Amábile-Cuevas and Chicurel, 1992). Many of the virulence factors and AMR genes described later in **Chapter 2** are plasmid-mediated.

1.2.2 Probiotics, prebiotics, and synbiotics

Probiotics, a term of Greek origin, means "for life" (AFRC, 1989). Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host", by the Food and Agriculture Organisation (FAO)/WHO (Group, 2001). Though probiotic activity is still unclear, these health beneficial bacteria work on so many levels, including providing microbiota with nutrients (AFRC, 1989), repressing signaling of innate immune system, activation of T-cell proliferation, and contributing to the elimination of pathogenic microbes (Schuppan et al., 2009). Probiotic bacteria should have the ability to colonise the gastrointestinal tract (GIT), endure GIT harsh conditions (low pH and presence of bile acids), and compete with other microorganisms (Nurmi et al., 1983). Examples of probiotic bacteria are *Lactobacillus* and *Bifidobacterium*, which are lactic acid bacteria (LAB), and they can restore the structure of the gut microbiota (Vyas and Ranganathan, 2012). LAB may restrict the growth of either Gram-positive or Gram-negative bacterial species, owing to their ability to produce by-products with antimicrobial activities including lactic acid, acetic acid, hydrogen peroxide, bacteriocins, and biosurfactants (Lee and Salminen, 1995). Other than bacteriocins, some LAB produce

reuterin, a compound with antimicrobial properties (Talarico et al., 1988). LAB as probiotics can be a single strain or several LAB strains that are ideally isolated from the gut microbiota of the animal to be targeted with this treatment. An *in vitro* study conducted on chickens supplied with *Lactobacillus salivarius* as a probiotic showed that there was an inhibition of *E. coli* and *Salmonella enterica* ser. Enteritidis growth, and specifically reducing their ability to adhere to the gut epithelium (Garriga et al., 1998). Other bacteria with such advantage include *Bacillus* spp., *Enterococcus faecium*, and fungus such as *Saccharomyces cerevisiae* (Simon et al., 2001).

The concept of prebiotics was introduced in 1907 by Elie Metchnikoff (Anukam and Reid, 2007), and ever since that time, these products have attracted attention along with probiotics (Vitetta et al., 2012). Prebiotics represent "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring health benefit(s) upon the host" (Gibson and Roberfroid, 1995). These ingredients that are often complex long chain oligosaccharides find their way to the large intestine, where certain numbers of the gut microbiota with the appropriate digestive enzymes can break them down to derive energy and proliferate (Bandyopadhyay and Mandal, 2014). In other words, they selectively nourish these gut microbiota to support their growth, increase their metabolic activities, and consequently suppress the growth of pathogens (Park and Floch, 2007). *Lactobacillus* and *Bifidobacterium* often have the capability to digest prebiotics and therefore become enriched in the large intestine. Prebiotics can be inulin and transgalacto-oligosaccharides (TOS) of carbohydrate and oligosaccharide nature (Bandyopadhyay and Mandal, 2014). Other examples of prebiotics are galacto-oligosaccharides (GOS) which can be found in human milk and can enrich the

growth of some gut microbiota (Macfarlane et al., 2008), mannan-oligosaccharides (MOS) that trigger immune responses, and bind to pathogens (*Salmonella* expressing type-1 fimbriae) found in the intestinal tract of young chicks (Spring et al., 2000), and fructo-oligosaccharides (FOS) improve digestion, increasing *Bifidobacterium* and *Lactobacillus* numbers but decreasing *E. coli* numbers, and mitigating *Salmonella enterica* ser. Typhimurium infection in broiler chickens (Choi et al., 1994).

Combining the administration of probiotics and prebiotics is called synbiotics (Vyas and Ranganathan, 2012). The synergy between probiotics and prebiotics allows probiotics to maximise their beneficial effects on the host (Bandyopadhyay and Mandal, 2014). This is because probiotics might not work effectively without supplying them with growth requirements (Apajalahti et al., 2004). An example on synergistic therapy is using both *Bifidobacterium lactis* and GOS that was effective in improving the gut microbiota (Jung et al., 2008), and controlling cellulitis caused by *E. coli* in broiler chickens (Estrada et al., 2001).

1.2.3 Phytochemicals

Another approach through which microbiota may be enhanced is with the use of natural plant products, called phytochemicals (Hashemi and Davoodi, 2010). Phytochemicals is a broad term that includes non-digestible carbohydrates and compounds such as lignin, resistant protein, polyphenols and carotenoids, some of which are considered anti-oxidants (Saura-Calixto et al., 2000). They differ in chemical structure, biological activity, plant source, and method of production. In other words, they are natural sources of growth promoters coming from plants, herbs, or spices (Hashemi and Davoodi, 2010). Generally,

phytochemicals are categorised into five main groups; terpenoids, polyphenols, organosulfur compounds, phytosterols, and alkaloids (Somani et al., 2015). The phytochemicals that infer good beneficial impacts on human health and are claimed to prevent and cure diseases are named nutraceuticals, that was first introduced as a term by DeFelice (DeFelice, 1995).

Presently, plant-based natural therapies are of increased popularity, because consumers are becoming aware of concerns regarding synthetic additives (Hammer et al., 1999) as well as the dangers of antibiotic use as discussed earlier. Scientific research changed the perception of food including phytochemicals from being an energy source to that of health promoting supplements because of their bioactive roles (Berner and O'Donnell, 1998). Therefore, it is crucial to understand the scientific background behind their beneficial roles as anti-microbial agents (Mitscher et al., 1987), and how can we use them as an alternative to antibiotics given the rising problem of ARB in poultry industry, consumers demands, and the EU on the usage of antibiotics for growth promotion.

Phytochemicals come from natural sources and are generally recognised as safe (GRAS) which make them good candidates to be used as feed additives in poultry production in comparison with antibiotics (Hashemi et al., 2008). The biological mechanism of action of phytochemicals depends on their chemical structure (Hashemi and Davoodi, 2010). Phytochemicals used as poultry feed additives can improve animal's health and performance because of their anti-microbial, anti-stress (Wang et al., 1998) and anti-oxidant properties (Valenzuela, 1995), and their ability to modulate gut microbiota (Hashemi et al., 2009) and enhance immune responses (Chowdhury et al., 2018). The

efficiency of these phytochemicals is determined by intrinsic and extrinsic factors such as animal's nutrition and health, type of diet and environment (Giannenas et al., 2003). On the other hand, phytochemicals used in humans have many functions including being anti-oxidant, anti-estrogenic, anti-inflammatory, immunomodulatory and anti-carcinogenic (Laparra and Sanz, 2010). They can act as prebiotics by enhancing the growth of beneficial bacteria and suppressing the growth of pathogenic bacteria (Cencic and Chingwaru, 2010). Thus, they reward the host by shaping gut microbiota in a beneficial way (Laparra and Sanz, 2010). Additional modes of action of phytochemicals include the decrease in mutagen and carcinogen formation (Bartsch et al., 1988); stimulation of healing intestinal mucosa damage (Asfar et al., 2003); decrease in colonic mucosa DNA oxidative damage (Dolara et al., 2005); alteration in colon microbiota; and regulation of metabolic, immunological, and adaptive gene expression (López-Oliva et al., 2010).

1.2.3.1 Polyphenols

Polyphenols are the key group of phytochemicals exhibiting nutritional importance to health (Saura-Calixto et al., 2007) with more than 8000 chemical compounds extracted and described from this group (Han et al., 2007). They are secondary metabolites produced by plants, and can be found in fruits, vegetables, cereals, and beverages (Puupponen-Pimia et al., 2002). They display anti-bacterial effects (Fukai et al., 1991) measurable by minimum inhibitory concentration (MIC) experiments (Eloff, 1998), anti-fungal effects (Larrondo et al., 1995), and anti-oxidant effects with free radical scavenging activity (González-Paramás et al., 2004) resorting to their chemical structure (Farag et al., 1989). As anti-bacterial agents, they can decrease the adhesion capability of pathogenic bacteria e.g. *Clostridium perfringens* and their numbers in chickens gut (Mitsch et al., 2004), and inhibit the growth

of food-borne pathogens such as *Staphylococcus aureus* and *Salmonella enterica* ser. Typhimurium (Paster et al., 1990). Also, they can improve the efficiency of feed utilisation (Hernandez et al., 2004), and therefore enhance the performance of host animals (Terada et al., 1993). The phytochemicals investigated in this thesis are carvacrol and oregano which are of polyphenol nature.

1.3 *Escherichia coli* (*E. coli*)

The German paediatrician, Theodor Escherich, was the first one to observe *E. coli* in 1885 (Escherich, 1886). *E. coli* is a facultative anaerobe (Finegold et al., 1983a), Gram-negative bacterium (Scheutz and Strockbine, 2005) belonging to the phylum Proteobacteria (Marchesi et al., 2016) and the family *Enterobacteriaceae* (Ewing, 1986), and it is part of the human and animal intestinal natural microbiota (Ørskov and Ørskov, 1992). The beneficial *E. coli* strains such as *E. coli* strain Nissle can be used as a probiotic (Wehkamp et al., 2004). Probiotics have been reported to have beneficial health effects, which include defence against enteric pathogens, stimulation and improvement of intestinal immune system and peristaltic activity, and enriched digestion through enzymes to break down indigestible food nutrients such as lactase in those who are lactose intolerant (Doyle et al., 2013). However, some *E. coli* strains have the ability to cause diseases, like diarrhoea, urinary tract infections (UTI), bacteraemia, and infant meningitis (Ørskov and Ørskov, 1992). These pathogenic strains attained virulence factors for producing toxins, protective cell covering, and iron-acquisition systems (Babai et al., 1997). The pathogenic strains spread among contaminated food mainly uncooked or undercooked meat (Gould et al., 2013) and municipal potable water (Brunkard et al., 2011).

1.3.1 Types of *E. coli*

E. coli is a complex specie with many variant types: there are three main groups of *E. coli* which are commensal, diarrhoeagenic, and extraintestinal pathogenic strains, classified based on genetic and clinical principles (Russo and Johnson, 2000). Commensal *E. coli* is part of the human and animal intestinal natural microbiota (Ørskov and Ørskov, 1992), and tend to harbour none or a very small number of virulence factors in comparison with the pathogenic ones (Selander et al., 1987) and also belongs to different phylogenetic groups (Herzer et al., 1990).

1.3.1.1 Human pathogenic *E. coli*

E. coli as a human pathogen is commonly responsible for most UTI clinical cases caused by the urinary pathogenic *E. coli* (UPEC) (Ronald, 2002). Under the diarrhoeagenic strains, there are different sub-pathotypes, and they are enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC) that induce diarrhoea, whereas extraintestinal pathogenic *E. coli* (ExPEC), enteroinvasive *E. coli* (EIEC) and UPEC are associated with non-intestinal infections. It should be pointed out that UPEC are often found in the gastro-intestine and colonise the urethra and potentially kidneys through faecal contamination. Each pathotype has its own virulence characteristics, and thereby different clinical behaviours (Kaper et al., 2004). The focus of this thesis will be on *E. coli* from avian sources; commensal and pathogenic types.

1.3.1.2 Avian pathogenic *E. coli* (APEC)

Pathogenic *E. coli* specific for poultry are defined as avian pathogenic *E. coli* (APEC), that are routinely found as part of the chicken or birds gut microbiota and not necessarily associated with an overt disease (Dho-Moulin and Fairbrother, 1999). APEC are considered to be a sub-type of human ExPEC as they share several virulence genes (Rodriguez-Siek et al., 2005), although there remains a debate whether APEC are true zoonotic microorganisms (Tivendale et al., 2010, Krishnan et al., 2015, Mohamed et al., 2018). APEC bacteria usually carry large plasmids which harbour some genes that contribute to their pathogenicity (Rodriguez-Siek et al., 2005), and some of these plasmids can be transmitted to other bacteria through conjugation (Trieu-Cuot et al., 1987). APEC are regarded as opportunistic bacteria that readily colonise intestine as well as lungs and air sac epithelium layer of poultry, due to their ability to produce adhesins (Stordeur et al., 2002b). Progression to causing disease is often associated with other stress-related stimuli such as high ammonia in the atmosphere (Oyetunde et al., 1978), or infections with *Mycoplasma* species (Weinack et al., 1984). It has been suggested that some APEC are transmitted vertically and colonise the yolk-sac of hatchlings which tend to progress to disease without other stimuli. As a result of APEC infections, the poultry industry suffered a lot from the loss of chickens and birds, which affected the food economy (Dho-Moulin and Fairbrother, 1999). These APEC infections were first controlled using antibiotics, but unfortunately, the misuse of the antibiotics led to the rise of ARB strains instead (Bass et al., 1999). The emergence of antibiotic resistance reflects the bacterial evolution at the genetic level (McArthur et al., 2013), due to environmental stresses (Herren et al., 2006) and the transfer of multiple ARG among enteric bacteria that occurs through transmission of integrons (Bass et al., 1999), plasmids and transposons (Diarrassouba et al., 2007).

Currently, APEC have a very high incidence of multiple antibiotic resistance (Magray et al., 2018, Subedi et al., 2018).

1.3.1.2.1 Avian colibacillosis caused by APEC

One of the diseases associated with APEC strains in poultry industry is a disease collectively named avian colibacillosis (Gross, 1994) responsible for worldwide poultry loss as a result of their contribution to high morbidity and mortality rates (Ewers et al., 2003) and can affect birds at any age (Otaki, 1995). Avian colibacillosis is a term that encompasses localised and systemic infections (Nolan et al., 2013), but systemic infections are the most prevalent among poultry (chicken and turkey) such as pericarditis, perihepatitis, peritonitis, and salpingitis (Dho-Moulin and Fairbrother, 1999). The common route of infection is through inhalation of dust particles containing APEC strains found in contaminated faeces (Barnes et al., 2003). APEC strains first adhere and colonise or establish themselves in the upper respiratory tract causing air sacculitis or air sac disease (Nolan et al., 2013). Depending on several factors such as possession of virulence genes by these APEC strains and the health status of the host, they might migrate via the blood stream causing septicaemia and spread to other internal organs resulting in systemic infections (Barnes et al., 2003). However, they eventually colonise the large intestine which becomes the main reservoir for these bacterial strains (Dho-Moulin and Fairbrother, 1999) which can be excreted with the faeces to repeat the cycle of infection.

1.3.2 *E. coli* as a model microorganism

The focus of this study was upon *E. coli* for the following reasons. As previously mentioned, *E. coli* is normally part of the human and animals' intestinal natural microbiota (Ørskov and Ørskov, 1992), being the most dominant aerobic bacterium (Savageau, 1983) with 10^6 to 10^9 colony forming unit (CFU) per cm of the poultry (chicken and turkey) intestine (Leitner and Heller, 1992), and it is one of the first species to colonise the gut of human (Mitsuoka, 1973) and animal (Hudault et al., 2001). Also, it is one of the best studied bacterial species and often used as a model microorganism because of their different commensal and pathogenic types, and with *E. coli* K12 being the most common reference strain (Hobman et al., 2007). The pathogenic type such as APEC strains are the causative agent of colibacillosis in poultry (Gross, 1994). Moreover, *E. coli* can be easily grown in the laboratory, as it needs simple growth requirements, grows at a fast rate, and extensive information is already provided in literature (Donachie and Begg, 1970).

In terms of antibiotic resistance, *E. coli* is the most common carrier of ESBL genes which are located on plasmids that facilitate their transfer (Donachie and Begg, 1970), and these genes are widespread among chickens (Machado et al., 2008). Food containing ARB of animals origin affect antibiotics action used among humans, because of the spread of ARB and their ARG, or provides a reservoir for ARB and their ARG in human gut that might cause opportunistic diseases in the future (Smith et al., 2002). In human hosts, it is the main causative agent and is responsible for most cases of UTI infection (Stamm and Hooton, 1993).

1.4 General overview of the thesis

This thesis is intended to shed light upon current issues in the poultry industry which are threatening health and welfare of birds and affecting their productivity. The first issue is the spread of antibiotic resistance among *E. coli* strains in poultry industry. As a result of the use and abuse of antibiotics and how we can control *E. coli* using alternatives. The second issue is controlling pathogenic *E. coli* strains in poultry; APEC strains are the causative agent of colibacillosis disease and responsible for economic losses in poultry industry. The focus was on using phytochemicals from polyphenol group (carvacrol and oregano) as potential feed additives in poultry production. Thus, the testable hypotheses of this thesis are:

- Phenotypic and genotypic characteristics can be used to establish differential criteria between APEC and commensal *E. coli* strains.
- Phytochemicals (carvacrol an oregano) used in this study can exhibit anti-bacterial properties against the *E. coli* strains.
- The mechanisms of action of these phytochemicals can be investigated.
- The use of combined treatment of sub-lethal concentrations of carvacrol and ampicillin can be used to increase the efficacy of ampicillin in controlling ampicillin-resistant *E. coli* strains.
- The effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin can be used to increase the efficacy of ampicillin in controlling a mixture of an APEC and two commensal *E. coli* strains.
- The effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin can be used to increase the efficacy of ampicillin in controlling

ampicillin-resistant *E. coli* strains in pooled caecal content challenged with an APEC strain.

1.5 General aims and objectives

The aims and objectives of this thesis were:

- To characterise different types of *E. coli* strains (commensal and APEC) coming from poultry sources (chicken and turkey) in terms of their phenotype and genotype (**see Chapter 2**).
- To study the efficacy of phytochemicals (carvacrol and oregano) as anti-bacterial agents on the growth of the *E. coli* strains, their biofilm formation, and their production of short-chain fatty acids (SCFAs) (**see Chapter 3**).
- To investigate the mechanistic roles of these phytochemicals on the *E. coli* strains by looking at their phenotypic and genotypic changes (**see Chapter 4**).
- To study the efficacy of carvacrol when combined with ampicillin on ampicillin-resistant *E. coli* strains by looking at their growth, and production of SCFAs as part of their metabolism, and to study the efficacy of combined treatment of carvacrol and ampicillin on a mixed *E. coli* culture of an APEC and two commensal *E. coli* strains by looking at their total numbers and ability to recover the starting strains (**see Chapter 5**).
- To study the efficacy of combined treatment of carvacrol and ampicillin on pooled caecal content challenged with an APEC strain by looking at total numbers of *E. coli* and ampicillin-resistant *E. coli*, production of SCFAs, and changes in bacterial populations at different taxonomic levels (**see Chapter 6**).

Note: This general introduction aimed at covering all the aspects and linking different angles related to this topic in brief without going into details of each section. More detail will be given in each research chapter (**see Chapters 2 to 6)** in relation to the discussed topic.

CHAPTER 2: Identification and characterisation of *E. coli* strains

2.1 Introduction

E. coli is normally part of the intestinal natural bacterial microbiota of humans and animals intestinal tract (Ørskov and Ørskov, 1992). It is a facultative anaerobe enabling it to live in both aerobic and anaerobic environments (Finegold et al., 1983b). It can overcome the strict anaerobic conditions of the gut by colonising the epithelium layer close to blood supply where oxygen is available (Savage, 1977). Moreover, it can utilise mucin within the mucous layer covering the epithelium layer as a sole carbon and energy source (Montagne et al., 2004). However, *E. coli* flourishes and thrives in many environmental conditions contaminated by faecal matter (Ibekwe et al., 2011). Thus, controlling effluent contaminated with faecal matter is essential (James, 2006) whether this is on farm, slaughterhouses, hospitals, workplaces or homes. It is particularly important to control faecal contamination from farm to fork along the human food chain. Irrespective of whether *E. coli* which we are highlighting in this study is defined as a harmless commensal or a pathogen, a major concern for *E. coli* and for that matter all bacteria along this farm to fork pathway is the carriage of ARG. Animal production systems have relied upon antibiotics for healthy animal development, but this comes with the known outcome of selection of resistant organisms, increasing the reservoir of resistance genes that may enter the food chain and ultimately the human microbiome (Sommer et al., 2010). ARB pose a major risk because of the potential to become MDR over time (Fair and Tor, 2014) and resistant to antibiotic therapy.

This chapter aims to study the characteristics of *E. coli* strains isolated from the gut of poultry (Chickens and Turkeys) as it is intended to use representative poultry *E. coli* to assess the potential of phytochemicals as alternatives to antibiotics as a way of controlling them given the fact that they may carry antibiotic resistance and virulence genes that might spread among the food chain.

2.2 Materials and methods

2.2.1 *E. coli* strains

A total of thirty-three (33) *E. coli* strains (**Table 2.1**) were used in this study:

- 12 APEC strains isolated from the respiratory system (lung and air sacs; where the colibacillosis disease usually starts) of the diseased birds (chickens) suffering from colibacillosis with clear signs of infection (generously provided by Prof. Roberto La Ragione, University of Surrey),
- 9 wild-type presumptive commensal *E. coli* strains isolated from healthy chicken gut (caecum) and 10 *E. coli* strains isolated from healthy turkey gut (caecum) (a gift from colleague, Fatemah Alkandhari; a previous PhD student who had undertaken poultry feed trials at the CEDAR farm, University of Reading),
- 2 *E. coli* strains wild-type K12 (MG 1655 WT) and its *acrAB* mutant (MG 1655M) (generously provided by Prof. Chris Thomas, University of Birmingham).

Table 2.1 *E. coli* strains

No.	Code	Source/ Host	Type
1	**7**	Chicken respiratory system	APEC
2	**14**	Chicken respiratory system	APEC
3	**43**	Chicken respiratory system	APEC
4	**45**	Chicken respiratory system	APEC
5	**46**	Chicken respiratory system	APEC
6	**47**	Chicken respiratory system	APEC
7	**48**	Chicken respiratory system	APEC
8	**49**	Chicken respiratory system	APEC
9	**51**	Chicken respiratory system	APEC
10	**52**	Chicken respiratory system	APEC
11	**53**	Chicken respiratory system	APEC
12	**54**	Chicken respiratory system	APEC
13	**C1**	Chicken gut (Caecum)	Wild-type commensal strain
14	**C2**	Chicken gut (Caecum)	Wild-type commensal strain
15	**C3**	Chicken gut (Caecum)	Wild-type commensal strain
16	**C4**	Chicken gut (Caecum)	Wild-type commensal strain
17	**C5**	Chicken gut (Caecum)	Wild-type commensal strain
18	**C6**	Chicken gut (Caecum)	Wild-type commensal strain
19	**C7**	Chicken gut (Caecum)	Wild-type commensal strain
20	**C8**	Chicken gut (Caecum)	Wild-type commensal strain
21	**C9**	Chicken gut (Caecum)	Wild-type commensal strain
22	**T1**	Turkey gut (Caecum)	Wild-type commensal strain
23	**T2**	Turkey gut (Caecum)	Wild-type commensal strain
24	**T3**	Turkey gut (Caecum)	Wild-type commensal strain
25	**T4**	Turkey gut (Caecum)	Wild-type commensal strain
26	**T5**	Turkey gut (Caecum)	Wild-type commensal strain
27	**T6**	Turkey gut (Caecum)	Wild-type commensal strain
28	**T7**	Turkey gut (Caecum)	Wild-type commensal strain
29	**T8**	Turkey gut (Caecum)	Wild-type commensal strain
30	**T9**	Turkey gut (Caecum)	Wild-type commensal strain
31	**T10**	Turkey gut (Caecum)	Wild-type commensal strain
32	**MG1655 WT**	University of Birmingham	Wild-type K12
33	**MG1655 *acrAB* M**	University of Birmingham	Mutant K12 (*acrAB* mutant)

APEC strains refer to *E. coli* strains isolated from infected birds (chickens); wild-type commensal strains refer to *E. coli* strains isolated from healthy birds (chickens and turkeys).

2.2.2 Confirmation of the identity of *E. coli* strains

2.2.2.1 Growth on general purpose and selective media

For preliminary identification, an inoculum (a loopful of colonies) from an overnight *E. coli* culture (10 – 12hr) grown on nutrient agar (NA) (Sigma-Aldrich, UK) plate at 37°C was streaked to give single colonies on MacConkey agar (Sigma-Aldrich, UK) and Eosin-methylene blue (EMB) agar (Sigma-Aldrich, UK) plates. The plates were incubated overnight at 37°C. After the incubation period, each plate was checked for growth and pink colonies on MacConkey agar, and metallic green colonies on EMB agar, gave a presumptive indication of being *E. coli* strains.

2.2.2.2 Catalase test

An inoculum (a loopful of colonies) from an overnight *E. coli* culture (10 – 12hr) grown on NA plate was placed on a glass slide. Then, a drop of Hydrogen Peroxide (3%) (Sigma-Aldrich, UK) was placed on top of the bacterial biomass. Appearance of bubbles over the bacterial biomass immediately and certainly no later than 3min indicated the presence of catalase enzyme.

2.2.2.3 Oxidase test

An inoculum (a loopful of colonies) from an overnight *E. coli* culture (10 – 12hr) grown on nutrient agar plate was placed on filter paper. Then a drop of 1% tetramethyl-p-phenylene diamine dihydrochloride (Sigma-Aldrich, UK) was placed on top of the bacterial biomass. Appearance of blue colour indicated the presence of oxidase enzyme.

2.2.2.4 Analytical Profile Index (API) 20E

An inoculum (a loopful of colonies) from an overnight *E. coli* culture (10 – 12hr) grown on NA plate was suspended in sterile-distilled water, and the optical density (OD) of the suspension was adjusted to 0.1 at 600nm (10^8 CFU/ml). Then, the bacterial suspension was introduced into API 20E (BioMèrieux, UK) strips according to the manufacturer's instructions. The strips were incubated overnight at 37°C. Results were taken as positive or negative following the manufacturer's instructions.

2.2.3 Phenotypic characterisation

2.2.3.1 Sugar utilisation

An inoculum (a loopful of colonies) from an overnight *E. coli* culture (10 – 12hr) grown on NA plate was streaked on M9 minimal medium (**see Appendix**) or M9 minimal medium supplemented with thiamine (25µg/ml) if mutant *E. coli* K12 strains were to be tested to which different sugars (sucrose, sorbose, and dulcitol) at a concentration of 0.2% w/v were provided as the sole carbon and energy source. The plates were incubated at 37°C for up to 48hr and scored as positive if growth was observed or negative if no growth was observed.

2.2.3.2 Antibiotic susceptibility testing (AST) using antibiotic discs

The antibiotic susceptibility of the *E. coli* strains was determined using Kirby-Bauer method (Barry et al., 1979). Briefly, an inoculum (a loopful of colonies) from an overnight *E. coli* culture (10 – 12hr) grown on NA plate was suspended in Luria-Bertani (LB) broth (Sigma-Aldrich, UK) and the OD of the suspension was adjusted to 0.100 at 600 nm (10^8 CFU/ml). Then, 0.1 ml of the suspension was transferred to NA or LB plates and spread

evenly. After that, antibiotic discs purchased from Thermo Fisher (cefotaxime 30µg, nalidixic acid 30µg, chloramphenicol 30µg, colistin 10µg, ampicillin 25µg, streptomycin 25µg, and tetracycline 30µg) were placed on the air-dried surface of the spread bacterial inocula, and plates were incubated overnight at 37°C. Diameters of inhibition zones were interpreted according to the guidelines of Clinical and Laboratory Standards Institute (CLSI), 2016. The zone diameter interpretive criteria for each antibiotic disk were the following; cefotaxime 30µg (S = ≥ 2.6 cm, I = 2.3-2.5 cm, R ≤ 2.2 cm), nalidixic acid 30µg (S = ≥ 1.9 cm, I = 1.4-1.8 cm, R ≤ 1.3 cm), chloramphenicol 30µg (S = ≥ 1.8 cm, I = 1.3-1.7 cm, R ≤ 1.2 cm),, colistin 10µg (S = ≥ 1.1 cm, R ≤ 1.0 cm), ampicillin 25µg (S = ≥ 1.7 cm, I = 1.4-1.6 cm, R ≤ 1.3 cm), streptomycin 25µg (S = ≥ 1.5 cm, I = 1.2-1.4 cm, R ≤ 1.1 cm), and tetracycline 30µg (S = ≥ 1.5 cm, I = 1.2-1.4 cm, R ≤ 1.1 cm).

2.2.3.3 Detection of biofilm using modified Congo red agar

The method of Milanov was followed for detection of biofilm in *E. coli* (Milanov et al., 2015). In brief, an inoculum of overnight *E. coli* culture (10 – 12hr) grown on NA plate at 37°C was spotted on modified Congo red agar plates. The plates were incubated overnight at 37°C, then for 4 days at 25°C. After the incubation period, each plate was checked for growth, colour of colonies, and appearance, to detect lacy colonies which are a correlate for biofilm formation.

2.2.4 Genotypic characterisation

2.2.4.1 DNA extraction and quantitative and qualitative assessments

Genomic DNA of the *E. coli* strains (**Table 2.1**) was extracted using Gentra Puregene Yeast/Bact. Kit (Qiagen®, UK) following the manufacturer's recommendations. For quantitative assessment of the genomic DNA extracts, DNA concentration was measured spectrophotometrically using a Nanodrop machine (NanoDrop Technology, USA) following the manufacturer's recommendation. And for qualitative assessment, a sample of the genomic DNA extracts (1µl) was separated on a 1% agarose gel in 0.5X tris-borate EDTA (TBE) buffer containing 0.03µg/ml ethidium bromide stain. The genomic DNA extracts were stored at -20^{0}C; to be used as templates for polymerase chain reaction (PCR) experiments.

2.2.4.2 Detection of virulence factors by PCR

The presence of virulence genes is listed in **Table 2.2** and was detected by PCR using GE Healthcare illustra™ PuReTaq Ready-To-Go™ PCR Beads (Thermo Fisher Scientific, UK) in the panel of *E. coli* strains (n= 31) and compared with the control strain MG1655 WT (*E. coli* K12). PCR reactions of the target genes using specific primers (**Table 2.2**) were performed in a volume of 25µl with different volumes of 10mM $MgCl_2$ (no $MgCl_2$ for *fim1*, *papC*, *csgA*, *iucD*, and *cvi/cva*; and 1.25µl of 10mM $MgCl_2$ for *crl*, *tsh*, *irp2*, *iss*, *astA*, *kps*, and *hlyA*). The PCR reactions were carried out in a Thermocycler (Thermo Scientific, UK) with the following program: 3 min initial denaturation at 94°C, followed by 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 58°C, 3 min extension at 68°C, and final extension of 72°C for 5 min. The amplified products were separated in 1.5% (wt/vol) agarose gel in 0.5X TBE buffer containing 0.03µg/ml ethidium bromide stain at 50V for 1hr.

Table 2.2 PCR primers used for detection of virulence genes

Gene ID	Virulence factor	Class	Primers sequence (5` --- 3`)	Product size (bp)	Genomic location	Reference* (primers)
fim1	Type 1 fimbriae	Adhesins	**F:** AGAACGGATAAGCCGTG **R:** GCAGTCACCTGCCCTCCGGTA	508	Chromosome	(Johnson and Stell, 2000)
papC	P fimbriae	Adhesins	**F:** TGATATCACGCAGTCAGTAG **R:** CCGGCCATATTCACATA	501	Chromosome	(Ewers et al., 2005)
csgA	Curli fimbriae	Adhesins	**F:** ACTCTGACTTGACTATTACC **R:** AGATGCAGTCTGGTCAAC	200	Chromosome	(Maurer et al., 1998)
crl	Curli fimbriae	Adhesins	**F:** TTTCGATTGTCTGGCTGTATG **R:** CTTCAGATTCAGCGTCGTC	250	Chromosome	(Maurer et al., 1998)
tsh	Temperature-sensitive hemagglutinin	Adhesins	**F:** ACTATTCTCTGCAGGAAGT **R:** CTTCCGATGTTCTGAACG	824	Plasmid	(Ewers et al., 2005)
iucD	Aerobactin protein	Iron acquisition system	**F:** ACAAAAAGTTCTATCGCTTC **R:** CCTGATCCAGATGATGCT	714	Plasmid	(Ewers et al., 2005)
irp2	Yersiniabactin protein	Iron acquisition system	**F:** AAGGATTCGCTGTTACCGGA **R:** AACTCCTGATACAGGTGG	413	Chromosome	(Ewers et al., 2005)
iss	Increased serum survival protein	Serum resistance	**F:** ATCACATAGGATTCTGCC **R:** CAGCGGAGTATAGATGCC	309	Plasmid	(Ewers et al., 2005)
astA	Heat-stable enteroaggregative toxin	Toxin	**F:** TGCCATCAACACAGTATATC **R:** TCAGGTCGCGAGTGACGG	116	Plasmid	(Ewers et al., 2005)
cvi/cva	Colicin V protein	Colicin	**F:** TCCAAGCGGACCCCTTATAG **R:** CGCAGCATAGTTCCATGCT	598	Plasmid	(Ewers et al., 2005)
kps	K1 capsular antigen	Capsule	**F:** TATAATTAGTAACCTGGGGC **R:** GGCGCTATTGAATAAGACTG	927	Plasmid	(Knöbl et al., 2012)
hlyA	α-Haemolysin	Toxin	**F:** AACAAGGATAAGCACTGTTCTGGCT **R:** ACCATATAAGCGGTCATTCCCGTCA	1177	Plasmid	(Yamamoto et al., 1995)

2.2.4.3 Detection of antimicrobial resistance (AMR) genes by PCR

The presence of AMR genes is listed in **Table 2.3** and was detected by PCR using GE Healthcare illustra™ PuReTaq Ready-To-Go™ PCR Beads (Thermo Fisher Scientific, UK) in the panel of *E. coli* strains (n= 31) and compared with the control strain MG1655 WT (*E. coli* K12). PCR reactions of the target genes using specific primers (**Table 2.3**) were performed in a volume of 25µl, and carried out in a Thermocycler (Thermo Scientific, UK) with the programs listed in (**Table 2.4**). The amplified products were separated in 1.5% (wt/vol) agarose gel in 0.5X TBE buffer containing 0.03µg/ml ethidium bromide stain at 75V for 1hr.

Table 2.3 PCR primers used for detection of AMR genes

Gene ID	Antimicrobial resistance gene	Primers sequence (5` --- 3`)	Product size (bp)	Genomic location	Reference (Primers)
CTX-M1	Cefotaxime	**F:** GGACGTACAGCAAAAACTTGC **R:** CGGTTCGCTTTCACTTTTCTT	624	Plasmid	(Ryoo et al., 2005)
CTX-M15 (*QnrA*)	Cefotaxime	**F:** AGAGGATTTCTCACGCCAGG **R:** TGCCAGGCACAGATCTTGAC	580	Plasmid	(Cattoir et al., 2007)
mcr-1	Colistin	**F:** ATGATGCAGCATACTTCTGTGTGG **R:** TCAGCGGATGAATGCGGTGC	320	Plasmid	(Cannatelli et al., 2016)
mcr-4	Colistin	**F:** ATTGGGATAGTCGCCTTTTT **R:** TTACAGCCAGAATCATTATCA	487	Plasmid	(Carattoli et al., 2017)
SHV	β-lactams	**F:** CACTCAAGGATGTATTGTG **R:** TTAGCGTTGCCAGTGCTCG	885	Plasmid	(Pitout et al., 1998)
TEM	β-lactams	**F:** TCGGGGAAATGTGCGCG **R:** TGCTTAATCAGTGAGGCACC	971	Plasmid	(Pitout et al., 1998)
IncF	Fertility factor	**F:** CCATGCTGGTTCTAGAGAAGGTG **R:** GTATATCCTTACTGGCTTCCGCAG	462	Plasmid	(Carattoli et al., 2005)
IncI	Cefotaxime	**F:** CGAAAGCCGGACGGCAGAA **R:** TCGTCGTTCCGCCAAGTTCGT	139	Plasmid	(Carattoli et al., 2005)
IncK	AMR genes	**F:** GCGGTCCGGAAAGCCAGAAAAC **R:** TCTTTCACGAGCCCGCCAAA	160	Plasmid	(Carattoli et al., 2005)
IncX	Colistin	**F:** AACCTTAGAGGCTATTTAAGTTGCTGAT **R:** TGAGAGTCAATTTTTATCTCATGTTTTAGC	376	Plasmid	(Carattoli et al., 2005)

Table 2.4 Thermocycler programs for the PCR primers used for detection of AMR genes

Gene ID	10mM $MgCl_2$ (µl)	Initial denaturation		No. of cycles	Denaturation		Annealing		Extension (72ºC)	Final Extension (72ºC)
		Temp.	Time		Temp.	Time	Temp.	Time	Time	Time
CTX-M1	1.25	95ºC	10min	35	95ºC	1min	54ºC	1min	1min	10min
CTX-M15 (*QnrA*)	1.25	94ºC	10min	35	94ºC	1min	56ºC	1min	1min	10min
mcr-1	3.75	95ºC	10min	30	95ºC	1min	55ºC	1min	1min	10min
mcr-4	1.25	95ºC	10min	30	95ºC	1min	50ºC	1min	1min	10min
SHV	6.25	96ºC	15sec	26	96ºC	15sec	50ºC	15sec	2min	2min
TEM	6.25	96ºC	15sec	24	96ºC	15sec	50ºC	15sec	2min	2min
IncF	-	95ºC	10min	30	95ºC	1min	60ºC	1min	1min	10min
IncI	1.25	95ºC	10min	30	95ºC	1min	62ºC	1min	1min	10min
IncK	1.25	95ºC	10min	30	95ºC	1min	62ºC	1min	1min	10min
IncX	3.75	95ºC	10min	30	95ºC	1min	55ºC	1min	1min	10min

2.2.4.4 Genotypic analysis by Enterobacterial Repetitive Intergenic Consensus – Polymerase Chain Reaction (ERIC-PCR)

PCR reactions were carried out using GE Healthcare illustra™ PuReTaq Ready-To-Go™ PCR Beads (Thermo Fisher Scientific, UK) with specific primers, ERIC primer 1: 5′-ATGTAAGCTCCTGGGGATTCAC-3′, and ERIC primer 2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′ and performed in a volume of 25µl: 1µl of DNA extract, 2µl of ERIC-PCR primers mix, 6.25µl of 10mM $MgCl_2$, and 15.75µl of de-ionised water. The PCR tubes were placed in a Thermocycler (Thermo Scientific, UK) with the following program: 2 min initial denaturation at 94°C, followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 52°C, 1 min extension at 72°C, and final extension of 72°C for 5 min. The amplified products were separated in 1.5% (wt/vol) agarose gel in 0.5X TBE buffer containing 0.03µg/ml ethidium bromide stain at 50V for 1hr. The resulted bands were identified and classified into different sizes to construct a dendrogram based on similarities and differences in number and sizes of bands using NTSYS software.

2.2.5 Statistical analyses

ERIC-PCR dendrogram was constructed using data in the form of binary matrix scored as 0 for absence of band or 1 for presence of band. The scores were analysed using SAHN program of the NTSYS-pc software version 2.2 based on simple matching coefficient and Unweighted Pair Group Method with Arithmetic Averages (UPGMA) to construct a cluster analysis in the form of a dendrogram.

Results of all tests (except for ERIC-PCR) were summarised in the form of binary data of 0 as negative, 1 as positive, and -1 as intermediate (AST results), and used to construct a cluster analysis in the form of multi-dimensional scaling (MDS) using R i386 3.4.3 software.

2.3 Results

2.3.1 Confirmation of the identity of *E. coli* strains

The *E. coli* strains (**Table 2.1**) gave pink colonies on MacConkey agar, and green metallic sheen colonies on EMB agar, which is highly indicative that they are *E. coli* strains (**Table 2.5**). As for catalase and oxidase tests, the results showed that all the strains were catalase positive and oxidase negative bacteria (**Table 2.5**). This gave us additional evidence that these strains are *E. coli* and gave us a clue on which API strip is to be used. Therefore, *E. coli* strains were characterised biochemically using API 20E strips which summarises 20 biochemical tests in one strip. The biochemical profile based on a seven-digit profile code confirmed that the identification of the bacterial identity according to the biochemical tests were *E. coli* strains (**Table 2.5**).

2.3.2 Phenotypic characterisation

The *E. coli* strains were tested for their ability to utilise different sugars (Sucrose, sorbose, and dulcitol). The *E. coli* strains (APEC = 12 strains, chicken strains = 9 strains, and turkey strains = 10 strains) showed various results; (30/31: 96.77%) utilised sucrose, (24/31: 77.42%) utilised sorbose, and (12/31: 38.71%) utilised dulcitol (**Table 2.5**). The high prevalence of sucrose utilisation was unexpected in comparison with the reference strain *E. coli* K12 MG 1655WT, whereas the distribution of dulcitol and sorbose utilisation was in keeping with known variability in these phenotypes (Edwards and Ewing, 1962).

Table 2.5 Growth on selective media, API 20E code, catalase test, oxidase test, and sugar utilisation

Code	API 20E Code	MCA	EMB	CAT	OXI	SAC	SOR	DUL
7	1044572	Pink	Metallic green	+	-	+	+	-
14	1044572	Pink	Metallic green	+	-	+	+	-
43	1044572	Pink	Metallic green	+	-	+	+	+
45	1044572	Pink	Metallic green	+	-	+	+	+
46	1044552	Pink	Metallic green	+	-	+	+	+
47	1044552	Pink	Metallic green	+	-	+	+	-
48	1044572	Pink	Metallic green	+	-	+	+	+
49	1044552	Pink	Metallic green	+	-	+	+	+
51	7144572	Pink	Metallic green	+	-	+	+	+
52	1044552	Pink	Metallic green	+	-	+	+	+
53	1044572	Pink	Metallic green	+	-	+	+	+
54	1044572	Pink	Metallic green	+	-	+	+	+
C1	5044552	Pink	Metallic green	+	-	+	+	-
C2	5044152	Pink	Metallic green	+	-	+	-	-
C3	7144572	Pink	Metallic green	+	-	+	+	+
C4	7044552	Pink	Metallic green	+	-	+	+	-
C5	5044552	Pink	Metallic green	+	-	+	+	+
C6	7044552	Pink	Metallic green	+	-	+	+	-
C7	7044552	Pink	Metallic green	+	-	+	+	-
C8	5044552	Pink	Metallic green	+	-	+	+	-
C9	7144572	Pink	Metallic green	+	-	+	+	+
T1	5144522	Pink	Metallic green	+	-	-	-	-
T2	5144572	Pink	Metallic green	+	-	+	+	-
T3	7044552	Pink	Metallic green	+	-	+	+	-
T4	7144572	Pink	Metallic green	+	-	+	+	-
T5	7044552	Pink	Metallic green	+	-	+	-	-
T6	5044552	Pink	Metallic green	+	-	+	-	-
T7	7044573	Pink	Metallic green	+	-	+	-	-
T8	5044552	Pink	Metallic green	+	-	+	+	-
T9	7044552	Pink	Metallic green	+	-	+	-	-
T10	7044552	Pink	Metallic green	+	-	+	-	-
MG 1655 WT	5044552	Pink	Metallic green	+	-	+	-	+
MG1655 *acrAB* M	4064500	Beige	Pink	+	-	-	-	+

Shaded cells represent positive for utilisation of sugars. MCA, MacConkey agar; EMB, Eosin-methylene blue agar. CAT, Catalase; OXI, Oxidase; SAC, Sucrose; SOR, Sorbose; DUL, Dulcitol; +, positive; -, negative.

The susceptibility of the *E. coli* strains to different classes of antibiotics was examined and the results are shown in **Table 2.6**. The bacterial strains (APEC = 12 strains, chicken strains = 9 strains, and turkey strains = 10 strains) showed very high levels of resistance to colistin (31/31: 100%) and cefotaxime (27/31: 87.10%). The lowest percentage of resistance was noted in chloramphenicol (2/31: 6.45%), that was only found in two APEC strains (APEC 7 and 46). Generally, most of the bacterial strains displayed multiple antibiotic resistance to several antibiotics. To compare the trend of antibiotic resistance among the strains, the highest percentages of multiple antibiotic resistance (resistance to at least 3 antibiotics) were encoded by the presumed commensal strains isolated from chicken (9 strains) and turkey (10 strains) which showed very similar prevalence of resistance (8/9: 88.89% and 10/10: 100%, respectively) followed by APEC strains (7/12: 58.33%). However, APEC strains were the only strains that exhibited resistance to 6 antibiotics out of the tested 7 antibiotics among the strains, and this was displayed by the strains APEC 46 and 52.

Table 2.6 AST profiling of the *E. coli* strains

Code	CTX 30µg	NA 30µg	C 30µg	CT 10µg	SAM 25µg	S 25µg	TE 30µg
7	R	R	R	R	S	S	R
14	I	R	S	R	S	S	S
43	R	S	S	R	S	I	S
45	R	S	S	R	R	S	S
46	S	R	R	R	R	R	R
47	R	S	S	R	S	S	S
48	I	R	I	R	R	R	R
49	S	S	S	R	S	S	S
51	R	S	S	R	R	S	S
52	R	R	S	R	R	R	R
53	R	R	S	R	S	S	S
54	R	S	S	R	S	I	S
C1	R	R	S	R	R	I	R
C2	R	S	S	R	R	S	S
C3	R	S	S	R	R	R	R
C4	R	R	S	R	I	I	R
C5	R	S	S	R	R	S	S
C6	R	S	S	R	R	S	S
C7	R	R	S	R	R	S	R
C8	R	R	S	R	R	S	R
C9	R	S	S	R	S	S	S
T1	R	S	S	R	R	S	S
T2	R	S	S	R	R	S	S
T3	R	S	S	R	R	R	R
T4	R	S	S	R	R	S	S
T5	R	S	S	R	R	S	R
T6	R	R	S	R	R	R	S
T7	R	R	S	R	R	S	S
T8	R	R	S	R	R	S	S
T9	R	R	S	R	R	S	S
T10	R	R	S	R	R	S	S
MG 1655 WT	S	R	I	R	I	R	S
MG1655 *acrAB* M	S	I	S	R	R	I	S

Shaded cells indicate resistance. CTX, Cefotaxime; **NA**, Nalidixic acid; **C**, Chloramphenicol; **CT**, Colistin; **SAM**, Ampicillin; **S**, Streptomycin; **TE**, Tetracycline; R, resistant; S, sensitive; I, intermediate.

The *E. coli* strains showed their ability to form lacy colonies on the modified Congo red agar plates. This phenotype is recognised as a surrogate assessment for the bacterial ability to produce curli fimbriae and/or cellulose (**Table 2.7**).

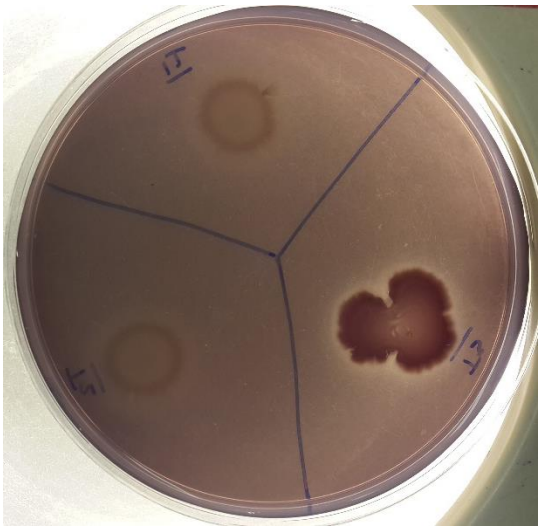

Figure 2.1 An example of the results of Congo red agar; red lacy colonies = positive for biofilm formation, and smooth beige colonies = negative for biofilm formation.

Table 2.7 Detection of biofilm on the modified Congo red agar

Code	Appearance	Colony color
All APEC strains	Lacy	Red
All chicken strains	Lacy	Red
T1, T2, T4	Smooth	Beige
T3, T5, T6, T7, T8, T9, T10	Lacy	Red
MG1655 WT	Lacy	Red
MG1655 *acrAB* M	Lacy	Red

2.3.3 Genotypic characterisation

2.3.3.1 Detection of virulence genes by PCR

The pathogenic potential of the *E. coli* strains from three categories of poultry sources were investigated by the detection of twelve virulence genes (**Table 2.8**). **Table 2.8** is constructed such that virulence genes were grouped according to the function encoded by the virulence factor; five genes responsible for adhesion (*fim1*, *papC*, *csgA*, *crl*, and *tsh*), two genes responsible for iron acquisition system (*iucD* and *irp2*), one gene responsible for serum resistance (*iss*), one gene responsible for colicin (*cvi/cva*), one gene responsible for capsule (*kps*), and two genes responsible for the production of toxins (*astA* and *hlyA*) located in either the bacterial chromosome or a plasmid. The reasons for choosing these virulence genes specifically were the following: 1) *fim1* deletion was associated with reduction of colonization and inflammation of UTI (Connell et al., 1996, Bahrani-Mougeot et al., 2002), 2) *papC* is required for the expression of P fimbriae (Nowrouzian et al., 2001), 3) *csgA* is the major structural subunit of the curli operon (Dueholm et al., 2011), 4) *crl* encodes for the transcriptional regulator of the *csgBA* operon which coordinates curli biosynthesis (Pratt and Silhavy, 1998), and activates biofilm formation (Gualdi et al., 2008), 5) *tsh* is associated with virulence of APEC strains and found in birds suffering from colibacillosis (Janßen et al., 2001), 6) *iucD* is a gene found in the aerobactin operon which regulates aerobactin biosynthesis (Neilands, 1992), 7) *irp2* is the promoter region of high-pathogenicity island which is yersinibactin region (Carniel, 1999), 8) *iss* gene was found to be associated with complement resistance and virulence of avian *E. coli* (Nolan et al., 2003), 9) *astA* encodes for enterotoxin conferring heat-stability (Savarino et al., 1993), 10) *cvi/cva* is part of the *colicin V* operon encoding for colicin protein with toxicity traits

(Ewers et al., 2007) and spread among APEC strains found in chickens suffering from mortality of chicken embryo (Oh et al., 2012), 11) *kps* encodes for the biosynthesis of the capsule or K-antigen which mediates capsular transport system (Smith et al., 1990), 12) *hlyA* encodes for one of the major virulence factors α-hemolysin protein which is an exotoxin that binds to the host red blood cells (Menestrina et al., 1994), and found in APEC strains in chickens suffering from colibacillosis (Janßen et al., 2001).

Generally, APEC strains harboured more virulence genes than the commensal strains, which is what was anticipated. APEC 47 possessed all virulence genes except *csgA* and *hlyA*. The two genes responsible for adhesion (*papC* and *tsh*) were absent in all presumptive commensal strains which may suggest they have less ability to colonise than the APEC strains. Among the virulence genes, the most prevalent genes were *fim1* and *irp2* which again were present in a very high percentage among APEC strains (12/12: 100% and 11/12: 91.67%, respectively). As a control, *E. coli* MG 1655 WT (*E. coli* K12) was included in this study, and it only showed the presence of one gene (*cvi/cva*). Also, it was noted that chromosome-related genes (*fim1*, *papC*, *csgA*, *crl*, *irp2* and *kps*) were more frequently spread among the *E. coli* strains than plasmid-related genes (*tsh*, *iucD*, *iss*, *astA*, *cvi/cva* and *hlyA*). Among the commensal strains, turkey strains showed a higher frequency of presence of *irp2* which encodes for iron-acquisition system in comparison with chicken strains (7/10: 70% and 1/9: 11.11%, respectively), but *iucD* was relatively more prevalent in chicken strains (2/9: 22.22%) and completely absent in turkey strains (0/10: 0%). The *csgA* and *crl* genes that govern curli fimbriae expression were present in all chicken strains (7/9: 77.78% and 9/9: 100%, respectively) but were less prevalent in turkey strains (5/10:

50% and 7/10: 70%, respectively). This may suggest that chicken strains are better colonisers than turkey strains, because expression of curli facilitates colonisation and formation of biofilm (Szabó et al., 2005).

In terms of the potential toxicity of the *E. coli* strains, the presence of *astA* gene as confirmed to be more prevalent among APEC strains (7/12: 58.33%) than chicken (6/9: 66.67%) and turkey (2/10: 20%) strains. There is a noticeable difference in the occurrence of *kps* gene that encodes for the synthesis of capsule with percentages of 33.33% (4/12) in APEC strains, 11.11% (1/9) in chicken strains, and 0% (0/10) in turkey strains. According to the presence of *cvi/cva* gene, the ColV plasmid is more prevalent among APEC strains (10/12: 83.33%), in comparison with chicken (2/9: 22.22%) and turkey (3/10: 30%) strains. Also, the *iss* gene responsible for serum resistance was present in (11/12: 91.67%) of APEC strains, (4/9: 44.44%) of chicken strains, and (3/10: 30%) of turkey strains.

Table 2.8 Virulence genes profile in the *E. coli* strains

Code	*fim1*	*papC*	*csgA*	*crl*	*tsh*	*iucD*	*irp2*	*iss*	*astA*	*cvi/cva*	*kps*	*hlyA*
7	+	-	+	-	-	-	-	-	-	-	-	-
14	+	-	+	+	+	+	+	+	+	-	+	+
43	+	-	+	-	-	+	+	+	+	+	-	+
45	+	-	+	+	-	+	+	+	+	+	-	+
46	+	-	+	+	+	+	+	+	+	+	-	+
47	+	+	-	+	+	+	+	+	+	+	+	-
48	+	-	-	+	+	+	+	+	-	+	+	-
49	+	+	+	+	+	+	+	+	+	+	-	+
51	+	-	-	-	-	-	+	+	+	+	-	+
52	+	-	+	+	+	-	+	+	-	+	-	-
53	+	-	+	+	-	+	+	+	-	+	-	+
54	+	-	-	+	-	-	+	+	-	+	+	-
C1	+	-	+	+	-	-	-	-	+	-	-	+
C2	+	-	+	+	-	-	-	+	-	+	-	-
C3	+	-	-	+	-	-	-	+	+	-	-	-
C4	+	-	+	+	-	-	-	-	+	-	-	-
C5	+	-	+	+	-	+	-	+	-	+	-	-
C6	+	-	-	+	-	-	-	-	-	-	-	+
C7	-	-	+	+	-	-	-	-	+	-	-	+
C8	+	-	+	+	-	-	-	-	+	-	-	-
C9	-	-	+	+	-	+	+	+	+	-	+	-
T1	+	-	+	+	-	-	+	+	+	+	-	-
T2	+	-	+	+	-	-	+	+	-	+	-	-
T3	-	-	-	-	-	-	-	-	-	-	-	-
T4	-	-	-	+	-	-	-	-	-	+	-	-
T5	+	-	+	+	-	-	-	+	-	-	-	+
T6	+	-	+	+	-	-	+	-	-	-	-	+
T7	+	-	-	+	-	-	+	-	+	-	-	-
T8	+	-	-	-	-	-	+	-	-	-	-	-
T9	+	-	+	+	-	-	+	-	-	-	-	-
T10	+	-	-	-	-	-	+	-	-	-	-	-
MG1655 WT	-	-	-	-	-	-	-	-	-	+	-	-

Shaded cells (+) represent positive for presence of the virulence genes and unshaded cells (-) represent negative for absence of the virulence genes. +, positive; -, negative.

2.3.3.2 Detection of AMR genes by PCR

The presence of antimicrobial resistance genes of the *E. coli* strains from three categories of poultry sources were investigated by the detection of twelve virulence genes (**Table 2.9**). **Table 2.9** focused on certain antimicrobial resistance genes (cefotaxime, colistin, and β-lactams) as these were the most prevalent ones from AST results (**Table 2.6**). Among the spread of AMR genes, *TEM* plasmid was present in all turkey strains (10/10: 100%) but with less occurrence in APEC and chicken strains (4/12: 33.33%, and 4/9: 44.44%, respectively). In general, APEC strains harboured higher percentages of AMR genes than the commensal strains. Moreover, there was a relatively higher percentage of occurrence of β-lactam plasmids (*SHV* and *TEM*) than the remaining AMR genes. Cefotaxime resistance plasmids were less present than colistin resistance plasmids among the *E. coli* strains.

Table 2.9 AMR genes profile in the *E. coli* strains

Code	*CTX-M1*	*CTX-M15 (QnrA)*	*mcr-1*	*mcr-4*	*SHV*	*TEM*	*IncF*	*IncI*	*IncK*	*IncX*
7	+	-	+	+	+	-	-	+	-	-
14	-	+	-	+	+	-	-	-	-	-
43	-	+	-	-	-	-	-	-	-	-
45	-	+	-	-	-	+	-	-	-	-
46	-	-	-	-	-	+	-	-	-	-
47	-	+	-	+	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-
49	-	+	-	-	+	-	-	+	+	-
51	+	+	-	+	+	+	-	-	-	-
52	-	+	+	+	+	+	+	-	-	+
53	-	+	+	+	+	-	+	-	-	-
54	-	-	-	+	-	-	-	+	-	-
C1	-	+	-	+	-	+	-	-	-	-
C2	-	-	-	-	-	+	-	-	-	-
C3	-	-	-	-	-	-	-	-	-	-
C4	-	-	-	-	-	-	-	-	-	-
C5	-	-	-	-	-	+	-	+	-	-
C6	-	+	+	+	+	+	-	-	+	-
C7	-	-	-	-	-	-	-	-	-	-
C8	-	+	-	+	-	+	-	-	-	-
C9	-	-	-	-	-	-	-	-	+	-
T1	+	-	+	+	+	+	-	-	+	-
T2	-	-	-	-	-	+	-	-	-	-
T3	-	+	-	+	+	+	-	-	-	-
T4	-	+	+	-	+	+	-	-	-	-
T5	-	+	-	-	+	+	-	-	-	-
T6	+	+	-	+	+	+	+	+	-	-
T7	-	+	-	-	+	+	+	-	-	-
T8	-	-	-	-	-	+	+	-	-	-
T9	-	-	-	-	-	+	+	-	-	-
T10	-	-	-	-	-	+	+	-	-	-
MG1655 WT	-	+	+	+	+	-	+	-	+	-

Shaded cells (+) represent positive for presence of the AMR genes and unshaded cells (-) represent negative for absence of the AMR genes. +, positive; -, negative.

2.3.3.3 Genotypic analysis by ERIC-PCR

ERIC-PCR patterns yielded different numbers and sizes of the amplified products, ranging from 100 to 10,000bp. UPGMA cluster analysis using NTSYS software combined all the profiles and placed them in the form of a dendrogram. Dendrogram analysis (**Figure 2.2**) placed the strains into 3 main discrete groups/nodes consisting of APEC strains (except for 49 and 52), turkey strains (excluding T1 and T2) with *E. coli* MG1655 WT (*E. coli* K12), and chicken strains with APEC 49 and 52, and an outlier of two turkey strains (T1 and T2). They were classified into different and variable clusters, reflecting an overlap between pathogenic and commensal strains.

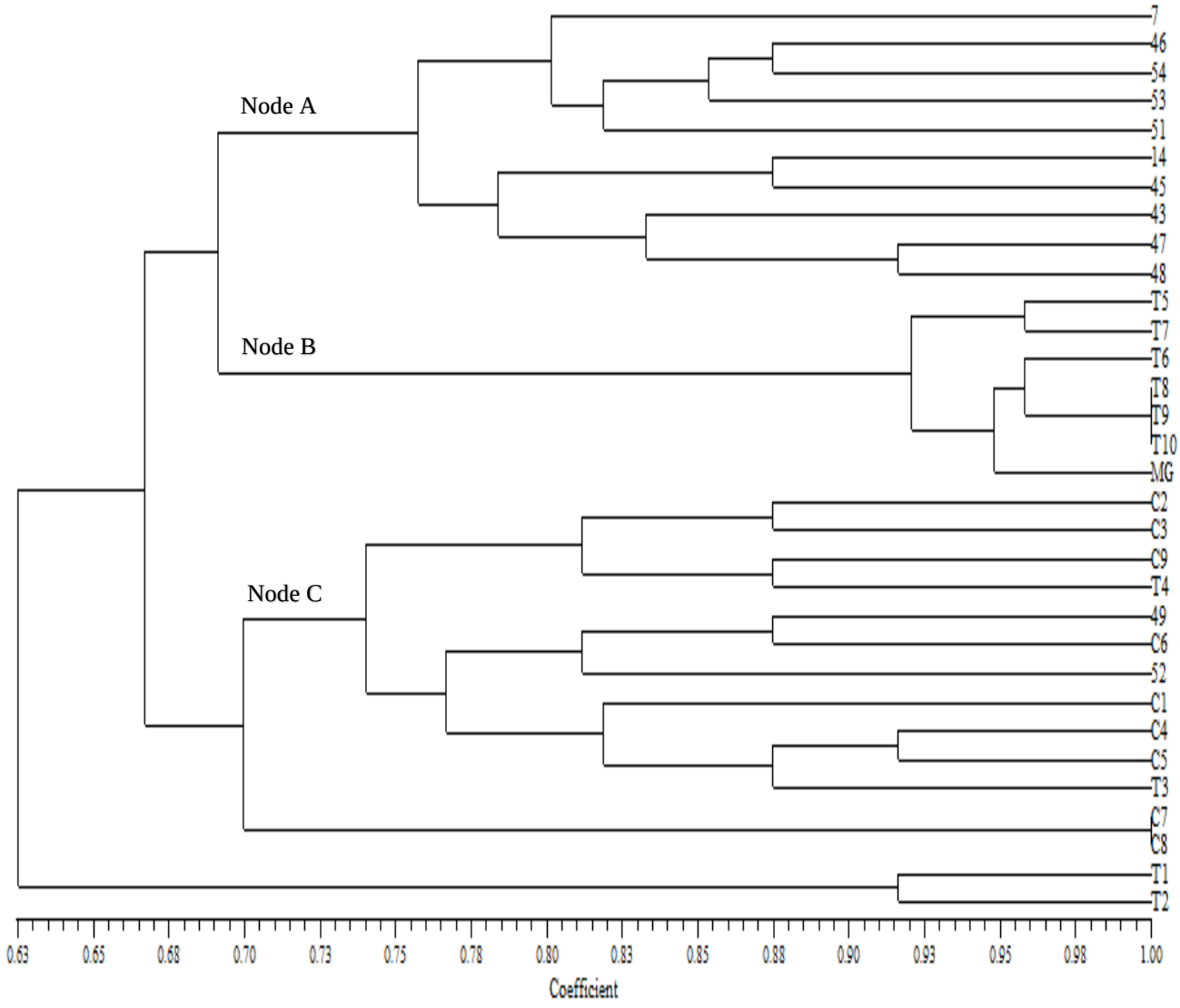

Figure 2.2 A dendrogram showing bacterial diversity of the *E. coli* strains based on UPGMA cluster analysis

2.3.4 MDS cluster analysis

The results of confirmation of *E. coli* strains' identity, phenotypic characterisation, and genotypic characterisation (except for ERIC-PCR) were summarised and used for construction of a cluster analysis in the form of MDS (**Figure 2.3**).

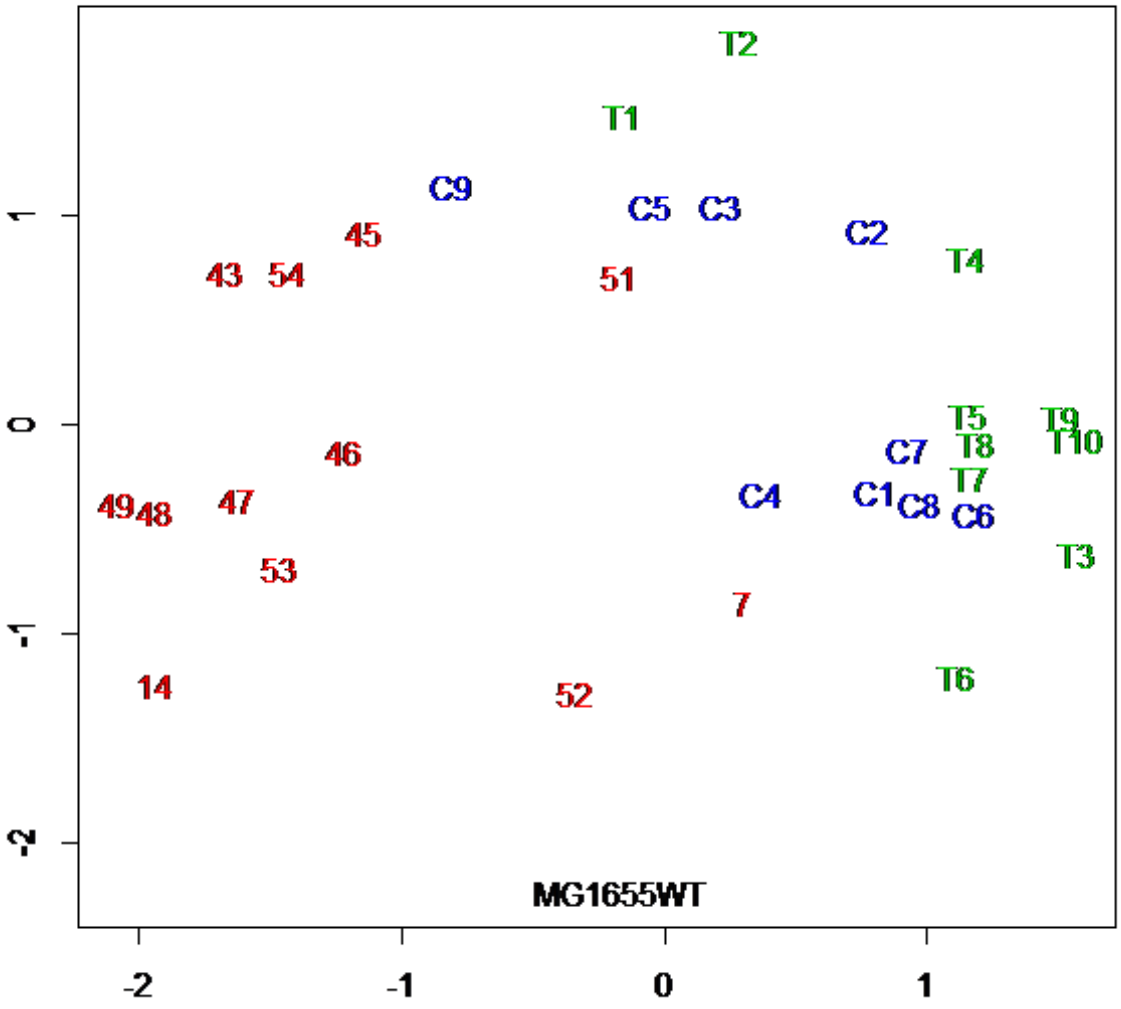

Figure 2.3 MDS showing bacterial diversity of the *E. coli* strains based on the results of all previous tests. The *E. coli* strains were grouped in different colours (APEC = red, commensal chickens = blue, commensal turkeys = green, and *E. coli* MG 1655 WT = black).

2.4 Discussion

An early objective of this study was to establish appropriate *E. coli* representatives of the poultry (chickens and turkeys) gut, to study the impact of the selected phytochemicals (carvacrol and oregano extract). *E. coli* are commensals in the gut, but some variants also have the potential to be pathogenic in poultry causing a variety of clinical signs as discussed in the introduction. Moreover, these *E. coli* strains reflect strains that potentially colonise the human gut after direct or indirect contamination during poultry consumption, which maybe a source of antibiotic resistance in humans (Linton et al., 1977a, Van den Bogaard et al., 2001, Liu et al., 2016). The 33 *E. coli* strains included different groups of *E. coli*; APEC strains (12 strains), wild-type commensal strains isolated from poultry gut of healthy chickens (9 strains) and turkeys (10 strains) and supported by mutant *E. coli* K12 MG1655 WT and MG1655 *acrAB* M strains to be used as negative controls (**Table 2.1**). The wild-type commensal strains were isolated from the caecum of the healthy chickens and turkeys where high counts of enteric bacteria are known to be colonising (Zhu et al., 2002). For the brevity in this thesis, the term "commensal" rather than 'putative/presumptive commensal' has been used to describe any *E. coli* that came from the gut of a healthy chicken or turkey. However, it is highly likely that as APEC can colonise the gut of poultry and not cause any disease until an appropriate predisposition causes induction, it is possible that the group called "commensal" could indeed have some APEC representatives. Without testing Koch's postulates in animal studies, it is not possible here to affirm true commensal or pathogen status. That said, the APEC strains were defined as pathogenic as these were isolated from tissues within the bird suffering colibacillosis.

For the sake of a quick phenotypic characterisation and differentiation among the strains, the second set of biochemical tests included sugar utilisation was done. Sugar utilisation tests (**Table 2.5**) demonstrated that the *E. coli* strains can utilise different sugar sources (sucrose, sorbose, and dulcitol). The reason for these simple tests was to establish how variable a group of strains were represented in the panel of 31 bacterial strains used in this study, as it is known that each of the previously mentioned sugars is used by a proportion of but not all strains (Edwards and Ewing, 1972). It was considered a simple method for rapid differentiation between strains if such availability was represented in this group of strains. The majority of the *E. coli* strains utilised sucrose (**Table 2.5**) as a carbon and energy source was found to be responsible for the increase in acetate and butyrate concentrations in the chicken caecum. This makes sucrose an important energy source found in poultry diet (Jozefiak et al., 2004). Also, this indicates that the *E. coli* strains break down sucrose by catalytic enzymes sucrose 6-phosphate hydrolases and sucrose phosphorylases (Reid and Abratt, 2005). The frequency of use of sucrose was considerably higher than anticipated when compared with the data of (Edwards and Ewing, 1972) and (Alaeddinoglu and Charles, 1979), suggesting that perhaps sucrose utilisation was a characteristic of poultry *E. coli*, and perhaps this characteristic gives some selective advantage over non-sucrose utilisers. Also, the use of sorbose was a little higher than anticipated compared with the previous work of (Woodward and Charles, 1982). The ability of these *E. coli* strains to utilise more than one type of sugar reflects their ability to compete for nutrients available in poultry gut (mainly in the caecum), which may enable them to successfully colonise poultry gut (Conway and Cohen, 2015). The majority of APEC strains (9/12: 75%) were able to utilise the three sugars, in comparison with only (3/9: 33.33%) of the chicken strains and none of the turkey strains (0/10: 0%). However, without much wider studies relating to metabolic competence with host species, any correlation proposed here is somewhat speculative. Additional phenotypic characterisation and differentiation was investigated

through profiling of antibiotic resistance using antibiotic disks and detection of biofilm formation on a modified Congo red agar plates.

Genotypic characterisation using molecular methods including PCR were included to determine their virulence determinants and check the correlation between metabolic type and virulence type and antimicrobial resistance genes. Antimicrobial resistance, although not being a virulence factor in the true sense of the term, is one of the factors that can contribute to the pathogenicity or virulence of the *E. coli* strains, by providing systems that prevent the effectiveness of antibiotics used in treatment.

One of the aims in this study is to assess the utility of phytochemicals to act against both antibiotic-resistant and pathogenic bacteria using *E. coli* as a suitable bacterial model. Hence, the *E. coli* strains were tested for their ability to resist antibiotics, and results showed a high prevalence of multi-resistance to several antibiotics (**Table 2.6**). The reason for an emphasis on ß-lactam antibiotics in particular, is due to the very frequent use of ampicillin in poultry production and the rapid emergence of resistance in poultry and the need to counter this rise (Hayes et al., 2004, Bortolaia et al., 2010, Dierikx et al., 2013). One question that arises regarding in the panel of 31 strains used in this study is whether they are representative of the *E. coli* found in poultry production or not. Each isolate was recovered from on-farm poultry production, and therefore are truly representatives of the type of *E. coli* in these specific animals, but whether representative of poultry production in this region or nationally is questionable. At the slaughterhouse, these strains can be easily spread at the evisceration stage resulting in cross-contamination of poultry meat that then enter the human food chain (Redmond et al., 2004). Humans will be exposed to such bacteria either by direct or indirect contact during food handling, processing, and preparation or cooking/eating, and therefore

increasing the spread of antibiotic resistant *E. coli* strains (Collignon, 2009). A previous study has shown that human guts are colonised by antibiotic resistant *E. coli* of chicken sources (Linton et al., 1977a). Another study confirmed that humans carry antibiotic resistant *E. coli* strains that mostly come from poultry sources (Collignon, 2009). Thus, this test demonstrates the high prevalence or high spread of antibiotic resistance of *E. coli* in poultry proved by AST (**Table 2.6**) and PCR (**Table 2.9**) results, and therefore alternatives (such as phytochemicals) other than antibiotics should be investigated for their use to control their spread among the food chain.

The second factor to be considered in this assessment was biofilm formation which was detected by two ways; on modified Congo red agar as a surrogate for curli production (**Table 2.7**) which is an important component of biofilm formation (Cookson et al., 2002), and by the crystal violet biofilm assay (**see Chapter 3**). The *E. coli* strains were initially tested for their ability to form "lacy colonies" on the modified Congo red agar as a rapid and simple estimation of the production of the main ingredients of biofilm; curli and/or cellulose (**Table 2.7**). Even though the culturing method was not very accurate in detecting the ability of the bacterial strains to form biofilm and its composition, it was a simple plate test to assess biofilm formation. It was noted that the vast majority of the *E. coli* strains (28/31: 90.32%) produced lacy colonies, with only three commensal turkey strains (T1, T2, and T4) unable to do so.

To profile the *E. coli* strains for the carriage of virulence determinants, PCR was performed (**Table 2.8**). There are no clear definitions for APEC strains though the Nolan laboratory in the USA suggests detection of as few as five virulence genes is sufficient to define an APEC (Johnson et al., 2008), even though this is challenged by the recent work from the Woodward

Laboratory (Cordoni et al., 2016). Also, there is an overlap between APEC strains and UPEC causing urinary tract infections in human and meningitis in newly-born babies (Ewers et al., 2007). Moreover, in terms of virulence genes content, there is a similarity between APEC and ExPEC in human, therefore virulence genes harboured by APEC strains may be a source of spread of these genes among ExPEC strains (Zhao et al., 2009). Although the number of genes tested was not extensive, the PCR data shows there is a high prevalence of virulence genes encoded in the chromosome or on plasmid among the APEC strains (Dho-Moulin and Fairbrother, 1999). Even though there is a higher prevalence of chromosome-related genes spread among the strains than plasmid-related genes, here is evidence that there is a reservoir of genes that are located on transmissible plasmids, providing a source for their dissemination among bacterial strains (Ewers et al., 2007). The collective set of adhesion genes (*fim1*, *papC*, *csgA*, *crl*, and *tsh*) are widespread among the *E. coli* strains allowing them to adhere to mucosal surfaces inside the host and are mediated by the expression of adhesin proteins (Stordeur et al., 2002a). A previous study proved that the presence of *fim1* responsible for expression of type 1 fimbriae increases the chances of colonising the urinary tract and therefore giving them an extra virulence advantage (Connell et al., 1996). Interestingly, the presence of *papC* gene is associated with septicemia in chicken and turkey, which was found among APEC strains only. Iron acquisition systems which are encoded by the two genes (*iucD* and *irp2*) (Khasheii et al., 2016) were highly prevalent in APEC strains, enabling them to survive and thrive in iron-deprived environments (Foxman et al., 2000) reflecting their pathogenicity nature (Zhao et al., 2009). The presence of *astA* gene encoding enterotoxic activity (Savarino et al., 1993) was widespread among APEC strains.

The following genes (*iss*, *kps*, and *cvi/cva*) encoding for different virulence factors were found encoded on the same ColV plasmid (Johnson et al., 2006). Increased serum resistance is encoded by the *iss* gene enabling the strain to fight immunological responses of the host (Johnson et al., 2002). The gene *kps* is responsible for expression of capsular antigen that has anti-immunological properties (Dho-Moulin and Fairbrother, 1999), was also prevalent in APEC and chicken strains but absent in turkey strains. The production of bacteriocin colicin V (ColV) mediated by *cvi/cva* genes, regulated by ColV operon (Clowes, 1972) was commonly widespread among APEC strains and confirmed here, also exhibit anti-immunological properties (Johnson et al., 2006). The last determinant included in the PCR tests was the production of α-haemolysin toxin encoded by the *hlyA* gene responsible for breaking down red blood cells in mammals and associated with extra-intestinal diseases (Cavalieri et al., 1984) was also widespread among APEC strains.

AST profiling (**Table 2.6**) was initially performed to screen for the antibiotic resistance among the *E. coli* strains and to focus on specific antibiotics to be further detected for using PCR (**Table 2.9**). The antibiotics of interest were cefotaxime, colistin, and β-lactams, with penicillin being the first antibiotic of use in poultry. Once more, APEC strains harboured more AMR genes than the commensal strains. These tests demonstrate that the percentage of AMR resistance spread among these *E. coli* strains is very high, despite the ban that was imposed in 2006 on the use of prophylactic antibiotics.

ERIC-PCR is a technique that generates multiple copies of conserved and repeated sequences that can be found in the *Enterobacteriaceae* family (Hulton et al., 1991) Bacteria possessing these 126bp sequences differ in the number and their location on the genome, and therefore the sizes of the amplicons generated by PCR using ERIC sequences for priming amplification

(Sharples and Lloyd, 1990). The fingerprinting profiles generated can be used to study the relationships between bacterial strains isolated from different groups (Mohapatra et al., 2007). Thus, ERIC-PCR is a quick surveillance tool to characterise bacterial strains at the molecular level (Millemann et al., 1996), and it is a favoured tool in typing/differentiating *E. coli* strains, because of its high discriminatory power and reproducibility, enabling the study of genetic relationships isolated from different origin (Oltramari et al., 2014). The data generated in **Figure 2.2** showed three distinct clusters (nodes) referring to different origin of the analysed strains, and at a similarity coefficient of 0.75. Two Turkey strains (T1 and T2) lie outside of these clusters which suggests a less homogenous group spread across two of three nodes excluding the two outliers already mentioned. However, except for APEC strains 49 and 52, the remaining APEC strains (7, 14, 43, 45, 46, 47, 48, 51, 53, and 54) clustered together in one node (node A **Figure 2.2**) and all chicken strains were found in a single cluster (node C **Figure 2.2**). The third cluster (node B **Figure 2.2**) combined the remaining turkey strains apart from the outliers T1 and T2. The APEC strains were potential pathogens as each was isolated as a pure culture from the internal tissues of a diseased chicken. The chicken *E. coli* strains were isolated from healthy animals and for this thesis the term presumptive commensal has been used to describe these. Previous experiments have shown the distribution of virulence genes and other markers (e.g. sugar utilisation and antibiotic resistance) and it has been difficult to readily differentiate the "presumptive commensals" from APEC virulent based on those characteristics alone. ERIC-PCR has however, demonstrated relatively distinct profiles suggesting distinct genomic backbone and distributions of repetitive elements. Given ERIC-PCR may reflect in part deeper ancestral lineages, although this can be challenged when compared with other methods such as multi-locus sequence typing (MLST), the separation between these two clusters appears to reflect relative pathogenicity. Is it possible that APEC 49 and 52 are commensals and characterised as APEC in error or could these commensals

carrying transient APEC traits; keeping in mind that some virulence factors (e.g. fimbriae are very common across all *E. coli* types and that some virulence factors (e.g. some toxins) are plasmid carried and therefore mobile.

MDS cluster analysis (**Figure 2.3**) grouped the APEC strains (red colour) away from the commensal ones, and this confirms their different phenotypic and genotypic background except for APEC 7, 51, and 52. The commensal chicken *E. coli* strains (blue colour) were in the middle between APEC and turkey strains (green colour), which may suggest that some of them may be APEC strains. While, the turkey strains were more distinctive commensals among the three groups as suggested by previous tests. However, there is an overlap between the accurate definition of APEC and commensal *E. coli* strains isolated from poultry sources.

In conclusion, phenotyping and genotypic characteristics can be used to establish differential criteria between APEC and commensal strains. The earlier experiments demonstrated that *E. coli* strains are so diverse in their phenotypic and genotypic characteristic, and most of the groups were either APEC or commensals suggesting genetic diversity. However, there was a bit of overlap between the two groups suggesting shared genetic similarities or partial relationships between APEC and commensal strains. Genetic typing may give us a clue into the complete definition of *E. coli* strains from APEC or commensal origin (Kilic et al., 2009). Future experiments may include differentiating the strains based on whole genome sequencing (WGS), to give us better understanding of their genetic identity and origin, Real-Time PCR to study gene expression, as the genes might be present but not expressed, and plasmids extraction to study their stability. To increase the accuracy and neglect biasedness of these tests, higher

numbers of *E. coli* strains should be included, and collected from different hatchery sources and different countries of the EU.

Collectively, the previously-mentioned studies confirmed that they are *E. coli* strains, characterised them phenotypically using biochemical tests, detected the occurrence of antimicrobial resistance, formation of biofilm by simple plating method, and presence of virulence genes, AMR genes, and genotypic typing using ERIC-PCR among APEC and commensal wild-type *E. coli* strains, which were isolated from poultry sources reared for human consumption. These traits are associated with possible health risks in poultry food chain, so it was important to know if these traits were spread or not, and how often are they are spread among the *E. coli* strains of different provenance. Therefore, this thesis will explore the potential of controlling these *E. coli* strains with natural plant products i.e. phytochemicals with an emphasis upon carvacrol and oregano extract, as a possible alternative to antibiotics in poultry feeds.

CHAPTER 3: Phytochemicals (carvacrol and oregano) as anti-bacterial agents

3.1 Introduction

3.1.1 Phytochemicals as feed additives in poultry production

Previous research has demonstrated that using phytochemicals as feed additives results in a decrease in the population of *E. coli* and also an increase in the activity of specific digestive enzymes (Jang et al., 2007) such as amylase in the intestinal system of female broiler chickens (Lee et al., 2003a) and maltase in the intestinal system of male broiler chickens (Xu et al., 2003). Carvacrol and thymol as feed additives showed enhanced growth promoting effects on anti-oxidant enzyme activities, immune responses, digestive enzyme activities among broiler chickens (Hashemipour et al., 2013). Oregano oil containing carvacrol and thymol is effective against *E. coli* in a dosage-dependent manner (Friedman et al., 2002). Furthermore, oregano oil exhibits high biological activities resulting in growth promotion when used as feed additives in poultry (Giannenas et al., 2005). Another study showed that oregano extract (*Origanum vulgare*) contains a high phenolic content that exhibits anti-oxidant properties (Gómez-Estaca et al., 2009). An older study showed that consuming oregano extract and other herbs as part of the human diet exhibited anti-microbial activities (Hammer et al., 1999). More recent studies showed that broiler chickens fed diet supplemented with oregano resulted in the following : 1) significant increase in the digestive enzyme chymotrypsin and enhanced protein digestion (Basmacioğlu Malayoğlu et al., 2010), 2) significant increase in body weight, higher anti-oxidant activity of serum, significant decrease in caecal *E. coli* population resulting in an increased growth performance (Roofchaee et al., 2011), 3) significant increase in body weight and significant decrease in FCR among broilers chickens infected with *Eimeria* species (PAJIĆ

et al., In press). Moreover, oregano and other herb extracts can suppress the growth of harmful coliform bacteria, but do not affect the growth of beneficial bacteria (Namkung et al., 2004).

3.1.2 Effect of phytochemicals on the growth of *E. coli*

Thymol and carvacrol are phenolic compounds and they are the main constituents of the essential oils of oregano. They are structural isomers, sharing the same chemical structure in the form of a phenolic ring but differing in the location of hydroxyl groups (Ultee et al., 2002b). Moreover, carvacrol is the key ingredient of oregano essential oil that is extracted from plants of the genus *Origanum* (Kintzios, 2002), but its abundance in *plantae* differs from one species to another (Gounaris et al., 2002). Thymol, carvacrol, and oregano share the same chief components which are monoterpenic phenols consisting of two main ingredients of γ-terpinene and p-cymeme (Kokkini, 1996). Carvacrol and oregano exhibit anti-microbial activities against pathogenic microorganisms whether from plant, animal or human sources, and these microorganisms include bacteria and fungi (Baricevic and Bartol, 2002).

In the field of pharmacology, phytochemicals have been shown to have many beneficial properties being anti-microbial and anti-oxidant agents (Kalemba and Kunicka, 2003). Generally, the phytochemicals (e.g. carvacrol) with a high percentage of other phenolic compounds display potent anti-bacterial properties (Guynot et al., 2003). As anti-bacterial agents, the main mechanism of action appears to be disruption of the integrity and functionality of the cell wall and cell membrane structures (Sikkema et al., 1995a). At MIC level, they disrupt the outer membrane structure of Gram-negative bacteria, increasing the permeability of cell membrane, leading to leakage of cellular energy sources in the form of adenosine tri-phosphate (ATP) (Gill and Holley, 2006) and may also result in the bursting of the bacterial cell (Sikkema et al., 1995a). The essential oils are highly hydrophobic and thus can readily integrate into and

transition across the bacterial cell membrane (Sikkema et al., 1995a). Interestingly, exposing bacteria to sub-lethal concentrations of these phytochemicals leads to changes in the ratio of unsaturated and saturated fatty acid component of the cell membrane (Di Pasqua et al., 2006) suggesting that bacteria develop an adaptive response upon exposure.

3.1.3 Biofilm formation in *E. coli*

Biofilm formation is a change in behavioural lifestyle that reflects a protective growth mode allowing bacteria to survive harsh conditions (Costerton et al., 1995), as planktonic cells attach to a surface and become covered with extracellular matrix of carbohydrate or exopolysaccharide nature (Wingender et al., 2012). Biofilm is defined as the collective attachment of bacterial cells to a solid surface (biotic or abiotic), whereby the process involves coverage with a bacterial derived exopolysaccharide matrix, creating a community of one or a variety of bacterial species (Costerton et al., 1995). The major components of the biofilm structure are exopolysaccharide, proteins, and nucleic acids (Davey and O'toole, 2000). The exopolysaccharide component is the result of bacterial metabolism (Davies et al., 1993), encompasses the large part of biofilm dry weight, and plays a key role in biofilm formation (Beauregard et al., 2013). It is known that specific genes of the bacterium are upregulated to enable this form of adherence, and that certain factors such as gravity and surface attachment are required to switch the relevant genes on. These complex structures form on many surfaces including host tissues (Costerton et al., 1987). Moreover, biofilm as a structural unit provides an ideal space for gene exchange through conjugation between cells (Hausner and Wuertz, 1999).

The first step in biofilm formation includes motility and adherence to surfaces enhanced by flagella (Harshey, 2003). Attachment to surfaces triggers genes expression and production of the extracellular matrix (Beloin et al., 2008). Biofilm formation enables sessile cells to lower their growth rate and utilise less nutrients (Costerton et al., 1995). Most bacteria live in the biofilm form rather than planktonic form as an adaptation strategy in the natural environment (Simões et al., 2010). There are many bacterial species associated with biofilm formation behaviour, but the significant ones are food-borne pathogens such as *Listeria monocytogenes*, *Salmonella* sp., *Bacillus* sp. (Palmer et al., 2007), *Escherichia coli, Pseudomonas* sp. (Stoodley et al., 2001) and *Campylobacter jejuni* (Kalmokoff et al., 2006).

Generally, biofilm-forming bacteria have greatly reduced sensitivity to anti-microbial compounds and other stressors, in comparison with non-formers (Høiby et al., 2010). The reason behind their reduced sensitivity is due to the difficulty with which anti-bacterial agents penetrate the bacterial cell, and also due to the presence of genes responsible for protection against stress (Stewart, 2002). Stopping bacteria from forming biofilm may improve susceptibility to antibiotics (Stewart, 2002). Thus, eradication of biofilm formation is through prevention of bacterial growth in the first place which can be achieved by using anti-bacterial agents (Zhao et al., 2017). Research has suggested that essential oils maybe be effective in attenuating bacterial biofilm formation (Gursoy et al., 2009).

3.1.4 Production of SCFAs by *E. coli*

As part of *E. coli* metabolism, SCFAs are produced, and they are defined as carboxylic acids with an aliphatic tail of varied length (McNaught and Wilkinson, 1997) of no more than 6 carbon atoms. They are produced as a result of bacterial fermentation which occurs anaerobically (Soergel, 1994) in the caecum or colon; the main site of anaerobic fermentation

(Reilly and Rombeau, 1993). Three SCFAs (propionate, butyrate, and acetate) play a role in intestinal health, especially butyrate as a direct energy source for epithelial cells (Wong et al., 2006), and is important in sustaining a good shape gastrointestinal tract (Meimandipour et al., 2010). The other SCFAs produced mostly in monogastric species in minor amounts include lactate, succinate, and branched chain fatty acids (Demigne et al., 1999). The quantity and type of SCFAs produced reflect the diversity and number of bacterial species present in the gut, and the type of substrates available (Macfarlane and Macfarlane, 2003).

The production rate of SCFAs depends mainly on the starch, fibre, and carbohydrates available in the diet (Demigné and Rémésy, 1985) that were not digested in the small intestine (Wong et al., 2006). SCFAs exert many beneficial effects by reducing gut inflammation, increasing caecal blood flow and thickening mucosal lining, and providing energy (Gorbach, 1996). Specifically, butyrate which makes up 15-20% of the SCFAs produced in the human colon (Hamer et al., 2008) is responsible for suppressing the proliferation of colonic cancer cells *in vitro* (Gibson et al., 1992). Moreover, SCFAs provide communication signals between the host and SCFA-producing gut bacteria lining the epithelium layer (Kelly et al., 2015).

In birds, SCFAs are available in different amounts throughout their gastrointestinal tract, but the highest concentration is in the caecum (Annison, 1991), as it harbours the highest concentrations of bacteria most of which are strict anaerobes that produce SCFAs as end products (Barnes et al., 1972). Also, the concentrations of SCFAs vary over the life cycle of the chicken (Barnes, 1979) but importantly are maintained at 5-15% equivalence of their daily energy requirements (Gasaway, 1976). They also provide protection against colonisation of pathogenic bacteria (Nurmi and Rantala, 1973). However, birds fed high fibre diets tend to

have an elevated level of fermentation in the caecum, and this may cause caecal hypertrophy (Redig, 1989).

3.1.5 Aims and objectives

This section of the thesis focuses on:

- Testing the potential of these phytochemicals as control agents in poultry production with a specific objective of controlling *E. coli*, that is both a natural commensal as well as an opportunistic pathogen (Hartl and Dykhuizen, 1984) and their ability to reduce or eliminate biofilm formation (Costerton et al., 1999).
- Studying the metabolic pathways of the *E. coli* strains upon addition of sub-lethal concentrations of aqueous carvacrol and oregano, by focusing on the production of SCFAs which can be formed during respiration and fermentation, as this approach may give us some clues about the mechanisms of action of these phytochemicals by checking which part of the metabolic pathways they are affecting, and if there are more effects on the by-products of respiration or fermentation of the metabolic reactions. Also, will the presence of sub-lethal concentrations lead to a shift in the production of SCFAs and to what extent?

3.2 Materials and methods

3.2.1 Determination of growth curves and MIC values of *E. coli* strains in the presence of different concentrations of aqueous phytochemicals

An inoculum of each pure culture of the *E. coli* strains (APEC = 12 strains, chicken strains = 9 strains, turkey strains = 10 strains, and 2 reference *E. coli* MG1655 K12 strains) was prepared by picking well-isolated colonies from an overnight bacterial culture (10 – 12hr) grown on NA plate and suspending in LB broth. The OD was adjusted to 0.01 at 600nm (10^7 CFU/ml) and a volume of 75µl of the bacterial suspension was added to the wells of the Greiner CELLSTAR® 96 well plate (sterile, F-bottom, with lid). Each well had 225µl of LB broth containing test phytochemicals (purchased as essential oils with assay of ~ 99% from Sigma Aldrich, UK) at the various concentrations previously added, so that the total final volume was 300µl in each well. For these initial studies, carvacrol or oregano (Sigma Aldrich, UK) were dissolved directly in the water-based LB broth, and dilutions made to add to the wells of the Greiner plate. For future reference, these dilutions will be referred to as aqueous carvacrol or aqueous oregano. The concentration ranges were given in the results section **3.3**. Then, the OD_{600} of the growing bacterial strain was taken spectrophotometrically every 1hr for 24hr under aerobic conditions and at a temperature of 37ºC using Fluostar Omega system. The OD_{600} readings were used to plot the relationship between time and OD. Plots were used to calculate bacterial growth to determine the MIC value of carvacrol or oregano against the *E. coli* strains.

3.2.2 Determination of *E. coli* biofilm formation using crystal violet method in the presence of different concentrations of ethanol-based phytochemicals

The method of O'Toole and Kolter was adapted for the determination of the potential of *E. coli* to form biofilm (O'Toole and Kolter, 1998). In brief, well-isolated colonies of each *E. coli* strains (APEC = 12 strains, chicken strains = 9 strains, turkey strains = 10 strains, and 2

reference *E. coli* MG1655 K12 strains) from an overnight culture (10 – 12hr) grown on nutrient agar were suspended in LB broth without salt, and the OD was adjusted to of 0.01 at 600nm (10^7 CFU/ml). Then, 225µl of LB broth medium without salt supplemented with differing concentrations of ethanol or phytochemical dissolved in ethanol, and 75µl of the bacterial suspension were loaded into individual wells of a polystyrene Greiner CELLSTAR® 96 well plate (sterile, F-bottom, with lid). In this experiment, the phytochemicals were first dissolved in ethanol (70%) and subsequently diluted in LB broth medium without salt. This was done to overcome false biofilm formation caused by small amounts of undissolved phytochemicals binding the well wall and absorbing crystal violet (see below).

The 96 well plates were incubated overnight at 37ºC, then for 2 days at 25°C. After incubation, the plate was inverted over a stack of absorbent paper to remove the bulk of the bacterial suspension in each well. Then, the wells were first washed with running tap water twice, stained with 300µl of 0.1% crystal violet for 30 min for biofilm detection. After that, the wells were washed with running tap water twice, dried, and for quantification of the formed biofilm, 300µl of (9:1) ethanol/acetone was added into each well, and then the absorbance was measured at 590nm using Fluostar Omega system.

3.2.3 Determination of SCFAs in *E. coli* strains in the presence of sub-lethal concentrations of aqueous phytochemicals by capillary gas chromatography (GC)

The method of Richardson was adapted to extract SCFAs using GC (Richardson et al., 1991). A sample of 1ml of overnight culture (10 – 12hr) from each *E. coli* strain (APEC = 12 strains, chicken strains = 7 strains and turkey strains = 6 strains with carvacrol and oregano MIC values ≥ 0.3µg/ml , and 1 reference *E. coli* MG1655 K12 strain) with OD of 1.00 at 600nm (10^9 CFU/ml) grown in LB broth (control), and LB broth containing 0.2µg/ml sub-lethal

concentrations of aqueous carvacrol or aqueous oregano with OD of 0.1 at 600nm (10^8 CFU/ml) at 37ºC was transferred into a flat bottomed 100mm x 16mm glass tube. Then, 1ml of external standard consisting of acetate, butyrate, formate, propionate, and lactate prepared at 1M each and in final concentration of 100mM, 50µl of 0.1M 2-ethylbutyric acid solution (internal standard), 500µl of concentrated hydrochloric acid (HCl), and 3ml of diethyl ether were added in this order to the tube. The tubes were vortexed in a multi-vortex for 1min, and then centrifuged at 2000 g for 10min in MSE Minstral 3000i centrifuge. A volume of 400µl of the resultant supernatant was transferred to a GC vial and 50µl of standard N-methyl-N-(t-butyldimethylsilyl) - trifluoroacetamide (MTBSTFA) was added to it. The GC vial was incubated at room temperature for 3 days, and then placed in a HP 5890 series II GC system (Hewlett Packard, Palo Alto, USA) using fused-silica capillary column 0.25µm film thickness (25m x 0.32mm) for the determination of SCFAs. The column temperature was set to be at 63ºC for 3min and then increased to 190ºC at 10ºC/min, and the carrier gas was helium.

3.2.4 Statistical analyses

For growth experiments, least square means for multiple comparisons was performed to compare between OD_{600} readings of control vs. OD_{600} readings of MIC level for each phytochemical at 9hr (exponential phase = highest OD reading). As for biofilm experiments, least square means for multiple comparisons was performed to compare between OD_{590} readings of control vs. OD_{590} readings of inhibition of biofilm level for each phytochemical.

For growth experiments, Pearson's Chi-square test was performed to study the significance level between MIC value of each phytochemical among the *E. coli* strains, and to study the levels of biofilm production by different *E. coli* strains. As for GC experiments, linear mixed model was performed to study the significance between control vs. production of each SCFA at sub-lethal concentrations of each phytochemical. Statistical analyses were performed using R i386 3.4.3 software.

3.3 Results

3.3.1 Effect of different concentrations of aqueous phytochemicals on the growth of *E. coli* strains

The effect of aqueous carvacrol and aqueous oregano on the growth of *E. coli* was investigated. OD_{600} measurements of growing broth cultures in the presence of increasing concentrations of aqueous carvacrol and oregano were taken over a 24hr incubation, and the results are shown in **Table 3.1**. These results were used to determine the MIC value under five different concentrations ranging from 0.1µg/ml to 0.5µg/ml in comparison with the control that lacked any phytochemical. The phytochemicals carvacrol and oregano showed concentration-dependent bactericidal effects on all of the *E. coli* strains tested. The MIC was determined as the lowest concentration where no growth was noted. The *E. coli* strains reacted variously to the different concentrations of aqueous carvacrol, and the MIC values ranged from 0.2µg/ml to 0.5µg/ml. In general, the most resilient *E. coli* strain was T4 with aqueous carvacrol MIC value of 0.5µg/ml. With aqueous oregano, the most resilient *E. coli* strains were APEC 48, APEC 49, and C8 with MIC values of 0.5µg/ml. As a generalisation, aqueous oregano showed higher MIC values in comparison with aqueous carvacrol. However, when least square means for multiple comparisons was performed, it showed that there was a significant difference (P-value < 0.0001) between OD_{600} readings of control vs. MIC level of aqueous carvacrol or aqueous oregano, but there were no significant differences (P-value = 0.9993) between MIC levels of aqueous carvacrol and aqueous oregano. This was supported by Pearson's Chi-square test which showed that there was no significant difference (P-value = 0.1252) between MIC values of aqueous carvacrol and aqueous oregano.

The growth curves were plotted and clearly showed that carvacrol and oregano showed a concentration- dependent inhibition of growth. Representative examples of growth curves of APEC 7 strains (sharing the same carvacrol and oregano MIC value = 0.3µg/ml) are shown in **Figures 3.1** and **3.2**. From this data, it was possible to establish the MIC value.

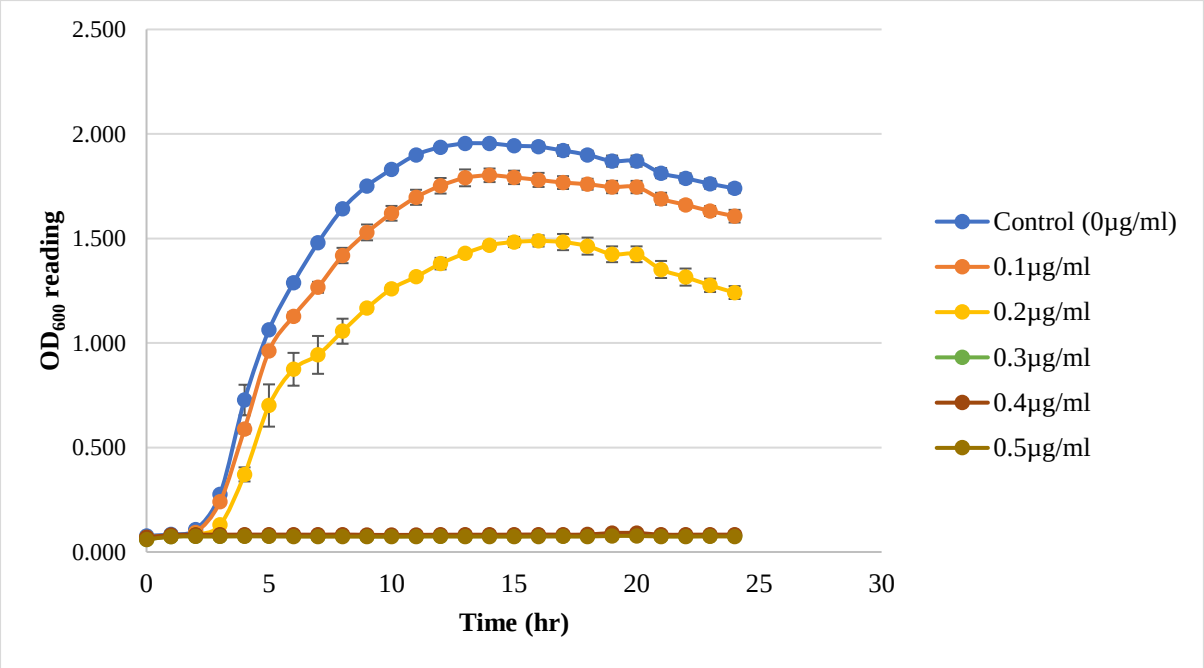

Figure 3.1 Representative growth curve showing the effect of different concentrations of aqueous carvacrol against strain APEC 7 (MIC = 0.3µg/ml). Results are expressed as means (n =3) with error bars.

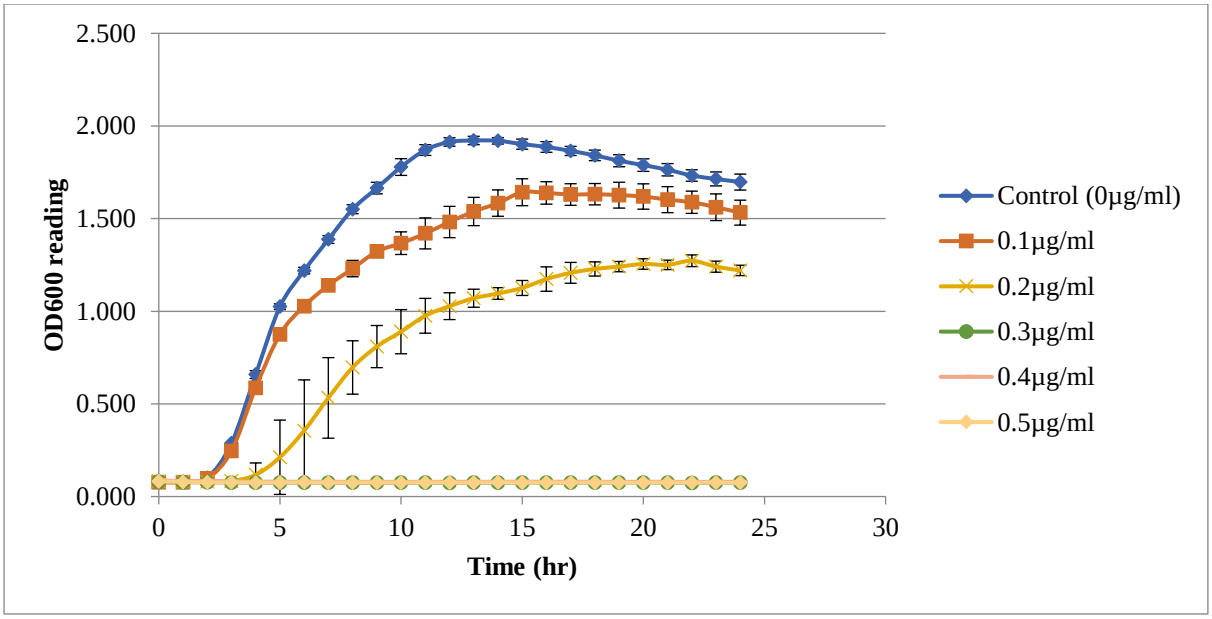

Figure 3.2 Representative growth curve showing the effect of different concentrations of aqueous oregano against strain APEC 7 (MIC = 0.3µg/ml). Results are expressed as means (n =3) with error bars.

Table 3.1 MIC values of aqueous carvacrol, and aqueous oregano against *E. coli* strains

Code	MIC value (μg/ml)		Minimum value (μg/ml)		Maximum value (μg/ml)		Mean (μg/ml)	
	C	O	C	O	C	O	C	O
7	0.3	0.3	0.3	0.3	0.5	0.5	0.358	0.342
14	0.3	0.3						
43	0.3	0.3						
45	0.4	0.4						
46	0.3	0.3						
47	0.4	0.3						
48	0.4	0.5						
49	0.4	0.5						
51	0.4	0.3						
52	0.3	0.3						
53	0.3	0.3						
54	0.3	0.3						
C1	0.3	0.3	0.2	0.3	0.3	0.5	0.278	0.367
C2	0.3	0.3						
C3	0.3	0.3						
C4	0.3	0.4						
C5	0.2	0.4						
C6	0.3	0.3						
C7	0.3	0.4						
C8	0.3	0.5						
C9	0.2	0.4						
T1	0.3	0.3	0.2	0.3	0.5	0.4	0.280	0.330
T2	0.2	0.4						
T3	0.2	0.3						
T4	0.5	0.3						
T5	0.2	0.3						
T6	0.3	0.4						
T7	0.2	0.4						
T8	0.3	0.3						
T9	0.3	0.3						
T10	0.3	0.3						
MG1655 WT	0.4	0.3						
MG1655 *acrAB* M	0.3	0.2						

Shaded cells refer to a high level of MIC values (greater than 0.3μg/ml). C, carvacrol; O, oregano. Results are expressed as means (n =3) ± STD = 0.00.

3.3.2 Effect of different concentrations of ethanol-based phytochemicals on *E. coli* biofilm

An initial study of biofilm formation showed that aqueous phytochemicals (carvacrol, and oregano) by themselves when prepared by direct dissolution in broth gave a false positive biofilm result, namely the aqueous phytochemicals bound to the wall of the well that bound to the crystal violet. This process was independent of any bacterial growth as demonstrated by positive readings for biofilm in the medium control where no bacterial suspension was given. Thus, to overcome this issue, carvacrol or oregano was first dissolved in 70% ethanol and then diluted in broth. Controls showed that carvacrol or oregano dissolved this way did not cause false positive results for biofilm formation.

Ethanol is a known inhibitor of bacteria, and it was considered possible even at the very low concentrations of ethanol in the ethanol - carvacrol or oregano mixture (max. 3.47%), the ethanol may impact on biofilm formation either more than or synergistically with carvacrol or oregano. Therefore, a control study was set up to test the impact of ethanol alone at the dilutions and a little above used in the phytochemical dilutions. The experiments were performed as described in materials and methods and the results are shown in **Table 3.2**. The data clearly showed that ethanol alone and at different concentrations used resulted in either stimulation or inhibition of biofilm formation. These experiments were repeated to generate statistical robustness in this analysis, with a significant P-value of less than 0.05. It was concluded that the concentrations of ethanol used to dissolve the phytochemicals were sufficient to stimulate biofilm formation in most strains. The collective data (**Tables 3.2, 3.3, and 3.4**) showed that biofilm was formed in the presence of different concentrations of ethanol, but it was significantly inhibited (P-value < 0.05) upon addition of different concentrations of ethanol-based carvacrol or ethanol-based oregano.

A layer of biofilm was formed on the walls of the used 96 well plate in the absence of phytochemicals, of which 41.7% of the tested bacteria were defined as strong biofilm formers which were mostly APEC strains, and the remaining 58.3% were defined as weak biofilm formers (**Tables 3.2, 3.3, and 3.4**). The effect of both ethanol-based phytochemicals resulted in an inhibition of the *E. coli* biofilm upon increasing their concentrations (**Tables 3.3 and 3.4)**. There was a general trend in attenuating *E. coli* biofilm formation by ethanol-based carvacrol and ethanol-based oregano, but *E. coli* strains exhibited different inhibitory effects reflected by different concentrations of ethanol-based carvacrol and ethanol-based oregano. It was noted that the very high biofilm former APEC 7; required a very high concentration (0.5µg/ml) of ethanol-based carvacrol to inhibit its biofilm formation (**Table 3.3**), but its biofilm was inhibited upon addition of 0.2µg/ml of ethanol-based oregano (**Table 3.4**).

Table 3.2 Effect of different concentrations of ethanol on *E. coli* biofilm

Code	Ethanol (%)					
	0	**0.5**	**1**	**2**	**3**	**3.5**
	Biofilm formation = OD_{590} – OD_{590} of LB broth					
7	3.263 ± 0.06	3.192 ± 0.00	3.192 ± 0.00	3.192 ± 0.00	0.000 ± 0.00*	0.000 ± 0.01*
14	1.755 ± 1.64	3.123 ± 0.00	3.061 ± 0.07	2.600 ± 0.61	2.782 ± 0.12	2.993 ± 0.15
43	3.168 ± 0.16	3.123 ± 0.91	3.088 ± 0.00	1.005 ± 1.67	1.692 ± 0.95	1.123 ± 0.00
45	2.535 ± 0.39	2.895 ± 0.02	2.896 ± 0.01	2.879 ± 0.04	2.261 ± 0.21	1.561 ± 0.68
46	1.782 ± 1.57	2.852 ± 0.09	2.883 ± 0.02	2.897 ± 0.01	2.904 ± 0.00	2.891 ± 0.02
47	2.091 ± 1.81	2.187 ± 0.52	2.864 ± 0.07	2.904 ± 0.09	2.279 ± 1.08	2.904 ± 0.00
48	1.347 ± 1.71	2.911 ± 0.02	2.919 ± 0.00	2.858 ± 0.07	2.508 ± 0.22	1.579 ± 0.70
49	1.472 ± 1.45	2.891 ± 0.05	2.884 ± 0.03	2.919 ± 0.00	2.889 ± 0.05	2.919 ± 0.00
51	2.529 ± 0.63	2.589 ± 0.44	2.994 ± 0.01	2.998 ± 0.09	2.505 ± 0.85	2.998 ± 0.00
52	1.095 ± 0.96	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.002 ± 0.57
53	2.170 ± 1.88	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00
54	1.287 ± 1.21	0.569 ± 0.62	1.096 ± 1.48	1.013 ± 0.01	1.227 ± 0.20	1.350 ± 1.72
C1	3.221 ± 0.14	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00
C2	1.508 ± 1.60	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00	3.330 ± 0.00
C3	3.287 ± 0.13	3.330 ± 0.00	3.330 ± 0.00	3.526 ± 0.86	3.330 ± 0.00	3.330 ± 0.00
C4	1.796 ± 1.63	3.325 ± 0.00	3.321 ± 0.01	3.325 ± 0.00	3.292 ± 0.06	3.325 ± 0.00
C5	0.829 ± 0.72	2.345 ± 0.87	3.023 ± 0.25	3.325 ± 1.21	2.020 ± 0.63	2.726 ± 0.65
C6	1.523 ± 1.57	3.137 ± 0.23	3.180 ± 0.04	3.301 ± 1.36	3.219 ± 0.09	3.322 ± 0.01
C7	3.260 ± 0.07	3.383 ± 0.00	3.350 ± 0.02	3.383 ± 0.00	3.333 ± 0.02	3.345 ± 0.03
C8	0.793 ± 0.83	3.311 ± 0.13	3.153 ± 0.14	3.106 ± 0.63	3.235 ± 0.26	3.304 ± 0.13
C9	1.671 ± 1.47	1.003 ± 0.00	1.104 ± 0.08	1.005 ± 0.00	1.238 ± 0.41	1.910 ± 1.71
T1	0.023 ± 0.02	0.082 ± 0.01	0.105 ± 0.01	0.000 ± 0.14	0.000 ± 0.10	0.000 ± 0.12
T2	0.003 ± 0.03	0.000 ± 0.09	0.002 ± 0.00	0.000 ± 0.11	0.000 ± 0.10	0.000 ± 0.11
T3	1.479 ± 1.35	2.182 ± 0.26	2.349 ± 2.02	2.081 ± 0.20	1.755 ± 1.77	1.349 ± 0.00
T4	0.000 ± 0.03	0.093 ± 0.05	0.066 ± 0.01	0.080 ± 0.05	0.041 ± 0.01	0.046 ± 0.01
T5	1.984 ± 1.73	3.385 ± 0.02	3.397 ± 0.00	2.981 ± 0.57	3.397 ± 0.00	3.397 ± 0.00
T6	0.024 ± 0.15	0.013 ± 0.00	0.060 ± 0.03	0.016 ± 0.00	0.123 ± 0.06	0.063 ± 0.04
T7	0.119 ± 0.10	0.278 ± 0.04	0.190 ± 0.04	0.189 ± 0.10	0.164 ± 0.05	0.055 ± 0.03
T8	0.000 ± 0.05	0.000 ± 0.00	0.076 ± 0.02	0.142 ± 0.19	0.056 ± 0.02	0.071± 0.02
T9	0.064 ± 0.11	0.004 ± 0.02	0.081 ± 0.02	0.000 ± 0.01	0.072 ± 0.06	0.098 ± 0.05
T10	0.000 ± 0.03	0.211 ± 0.05	0.350 ± 0.07	0.160 ± 0.14	0.071 ± 0.01	0.052 ± 0.02
MG1655 WT	0.053 ± 0.02	3.225 ± 0.01	3.131 ± 0.03	3.336 ± 0.01	2.567 ± 0.01	2.366 ± 0.01
MG1655 *acrAB* M	0.027 ± 0.01	0.053 ± 0.01	0.060 ± 0.01	0.014 ± 0.01	0.073 ± 0.01	0.154 ± 0.01

Shaded cells refer to strong biofilm formers (greater than 1.500), considering 1.500 as a cut-off value. Values represent corrected OD readings at 590nm by subtracting the resulted OD readings from OD readings value of LB broth without salt as a negative control. Results are expressed as means (n =3).

Table 3.3 Effect of different concentrations of ethanol-based carvacrol on *E. coli* biofilm

Code	Carvacrol (µg/ml) (Ethanol %)					
	0 (0%)	0.1 (0.7%)	0.2 (1.4%)	0.3 (2.1%)	0.4 (2.8%)	0.5 (3.4%)
	Biofilm formation = OD_{590} – OD_{590} of LB broth					
7	3.263 ± 0.06	3.335 ± 0.00	3.335 ± 0.00	3.335 ± 0.17	1.310 ± 0.12*	0.533 ± 0.09*
14	1.755 ± 1.64	2.026 ± 1.30	2.363 ± 0.90	0.000 ± 0.03*	0.226 ± 0.30*	0.007 ± 0.02*
43	3.168 ± 0.16	2.986 ± 0.21	2.764 ± 0.57	0.038 ± 0.07*	0.678 ± 0.27*	0.016 ± 0.03*
45	2.535 ± 0.39	2.583 ± 0.69	1.964 ± 0.32	0.030 ± 0.08*	0.544 ± 0.89*	0.000 ± 0.01*
46	1.782 ± 1.57	0.232 ± 0.09*	0.000 ± 0.00*	0.000 ± 0.00*	0.000 ± 0.00*	0.000 ± 0.01*
47	2.091 ± 1.81	0.000 ± 0.07*	0.000 ± 0.06*	0.000 ± 0.06*	0.000 ± 0.07*	0.000 ± 0.07*
48	1.347 ± 1.71	0.025 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.00*
49	1.472 ± 1.45	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.01*
51	2.529 ± 0.63	0.129 ± 0.26*	0.097 ± 0.24*	0.000 ± 0.01*	0.000 ± 0.02*	0.000 ± 0.00*
52	1.095 ± 0.96	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.00*	0.000 ± 0.02*	0.000 ± 0.00*
53	2.170 ± 1.88	0.052 ± 0.04*	0.009 ± 0.13*	0.000 ± 0.00*	0.000 ± 0.00*	0.000 ± 0.00*
54	1.287 ± 1.21	0.010 ± 0.01*	0.012 ± 0.02*	0.019 ± 0.01*	0.000 ± 0.00*	0.000 ± 0.00*
C1	3.221 ± 0.14	0.269 ± 0.89*	0.063 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.00*
C2	1.508 ± 1.60	0.000 ± 0.01*	0.003 ± 0.01*	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.01*
C3	3.287 ± 0.13	0.080 ± 0.04*	0.102 ± 0.04*	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.00*
C4	1.796 ± 1.63	0.275 ± 0.08*	0.017 ± 0.02*	0.000 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.00*
C5	0.829 ± 0.72	0.000 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.00*	0.000 ± 0.00*	0.000 ± 0.00*
C6	1.523 ± 1.57	0.000 ± 0.02*	0.000 ± 0.03*	0.000 ± 0.01*	0.000 ± 0.00*	0.000 ± 0.00*
C7	3.260 ± 0.07	0.865 ± 0.93*	0.128 ± 0.07*	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.01*
C8	0.793 ± 0.83	0.058 ± 0.05*	0.000 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.01*	0.000 ± 0.00*
C9	1.671 ± 1.47	0.216 ± 0.12*	0.203 ± 0.19*	0.000 ± 0.01*	0.004 ± 0.00*	0.000 ± 0.00*
T1	0.023 ± 0.02	0.068 ± 0.01	0.000 ± 0.01	0.000 ± 0.00	0.000 ± 0.01	0.000 ± 0.00
T2	0.003 ± 0.03	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.01
T3	1.479 ± 1.35	0.027 ± 0.05*	0.653 ± 0.96*	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.01*
T4	0.000 ± 0.03	0.000 ± 0.01	0.000 ± 0.03	0.000 ± 0.00	0.000 ± 0.01	0.000 ± 0.01
T5	1.984 ± 1.73	0.000 ± 0.00*	0.000 ± 0.04*	0.000 ± 0.01*	0.000 ± 0.04*	0.000 ± 0.01*
T6	0.024 ± 0.15	0.000 ± 0.01	0.000 ± 0.02	0.000 ± 0.02	0.000 ± 0.01	0.000 ± 0.02
T7	0.119 ± 0.10	0.008 ± 0.01	0.054 ± 0.05	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.01
T8	0.000 ± 0.05	0.000 ± 0.00	0.000 ± 0.01	0.000 ± 0.00	0.000 ± 0.01	0.000 ± 0.01
T9	0.064 ± 0.11	0.038 ± 0.02	0.072 ± 0.09	0.000 ± 0.01	0.000 ± 0.01	0.000 ± 0.00
T10	0.000 ± 0.03	0.000 ± 0.00	0.000 ± 0.02	0.000 ± 0.00	0.000 ± 0.01	0.000 ± 0.00
MG1655 WT	0.053 ± 0.02	0.084 ± 0.01	0.029 ± 0.02	0.022 ± 0.01	0.036 ± 0.02	0.012 ± 0.02
MG1655 *acrAB* M	0.027 ± 0.01	0.021 ± 0.01	0.000 ± 0.01	0.000 ± 0.01	0.000 ± 0.00	0.020 ± 0.01

Shaded cells refer to strong biofilm formers (greater than 1.500), considering 1.500 as a cut-off value. Values represent corrected OD readings at 590nm by subtracting the resulted OD readings from OD readings value of LB broth without salt as a negative control. Results are expressed as means (n =3).

Table 3.4 Effect of different concentrations of ethanol-based oregano on *E. coli* biofilm

Code	Oregano (µg/ml) (Ethanol %)					
	0 (0%)	**0.1 (0.7%)**	**0.2 (1.4%)**	**0.3 (2.1%)**	**0.4 (2.8%)**	**0.5 (3.4%)**
	Biofilm formation = OD_{590} – OD_{590} of LB broth					
7	3.263 ± 0.06	1.970 ± 1.08*	0.000 ± 0.15*	0.000 ± 0.09*	0.018 ± 0.13*	0.000 ± 0.00*
14	1.755 ± 1.64	0.000 ± 0.08*	0.000 ± 0.10*	0.000 ± 0.11*	0.000 ± 0.08*	0.000 ± 0.07*
43	3.168 ± 0.16	2.680 ± 1.73	0.000 ± 0.11*	0.000 ± 0.08*	0.000 ± 0.09*	0.000 ± 0.08*
45	2.535 ± 0.39	0.000 ± 0.01*	0.000 ± 0.06*	0.000 ± 0.05*	0.000 ± 0.00*	0.000 ± 0.00*
46	1.782 ± 1.57	1.465 ± 0.65	0.000 ± 0.11*	0.000 ± 0.08*	0.000 ± 0.09*	0.000 ± 0.08*
47	2.091 ± 1.81	0.096 ± 0.15*	0.000 ± 0.10*	0.000 ± 0.10*	0.000 ± 0.08*	0.000 ± 0.08*
48	1.347 ± 1.71	0.000 ± 0.12*	0.020 ± 0.13*	0.000 ± 0.08*	0.000 ± 0.09*	0.000 ± 0.08*
49	1.472 ± 1.45	0.000 ± 0.00*	0.000 ± 0.13*	0.000 ± 0.10*	0.000 ± 0.09*	0.000 ± 0.09*
51	2.529 ± 0.63	1.894 ± 1.16	0.034 ± 0.15*	0.000 ± 0.07*	0.000 ± 0.08*	0.000 ± 0.07*
52	1.095 ± 0.96	0.000 ± 0.08*	0.000 ± 0.12*	0.000 ± 0.09*	0.000 ± 0.09*	0.000 ± 0.01*
53	2.170 ± 1.88	1.487 ± 0.94	0.000 ± 0.15*	0.000 ± 0.01*	0.000 ± 0.11*	0.000 ± 0.09*
54	1.287 ± 1.21	0.000 ± 0.14*	0.000 ± 0.12*	0.000 ± 0.00*	0.000 ± 0.10*	0.000 ± 0.11*
C1	3.221 ± 0.14	2.208 ± 2.02	0.061 ± 0.16*	0.017 ± 0.01*	0.000 ± 0.07*	0.003 ± 0.00*
C2	1.508 ± 1.60	0.016 ± 0.00*	0.000 ± 0.07*	0.018 ± 0.00*	0.000 ± 0.07*	0.003 ± 0.00*
C3	3.287 ± 0.13	1.051 ± 1.19*	0.000 ± 0.10*	0.045 ± 0.01*	0.000 ± 0.09*	0.000 ± 0.08*
C4	1.796 ± 1.63	0.359 ± 0.42*	0.285 ± 0.36*	0.131 ± 0.01*	0.000 ± 0.10*	0.000 ± 0.10*
C5	0.829 ± 0.72	0.106 ± 0.23*	0.000 ± 0.10*	0.000 ± 0.09*	0.000 ± 0.09*	0.000 ± 0.07*
C6	1.523 ± 1.57	0.124 ± 0.24*	0.024 ± 0.13*	0.000 ± 0.10*	0.000 ± 0.09*	0.000 ± 0.08*
C7	3.260 ± 0.07	3.084 ± 0.07	1.382 ± 0.56*	0.133 ± 0.03*	0.057 ± 0.01*	0.000 ± 0.01*
C8	0.793 ± 0.83	1.014 ± 1.06	0.191 ± 0.27*	0.017 ± 0.11*	0.000 ± 0.10*	0.000 ± 0.09*
C9	1.671 ± 1.47	0.638 ± 0.67*	0.010 ± 0.10*	0.055 ± 0.01*	0.000 ± 0.08*	0.023 ± 0.00*
T1	0.023 ± 0.02	0.276 ± 0.01	0.222 ± 0.04	0.229 ± 0.03	0.185 ± 0.01	0.195 ± 0.01
T2	0.003 ± 0.03	0.000 ± 0.01	0.000 ± 0.04	0.000 ± 0.01	0.000 ± 0.01	0.000 ± 0.02
T3	1.479 ± 1.35	0.237 ± 0.17*	0.000 ± 0.02*	0.000 ± 0.00*	0.000 ± 0.01*	0.000 ± 0.00*
T4	0.000 ± 0.03	0.000 ± 0.01	0.000 ± 0.02	0.000 ± 0.01	0.000 ± 0.01	0.000 ± 0.01
T5	1.984 ± 1.73	0.856 ± 0.00*	0.824 ± 0.06*	0.498 ± 0.34*	0.856 ± 0.00*	0.366 ± 0.34*
T6	0.024 ± 0.15	0.000 ± 0.13	0.000 ± 0.01	0.000 ± 0.06	0.000 ± 0.04	0.000 ± 0.05
T7	0.119 ± 0.10	0.000 ± 0.03	0.000 ± 0.03	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00
T8	0.000 ± 0.05	0.000 ± 0.02	0.000 ± 0.01	0.000 ± 0.02	0.000 ± 0.07	0.000 ± 0.01
T9	0.064 ± 0.11	0.077 ± 0.03	0.051 ± 0.05	0.021 ± 0.01	0.000 ± 0.01	0.000 ± 0.01
T10	0.000 ± 0.03	0.000 ± 0.00	0.000 ± 0.08	0.000 ± 0.10	0.000 ± 0.07	0.000 ± 0.09
MG1655 WT	0.053 ± 0.02	0.014 ± 0.02	0.000 ± 0.01	0.005 ± 0.04	0.000 ± 0.00	0.000 ± 0.00
MG1655 *acrAB* M	0.027 ± 0.01	0.013 ± 0.01	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.01	0.000 ± 0.01

Shaded cells refer to strong biofilm formers (greater than 1.500), considering 1.500 as a cut-off value. Values represent corrected OD readings at 590nm by subtracting the resulted OD readings from OD readings value of LB broth without salt as a negative control. Results are expressed as means (n =3).

3.3.3 Effect of sub-lethal concentrations of aqueous phytochemicals on the production of SCFAs by *E. coli* strains

Results showed that *E. coli* strains produce variable amounts of SCFAs (**Tables 3.5 and 3.6**) and there were subtle differences in the production of SCFAs among the three groups of *E. coli* strains. In general, the highest SCFA produced was lactate (fermentation by-product), and the lowest SCFA produced was succinate (respiration by-product), interestingly produced by APEC and chicken strains. Whereas, turkey strains produced acetate as the highest SCFA produced, and succinate as the lowest produced SCFA. Statistically speaking, there were no significant differences (P-value > 0.05) between each phytochemical and the production of each SCFA when performing linear mixed model. Also, there were no significant differences (P-value > 0.05) between each phytochemical and the production of SCFAs as respiration by-products (propionate, butyrate, and succinate) against different types of *E. coli* strains.

There was a reduction in the production of lactate (fermentation by-product) in most APEC strains and some chicken strains incubated in sub-lethal concentrations of aqueous oregano in comparison with the control sets. Since lactate was the highest SCFA produced, there was a more focus on it. Linear mixed model showed that there was a significant difference between each phytochemical and production of lactate between APEC and turkey strains (P-value = 0.011), and between chicken and turkey strains (P-value = 0.0028). The other by-product was acetate showed that there was a significant difference between each phytochemical and its production against APEC and turkey (P-value = 0.0002), and between chicken and turkey (P-value = 0.0004), but there was no significant difference between APEC and chicken (P-value = 0.995).

Bacteria were grouped according to their MIC value of carvacrol, and this showed that bacteria with high MIC value had lower lactate production than bacteria with low MIC value. However, there was a direct relationship between amount of lactate production and oregano MIC value.

Table 3.5 Effect of sub-lethal concentrations of aqueous carvacrol and aqueous oregano (0.2µg/ml) on the production of SCFAs as respiration by-products

Code	Propionate			Butyrate			Succinate		
	Control	C	O	Control	C	O	Control	C	O
7	125.135±0.40	118.009±1.82	97.942±25.83	111.382±0.29	110.288±1.62	96.621±15.23	7.372±1.36	6.316±0.30	16.430±22.22
14	167.340±6.57	166.460±1.75	161.040±4.42	149.804±5.42	149.455±0.67	146.953±2.48	4.765±0.20	5.361±0.13	4.847±0.33
43	174.547±1.56	171.711±2.28	164.450±12.10	155.690±4.38	153.919±1.58	155.097±7.70	6.404±0.10	6.937±0.24	7.027±0.49
45	149.099±4.18	154.595±0.13	103.480±0.13	130.163±0.13	137.055±0.13	91.857±0.13	5.427±0.14	6.710±0.15	3.613±3.13
46	157.912±3.04	165.677±1.75	161.361±1.73	144.582±5.56	164.129±6.16	142.102±0.84	5.182±0.11	5.841±0.35	5.476±0.08
47	166.831±8.66	165.352±1.05	175.824±6.51	144.694±7.55	149.040±1.42	158.487±6.60	5.362±0.31	5.264±0.14	5.823±0.09
49	42.116±72.95	124.153±11.64	97.646±85.92	41.309±71.55	121.966±12.08	96.557±84.95	0.961±1.67	2.527±1.01	1.319±1.36
51	120.008±15.13	34.585±59.90	122.196±22.24	119.408±15.36	34.904±60.45	122.020±19.72	2.417±1.30	0.253±0.44	7.958±10.67
52	93.552±81.52	97.109±84.84	100.373±88.11	93.165±81.22	96.155±84.37	100.018±87.04	1.484±1.53	1.438±1.57	5.831±8.58
53	151.237±4.55	102.046±88.67	149.550±15.88	156.835±6.66	103.037±89.75	148.972±13.07	3.707±0.88	2.150±2.05	3.168±1.45
54	106.455±92.21	104.692±90.67	47.579±82.41	102.261±88.60	105.272±91.19	46.438±80.43	2.653±2.32	2.543±2.20	1.143±1.98
C1	58.824±101.89	107.748±93.31	55.308±95.80	54.011±93.55	104.141±90.23	52.509±90.95	1.634±2.83	0.986±0.85	1.230±2.13
C2	160.649±4.69	164.448±2.58	159.067±2.22	166.259±19.36	180.379±8.36	170.999±8.07	4.737±0.42	6.446±0.16	4.705±0.22
C3	154.772±0.74	161.604±0.82	160.907±0.65	153.536±6.25	146.095±1.60	147.653±3.72	4.791±0.09	3.959±0.10	4.688±0.10
C4	170.555±2.74	146.762±17.10	155.231±10.53	177.066±21.13	160.478±24.71	164.866±13.95	3.959±0.05	1.997±1.12	3.000±1.19
C6	140.304±25.14	157.995±3.04	171.519±2.05	134.457±25.40	153.078±4.97	166.199±3.25	2.991±1.60	2.699±1.29	3.832±0.05
C7	155.242±0.40	145.169±8.48	152.754±3.83	150.125±0.33	141.251±10.55	146.745±5.63	3.796±0.05	2.750±0.62	3.570±0.56
C8	112.537±4.41	130.931±23.17	122.199±24.70	118.364±4.20	141.221±24.49	131.877±26.26	0.969±0.17	2.227±1.16	1.467±1.16
T1	131.893±12.20	140.088±15.35	145.919±18.49	127.114±11.51	135.584±14.14	140.391±17.48	1.186±0.19	2.313±1.52	2.568±1.36
T4	147.680±18.90	140.787±7.03	143.870±11.91	144.789±18.38	142.295±6.93	135.159±11.24	2.181±1.07	1.724±0.27	2.069±0.69
T6	143.844±19.12	125.719±10.17	132.201±13.04	133.284±17.65	121.365±9.26	125.972±10.25	2.960±1.44	1.501±0.51	7.176±8.90
T8	106.720±6.46	107.830±5.84	102.040±21.97	109.807±6.76	108.457±8.12	102.260±18.78	1.316±0.60	1.660±1.06	10.330±13.33
T9	82.341±7.96	83.998±9.72	93.186±11.73	88.028±6.24	87.715±5.98	96.488±8.24	221.772±171.40	115.176±178.38	14.119±11.35
T10	87.326±14.47	84.211±26.15	103.300±17.18	93.046±5.88	98.481±15.99	103.768±12.65	14.170±12.37	11.100±8.70	9.563±12.49
MG1655 WT	60.377±52.85	272.533±0.23	171.221±46.25	63.116±54.67	263.671±23.63	184.929±19.76	7.733±12.66	20.206±23.49	30.566±24.68

Numbers refer to an average of three values in millimolar (mM) unit. Control, refers to the *E. coli* production of SCFAs without phytochemicals. C, aqueous carvacrol; O, aqueous oregano. Results are expressed as means (n =3) ± STD.

Table 3.6 Effect of sub-lethal concentrations of aqueous carvacrol and aqueous oregano (0.2µg/ml) on the production of SCFAs as by-products of anaerobic pathway

Code	Acetate			Lactate		
	Control	C	O	Control	C	O
7	122.398±2.02	122.497±2.64	115.580±151.82	394.209±6.77	386.057±12.33	246.232±213.37
14	160.081±5.85	160.034±3.37	149.599±7.85	348.927±14.23	353.877±10.38	320.683±23.03
43	162.942±3.16	163.486±3.16	163.400±10.93	334.561±6.17	338.238±10.88	333.245±31.49
45	144.031±3.61	151.725±2.59	100.310±86.89	244.553±2.44	255.243±1.83	171.256±0.15
46	155.059±5.60	174.660±4.47	161.996±2.01	265.483±9.97	297.774±12.89	162.315±1.92
47	173.510±7.79	175.550±1.76	184.409±5.26	181.182±5.24	179.510±2.63	187.656±1.64
49	34.797±60.27	104.360±16.53	74.660±64.68	24.374±42.22	75.750±12.29	55.908±48.45
51	103.661±21.08	23.731±41.10	101.865±30.79	131.794±36.26	31.834±55.14	118.351±25.12
52	70.689±62.95	74.292±67.07	79.012±71.34	47.935±42.37	50.945±45.33	35.563±61.60
53	120.209±8.48	81.155±71.10	118.461±26.19	127.911±9.21	85.668±75.29	107.809±10.88
54	92.098±79.81	87.760±76.00	40.419±70.01	78.561±68.18	73.972±64.07	47.343±82.00
C1	53.445±92.57	97.955±84.83	51.709±89.56	94.437±163.57	175.035±151.59	96.812±167.68
C2	138.069±3.62	146.838±3.02	139.620±1.58	291.116±19.36	332.630±8.36	306.454±8.07
C3	134.402±0.03	145.393±0.86	144.395±1.15	300.529±6.25	262.326±1.60	258.782±3.72
C4	146.829±1.46	120.258±25.35	125.618±17.24	238.355±80.79	144.643±44.28	170.355±38.42
C6	104.836±27.95	125.271±13.74	143.467±1.34	152.122±74.78	202.401±51.89	250.396±1.49
C7	129.376±3.05	121.811±10.13	127.040±6.33	213.539±4.08	207.219±31.73	233.530±19.63
C8	83.931±3.60	110.479±26.88	94.937±27.82	120.312±11.35	175.434±59.16	139.852±52.38
T1	91.027±7.24	104.621±18.78	111.799±20.65	107.420±8.01	139.795±35.11	151.655±36.71
T4	118.180±24.63	108.610±8.01	109.566±14.66	184.619±44.03	161.170±9.36	167.008±33.00
T6	114.030±23.43	94.765±12.55	355.749±436.75	191.425±41.37	151.190±20.30	157.405±24.50
T8	90.106±13.44	89.344±17.21	359.241±448.46	67.706±34.52	69.614±39.53	39.430±45.73
T9	1071.762±553.28	774.934±534.78	560.872±424.57	0.000±0.00	28.519±49.40	31.535±54.62
T10	669.569±384.59	471.660±356.20	481.924±72.09	29.271±50.70	24.554±42.53	52.195±64.49
MG1655 WT	425.135±42.51	277.542±105.07	409.317±249.44	31.083±53.84	373.841±320.39	227.169±250.59

Numbers refer to an average of three values in mM unit. Control, refers to the *E. coli* production of SCFAs without incubating it in phytochemicals. C, aqueous carvacrol; O, aqueous oregano. Results are expressed as means (n =3) ± STD.

3.4 Discussion

MIC values for aqueous phytochemicals (carvacrol and oregano) were determined against APEC strains, commensal *E. coli* isolated from chicken and turkey, and *E. coli* MG1655 K12 strains (**Table 3.1**). Aqueous carvacrol and aqueous oregano demonstrated their bactericidal activity against these *E. coli* strains. The MIC values of aqueous carvacrol varied between the different types of *E. coli* strains (**Table 3.1**), with APEC strains giving the highest MIC values. Whether there is a genuine correlation between relative insensitivity to carvacrol and/or oregano and their pathogenicity (e.g. APEC 48 has an MIC value of 0.4µg/ml and 0.5µg/ml, respectively) is open to question but is worthy of a wider population study. The resulting MIC values cannot be compared directly with values given in the scientific literature or from previous studies for reasons that relate largely to differing preparation methods of the phytochemicals: 1) extraction method of the essential oil; 2) essential oil content of the stock; 3) preparation of stock in different solvents; 4) volume and number of bacterial in the inoculum; 5) different bacterial cultures and broth media; 6) OD measurements at different wavelengths. However, irrespective of the differences in approaches and techniques, the data generated in this study and elsewhere in the literature suggest that the sensitivity of the *E. coli* towards these phytochemicals is promising for their potential use as control measures.

The trend from the data generated in this study needs further investigation. The relative lower sensitivity of APEC compared with presumed poultry commensal strains is of concern, as APEC strains showed the highest MIC values. A question that could be asked is whether reduced sensitivity is related to virulence and possession of virulence factors associated with avian colibacillosis (Delicato et al., 2003). Many virulence factors such as fimbriae and other protein components of the cell wall structure may alter the properties of the cell membrane resulting in the relative insensitivity of APEC strains. It is possible that the use of these

phytochemicals at low level will select APEC and reduce commensals. This will need further investigation at the population level in caecal contents of broiler chickens which will be presented in **Chapter 6**.

The mechanism of action of these phytochemicals is most likely related to their hydrophobic nature that allows them to diffuse through the cell wall structure (Sikkema et al., 1995a) and this represents the first target site within the bacterial cell. The exact mechanism of action of these phytochemicals is not well-known, so it is still not clear whether there might be single or multiple target sites within the bacterial cells such as cell membrane, and nucleoid structures. In order to interrogate the mechanisms of action, one set of future studies will be the derivation of mutants showing reduced susceptibility, selected by continuous exposure to sub-MIC level of phytochemicals in growth medium, in order to study mutational changes by WGS and thereby get a hint into the changes in genetic makeup which will be presented in the following **Chapter 4**.

When comparing MIC values of aqueous carvacrol and aqueous oregano, results showed that aqueous carvacrol has a lower MIC than aqueous oregano against *E. coli* strains which agrees with previous research (Cosentino et al., 1999), even though both phytochemicals come from the same polyphenol group. This might be due to the differences in their chemical structures as it is known that the anti-bacterial activity and its mode of action of the essential oil is determined by its chemical structure (Dorman and Deans, 2000). However, when least square means for multiple comparisons was performed, it showed that there was a significant difference (P-value < 0.0001) between OD_{600} readings of control vs. MIC level of aqueous carvacrol or aqueous oregano, but there was no significant difference (P-value = 0.9993)

between MIC levels of aqueous carvacrol and aqueous oregano. This was supported by Pearson's Chi-square test which showed that there was no significant difference (P-value = 0.1252) between MIC values of aqueous carvacrol and aqueous oregano, even though carvacrol is the active ingredient of oregano oil, and that both phytochemicals are active in controlling *E. coli* strains (i.e. effective in inhibiting their growth).

Results confirmed the bactericidal activity of the aqueous carvacrol and aqueous oregano at the concentrations ranges used against a variety of *E. coli* strains including those strains with multi-antibiotic resistance, biofilm formation, and a variety of virulence genes isolated from poultry sources. This *in vitro* study provides promising data that the use of carvacrol and oregano may be potent against *E. coli* strains that inhabit poultry guts reared for human consumption. This opens additional avenues in reducing the burden of antibiotic resistant *E. coli* strains *in vivo* with phytochemicals instead of antibiotics in poultry industry. However, as stated, there is a concern that removal of commensal *E. coli* strains may have a deleterious impact on performance, but this may outweigh by other benefits; better performance in broiler production (Personal communication, St. David's Veterinary Practice).

In the growth experiments **3.3.1**, it was noted that phytochemicals were effective in inhibiting the growth of *E. coli* bacteria (**Table 3.1 and Figure 3.1**). Given the overall aim of this thesis, it was of interest to investigate whether carvacrol and oregano have any influence on biofilm formation too, as biofilm formation is a virulence factor associated with *in vivo* colonisation and associated with bacterial survival in the environment. Bacterial biofilms are known to be formed with a sophisticated architecture and comprise of the bacterial cells themselves with extensive bacterially-derived proteins, often fimbriae and flagellae, and exopolysaccharides, that can include cellulose for example (Costerton et al., 1987). Results showed that most of the

strong biofilm formers were APEC strains (**Tables 3.2, 3.3, and 3.4**), and this supports an earlier study that biofilm formation is one of the virulence determinants of APEC strains (Skyberg et al., 2007). Also, the data presented in **Tables 3.3** and **3.4** demonstrated that ethanol-based carvacrol and ethanol-based oregano display an anti-biofilm activity.

Fimbriae and flagellae are proteinaceous biofilm components, and there is a possibility that interaction between carvacrol and these proteins will inhibit biofilm formation as suggested by the studies of Burt in *Chromobacterium violaceum* (Burt et al., 2014). If this is the case, the finding has implications on the ability of the *E. coli* to colonise poultry. Thus, bacterial biofilm formation and the effect of carvacrol, and oregano on biofilm formation were further investigated using the previously mentioned method of O'Toole and Kolter in **3.2.2**. Ethanol-based carvacrol, showed an ability to inhibit the biofilm formation of the *E. coli* strains, and this confirms a previous study that carvacrol mitigated bacterial biofilm formation at sub-lethal concentrations (Burt et al., 2014). This indicates that carvacrol which is of phenolic nature might disrupt biofilm formation, by binding and interfering with its key structures (flagella and curli fimbriae) that are proteinaceous in nature. Another study revealed that the interaction of carvacrol with bacterial surface proteins leads to exerting changes in their morphological structures, and thereby preventing initial attachment stage of biofilm formation (Nostro et al., 2007).

Oregano showed an increased ability to prevent biofilm formation of the tested *E. coli* strains in comparison with carvacrol. This is may be due to the differences in their chemical structures. The reason is that the anti-bacterial activity and its mode of action of the essential oil is determined by its chemical structure (Dorman and Deans, 2000). Also, the diffusion of oregano oil containing carvacrol and thymol through the exopolysaccharide matrix component of the

biofilm resulting in its destabilization and anti-bacterial activity (Nostro et al., 2007). As a conclusion to this *in vitro* study, the phytochemicals were successful in inhibiting biofilm formation of the previously characterised *E. coli* strains, but the exact mechanisms of these inhibitory actions are still unclear, but these phytochemicals maybe considered as a potential control measure.

It is necessary to be aware of the evidence that suggests virulent APEC strains are better biofilm formers than the presumptive commensals at this level of investigation. Thus, any control measure that uses carvacrol or oregano might have an impact upon presumptive commensals than upon APEC. This may have consequences upon the host chicken, as oregano which is currently used extensively in poultry production (St. David's Veterinary Practice, UK, personal communication) and this may select APEC *in vivo*. Another issue is that one APEC strain (APEC 7) was highly tolerant of carvacrol. This begs the question regarding the mechanism for comparative resistance. Hence, this strain is somewhat unique and perhaps WGS may give clues by identifying gene of regulatory mutations that have arisen to generate high biofilm formation capability.

The production of SCFAs reflects how metabolically active these *E. coli* cells are and characterise them according to their type or group, and this might give us an insight into their metabolic profile. *E. coli* is a facultative anaerobe (Finegold et al., 1983a). Generally, results showed that there were higher fermentation by-products than respiration by-products, and this suggests that *E. coli* relies on both pathways to generate energy despite the anaerobic conditions of the gut.

Generally, the highest SCFA produced was lactate, and the lowest SCFA produced was succinate and these results were proven to be significant (P-value < 0.05), and this is in agreement with a previous study that *E. coli* produces acetate as a major fermentation by-product and succinate as a minor fermentation by-product which proves performing mixed acid fermentation (Lee et al., 2005), and this may give an advantage to the *E. coli* strains in the real gut environment. Generally, the production of lactate was reduced in most APEC strains and some chicken strains incubated in sub-lethal concentrations of aqueous oregano in comparison with the control sets, and this may indicate that oregano as a mixture of essential oils containing carvacrol (Russo et al., 1998) is responsible for the reduction of fermentation levels in *E. coli* and may suppress its activity which was in agreement with this study (Varel, 2002). Aqueous carvacrol and aqueous oregano decreased the production of acetate in some turkey strains, which may suggest that one of their target sites works against the by-product of glycolysis and stops the tricarboxylic acid (TCA) cycle (Wolfe, 2005) and this may indicate one of their mechanisms of action. Reduction in the production of volatile fatty acids in response to the phytochemicals may lead to a stabilised intestinal pH, and this would enhance the activity of digestive enzymes (Jamroz et al., 2003). The previously mentioned comments were not proved statistically, as linear mixed model showed insignificant differences (P-value > 0.05) between production of SCFAs and each phytochemical. This may suggest that incubating the *E. coli* strains in sub-lethal concentrations of aqueous phytochemicals were more effective in inhibiting the anaerobic pathways which requires less energy in comparison with the aerobic pathways. To check whether the incubation period in sub-lethal concentrations of phytochemicals were important in suppressing the aerobic pathways of the *E. coli* strains, different time points representing different incubation periods must be included for future experiments.

In conclusion, carvacrol and oregano showed their ability in inhibiting growth and biofilm formation associated with virulence and survival mechanisms. Carvacrol and oregano showed their bactericidal activity at very low concentrations (MIC values ranging between 0.2 – 0.5μg/ml) in comparison with antibiotics. However, at sub-MIC levels of carvacrol and oregano, these phytochemicals did not induce or show any metabolic changes among the *E. coli* strains.

CHAPTER 4: *In vitro* investigations into the anti-bacterial mechanisms of the phytochemicals

4.1 Introduction

Several studies have investigated the anti-bacterial properties and mechanisms of action of phytochemicals which have been mentioned in the previous chapter (**see Chapter 3**), but overall, they remain ill-defined. What has been proven already is the anti-bacterial properties of phytochemicals (Lambert et al., 2001, Lopez-Romero et al., 2015, Umaru et al., 2019) and the contribution of their chemical structure such as the hydroxyl group of carvacrol as an important requirement for that activity (Ben Arfa et al., 2006). The interaction between phytochemicals and bacteria at the cellular level is due to the hydrophobic nature of phytochemicals which enables their entry into the lipid bilayer of the cytoplasmic membrane; hence acting as a membrane destabilising agent (Sikkema et al., 1995b, Luz et al., 2014, Yuan et al., 2019), inducing structural changes that results in modifying the function of membrane proteins of the lipid bilayer. Examples of theses membrane proteins are heat shock proteins; universal chaperone proteins expressed under stressful conditions (Richter et al., 2010); GroEL and DnaK which are required for bacterial cell adaptation in response to environmental stress such as thermal stress (Di Pasqua et al., 2013). Moreover, GroEL plays a regulatory role in bacterial conjugation in response to stress (Zahrl et al., 2007).

These phytochemicals can also affect the lipid moiety of the cell membrane and change properties of the cell membrane due to changes in its surface charge (Cristani et al., 2007) and that affects ion (H^+ and K^+) transport across the membrane (Ultee et al., 2002a). There are many other target sites inside the bacterial cell which could be affected by phytochemicals that

need to be thoroughly studied. To interrogate which components of the bacterial cell could be affected by phytochemicals, the approach chosen was training bacteria to grow at sub-MIC concentrations of phytochemicals to generate derivatives that have reduced sensitivity (increased resistance) to the phytochemicals. The purpose of this approach was to determine whether this resistance might be the result of a temporary physiological adaptation or a mutation. Genetic analysis of these trained strains should identify genes encoding cellular functions involved in response to the stress of phytochemicals. Recent results from previous PhD thesis showed that training *E. coli* strains to grow at sub-MIC levels of thymol (similar to carvacrol in chemical structure and its anti-bacterial activity) resulted in a non-sense mutation in *acrR* gene encoding for the AcrAB repressor which is a multi-drug efflux system (Alkhandari, 2017). Therefore, this chapter aimed at further investigations into the anti-bacterial role of carvacrol and oregano at the genetic level, in order to fill the gaps and increase our understanding in the area of mechanistic studies. The testable hypotheses were: 1) Whether any changes will be detected suggesting a temporary adaptation to stress or not?, 2) Whether mutation(s) will be selected as a result of continuous exposure to sub-lethal concentrations of carvacrol and oregano or not?, 3) If mutation(s) did arise, what are the kind of changes at the genetic level?, 4) Whether mutant *E. coli* strains will result in different phenotypic changes under the presence of different concentrations of aqueous carvacrol or not?

The aims and objectives behind this chapter were:

- To create selective pressure in result of continuous exposure to sub-lethal concentrations of phytochemicals to understand what is really happening at the cellular level of *E. coli*.
- To detect whether the trained isolates came from the same starting wild-type strains by checking their API 20E, AST, and PCR profiles.
- To demonstrate the effect of continuous exposure to sub-lethal concentrations of phytochemicals on the growth of *E. coli*, antibiotic resistance profile using antibiotic disks, and virulence genes profiling using PCR.
- To test the effect of different concentrations of aqueous carvacrol on different *E. coli* MG1655 strains with a single defined mutation using growth experiment, to further investigate the anti-bacterial role of carvacrol against efflux pump systems and cell wall synthesis.

4.2 Materials and methods

4.2.1 Training *E. coli* strains to tolerate sub-lethal concentrations of aqueous phytochemicals

Three *E. coli* strains; APEC 7, C1, and T8 that share the same MIC value (0.3µg/ml) against aqueous carvacrol and oregano were selected for these studies. Each *E. coli* strain was grown overnight (10 – 12hr) in LB broth at 37ºC to yield approximately 10^9 CFU/ml (OD_{600} = 1.00). Three sets of tubes were prepared for these three strains: control containing *E. coli* strain in LB broth, a tube containing *E. coli* culture and 0.2µg/ml aqueous carvacrol, and a tube containing *E. coli* culture and 0.2µg/ml aqueous oregano. The tubes (**Table 4.1**) were incubated at 37ºC with gentle shaking. Bacterial growth in the form of turbidity was observed visually, and an aliquot of 100µl was sub-cultured into new sets of tubes containing the same media as described

above. These procedures were repeated continuously for two consecutive months until there was an obvious increase in turbidity with the incubation period being 48hr exposure to phytochemicals.

Table 4.1 Components of each tube

Starting concentration of each component	Final concentration of each treatment		
	Control	**0.2µg/ml carvacrol**	**0.2µg/ml oregano**
***E. coli* strain culture (10^9 CFU/ml)**	100µl	100µl	100µl
5µg/ml carvacrol	-	400µl	-
5µg/ml oregano	-	-	400µl
LB broth	9.900ml	9.500ml	9.500ml
Total volume	10ml	10ml	10ml

Total volume of each tube was 10ml.

4.2.2 Biochemical characterisation and AST profiling of the representative trained isolates

After the period of two months of training, a volume of 100µl from each sample was spread on LB agar, and then a total of 36 representative colonies (**Table 4.2**) were picked, purified, stocked, and stored in cryotubes at -80ºC for further experiments. These trained isolates were cultured on MacConkey and EMB agar plates, biochemically characterised using catalase, oxidase, and API 20E strips; to ensure they are *E. coli*. Also, the 36 representative *E. coli* strains were used to determine their antibiotic susceptibility using the same protocol performed as mentioned earlier in **Chapter 2**; section **2.2.3.2**. Representative strains (highlighted in blue) were chosen for further experiments.

Table 4.2 Biochemical characteristics of the representative trained isolates

Trained strain	Original strain	Source / treatment
1M	APEC 7	Control
2M	APEC 7	Control
3M	APEC 7	Control
4M	APEC 7	Control
5M	C1	Control
6M	C1	Control
7M	C1	Control
8M	C1	Control
9M	T8	Control
10M	T8	Control
11M	T8	Control
12M	T8	Control
13M	APEC 7	0.2µg/ml carvacrol
14M	APEC 7	0.2µg/ml carvacrol
15M	APEC 7	0.2µg/ml carvacrol
16M	APEC 7	0.2µg/ml carvacrol
17M	APEC 7	0.2µg/ml oregano
18M	APEC 7	0.2µg/ml oregano
19M	APEC 7	0.2µg/ml oregano
20M	APEC 7	0.2µg/ml oregano
21M	C1	0.2µg/ml carvacrol
22M	C1	0.2µg/ml carvacrol
23M	C1	0.2µg/ml carvacrol
24M	C1	0.2µg/ml carvacrol
25M	C1	0.2µg/ml oregano
26M	C1	0.2µg/ml oregano
27M	C1	0.2µg/ml oregano
28M	C1	0.2µg/ml oregano
29M	T8	0.2µg/ml carvacrol
30M	T8	0.2µg/ml carvacrol
31M	T8	0.2µg/ml carvacrol
32M	T8	0.2µg/ml carvacrol
33M	T8	0.2µg/ml oregano
34M	T8	0.2µg/ml oregano
35M	T8	0.2µg/ml oregano
36M	T8	0.2µg/ml oregano

Representative strains (highlighted in blue) were chosen for further experiments.

4.2.3 Determination of MIC values of the trained *E. coli* strains against aqueous phytochemicals

Representative *E. coli* strains (13M, 19M, 22M, and 26M) were used to determine their MIC values against aqueous carvacrol and oregano. The same method was followed as described earlier in **Chapter 3**; section **3.2.1**. The same procedure was repeated after two weeks, to ensure that the increase in MIC values was stable and not a result of an adaptational change.

4.2.4 DNA extraction and detection of virulence genes in the trained *E. coli* strains by PCR

Representative *E. coli* strains (22M and 26M) were used to detect their virulence genes as listed in **Table 2.2** (**see Chapter 2;** section**s 2.2.4.1** and **2.2.4.2**) by PCR.

4.2.5 WGS of the trained *E. coli* strains

Representative trained *E. coli* strains from APEC group (13M and 19M) and commensal chicken strain (22M and 26M) with their original wild-type (APEC 7 and C1, respectively) were sent to MicrobesNG at the University of Birmingham for WGS. *In silico* serotyping analysis involved the following three genes (*fliC*, *wzy*, and *wzx*) (Joensen et al., 2015) and were performed using Serotype Finder 1.1 website (https://cge.cbs.dtu.dk/services/SerotypeFinder/). MLST analysis involved seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) (Sepehri et al., 2009) and were performed using MLST 2.0 software according to the protocol mentioned in (https://pubmlst.org/). I am grateful to Geoffrey Woodward of the NHS, Southmead Hospital, Genetic Analysis Department for carrying out the bioinformatics analyses.

4.2.6 Determination of MIC values of the *E. coli* K12 (MG1655 WT) and its mutant strains against aqueous carvacrol

Representative mutant *E. coli* strains (generously provided by Dr. Kimon-Andreas Karatzas, University of Reading) were obtained from Keio collection and used to determine their MIC values against aqueous carvacrol. The representative mutant *E. coli* strains were picked from two different groups; multi-drug efflux pump genes (*ΔacrA*, *ΔacrB,* and *ΔtolC*) and genes encoding for penicillin-binding proteins (PBPs) (*ΔmrcA*, *ΔmrcB*, *ΔampC, ΔampH*, *ΔpbpC*, *ΔpbpG*, *ΔdacC,* and *ΔdacD*). The same method was followed as described earlier in **Chapter 3**; section **3.2.1**.

4.2.7 Statistical analyses

For growth experiments, least square means for multiple comparisons was performed to compare between OD_{600} readings of control vs. OD_{600} readings of MIC level for each phytochemical at 9hr (exponential phase = highest OD_{600} reading) using R i386 3.4.3 software.

4.3 Results

4.3.1 Biochemical characterisation of the trained isolates

In order to make sure that the resultant trained isolates came from the same original strains, simple biochemical characterisation tests were done. API 20E (**Table 4.3**) indicated that these were all *E. coli*, but there were some very minor differences between the wild-type and trained strains. It is possible that these notable differences reflect the phenotype of the trained strains or the inaccuracy of the API test system as it is susceptible to technical variation. Subsequent tests were carried out to inform on the strain integrity.

Table 4.3 Biochemical characteristics of the trained isolates

Trained strain	Original strain	Source	API 20E / original strain	API 20E / trained strain
1M	APEC 7	Control	1044572	1044553
2M	APEC 7	Control	1044572	1244572
3M	APEC 7	Control	1044572	1044572
4M	APEC 7	Control	1044572	1044553
5M	C1	Control	5044552	1044572
6M	C1	Control	5044552	1044572
7M	C1	Control	5044552	1044572
8M	C1	Control	5044552	1044552
9M	T8	Control	5044552	1044572
10M	T8	Control	5044552	1044553
11M	T8	Control	5044552	1044552
12M	T8	Control	5044552	1044572
13M	APEC 7	0.2µg/ml carvacrol	1044572	1044572
14M	APEC 7	0.2µg/ml carvacrol	1044572	1044572
15M	APEC 7	0.2µg/ml carvacrol	1044572	1044572
16M	APEC 7	0.2µg/ml carvacrol	1044572	1044572
17M	APEC 7	0.2µg/ml oregano	1044572	1044152
18M	APEC 7	0.2µg/ml oregano	1044572	1044572
19M	APEC 7	0.2µg/ml oregano	1044572	1044572
20M	APEC 7	0.2µg/ml oregano	1044572	1044572
21M	C1	0.2µg/ml carvacrol	5044552	1044552
22M	C1	0.2µg/ml carvacrol	5044552	1044152
23M	C1	0.2µg/ml carvacrol	5044552	1044152
24M	C1	0.2µg/ml carvacrol	5044552	1044552
25M	C1	0.2µg/ml oregano	5044552	1044552
26M	C1	0.2µg/ml oregano	5044552	1044552
27M	C1	0.2µg/ml oregano	5044552	1044552
28M	C1	0.2µg/ml oregano	5044552	1044152
29M	T8	0.2µg/ml carvacrol	5044552	1044553
30M	T8	0.2µg/ml carvacrol	5044552	1044552
31M	T8	0.2µg/ml carvacrol	5044552	1044553
32M	T8	0.2µg/ml carvacrol	5044552	1044553
33M	T8	0.2µg/ml oregano	5044552	1044553
34M	T8	0.2µg/ml oregano	5044552	1044552
35M	T8	0.2µg/ml oregano	5044552	1044553
36M	T8	0.2µg/ml oregano	5044552	1044552

4.3.2 Antimicrobial susceptibility profiling of the trained *E. coli* strains

To confirm that the trained strains came from the same starting wild-type strains, and to study the effect of long-term and continuous exposure to sub-lethal concentrations of phytochemicals on the carriage of antibiotic resistance, AST was performed on the representative 36 trained *E. coli* strains. Two observations were noticed (**Table 4.4)**; a change from R to I or S in control strains and this was observed in cefotaxime, nalidixic acid, colistin, and tetracycline, and change from R to I or S in the trained strains. Results in **Table 4.4 A** showed that there is a relative decrease (14M, 16M, 18M, and 20M) in ampicillin resistance (by changing from R to I) after exposure to sub-lethal concentrations carvacrol and oregano. This was observed in tetracycline too (changing from R to S) and on a larger number of strains (13M, 15M, 21M, 24M, 25M, and from 29M to 36M), and in the control strains (no phytochemicals were added) as well (**Tables 4.4 A, B and C**).

Table 4.4 Antimicrobial susceptibility profiling of the representative trained strains
A) APEC 7 wild-type (originals strain) and its derivatives (trained strains)

Trained strain	Original strain	Source	CTX 30µg	NA 30µg	C 30µg	CT 10µg	SAM 25µg	S 25µg	TE 30µg
	APEC 7	Wild-type	R	R	R	R	S	S	R
1M	APEC 7	Control	S	R	S	S	R	S	S
2M	APEC 7	Control	S	S	S	S	R	S	S
3M	APEC 7	Control	S	S	S	S	R	S	S
4M	APEC 7	Control	S	R	S	R	R	S	S
13M	APEC 7	0.2µg/ml carvacrol	S	R	I	S	R	S	S
14M	APEC 7	0.2µg/ml carvacrol	S	R	R	S	I	I	R
15M	APEC 7	0.2µg/ml carvacrol	S	R	I	S	R	I	S
16M	APEC 7	0.2µg/ml carvacrol	S	R	R	S	I	I	R
17M	APEC 7	0.2µg/ml oregano	S	R	S	S	R	I	R
18M	APEC 7	0.2µg/ml oregano	S	R	R	S	I	I	R
19M	APEC 7	0.2µg/ml oregano	I	R	R	S	R	I	R
20M	APEC 7	0.2µg/ml oregano	S	R	R	R	S	S	R

Shaded cells indicate resistance. CTX, Cefotaxime; NA, Nalidixic acid; C, Chloramphenicol; CT, Colistin; SAM, Ampicillin; S, Streptomycin; TE, Tetracycline; R, resistant; S, sensitive; I, intermediate. Representative trained strains (highlighted in blue) were chosen for further experiments.

B) C1 wild-type (originals strain) and its derivatives (trained strains)

Trained strain	Original strain	Source	CTX 30µg	NA 30µg	C 30µg	CT 10µg	SAM 25µg	S 25µg	TE 30µg
	C1	Wild-type	R	R	S	R	R	I	R
5M	C1	Control	S	S	S	S	R	S	S
6M	C1	Control	S	S	S	S	R	S	S
7M	C1	Control	S	S	S	S	R	S	S
8M	C1	Control	S	R	S	S	R	S	R
21M	C1	0.2µg/ml carvacrol	S	R	S	S	R	S	S
22M	C1	0.2µg/ml carvacrol	S	R	I	S	R	S	R
23M	C1	0.2µg/ml carvacrol	S	R	S	R	R	S	R
24M	C1	0.2µg/ml carvacrol	S	R	S	R	R	S	S
25M	C1	0.2µg/ml oregano	I	R	S	R	R	S	S
26M	C1	0.2µg/ml oregano	S	R	S	S	R	S	R
27M	C1	0.2µg/ml oregano	S	R	S	R	R	S	R
28M	C1	0.2µg/ml oregano	S	R	S	R	I	I	R

Shaded cells indicate resistance. CTX, Cefotaxime; NA, Nalidixic acid; C, Chloramphenicol; CT, Colistin; SAM, Ampicillin; S, Streptomycin; TE, Tetracycline; R, resistant; S, sensitive; I, intermediate. Representative trained strains (highlighted in blue) were chosen for further experiments.

C) T8 wild-type (originals strain) and its derivatives (trained strains)

Trained strain	Original strain	Source	CTX 30µg	NA 30µg	C 30µg	CT 10µg	SAM 25µg	S 25µg	TE 30µg
	T8	Wild-type	R	R	S	R	R	S	S
9M	T8	Control	S	R	S	S	R	S	S
10M	T8	Control	S	R	S	S	R	S	S
11M	T8	Control	S	R	S	S	R	S	S
12M	T8	Control	S	S	S	R	R	R	S
29M	T8	0.2µg/ml carvacrol	I	R	I	R	R	S	S
30M	T8	0.2µg/ml carvacrol	S	R	S	S	R	S	S
31M	T8	0.2µg/ml carvacrol	S	R	S	R	R	S	S
32M	T8	0.2µg/ml carvacrol	S	R	S	R	R	S	S
33M	T8	0.2µg/ml oregano	S	R	S	R	R	S	S
34M	T8	0.2µg/ml oregano	S	R	S	R	R	S	S
35M	T8	0.2µg/ml oregano	S	R	S	R	R	S	S
36M	T8	0.2µg/ml oregano	S	R	S	S	R	S	S

Shaded cells indicate resistance. CTX, Cefotaxime; NA, Nalidixic acid; C, Chloramphenicol; CT, Colistin; SAM, Ampicillin; S, Streptomycin; TE, Tetracycline; R, resistant; S, sensitive; I, intermediate. Representative trained strains (highlighted in blue) were chosen for further experiments.

4.3.3 Effect of different concentrations of phytochemicals on the trained *E. coli* strains

To study the increased resistance (reduced sensitivity) to carvacrol that resulted from the training method, the representative *E. coli* strains (13M, 19M, 22M, and 26M) were used to determine their MIC values against aqueous carvacrol and oregano. The MIC values were increased from 0.3µg/ml to 0.6µg/ml to both phytochemicals (**Table 4.5**). Least square means for multiple comparisons was performed and it showed that there was a significant difference (P-value < 0.0001) between OD_{600} readings of control versus MIC level of aqueous carvacrol or aqueous oregano. To ensure that the resultant increase was stable and was not a result of an adaptational change, the same procedure was done after two weeks, and the same MIC values were achieved. During these two periods, the isolates were stored in cryotubes and not exposed to the phytochemicals.

Table 4.5 MIC values of phytochemicals against the starting original strains with their trained strains

Trained strain	Original Strain	Source	Original MIC value (µg/ml)	New MIC value (µg/ml)
13M	APEC 7	0.2µg/ml carvacrol	0.3 ± 0.00	0.6 ± 0.00
19M	APEC 7	0.2µg/ml oregano	0.3 ± 0.00	0.6 ± 0.00
22M	C1	0.2µg/ml carvacrol	0.3 ± 0.00	0.6 ± 0.00
26M	C1	0.2µg/ml oregano	0.3 ± 0.00	0.6 ± 0.00

Results are expressed as means (n =3) ± STD.

4.3.4 Detection of virulence genes in the trained strains

This study involved two trained *E. coli* (22M and 26M) strains only, as they showed more reliable results based on WGS data. The same set of results (**Table 2.6**; **see Chapter 2**) were achieved after carrying out PCR, and this ensures that the trained *E. coli* strains (22M and 26M) came from the same starting original strains. There were no observed changes at all at chromosome and plasmid levels of the virulence genes included in this study.

4.3.5 WGS of the trained *E. coli* strains

After confirming the identity of the trained strains and their elevated MIC values, WGS was considered to complete this study. The main focus of this thesis was on APEC and commensal chicken strains, so representative strains from APEC group (APEC 7, 13M, and 19M) and commensal chicken strain (C1, 22M, and 26M) (**Table 4.2**) were sent for WGS, to study the genetic changes upon exposure to sub-lethal concentrations of phytochemicals for a long time and to link between genotype and phenotype. *In silico* serotyping analysis involved the following three genes (*fliC*, *wzy*, and *wzx*) (Joensen et al., 2015) was performed to ensure that the trained *E. coli* strains came from the same starting *E. coli* strains. Also, it was supported by MLST analysis which involved seven housekeeping genes (*adk, fumC*, *gyrB, icd, mdh, purA*, and *recA*) (Sepehri et al., 2009). All of the trained strains showed the same *in silico* and MLST profiles in comparison with their wild-type except for 13M, and this could be due to possible contamination as a result of long period of training. The *in silico* profile of 13M showed different results in two genes (*wzy*, and *wzx*) and the MLST profile showed different results in the previously mentioned seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*).

Bioinformatics analysis showed that there were missense mutations detected in two genes; *CadC* which encodes for a transcriptional activator of *cad* operon (Küper and Jung, 2005) and *marR* which encodes for a repressor of *mar* operon (Cohen et al., 1993). The mutations resulted in an amino acid substitution and identified as a change from tyrosine to histidine at position 504 base in *CadC*, and a change from arginine to histidine at position 94 base in *marR*.

4.3.6 Effect of different concentrations of aqueous carvacrol on *E. coli* K12 (MG1655 WT) strain and its mutant strains with single defined mutation

Based on recent results from previous PhD thesis (Alkhandari, 2017), mutant *E. coli* K12 strains with mutations in efflux pump genes were considered. Also, β-lactam resistance in *Enterobacteriaceae* might be due to mutations in genes encoding for PBPs. Thus, to study the resistance effect of carvacrol on these specific mutations, growth experiments were performed in order to determine the MIC value and compare it with the reference strain MG1655 WT as an indirect way to investigate the mechanism of action of carvacrol and oregano. Representative strains with a specific single mutation from Keio library were used in this experiment. Results in **Table 4.6** showed that there was a decrease in MIC values in *E. coli* strains with a mutation in multi-drug efflux pump genes (*ΔacrA,* and *ΔtolC*), and in *E. coli* strains with a mutation in genes encoding for PBPs (*ΔampC, ΔampH*, *ΔpbpC*, and *ΔpbpG*). Interestingly, the same MIC values or relatively higher MIC values were achieved in one *E. coli* strain with mutation in multi-drug efflux pump genes (*ΔacrB*), and in *E. coli* strains with a mutation in genes encoding for PBPs (*ΔmrcA, ΔmrcB*, *ΔdacC*, and *ΔdacD*).

Table 4.6 MIC values of aqueous carvacrol against the reference strain *E. coli* MG1655 WT and its mutant strains

Type	Strain	Carvacrol MIC value (µg/ml)
Wild-type	MG1655 WT	0.4 ± 0.00
E. coli strains with a mutation in multi-drug efflux pump genes	*ΔacrA*	0.1 ± 0.00
	ΔacrB	0.4 ± 0.00
	ΔtolC	0.1 ± 0.00
E. coli strains with a mutation in genes encoding for PBPs	*ΔmrcA*	0.4 ± 0.00
	ΔmrcB	0.5 ± 0.00
	ΔampC	0.3 ± 0.00
	ΔampH	0.3 ± 0.00
	ΔpbpC	0.3 ± 0.00
	ΔpbpG	0.3 ± 0.00
	ΔdacC	0.5 ± 0.00
	ΔdacD	0.4 ± 0.00

Results are expressed as means (n =3) ± STD.

4.4 Discussion

This chapter involved training representative strains from different groups (APEC 7, C1, and T8) in the presence of sub-lethal concentrations of aqueous carvacrol and oregano for 60 days until there was an obvious increase in turbidity. Simple plating methods were performed to pick representative colonies for further investigation, and this was followed by simple biochemical characterisation (**Table 4.3**) as a quick way to ensure that these colonies were *E. coli* strains and derived from the same starting strains.

One of the issues tackled in this thesis is the carriage of antibiotic resistance in *E. coli* strains which is plasmid-based, and how can we control them using phytochemicals, so a simple AST method (**Table 4.4**) was performed to determine whether there was a change in their antibiotic resistance profile. Changes from R to I or S in control strains (no phytochemicals were added)

were observed in cefotaxime, nalidixic acid, colistin, and tetracycline, which indicates that the loss of the plasmid might be due to sub-culturing and not due to being exposed to sub-lethal concentrations of phytochemicals. This also indicates that the plasmid carrying these antibiotic resistances is less stable than the plasmid carrying ampicillin resistance, as there was a relative decrease in ampicillin resistance (changing from R to I) in four strains (14M, 16M, 18M, and 20M) after exposure to sub-lethal concentrations of carvacrol and oregano. Also, there was a decrease in tetracycline resistance (changing from R to S) in thirteen strains (13M, 15M, 21M, 24M, 25M, and from 29M to 36M). The loss of resistance trait was expected in plasmid-based antibiotic resistance, but not in chromosome-based antibiotic resistance as was observed in nalidixic acid which might be due to cross-contamination or the insensitivity of the AST test, as it is based on a scale measured in cm. Moreover, the loss of resistance trait was expected, but the gain of resistance (found in ampicillin in 13M which showed unreliable results based on WGS data later) was not, and this also could be due to cross-contamination or the insensitivity of the AST test.

Four representative trained strains (13M, 19M, 22M, and 26M) were chosen randomly; two from each group, to determine their MIC values against carvacrol and oregano and ensure there was elevation in their MIC values before sending them for WGS. Results in **Table 4.5** showed there was two-fold increase in their MIC values from 0.3µg/ml to 0.6µg/ml towards both phytochemicals. This experiment was repeated after two weeks, to ensure that this increase was stable and not the result of physiological adaptation to the presence of sub-lethal concentrations of phytochemicals as a source of stress. If time permitted, it would be of value to repeat this experiment after longer term storage of the trained strains. However, as the WGS data analysis indicates (see below), the phenotypes were the result of a chromosomal mutation

(chromosomal genes; *cadC* (Küper and Jung, 2005) and *marR* (Alekshun and Levy, 1997)) and thus this stability test may be considered redundant.

WGS is a genetic approach that enabled linkage between phenotype and genotype (Feil, 2004). Looking at the genomic variations among the strains of interest might benefit us and give us more information from an evolutionary point of view (Bryant et al., 2012). As these genetic variations are bacterial strategies to adapt in new environments which come in result of mutation(s) mostly and recombination of these resulted mutations (Tenaillon et al., 2001). WGS data analysis revealed the presence of a missense mutation in two genes; *cadC* and *marR*.

The *cad* operon is one of the survival mechanism systems in *E. coli* that is triggered in response to unfavourable acidic conditions (Gale and Epps, 1942). This system is composed of a cytoplasmic protein (CadA) and two transmembrane proteins (CadB and CadC) (Watson et al., 1992). CadC has a dual function as a transcriptional activator of the *cad* operon in *E. coli* (Küper and Jung, 2005) and as a sensor to external changes in pH in the environment (Dell et al., 1994). The missense mutation in *cadC* gene resulted in a substitution in amino acid from tyrosine which is partially hydrophobic with an aromatic side chain to histidine which is hydrophilic (Betts and Russell, 2003). Thus, this mutation might be a non-silent one with an effect on the Cad system.

The *mar* operon is responsible for chromosome-mediated multiple antibiotic resistance as a protective mechanism in response to environmental stresses such as presence of antibiotics and oxidative stress (Ariza et al., 1994). This operon which is short for multiple antibiotic resistance consists of four genes; *marA* (encoding for the activator protein of *mar* operon), *marR* (encoding for a repressor protein of *mar* operon) (Cohen et al., 1993), *marB* and *marC* (with

unknown function) (Alekshun and Levy, 2004). It was first found in resistant *E. coli* to low concentrations of tetracycline or chloramphenicol of chromosome origin. The presence of the *mar* operon is the reason behind increased resistance in *E. coli* strains to different classes of antibiotics involving tetracycline, chloramphenicol, β-lactams, and fluoroquinolones (George and Levy, 1983). This resistance is the result of the pumping out of these antibiotics in association with and dependent upon outer membrane porins (Cohen et al., 1988). Interestingly, the MarR repressor in *E. coli* found in the gut of animal hosts has another function which is detecting phenolic compounds of plant products (Sulavik et al., 1995). The detected missense mutation in *marR* gene was a substitution in amino acid from arginine which is of amphipathic nature to histidine which is of hydrophilic nature (Betts and Russell, 2003). Given the resistance phenotype of the *E. coli* strains, this amino acid substitution is probably a non-silent mutation resulting in an increased activity of the *mar* efflux system due to repressor failing to repress the *mar* operon, and therefore an increase in its resistance nature as was recently discovered (Chueca et al., 2018).

This was a single training study, so future experiments might be improved by: 1) repeating the training experiment again to determine whether the same mutations arise again or not?, 2) repeating training experiment again but at different concentrations of phytochemicals and check the genetic variations among these strains. To confirm the previously mentioned results, it would be essential to make these mutations in a test strain and undertake complementation studies to verify the phenotypes and their recovery. However, this will take long time, so the shortcut step was to use representative strains from Keio library of *E. coli* that will enable us to perform a wider study of phytochemical sensitivity studies as discussed below.

Further investigations at the genetic level involved screening the effect of different concentrations of carvacrol on *E. coli* strains with a mutation in multi-drug efflux pump genes (*ΔacrA*, *ΔacrB,* and *ΔtolC*) and in *E. coli* strains with a mutation in genes encoding for PBPs genes (*ΔmrcA*, *ΔmrcB*, *ΔampC, ΔampH*, *ΔpbpC*, *ΔpbpG*, *ΔdacC,* and *ΔdacD*) in comparison with the reference strain *E. coli* MG1655 WT. This was a reverse way to determine the mechanisms of action of carvacrol, and one of the common approaches used currently to study the resultant phenotypic changes based on specific mutations (Rainey et al., 2017).

The decreased MIC values as shown in **Table 4.6** in efflux pump genes (*ΔacrA* and *ΔtolC*) determined that the disrupted efflux pump system cannot pump carvacrol out of the cell, because acrAB is the main efflux pump system in *E. coli* with the aid of TolC (Ma et al., 1993), and that any disturbance in one of the three components (acrA, acrB, and TolC) of this system will result in susceptibility of *E. coli* to antibiotics and other toxic chemicals (Okusu et al., 1996). The decreased MIC value was an indicator of the susceptibility of these *E. coli* strains. However, there was an exception and it was observed in the *E. coli* with *ΔacrB* showing an increased MIC value in comparison with the other efflux pump mutations, but the same MIC value with the control strain (MG1655 WT), and this is due to its regulatory function in maintaining the structure of acrA (Pagès and Amaral, 2009).

The decreased MIC value in genes encoding for PBPs (*ΔampC, ΔampH*, *ΔpbpC*, and *ΔpbpG*) showed that carvacrol may inhibit cell wall synthesis in *E. coli* causing cell lysis and death which is similar to the mechanism of action of β-lactam antibiotics (Zeng and Lin, 2013), and that disruption in some of the PBPs will affect cell morphology (Vijayan et al., 2014). However, the phenotype generated by other genes (*ΔacrB*, *ΔmrcA, ΔmrcB*, *ΔdacC*, and *ΔdacD*) was not

affected, suggesting that the target site of carvacrol is specific and limited to some of the genes but not all of them. Interestingly, the results from previous PhD thesis (Alkhandari, 2017) and the results in this chapter showed that similar phytochemicals (thymol and carvacrol) have similar functions against antibiotic resistance, but different genes were responsible for this change. Thus, in order to study all the possible mechanisms of actions of these phytochemicals, all of the mutants included in the Keio collection must be involved in this study, as this will enable us to start classifying different phytochemicals according to their mechanisms of action as antibiotics are classified.

In conclusion, the possible anti-bacterial roles of these phytochemicals were investigated and were associated with missense mutations in genes responsible for multiple antibiotic resistance (*marR*) and survival mechanisms under unfavorable acidic conditions (*cadC*), and possible mutations associated with efflux pumps genes (*acrA* and *TolC*) and genes associated with cell wall inhibition (*ampC*, *ampH*, *pbpC* and *pbpG*) as bacterial strategies to overcome antibiotics.

CHAPTER 5: Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the growth, antibiotic resistance, and metabolism of ampicillin-resistant *E. coli* strains

5.1 Introduction

The discovery of the first natural β-lactam antibiotic, penicillin, by Alexander Fleming in 1928 paved the way for the use and development of antibiotics in various personal and industrial uses (Bennett and Chung, 2001). Overuse of antibiotics for prophylactic or therapeutic reasons has led to the emergence and spread of antibiotic-resistance bacteria, and loss in the value of using antibiotics especially in treating persistent infections (Reilly and Rombeau, 1993). Bacteria evolved survival strategies to antibiotic treatment and acquired resistance by different mechanisms such as HGT which involves transfer of resistance through 1) plasmids by a conjugation process, 2) gene exchange by bacteriophages, and 3) DNA uptake by a transformation process (Frost et al., 2005).

The most commonly emerging type of resistance among Gram-negative bacteria is due to the production of ESBLs (Bush and Jacoby, 2010), which are β-lactamase enzymes able to resist the anti-bacterial mechanisms of several natural and synthetic β-lactam classes of antibiotics (Lee et al., 2012). Also, these β-lactamase enzymes have the ability to inhibit the biological activity of transpeptidase or penicillin-binding proteins (PBPs) involved in the final stages of bacterial cell wall synthesis (Kelly et al., 1986). The rapid emergence of ESBL resistance acquired by large plasmids encoding for ESBLs production (Eckert et al., 2004), led to the failure of the development of novel synthetic antibiotics such as the first, second, third and fourth-generation ESBL which only provided a temporary solution (Docquier and Mangani, 2017). Currently, plasmid-mediated ESBL resistance is the most widely spread resistance

among bacteria coming from poultry sources (Dierikx et al., 2010). ESBL and AmpC-producing bacteria usually inhabit the GIT of animals (Carattoli, 2008), and high levels of *E. coli* harbouring ESBL resistance were isolated from poultry (Brinas et al., 2005). Certainly, efforts to develop new structures out of the original antibiotics to enhance their activity has become limited (Wong et al., 2006). Thus, other approaches to look for alternative novel products is essential (Barrow, 1992). Phytochemicals are natural plant products produced as secondary metabolites of which some possess antimicrobial effects (Metabolhtes, 2004), and therefore, may present a promising alternative strategy to antibiotics especially against antibiotic-resistant bacteria (Wong et al., 2006).

In poultry production, the rising issue of bacterial antibiotic resistance has prompted a search for alternatives to control diseases (Si et al., 2006). In order to investigate the application of phytochemicals as a potential alternative to antibiotics on the gut bacteria, *in vitro* gut models in the form of batch cultures were established to help in the study of gut bacteria in terms of their interaction and metabolism (Macfarlane and Macfarlane, 2007). These models simulate the conditions of different parts of the gut, as certain parameters such as gas phase, pH, temperature can be controlled by, and therefore permit to interrogate what may happen *in vivo* (Van den Abbeele et al., 2010). The guts of different species can be studied depending on the added broth medium that supplies the bacterial communities with nutritional substrates required for their metabolism (Lei et al., 2012). Previous studies looked at the effectivity of phytochemicals focusing specifically on oregano to control bacterial pathogens in broilers, but all of them were narrow approaches as they looked at certain parameters: 1) daily feed intake, weight and FCR (Ertas et al., 2005), 2) weight, FCR and percentage of mortality (Karimi et al., 2010), 3) FCR, carcass characteristics, villus height, crypt depth and ratio of villus height to

crypt depth (Peng et al., 2016). In this section, the intention was to undertake preliminary studies that evaluate the impact of carvacrol on a simple three strains batch culture model.

The emergence of MDR bacteria requires a combination approach; the use of more than one antibiotic to broaden the scope of inhibitory mechanism of action by affecting different target sites within the bacterial cells or different pathways within the same target site (Fischbach, 2011) in order to mitigate the resistance phenotype (Michel et al., 2008). In this chapter, the combination approach was the use of both carvacrol and ampicillin together and testing their efficacy in comparison with the efficacy of carvacrol alone which was demonstrated earlier in **Chapter 3**. Previous *in vitro* studies have demonstrated the effectiveness of combined phytochemicals with antibiotics when antibiotics become impractical (Palaniappan and Holley, 2010, Yap et al., 2013). But to be more specific to our area of investigation, very limited knowledge is available on the effect of combined treatment of polyphenols and ampicillin against β-lactam resistant bacterial strains (Gallucci et al., 2006). Ampicillin is known to inhibit bacterial cell wall synthesis by preventing peptide cross-linking of peptidoglycan units (Wise and Park, 1965), whereas carvacrol affects the structure and function of bacterial membranes as previously mentioned in **Chapter 4**. Thus, the testable hypotheses of this chapter were: 1) whether the combined treatment of carvacrol and ampicillin will increase the efficacy of ampicillin in controlling ampicillin-resistant *E. coli* strains or not?, 2) whether the combined treatment will result in synergistic or antagonistic interaction between carvacrol and ampicillin against ampicillin-resistant *E. coli* strains, 3) whether the combined treatment will affect the biochemicals reactions, AST profiling, and presence of antibiotic-resistant plasmids or not?, 4) whether the combined treatment will decrease the antibiotics residue in broiler chickens or not after long-term treatment, 5) whether the application of sub-lethal concentrations of carvacrol (the active ingredient of oregano) will have an inhibitory effect on a mixed *E. coli* culture

composed of a single APEC strains and two commensal strains, and 6) whether the application of combined treatment of sub-lethal concentrations of carvacrol and ampicillin will increase the efficacy of ampicillin given the fact that carvacrol is a phenolic compound that affects the permeability of cell wall and cell membrane structures.

The aims and objectives behind performing this study were the following:

- To study the different effect of each treatment and see how different they are from each other, and which one is more effective than the other.
- To study the effect of combined treatment of carvacrol and ampicillin on the growth and metabolic activities of very high ampicillin-resistant *E. coli* strains.
- To assess whether sub-MIC concentrations of carvacrol in the presence of ampicillin (70µg/ml) is inhibitory whilst carvacrol and ampicillin alone are not.
- To study the relative fitness of two commensal chicken *E. coli* strains and a single APEC strain using simple three strains batch culture, how they will interact with each other, and whether there will be any competition between them.
- To study the effect of combined treatment of carvacrol and ampicillin and test whether it is an alternative way to control or decrease the residues of very high ampicillin-resistant *E. coli* strains in the chicken gut and enhance the activity of ampicillin.
- Will the single APEC strains be able to survive and grow at a higher rate than the two commensal strains?
- To determine and compare between the efficacy and selectivity of each treatment (sub-lethal concentrations of carvacrol and ampicillin; combined and separately) on a larger scale in terms of their inhibitory or modulatory effects.

5.2 Materials and methods

5.2.1 Determination of bacterial number in terms of OD_{600} of ampicillin-resistant *E. coli* strains in the presence of combined treatment of sub-lethal concentrations of carvacrol and ampicillin

A) 96 well plate format

The same protocol was followed as described earlier in **Chapter 3**; section **3.2.1**. The *E. coli* strains included in this study were of moderate and high carvacrol MIC values (MIC values of 0.3µg/ml and 0.5µg/ml; **see Chapter 3**; **Table 3.1**) and highly ampicillin resistant (MIC value more than 70µg/ml). Each well of a 96 well plate had 225µl of LB broth containing carvacrol and ampicillin at sub-lethal concentrations were added together and 75µl of bacterial suspension, so that the total final volume was 300µl in each well. OD_{600} readings were measured at 9hr of the exponential phase when the highest OD_{600} reading was achieved.

B) Tube format

Four ampicillin-resistant *E. coli* strains with MICs of higher than 70 µg/ml (APEC 46, APEC 48, and commensal *E. coli* strains; C2, and T4) were selected for this study. These candidates were chosen based on certain criteria in terms of carvacrol MIC values: APEC 46 and C2 had an intermediate MIC value of 0.3µg/ml, and APEC 48 and T4 had a high MIC value of 0.4µg/ml and 0.5µg/ml, respectively (**see Chapter 3**; **Table 3.1**). Each of the strains were genetically distinct possessing different ampicillin resistance plasmid profiles (**see Chapter 2**; **Table 2.9**). Overnight cultures of the four *E. coli* strains were given OD_{600} of 0.9 (10^9 CFU/ml) in 10ml LB broth (Sigma Aldrich, UK) and incubated at 37ºC with gentle shaking. Four treatments (**Table 5.1**) were included in this study at sub-MIC levels of carvacrol and ampicillin and tubes were incubated again overnight at 37ºC with gentle shaking. Samples were taken to measure OD_{600} readings using spectrophotometer and spread on LB agar (Sigma Aldrich, UK) to monitor the changes in bacterial numbers (CFU/ml) in each treatment.

Representative colonies were picked, purified, and used for AST test as mentioned earlier in **Chapter 2**; section **2.2.3.2**.

Table 5.1 Components of each tube

Starting concentration of each component	**Final concentration of each treatment**			
	Control	**0.2µg/ml C**	**70µg/ml A**	**Combined treatment (0.2µg/ml C + 70µg/ml A)**
***E. coli* strain culture (10^9 CFU/ml)**	100µl	100µl	100µl	100µl
5µg/ml carvacrol	-	400µl	-	400µl
1000µg/ml ampicillin	-	-	700µl	700µl
LB broth	9.900ml	9.500ml	9.200ml	8.800ml
Total volume	10ml	10ml	10ml	10ml

C, carvacrol; A, ampicillin. Total volume of each tube was 10ml.

5.2.2 Screening for metabolic by-products using gas chromatography-mass spectrometry (GC-MS)

Overnight cultures (10 – 12hr) of the three *E. coli* strains (APEC 46, and commensal *E. coli* strains; C2, and T4) were given OD_{600} of 0.9 (10^9 CFU/ml) in 10ml LB broth and incubated at 37ºC with gentle shaking. Four treatments (**Table 5.1**) were included in this study at sub-MIC levels of carvacrol and ampicillin and tubes were incubated again at 37ºC with gentle shaking. Each treatment was carried out in triplicate set for each *E. coli* strain. A volume of 5ml of each sample was transferred into a head-space vial, and 100µl of 0.1ppm of internal standard (IS = dichlorobenzene) added to it, and then placed in a 7890A GC-MS system (Agilent

Technologies, UK) using DB-Wax column 0.25µm film thickness (30m x 0.25mm) for the determination of SCFAs, alcohol and indole. The column temperature was set to be at 35ºC for 5min and then increased to 220ºC at 10ºC/min, the carrier gas was helium with flow rate of 1.5ml/min, and the electron ionisation was at 70eV. I am thankful to Dr. Stephen Elmore of the Flavour Centre, for guidance with this technique.

5.2.3 *In vitro* batch cultures set-up

Three starting *E. coli* strains (APEC 46, and commensal *E. coli* strains; C2, and C4) were selected for this study. These candidates were chosen based on certain criteria in terms of ampicillin resistance: APEC 46 and C2 were highly ampicillin resistant with MICs of higher than 70µg/ml, and C4 had an intermediate MIC value of 25µg/ml. Sub-lethal concentrations of both carvacrol and ampicillin were used, because carvacrol may disrupt the outer membrane permitting greater access of ampicillin and perhaps reduce the efficiency of periplasmic penicillinases. Each of the strains was genetically distinct possessing different virulence gene profiles and ERIC-PCR profiles (**see Chapter 2**) but sharing the same MIC value (0.3µg/ml) against carvacrol. Overnight cultures (10 – 12hr) of the three *E. coli* strains that were grown separately in VL medium, as this medium was designed to reflect poultry gut luminal content (Lei et al., 2012). All of the chemicals and ingredients were prepared in VL medium. Four vessels (**Table 5.2**) were included in this study; vessel 1 (V1) contained the control of the mixture of the three *E. coli* strains (100µl of each strain with 10^7 CFU/ml) completed with Viande-Luvre (VL) medium to 20ml, vessel 2 (V2) contained the same strains mixture in addition to sub-lethal concentration of ampicillin treatment (20µg/ml), vessel 3 (V3) contained the same strains mixture in addition to sub-lethal concentration of carvacrol (0.2µg/ml), and vessel 4 (V4) contained the same strains mixture in addition to combined treatment of both ampicillin (20µg/ml) and carvacrol (0.2µg/ml). The set conditions of the 20ml vessels were

controlled throughout the 48hr period as such; anaerobic conditions (80% N_2, 10%H_2, and 10% CO_2), pH value in between 5.8 to 6.0 monitored by pH meter, and a temperature of 41°C maintained by water bath. The batch cultures were grown for 48hr and samples of 1.5ml were collected at three different time points of 0, 24, and 48hr. Serial dilutions of the samples were spread on LB agar to monitor the change in bacterial numbers during the study from which representative colonies were picked and then purified by sub-culturing on MacConkey and EMB agar. DNA from 36 purified strains was extracted for ERIC-PCR and detection of certain virulence genes (*iss* and *astA*) for quick differentiation and identification purposes (**see Chapter 2**). I am grateful to Dr. Vasiliki Kachrimanidou for her help in setting up the batch culture.

Table 5.2 Components of each batch culture vessel

Starting concentration of each component	**Final concentration in each vessel**			
	V1 (Control)	**V2 (20µg/ml ampicillin)**	**V3 (0.2µg/ml carvacrol)**	**V4 (20µg/ml ampicillin and 0.2µg/ml carvacrol)**
APEC 46 strain culture (10^7 CFU/ml)	100µl	100µl	100µl	100µl
Commensal C2 strain culture (10^7 CFU/ml)	100µl	100µl	100µl	100µl
Commensal C4 strain culture (10^7 CFU/ml)	100µl	100µl	100µl	100µl
1000µg/ml ampicillin	-	400µl	-	400µl
5µg/ml carvacrol	-	-	800µl	800µl
VL broth	19.700 ml	19.300 ml	18.900ml	18.500ml
Total volume	20ml	20ml	20ml	20ml

Total volume of each vessel (V) was 20ml.

5.2.4 Statistical analyses

For growth experiments in 96 well plate, least square means for multiple comparisons was performed to compare between OD_{600} readings of control vs. OD_{600} readings of sub-MIC level for each treatment at 9hr (exponential phase = highest OD_{600} reading). As for growth experiments in the tube format, least square means for multiple comparisons was performed to compare between CFU/ml of control vs. CFU/ml of sub-MIC level for each treatment. Linear model was performed to study the significance difference between effect of each treatment on the number of bacterial strains vs. control, and to study the effect of each treatment on the bacterial numbers between different time points. Statistical analyses were performed using R i386 3.4.3 software.

5.3 Results

5.3.1 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the OD_{600} readings of ampicillin-resistant *E. coli* strains

Carvacrol alone inhibited bacterial growth when provided in concentrations at MIC level (**see chapter 3**; **Table 3.1**). All the ampicillin-resistant *E. coli* strains (MIC higher than 70µg/ml) used in this study had an MIC to carvacrol in the region between 0.3 to 0.5µg/ml. However, this preliminary study on the effects of carvacrol and ampicillin was performed at sub-MIC levels. The aim here is to compare between OD_{600} readings at 9hr and at sub-MIC levels of carvacrol and ampicillin, separately and in combination. There were two observations in this experiment when comparing OD_{600} readings in the presence of 70µg/ml ampicillin alone, and in the presence of combined treatment of 0.2µg/ml carvacrol and 70µg/ml ampicillin (**Table 5.3**). There was either a significant decrease (P-value < 0.0001) in OD_{600} readings and this was found in eleven strains (45, 46, 51, 52, C1, C2, C6, T1, T4, T6 and T8) or insignificant decrease (P-value > 0.05) in the OD_{600} readings in the remaining six strains (48, C3, C7, C8, T9 and

T10). Also, there were two observations when comparing OD_{600} readings in the presence of 0.2µg/ml carvacrol alone, and in the presence of combined treatment of 0.2µg/ml carvacrol and 70µg/ml ampicillin (**Table 5.3**). There was a significant decrease (P-value < 0.0001) in OD_{600} readings in all strains except for C2. However, when comparing the results of all strains together, there were: 1) significance (Overall P-value < 0.0001) between using 0.1µg/ml carvacrol treatment and combined treatment of 0.1µg/ml carvacrol and 70µg/ml ampicillin, 2) significance (Overall P-value < 0.0001) between using 0.2µg/ml carvacrol treatment and combined treatment of 0.2µg/ml carvacrol and 70µg/ml ampicillin, 3) significance (Overall P-value = 0.0290) between using 70µg/ml ampicillin treatment and combined treatment of 0.1µg/ml carvacrol and 70µg/ml ampicillin, 4) significance (Overall P-value < 0.0001) between using 70µg/ml ampicillin treatment and combined treatment of 0.2µg/ml carvacrol and 70µg/ml ampicillin. Overall, there seem to be a synergistic inhibitory effect with combined treatment which might be effective in controlling highly ampicillin-resistant strains.

Table 5.3 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the growth of ampicillin-resistant *E. coli* strains

Code	OD_{600} reading at 9hr of the exponential phase					
	Control	**A**	**C**	**C + A**	**C**	**C + A**
	0	**70**	**0.1**	**0.1 + 70**	**0.2**	**0.2 + 70**
45	1.667 ± 0.00	0.863 ± 0.01	1.432 ± 0.06	0.845 ± 0.04	0.812 ± 0.10	0.402 ± 0.13*
46	1.602 ± 0.06	0.953 ± 0.06	1.553 ± 0.05	0.931 ± 0.06	1.116 ± 0.18	0.376 ± 0.11*
48	1.408 ± 0.04	0.265 ± 0.02	1.355 ± 0.04	0.318 ± 0.01	1.027 ± 0.19	0.290 ± 0.02*
51	1.704 ± 0.03	0.857 ± 0.02	1.553 ± 0.03	0.839 ± 0.04	1.241 ± 0.03	0.667 ± 0.06*
52	1.576 ± 0.02	0.448 ± 0.01	1.338 ± 0.01	0.495 ± 0.02	1.067 ± 0.04	0.220 ± 0.02*
C1	1.762 ± 0.06	0.760 ± 0.04	1.613 ± 0.01	0.882 ± 0.10	1.307 ± 0.07	0.423 ± 0.28*
C2	1.547 ± 0.01	0.866 ± 0.06	1.535 ± 0.02	0.911 ± 0.04	0.680 ± 0.41	0.608 ± 0.10
C3	1.364 ± 0.05	0.679 ± 0.03	1.314 ± 0.05	0.793 ± 0.03	1.230 ± 0.05	0.659 ± 0.01*
C6	1.657 ± 0.02	0.951 ± 0.01	1.586 ± 0.01	0.995 ± 0.09	1.212 ± 0.03	0.559 ± 0.11*
C7	1.699 ± 0.09	0.783 ± 0.04	1.515 ± 0.03	0.838 ± 0.04	1.063 ± 0.23	0.623 ± 0.05*
C8	1.795 ± 0.04	0.853 ± 0.07	1.624 ± 0.01	0.902 ± 0.05	1.414 ± 0.02	0.679 ± 0.02*
T1	1.524 ± 0.00	0.736 ± 0.06	1.357 ± 0.04	0.730 ± 0.02	0.993 ± 0.11	0.340 ± 0.08*
T4	1.754 ± 0.10	0.813 ± 0.06	1.503 ± 0.04	0.875 ± 0.04	1.168 ± 0.16	0.409 ± 0.08*
T6	1.598 ± 0.03	0.807 ± 0.03	1.573 ± 0.02	0.892 ± 0.05	1.447 ± 0.15	0.580 ± 0.10*
T8	1.587 ± 0.02	0.844 ± 0.03	1.487 ± 0.02	0.931 ± 0.05	1.331 ± 0.09	0.629 ± 0.03*
T9	1.607 ± 0.00	0.835 ± 0.01	1.565 ± 0.04	0.916 ± 0.03	1.491 ± 0.04	0.752 ± 0.04*
T10	1.580 ± 0.01	0.909 ± 0.06	1.581 ± 0.03	0.951 ± 0.04	1.448 ± 0.02	0.760 ± 0.03*

A, ampicillin at 70µg/ml; C, carvacrol at 0.1 and 0.2µg/ml. Numbers refer to average of triplicate values (n =3). * indicates significant decrease in growth when comparing control to each treatment.

5.3.2 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the number of ampicillin-resistant *E. coli* strains in terms of OD_{600} and CFU/ml

Four representative ampicillin-resistant *E. coli* strains (APEC 46, APEC 48, and commensal *E. coli* strains; C2, and T4) were selected to be studied further. First, bacterial counting was measured using two ways; taking OD_{600} readings and then plate counts to generate CFU/ml, and from those plates, representative colonies were randomly picked to be used later. Generally, bacterial counting in terms of OD_{600} and CFU/ml (**Table 5.4**) in control were similar to 70µg/ml ampicillin treatment, and this was anticipated, because these bacteria were ampicillin-resistant with an MIC above the concentration used in this study. There were lower bacterial counts in the treatment containing 0.2µg/ml carvacrol and the combined treatment which showed similar counts. This would suggest that the inhibitory effect of carvacrol resulted in lower number of bacterial cells which is also observed in bigger volume (10ml volume).

Overall, bacterial numbers in terms of CFU/ml in all strains was: 1) significantly reduced (Overall P-value < 0.0001) in 0.2µg/ml carvacrol treatment in comparison with the control, 2) insignificantly reduced (Overall P-value = 0.3116) in 70µg/ml ampicillin treatment in comparison with the control, 3) significantly reduced (Overall P-value < 0.0001) in combined treatment in comparison with the control. Also, there was significant reduction (Overall P-value < 0.0001) in bacterial numbers in treatment containing carvacrol or combined in comparison with ampicillin treatment.

Table 5.4 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the bacterial numbers in terms of OD_{600} readings and CFU/ml

Starting strain	Control		70µg/ml A		0.2µg/ml C		Combined treatment (0.2µg/ml C + 70µg/ml A)	
	OD_{600}	CFU/ml	OD_{600}	CFU/ml	OD_{600}	CFU/ml	OD_{600}	CFU/ml
46	0.927	$2.0x10^9 \pm 11.79$	0.972	$2.1x10^9 \pm 18.56$	0.660	$7.7x10^8 \pm 51.79$*	0.729	$7.0x10^8 \pm 60.70$*
48	0.878	$1.8x10^9 \pm 8.89$	0.887	$1.5x10^9 \pm 19.43$	0.435	$4.3x10^8 \pm 40.51$*	0.422	$4.2x10^8 \pm 33.53$*
C2	0.704	$1.7x10^9 \pm 13.58$	0.725	$1.3x10^9 \pm 13.75$	0.533	$5.6x10^8 \pm 10.02$*	0.475	$4.8x10^8 \pm 7.00$*
T4	0.780	$2.0x10^9 \pm 18.33$	0.742	$1.8x10^9 \pm 3.21$	0.390	$4.6x10^8 \pm 43.11$*	0.395	$4.2x10^8 \pm 23.90$*

A, ampicillin; C, carvacrol. Numbers refer to either OD readings of a single value (n =1) or CFU/ml as a mean of triplicate values (n =3).
*indicates significant decrease in growth when comparing control to each treatment.

5.3.3 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the antibiotic resistance profile of ampicillin-resistant *E. coli* strains

Representative colonies were picked after bacterial counting and purified to be used in AST test (**Table 5.5**). It was a very quick way to check any changes in their antibiotic susceptibility profile in result of different treatments. AST results showed that there was a change from R to I or S in cefotaxime mostly and in ampicillin in all treatments. Cefotaxime resistance in T4 was confirmed by the presence of *CTX-M15* plasmid (**see Chapter 2**; **Table 2.9**), but the changes from R to I or S in all treatments including control may suggest that the loss of *CTX-M15* plasmid was due to sub-culturing and not because of the effect of each treatment. Ampicillin resistance in 46 was confirmed by the presence of *TEM* plasmid (**see Chapter 2**; **Table 2.9**), but changes from R to I were not anticipated in ampicillin treatment and would be the result of cross-contamination. Also, ampicillin resistance C2 was confirmed by the presence of *TEM* plasmid (**see Chapter 2**; **Table 2.9**), but changes from R to I were found in all treatments suggesting the loss of TEM plasmid was in result of sub-culturing and not because of treatment.

Table 5.5 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the antibiotic resistance profile

Starting strain	Source	AST		
		CTX 30µg	CT 10µg	SAM 25µg
46	Wild-type	S	R	R
	Control – A & B	R	R	R
	Control - C	I	R	R
	0.2µg/ml carvacrol – A, B & C	I	R	R
	70µg/ml ampicillin - A	I	R	R
	70µg/ml ampicillin - B	I	R	I
	70µg/ml ampicillin - C	I	S	I
	Combined treatment – A, B & C	R	R	R
48	Wild-type	I	R	R
	Control – A, B & C	S	R	I
	0.2µg/ml carvacrol – A, B & C	S	R	R
	70µg/ml ampicillin – A & B	S	R	I
	70µg/ml ampicillin - C	I	R	R
	Combined treatment – A & C	I	R	R
	Combined treatment - B	S	R	I
C2	Wild-type	R	R	R
	Control – A, B & C	S	R	I
	0.2µg/ml carvacrol – A, B & C	S	R	I
	70µg/ml ampicillin - A	S	R	I
	70µg/ml ampicillin - B	S	R	R
	70µg/ml ampicillin - C	I	R	I
	Combined treatment - A	S	R	I
	Combined treatment – B & C	I	R	I
T4	Wild-type	R	R	R
	Control – A, B & C	I	R	R
	0.2µg/ml carvacrol – A & B	I	R	R
	0.2µg/ml carvacrol - C	S	R	R
	70µg/ml ampicillin – A & B	I	R	R
	70µg/ml ampicillin - C	S	R	R
	Combined treatment - A	I	R	R
	Combined treatment – B & C	S	R	R

Shaded cells indicate resistance. CTX, Cefotaxime; CT, Colistin; SAM, Ampicillin; R, resistant; S, sensitive; I, intermediate. This table shows the comparison between antibiotic resistance profile in the original strains vs. the effect of each treatment on the loss of cefotaxime, colistin and ampicillin plasmids using antibiotic disks. Results are expressed as n = 1.

5.3.4 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on the production of metabolic by-products in ampicillin-resistant *E. coli* strains

Representative *E. coli* strains (APEC 46, and commensal *E. coli* strains; C2, and T4) were used in this experiment for metabolic screening in order to detect any changes in the production of metabolic by-products in different treatments. The detected metabolic by-products were carbon dioxide (CO_2), ethanol, thymol, acetate, and indole in all treatments. However, acetate peak was not visible in **Figure 5.1** at this level, but it was visible upon zooming in the chromatogram. Results in **Figure 5.1** (showing APEC 46 as a representative example) were presented in chromatograms according to different treatments. All of the representative strains showed the same chromatogram in all treatments. The only difference was the presence of thymol in samples containing carvacrol and combined treatment which was absent in the remaining samples.

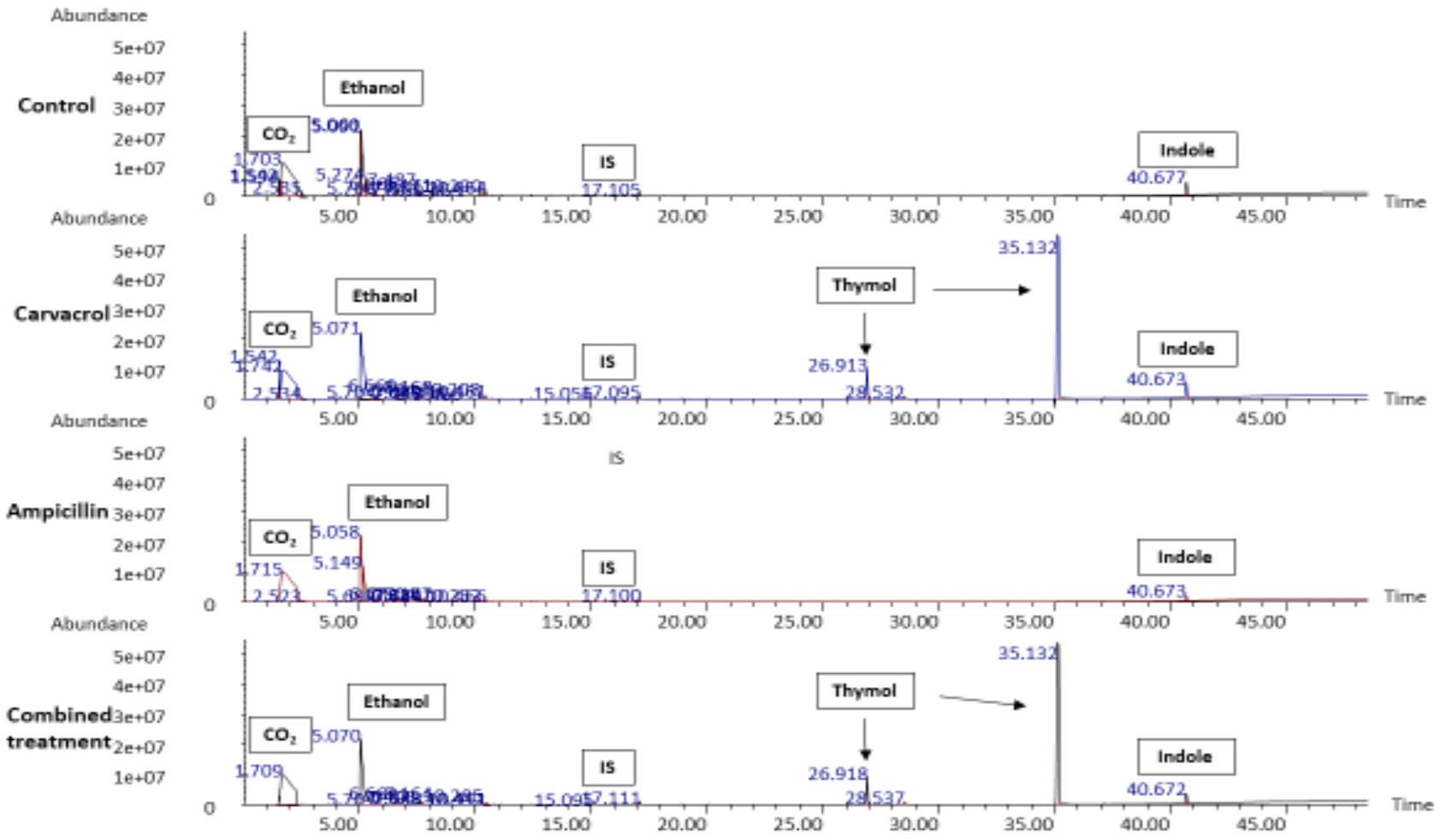

Figure 5.1 Chromatograms of metabolic by-products of APEC 46 in the presence of combined treatment of sub-lethal concentrations of carvacrol and ampicillin.

5.3.5 Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on a mixed *E. coli* culture

As shown in a previous chapter (**see Chapter 3**), carvacrol inhibited the growth of *E. coli* strains in a dose-dependent manner, and here the data set was expanded to study the impact of carvacrol on a mixed *E. coli* culture as well. The findings in **Table 5.6** showed there was a general increase in the total number of viable *E. coli* in all vessels after 48hr. The batch culture system was left to run for 48hr and samples were taken at different time points (0, 24, and 48hr), cultured, and 36 purified representative *E. coli* strains (**Table 5.7**) were randomly chosen for further characterisation such as ERIC-PCR (**Figure 5.2**), and detection of virulence genes by PCR (**Table 5.8**) in order to establish the relative fitness value for each starting strain (APEC 46, C2, and C4), and study the effectivity of each treatment. Linear model showed that there was a significant difference (P-value < 0.05) between bacterial numbers at different time points. However, it was not possible to study the effect between each treatment on the bacterial numbers because of the limited numbers of the vessels used (n = 1) for each treatment.

Table 5.6 Bacterial counting (total viable counts) on LB agar from the collected samples

Vessel / Time	0hr	24hr	48hr
V1	$3x10^6 \pm 1.53$	$3x10^8 \pm 5.03$	$1x10^9 \pm 16.80$
V2	$1x10^5 \pm 1.00$	$6x10^8 \pm 5.29$	$2x10^9 \pm 19.50$
V3	$1x10^6 \pm 11.93$	$1x10^8 \pm 4.73$	$7x10^8 \pm 10.60$
V4	$4x10^5 \pm 2.00$	$2x10^8 \pm 4.58$	$8x10^8 \pm 5.69$

Numbers refer to CFU/ml of triplicate values (n =3).

Table 5.7 Representative *E. coli* strains isolated from each fermentation vessel at different time points

No.	Code	Sample
1	V1A0	Vessel 1 at 0hr
2	V1B0	Vessel 1 at 0hr
3	V1C0	Vessel 1 at 0hr
4	V2A0	Vessel 2 at 0hr
5	V2B0	Vessel 2 at 0hr
6	V2C0	Vessel 2 at 0hr
7	V3A0	Vessel 3 at 0hr
8	V3B0	Vessel 3 at 0hr
9	V3C0	Vessel 3 at 0hr
10	V4A0	Vessel 4 at 0hr
11	V4B0	Vessel 4 at 0hr
12	V4C0	Vessel 4 at 0hr
13	V1A24	Vessel 1 after 24hr
14	V1B24	Vessel 1 after 24hr
15	V1C24	Vessel 1 after 24hr
16	V2A24	Vessel 2 after 24hr
17	V2B24	Vessel 2 after 24hr
18	V2C24	Vessel 2 after 24hr
19	V3A24	Vessel 3 after 24hr
20	V3B24	Vessel 3 after 24hr
21	V3C24	Vessel 3 after 24hr
22	V4A24	Vessel 4 after 24hr
23	V4B24	Vessel 4 after 24hr
24	V4C24	Vessel 4 after 24hr
25	V1A48	Vessel 1 after 48hr
26	V1B48	Vessel 1 after 48hr
27	V1C48	Vessel 1 after 48hr
28	V2A48	Vessel 2 after 48hr
29	V2B48	Vessel 2 after 48hr
30	V2C48	Vessel 2 after 48hr
31	V3A48	Vessel 3 after 48hr
32	V3B48	Vessel 3 after 48hr
33	V3C48	Vessel 3 after 48hr
34	V4A48	Vessel 4 after 48hr
35	V4B48	Vessel 4 after 48hr
36	V4C48	Vessel 4 after 48hr

A total of 3 representative strains were picked randomly from spread plates from each vessel at each time point.

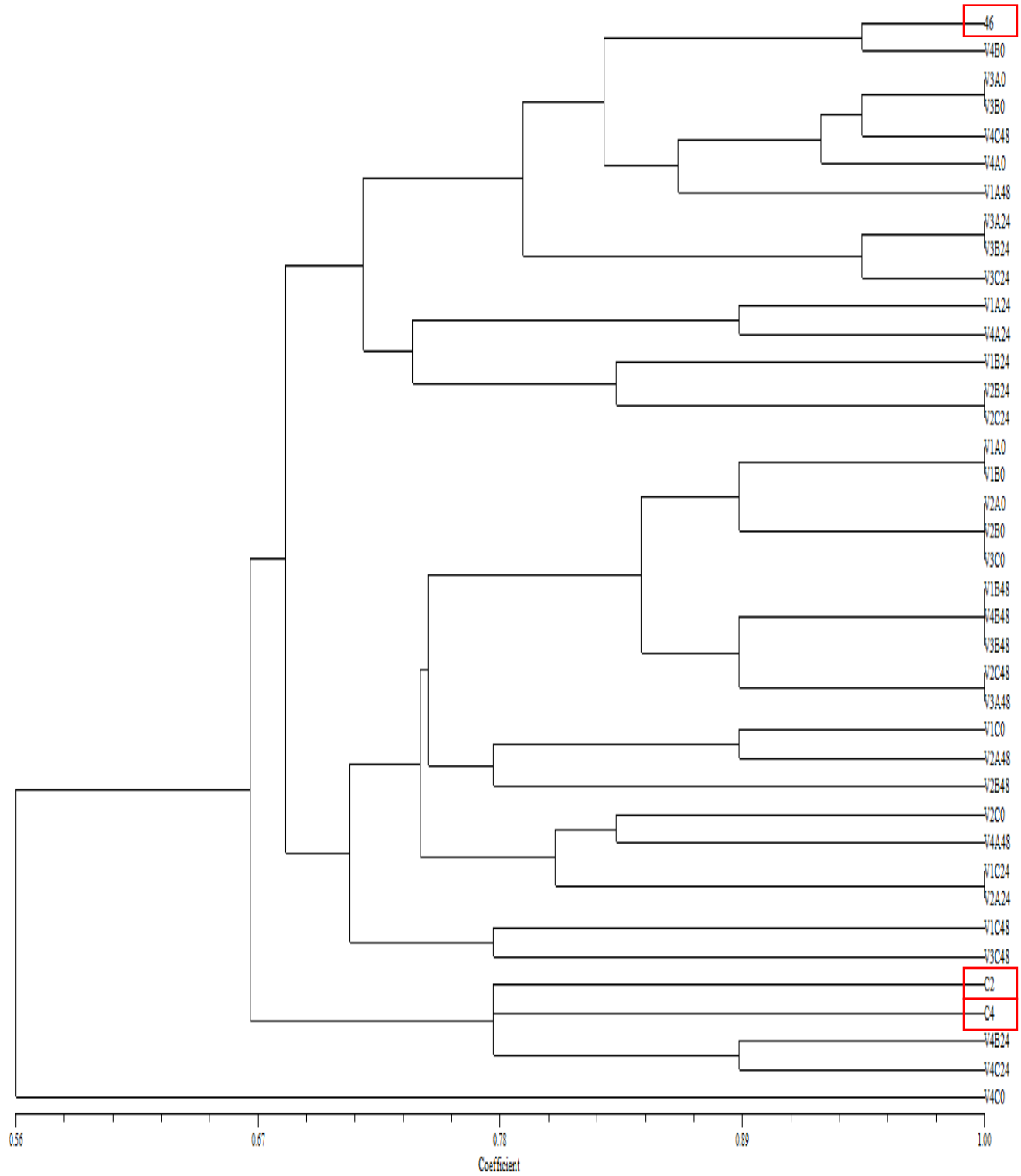

Figure 5.2 A dendrogram showing bacterial diversity of the representative *E. coli* strains isolated from batch culture based on UPGMA cluster analysis. The three original strains (APEC 46, C2 and C4) are marked in a red rectangle.

ERIC-PCR provides a quick tool to differentiate between the *E. coli* strains, so in theory, there should be three or four discrete nodes reflecting three representative strains coming from each treatment and/or reflecting strains adapting to different treatments because of a possible recombination event. However, **Figure 5.2** which demonstrated the variability of the results of ERIC-PCR analysis (**Table 5.7)** with the original strains used in this study showed different results. Even though, APEC 46 clustered in a different clade than the commensal chicken strains (C2 and C4) which comes in agreement with the results of ERIC-PCR from **Chapter 2**. Unfortunately, strains coming from different vessels clustered together in the same node. Thus, it was not possible to cluster strains coming from the same vessel in the same node sharing similar characteristics of the starting strains. ERIC-PCR was partly successful in showing how different the strains are, but it was not an effective technique in differentiating between the starting strains and representative strains, and between each vessel in order to understand which strains came from which vessel.

In order to differentiate between the *E. coli* strains effectively, detection of virulence genes (*iss* and *astA*) by PCR was used as a marker of the starting strains. **Table 5.8** showed that all of the *E. coli* strains were able to grow throughout the study, but at different fitness values. Despite the limitation of this study, C2 was detected at a higher rate than the other two strains with a percentage of 47.22% (17/36), followed by C4 with a percentage of 36.11% (13/36). However, APEC 46 was the strain which was the least detected strain, and at a very low percentage of 11.11% (4/36).

Table 5.8 Detection of virulence genes by PCR for the representative *E. coli* strains

No.	Code	*iss*	*astA*	Expected strain
1	V1A0	+	-	**C2**
2	V1B0	+	-	**C2**
3	V1C0	+	-	**C2**
4	V2A0	-	+	**C4**
5	V2B0	-	+	**C4**
6	V2C0	+	-	**C2**
7	V3A0	-	-	-
8	V3B0	+	-	**C2**
9	V3C0	-	+	**C4**
10	V4A0	+	-	**C2**
11	V4B0	+	-	**C2**
12	V4C0	-	+	**C4**
13	V1A24	+	-	**C2**
14	V1B24	+	-	**C2**
15	V1C24	-	-	-
16	V2A24	-	+	**C4**
17	V2B24	+	-	**C2**
18	V2C24	+	+	**APEC 46**
19	V3A24	+	-	**C2**
20	V3B24	+	+	**APEC 46**
21	V3C24	-	+	**C4**
22	V4A24	+	-	**C2**
23	V4B24	-	+	**C4**
24	V4C24	-	+	**C4**
25	V1A48	+	-	**C2**
26	V1B48	-	+	**C4**
27	V1C48	+	+	**APEC 46**
28	V2A48	+	-	**C2**
29	V2B48	-	+	**C4**
30	V2C48	+	+	**APEC 46**
31	V3A48	-	+	**C4**
32	V3B48	-	+	**C4**
33	V3C48	+	-	**C2**
34	V4A48	+	-	**C2**
35	V4B48	-	+	**C4**
36	V4C48	+	-	**C2**
37	APEC 46 (Amp R)	+	+	**APEC 46**
38	C2 (Amp R)	+	-	**C2**
39	C4 (Amp I)	-	+	**C4**

Shaded cells refer to the original *E. coli* strains used in this study. Results are expressed as n = 1.

5.4 Discussion

An interesting question posed in this section of the thesis was whether carvacrol will work synergistically or antagonistically with ampicillin to control highly ampicillin-resistant *E. coli* strains (MIC higher than 70µg/ml). These interactions might result from affecting different target sites within the bacterial cell due to different mechanisms of action (Esimone et al., 2006). The approach used was a checkerboard method (carvacrol concentration vs. ampicillin concentration), OD_{600} readings taken during 9hr of the exponential phase, and on a miniaturized (96 well plate) version of the original growth protocol (**Table 5.3**). This preliminary study showed an overall synergistic effect of carvacrol when combined with ampicillin at sub-lethal concentrations of 0.1µg/ml or 0.2µg/ml and 70µg/ml, respectively, but in only 11 strains out of the 17 strains. These eleven strains had MIC values of 0.3µg/ml in the following eight strains (46, 52, C1, C2, C6, T1, T6, and T8), 0.4µg/ml in two bacterial strains (45 and 51), and 0.5µg/ml in T4 only.

Synergy is when the effect of combined treatment of two chemicals is greater than the effect of each chemical separately (Rand et al., 1993). In the present study (**Table 5.3**), the synergistic effect was identified as a significant decrease (Overall P-value < 0.0001) in OD_{600} readings when using combined treatment of 0.1µg/ml or 0.2µg/ml of carvacrol and 70µg/ml ampicillin in comparison with each separately across all strains. This combined treatment might be effective in controlling highly ampicillin-resistant *E. coli* strains coming from poultry sources, and hence it should be evaluated in a batch culture model using mixed *E. coli* cultures (**see Chapter 5**), and chicken caecal contents (**see Chapter 6**). However, at this point, one cannot predict what might happened in such an interaction. Possible scenarios include: 1) Carvacrol may bind to the β-lactamase binding site instead of the β-lactamase as a result of competitive inhibition, 2) Carvacrol may bind to the β-lactamase enzyme and modifying its structure

making it less effective or inactive (Eliopoulus and Moellering, 1996) and allowing ampicillin activity, or 3) Metabolic burden (creating metabolic stress in the bacterial cell as a result of presence of high copy numbers of plasmids (Silva et al., 2012)) might result from the activities of both chemicals (carvacrol and ampicillin) at the same time, or 4) Carvacrol increases the permeability of the bacterial membrane (Ultee et al., 2002a) allowing more ampicillin to enter the cell (Johny et al., 2010) and perhaps causing burden on the activity of trans-peptidase enzyme. Thus, it is not clear what the physiological changes are that resulted from the addition of both chemicals together. So, a future experiment might include training these strains at different sub-MIC levels of carvacrol and ampicillin and then carry out WGS. This would be novel work and will provide more information of what are the genetic changes that might be selected in this study. Also, to expand this study, more antibiotics from different classes should be included to identify whether this synergy effect is limited or not.

Ampicillin is often the first antibiotic of choice in response to many clinical signs in poultry where a bacterial infection is considered to be the likely cause of disease or loss of productivity. However, we have data suggesting that 70.97% of the *E. coli* isolates made from poultry **(see Chapter 2**; **Table 2.6**) are already highly resistant to ampicillin rendering this treatment non-viable. The data of this initial study suggests that even a resistant strain may be susceptible to treatment when sub-lethal doses of carvacrol and ampicillin are administered at the same time, and this is in agreement with a previous study which synergy was identified using fractional inhibitory concentration (FIC) index (Palaniappan and Holley, 2010). This is an area for deeper exploration both in terms of application of carvacrol in the poultry industry and the mechanism of bacterial number reduction. This might suggest decreasing the ampicillin dose of that used regularly when administered with carvacrol to chickens as feed additives or use carvacrol alone and in turn this may decrease the ampicillin resistance *E. coli* and its ampicillin residue in

poultry gut in the long term. *In vitro* (**see Chapters 5 and 6**) and *in vivo* studies need to be performed in order to confirm these observations on a larger scale. This represents a new avenue by which carvacrol can control highly ampicillin-resistant *E. coli* strains and may increase their sensitivity to ampicillin in poultry industry.

In the tube format, bacterial counting was measured using two methods; taking OD_{600} readings and then CFU/ml. Determining CFU is a basic standard in microbiology laboratories, which counts the viable cells only (Miller, 1972). Though taking OD_{600} readings is also commonly used, but it measures viable and non-viable cells (Hazan et al., 2012). Thus, using both methods will increase the level of accuracy, and allow us to compare between the two measurements. Results in **Table 5.4** showed that the significant decrease (P-value < 0.05) in bacterial numbers in the presence of carvacrol and combined treatment was due to the inhibitory effect of carvacrol when compared to the control and ampicillin treatment.

Both of 96 well plate and tube format showed significant decrease (P-value < 0.05) in OD_{600} readings in carvacrol and combined treatment. However, when comparing results in **Table 5.3** and **Table 5.4**, there were differences in some OD_{600} readings in different treatments, and this may be due to the following: 1) difference in surface area in the 96 well plate and 15ml tube 2) differences in sensitivity of the microplate reader (96 well format) and spectrophotometer (tube format), 3) differences in shaking speed in the microplate reader and tube shaker, 4) measurements of OD readings were taken at different times (9hr in 96 well plate, and after 12hr in tube format), 4) differences in number of repeats (n=3 in 96 well plate, and n=1 in tube format).

Considering the differences in bacterial numbers in each treatment, it was of interest to pick colonies and use AST as a quick way to detect any changes in antibiotic susceptibility profile. AST results in **Table 5.5** were not successful in determining the effect of each treatment on the phenotypic changes of antibiotic susceptibility profile in the representative strains. The reason for focusing on cefotaxime, colistin, and β-lactams was because they showed resistance among the representative strains, and they gave the highest percentage of resistance among all of the 32 strains (**see Chapter 2**; **Table 2.6**), and moreover, ampicillin is the first antibiotic of choice in poultry. Changes from R or I to S would be anticipated as result of loss of plasmid in case of plasmid-mediated antibiotic resistance. However, changes from S to I or R would be the result of cross-contamination or conjugal transfer or the insensitivity of the AST test such as in control strains (A & B) and combined treatment (A, B, & C) derived from APEC 46 strain. These experiments were done only once and after overnight incubation, so this needs more repeats and more strains in order to determine whether there was any treatment effect or not. AST test is an inaccurate measurement of antibiotic resistance, so in order to determine the loss of plasmid responsible for antibiotic resistance, this should be investigated using WGS approach and then detected using PlasmidSeeker tool or using agarose gel electrophoresis approach.

GC-MS is an analytical technique to thoroughly investigate the metabolomic profile of the host or any biological sample, which became an important tool to understand the link between genotype and phenotype (Bino et al., 2004). As a method, GC-MS was able to detect some of the metabolic by-products such CO_2, ethanol, thymol, acetate and indole, which are normally produced by *E. coli* strains except for thymol. The added carvacrol in carvacrol and combined treatments was detected as thymol, due to their similarities in chemical structure. However, GC was not successful in detecting any changes in the production of each metabolic by-product in

comparison with GC method (**see Chapter 3**). Indole is a signaling molecule associated with regulation of biofilm formation (Di Martino et al., 2003) and stability of multi-copy plasmids in *E. coli* (Chant and Summers, 2007). Thus, it was of interest to check whether there is an effect of carvacrol or combined treatment on the production of indole. However, at different treatments, all of the chromatograms showed the same profile and in all *E. coli* strains (**Figure 5.1**). There might be an increase or decrease in the metabolic by-products, but they were not be detectable by this semi-quantitative method. Thus, it could be coupled with GC method which was shown to be a quantitative method or use a different protocol using GC-MS, to quantify these changes in concentrations.

To evaluate the efficacy of carvacrol as a phytochemical, we investigated initial parameters such as total viable counts of bacteria upon addition of sub-lethal concentrations of ampicillin and carvacrol. Results of bacterial counting (**Table 5.6**) showed a general increase in total viable counts after 48hr in all vessels, and this was proved to be statistically significant (P-value < 0.05) between different time points. V1 showed a gradual increase in *E. coli* total counts, and this was expected. This also, confirms the validity of using VL medium to grow *E. coli* batch culture, as it encompasses nitrogen and sugar sources needed as substrates for *E. coli* metabolism (Lei et al., 2012). Moreover, the continuous increase in bacterial numbers in V2 containing sub-lethal concentration of ampicillin was anticipated, because two of the added strains (APEC 46 and C2) were highly ampicillin resistant with MIC value of more than 70µg/ml. Being highly resistant, it may be reasoned that there was sufficient β-lactamase enzyme activity to inactivate the ampicillin allowing the third strain C4 to survive and grow despite being ampicillin intermediate. Also, it was noted that there was a possible trend in the total viable counts in V1 and V2 in comparison with V3 and V4, which suggests that there is a slightly slower growth in V3 and V4 after 48hr (**Table 5.6**), but without multiple repeats and

robust statistics, these differences are very marginal and insufficient to suggest that carvacrol may show a trend to a relatively reduced growth. Therefore, it was not possible to study the efficacy of each treatment due to the limited number of vessels for each treatment (n =1), and so we could not confirm whether carvacrol was more effective than ampicillin on a mixed culture of APEC and commensal *E. coli* strains or not. Also, the hypothesis that exposure to sub-lethal concentrations of carvacrol may increase sensitivity to ampicillin by membrane and enzyme disruption even in resistant strains was shown unfounded, and at the concentrations used in this experiment. As no obvious effects were seen, this study was not taken any further.

ERIC-PCR data was not successful in placing the different *E. coli* strains in different nodes/clades (**Figure 5.2**), and it was not possible to relate which starting strain was better able to grow in a certain vessel or not. Though ERIC-PCR is rapid, it would appear to be extremely unreliable in generating consistent profiles reflecting major genomic re-arrangements in the different *E. coli* strains used, given they were grown for 48hr in batch cultures supplied with different treatments. This might be solved through including more characterisation tests such as plating on different selective media based on their phenotypic characteristics, so we can easily differentiate between them and know which starting strain was better able to grow in a certain vessel and the efficacy of each treatment. As this will reveal the efficacy of each treatment and how *E. coli* strains might change throughout the course of each treatment.

Virulence gene profiling of *iss* and *astA* genes (**Table 5.8**) showed the ability to differentiate the representative strains based on the profile of the original starting *E. coli* strains used in this study. These virulence genes are located in plasmid and chromosome respectively, and generally *iss* plasmid could be spread by conjugation at a higher rate (58.33%: 21/36) than *astA*

gene (47.22%: 17/36) among the representative strains. Regardless of the limitation of this study, interestingly, C2 and C4 were detected more frequently than APEC 46, and this may suggest that the commensal strains have higher fitness value than the APEC strain. Whether this observation indicates the ability of the commensal strains to outcompete the APEC strain is open to question and requires more repeats in order to confirm this very preliminary finding. Moreover, this method was not able to reveal the ability of each treatment to stop or encourage the dissemination of the two virulence genes and their mobility between the strains. Whether these observations were really true or happened randomly, this requires further investigation.

Collectively, the simple three strains batch culture model could not demonstrate the ability carvacrol to decrease or suppress the growth of a mixed *E. coli* culture at sub-lethal concentrations in comparison with the control and ampicillin treatment. Generally, from these initial data of the representative 36 strains (**Table 5.7**), commensal strains (C2 and C4) were found at a higher rate than the APEC 46 strain (**Table 5.8**), and this may suggest the ability of these commensal strains to maintain normobiosis, as the number of commensal strains were higher than the APEC strain. Moreover, *E. coli* may enter viable but non-culturable (VNC) state (Van Elsas et al., 2011), and this will affect the true number of isolates in each vessel. These are only speculations, due to the limited number of vessels (n = 1 for each treatment) and small sample size of representative *E. coli* strains (n = 36), as it was impossible to cover all the strains. This initial study could have been improved by: 1) including more vessels for each treatment, and therefore result in larger number of strains, 2) detecting the presence of AMR genes beside virulence genes to monitor the effect of each treatment on the carriage of antimicrobial resistance mediated plasmids, 3) labelling the starting *E. coli* strains with fluorescence labelling and then track them, but this method might create extra stress on the cellular level on top of the treatment stress.

This was a preliminary study that opens the door to future studies and set a baseline to **Chapter 6** and for other experiments such as: 1) the effect of each treatment at different combinations of sub-lethal concentrations, 2) the effect of each treatment on *E. coli* population from chicken caecal content, as this will increase our understanding on what happens inside the actual chicken gut (**see Chapter 6**), 3) the effect of each treatment on the bacterial population of the chicken gut, as carvacrol might cause dysbiosis resulted from the decrease in *E. coli* population (**see Chapter 6**) , 4) the effect of each treatment on the number of Gram-positive and Gram-negative bacterial population in the chicken gut, 5) the effect of each treatment on the bacterial growth rate vs. bacterial growth yield. These *in vitro* studies were performed because they have to be carried out before going to *in vivo* experiments for ethical and safety reasons (Van den Abbeele et al., 2010). *In vivo* studies come at the last step after making sure that these treatments are safe to be used on animal models.

In conclusion, synergistic interactions between carvacrol and ampicillin were observed at sub-MIC levels to control the growth of ampicillin-resistant *E. coli* strains. However, it was not possible to detect any changes in the metabolic by-products of these *E. coli* strains in the presence of the combined treatment of sub-lethal concentrations of carvacrol and ampicillin. Taken these results into a simple three strains batch culture model, there was a significant increase (P-value < 0.05) in the total viable numbers of *E. coli* strains over time in all treatments. Therefore, it was not possible to observe the synergistic effect on a mixed *E. coli* culture.

CHAPTER 6: Effect of combined treatment of sub-lethal concentrations of carvacrol and ampicillin on *E. coli* isolated from caecal contents of broiler chicken

6.1 Introduction

The chicken gut is colonised directly after hatching (Brisbin et al., 2008) predominantly with environmental facultative anaerobes from the phylum Proteobacteria, but this population changes rapidly over time to become predominant with anaerobic Firmicutes after two weeks of age (Ballou et al., 2016). The gut microbiota has important roles in digestion and may be included in maintaining normobiosis state and contributing to the health of the host (Stanley et al., 2014). The chicken caecum is one of the main locations for colonisation by commensal bacteria (Fuller and Turvey, 1971), where they occur in large numbers, giving a dense bacterial ecosystem, ranging from 10^7 to 10^{11} bacteria per gram of caecal content (Apajalahti et al., 2004). Caeca are a pair of tubes (right and left), located at the intersection of ileum and colon (McLelland, 1989), and are considered to be the first part of large intestine (Nasrin et al., 2012). They are commonly present among avian species, but absent in some, and come in different sizes and shapes (Clench and Mathias, 1995). They are very adaptable in terms of their size; they increase in size as a result of high metabolic products included in the diet, and this affects their functionality (Pulliainen and Tunkkari, 1983). Though the full function of caeca is not well understood, they are responsible for being the location for important roles such as absorption of electrolytes and water (Thomas and Skadhauge, 1989), digestion of cellulose, proteins, and carbohydrates (JøRgensen et al., 1996), vitamin synthesis and absorption (Coates et al., 1968).

Caeca have a slower passage rate in comparison to the remaining gastrointestinal tract of poultry (Pan and Yu, 2014), which results in large numbers of bacteria accumulating with a consequence being that the caeca are the major location for microbial fermentation (Józefiak et al., 2004), mostly by anaerobic bacteria (Barnes and Impey, 1972). The production of SCFAs as the end products of fermentation lowers the pH of the caeca (Józefiak et al., 2004) and this prevents pathogenic bacteria from colonising (Apajalahti, 2005). Moreover, the caecal microbiota plays an important role in the bird's health status (Dunkley et al., 2009) by establishing a protective immunity against bacterial infections (Barrow, 1992). However, the microbiota is affected by a number of intrinsic factors such as the bird's age (Knarreborg et al., 2002), genetics (Zhao et al., 2013) and gender (Lee et al., 2003b), and extrinsic factors such as diet, antibiotic use (Knarreborg et al., 2002), presence of infection (Kimura et al., 1976), feed additives (Lee et al., 2003b), housing (Zhao et al., 2013) and other environmental factors such as build-up of ammonia in the atmosphere of the house (Apajalahti and Vienola, 2016).

Phytochemicals are natural sources of feed additives and have been proven to be GRAS (Hashemi et al., 2008). They provide an effective alternative to synthetic products, and they have shown to improve animals' well-being and increase productivity (Wray and Davies, 2000). Previous studies have demonstrated the *in vivo* efficacy of oregano as feed additives in broiler chickens (Ertas et al., 2005, Karimi et al., 2010, Peng et al., 2016) but without in-depth investigation of what is happening at the gut microbiota level. An *in vitro* batch culture study on the effect of carvacrol on caecal microbiota showed that using high concentrations of carvacrol affected fermentation processes performed by the gut microbiota due to its anti-bacterial role on growth and metabolism (Grilli et al., 2006), but without investigating the totality of the microbial population or their metabolic by-products.

In this chapter, the aim was to study the impact of the combined treatment of carvacrol with ampicillin, which to our knowledge has not been investigated before. Also, this study was performed to assess wider impacts on the microbiota that was not possible to test in a pure and mixed pure culture experiments (**see Chapter 5**). The testable hypotheses of the current study were: 1) Whether the use of sub-lethal concentrations of carvacrol (the active ingredient of oregano) will have a modulatory effect on a mixed population found in pooled caecal contents challenged with an APEC strain or not?, 2) Whether the added APEC strain will be suppressed or dominate the growth, and suppress the beneficial effects of the bacterial community irrespective of the treatments, and 3) Whether the application of the combined treatment of sub-lethal concentrations of carvacrol and ampicillin on pooled caecal contents challenged with an ampicillin-resistant APEC strain will increase the efficiency of ampicillin or not?

The aims and objectives of this study were:

- To test whether there is an observable effect of sub-lethal concentrations of carvacrol and ampicillin separately and in combination on the *E. coli* population and whole bacterial community present in chicken caecal content.
- To study the effect of each treatment upon the ampicillin-resistant APEC strain added to the healthy caecal content, and whether this strain can be recovered or not using whole population sequencing data.
- To test whether carvacrol inhibit/modulate the growth of the whole bacterial community resulting in a shift in the total bacterial caecal community or does it work selectively against certain bacterial population?
- To determine whether carvacrol fit into the criterion of a feed additive by its ability to suppress pathogenic bacteria and nourish the growth of beneficial bacteria?

- To demonstrate whether the combination of carvacrol and ampicillin increases the efficacy of ampicillin among ampicillin-resistant bacteria?
- To test the effect of each treatment on the production of SCFAs using GC.

6.2 Materials and methods

6.2.1 *In vitro* batch culture set-up

Fresh caecal contents were collected from five 28 days-old male broiler chickens slaughtered and handled at CEDAR farm (University of Reading) by the University veterinary officer following approved ethical standards and procedures. The farm premises and prodecures were regulated and in comply with the Home Office (Animal Procedures Act). Prior to killing, the chickens were housed and fed a standard commercial diet of corn-soy and fresh water ad libitum and handled according to the recommendations mentioned in the Code of Recommendations for the Welfare of Livestock: Meat chickens and breeding chickens (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/69372/pb7275meat-chickens-020717.pdf) and the Ross Broiler Management Handbook (http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-Broiler-Handbook-2014iEN.pdf). Birds/chickens were selected randomly for this experiment. The caecal samples were collected in sterile vials and stored on ice until they were transported to the laboratory, where they were used to prepare a pooled caecal slurry (1:10) dilution in VL medium to inoculate the system. Also, an overnight culture (10 – 12hr) of the APEC 46 strain was diluted to OD_{600} of 0.01 (10^7 CFU/ml) in fresh VL broth, and then added to each vessel (separately and after caecal slurry inoculation step) to give a final concentration of 10^5 CFU/ml in the 20ml vessel. VL broth was used as this medium was designed to reflect poultry gut luminal content (Lei et al., 2012). All of the chemicals and ingredients were prepared in VL medium. Eight vessels (**Table 6.1**) were included in this study; vessels 1 (V1) and 2 (V2) contained the control of the caecal

slurry and 100µl of APEC 46 strain completed with VL medium to 20ml, vessels 3 (V3) and 4 (V4) contained the same mixture in addition to sub-lethal concentration of ampicillin treatment (20µg/ml), vessels 5 (V5) and 6 (V6) contained the same mixture in addition to sub-lethal concentration of carvacrol (0.2µg/ml), and vessels 7 (V7) and 8 (V8) contained the same mixture in addition to combined treatment of both ampicillin (20µg/ml) and carvacrol (0.2µg/ml). The set conditions of the 20ml vessels were controlled throughout the 72hr as such; anaerobic conditions (80% N_2, 10%H_2, and 10% CO_2), pH value in between 5.8 to 6.0 monitored by pH meter, and a temperature of 41°C maintained by water bath. The system was left to run for 72hr, and a total sample of 1.5ml was collected at 4 different time points of 0, 24, 48, and 72hr. I am grateful to Dr. Vasiliki Kachrimanidou for her help in setting up the batch culture.

Table 6.1 Components of each batch culture vessel

Starting concentration of each component	**Final concentration in each treatment**			
	V1 &V2 (Control)	**V3 & V4 (20µg/ml ampicillin)**	**V5 & V6 (0.2µg/ml carvacrol)**	**V7 & V8 (Combined treatment of 20µg/ml ampicillin & 0.2µg/ml carvacrol)**
APEC 46 strain culture (10^7 CFU/ml)	100µl	100µl	100µl	100µl
Caecal slurry (1:10)	2ml	2ml	2ml	2ml
1000µg/ml ampicillin	-	400µl	-	400µl
5µg/ml carvacrol	-	-	800µl	800µl
VL broth	17.900ml	17.500ml	17.100ml	16.700ml

Total volume of each vessel (V) was 20ml.

6.2.2 Simple plate counting of *E. coli*

Samples of 300µl collected at 3 different time points of 0, 24, and 48hr were serially-diluted and spread on MacConkey agar and MacConkey agar containing 50µg/ml ampicillin. The plates were incubated overnight (10 – 12hr) at 37ºC, to monitor the changes in *E. coli* numbers and count the numbers of ampicillin-resistant *E. coli* during the study.

6.2.3 Production of SCFAs by GC

A volume of 1.2ml from each sample collected from 4 different time points of 0, 24, 48, and 72hr was centrifuged, and then 1ml of the supernatant was used for SCFAs analysis by GC using Richardson's protocol as described earlier in **Chapter 3;** section **3.2.3**.

6.2.4 Whole population profiling using next generation sequencing (NGS) technology

The pellet from section **7.2.3** above was retained and 200µl of the pellet was used for DNA extraction of whole bacterial community using DNeasy PowerSoil Kit (Qiagen®, UK) according to the manufacturer's recommendations, and samples of 50µl at 10ng/µl were sent to the Animal and Plant Health Agency (APHA) in Surrey for whole bacterial community sequencing using Illumina sequencing technology. The extracted DNA was used for amplification of V4 and V5 regions of the 16S rRNA gene using forward primer U515F (5'-GTGYCAGCMGCCGCGGTA-3') and reverse primer U927R (5'-CCCGYCAATTCMTTTRAGT-3'); universal primers to target bacterial and archaeal ribosomal RNA gene regions at a high taxonomic resolution (Wang and Qian, 2009). Fusion primers with overhang adapter; forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTAATAAGAGACAG-3') were used for barcoding.

PCR was performed using FastStart HiFi DNA polymerase (Roche Diagnostics Ltd, UK) with the following program: 3 min initial denaturation at 95°C, followed by 25 cycles of 30 sec denaturation at 95°C, 35 sec annealing at 55°C, 1 min extension at 72°C, and final extension of 72°C for 8 min. The amplified 16S rRNA gene products were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). Then, each sample was tagged with a unique pair of indices and sequencing primers by Nextera XT v2 Index Kits and 2x KAPA HiFi HotStart ReadyMix using the following program: 3 min initial denaturation at 95°C, followed by 12 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 55°C and 30 sec extension at 72°C, and final extension of 72°C for 5 min. The index-tagged amplified 16S rRNA gene products were further purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). The concentration of each sample was quantified using PicoGreen fluorescent assay (Invitrogen). Concentrations were normalised before pooling all samples, each of which would be later identified by its unique code of Merchant Identification Number (MID). Sequencing was performed on the Illumina MiSeq system with 2 x 300 base reads according to the manufacturer's instructions (Illumina, Cambridge, UK).

6.2.5 Bioinformatics analyses

Raw data were submitted to the bioinformatics services team provided by the University of Reading. I thank Dr. Bajuna Salehe from School of Biological Sciences for performing bioinformatics analyses and their associated statistical analyses using the Quantitative Insights Into Microbial Ecology (QIIME) 2 platform.

6.2.6 Statistical analyses

Linear mixed model was performed to study the significance of differences between the effect of each treatment on the number of bacterial strains vs. control, to study the effect of each treatment on the bacterial numbers, and to study the significance between control vs. production of each SCFA at sub-lethal concentrations of each treatment. Statistical analyses were performed using R i386 3.4.3 software.

6.3 Results

6.3.1 Total viable counts of *E. coli*

The first parameter involved in this study was to look at the differences in numbers of *E. coli* using a simple plating technique as described above and the counts are shown in **Figures 6.1** and **6.2**. It would appear that a very large proportion of *E. coli* or possibly even all of the *E. coli* were ampicillin-resistant at the beginning of the experiment, as the number of the added APEC strain would result in a similar number of *E. coli* at time 0hr. The findings in **Figures 6.1** and **6.2** showed that there was a significant increase (P-value < 0.05) in the total number of viable *E. coli* and ampicillin-resistant *E. coli* after 48hr in all vessels. Linear mixed model showed that there was a significant difference (P-value < 0.05) between numbers of *E. coli* and ampicillin-resistant *E. coli* at different time points (0hr and 24hr, 0hr and 48hr) in **Figures 6.1** and **7.2**. However, there was an insignificant difference (P-value > 0.05) on the effect of each treatment on the numbers of *E. coli* and ampicillin-resistant *E. coli* (**Figures 6.1** and **6.2**).

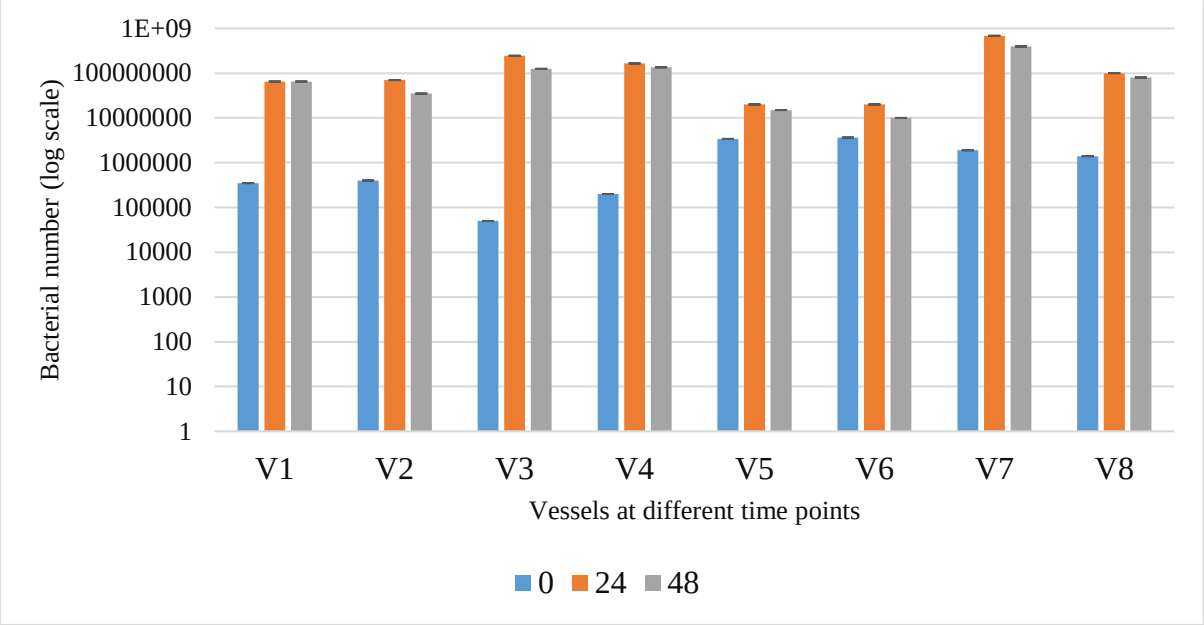

Figure 6.1 Total viable counts of *E. coli* on MacConkey agar from the collected samples (Numbers refer to log scale of CFU/ml of duplicate values).

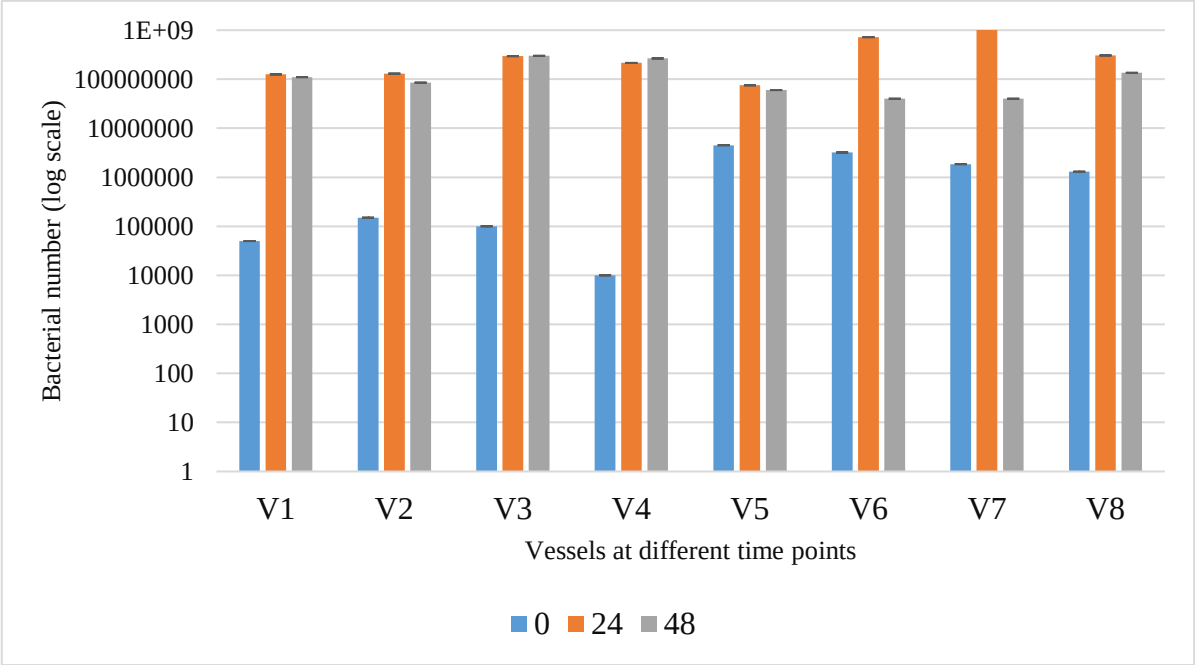

Figure 6.2 Total viable counts of *E. coli* on MacConkey agar containing 50µg/ml ampicillin from the collected samples (Numbers refer to log scale of CFU/ml of duplicate values).

6.3.2 Production of SCFAs using GC

The second measurement was to look at the effect of each treatment on the production of SCFAs (**Table 6.2A**) and (**Table 6.2B**), and this was monitored at four time points (0, 24, 48 and 72hr). Generally, lactate was the highest SCFA produced, followed by butyrate and propionate in all vessels. At time 0hr, all of the SCFAs were available in high concentrations (except for succinate) in all the vessels irrespective of the treatment, but changes were noticed after different time points. However, there were noticeable variations in the concentrations of SCFAs present at time 0hr: 1) lower concentrations of propionate in V7 and V8 in comparison with the remaining vessels **(Table 6.2A**), 2) lower concentrations of succinate in V4 and V7 in comparison with the remaining vessels (**Table 6.2A**), 3) higher concentration of acetate in V8 in comparison with the remaining vessels **(Table 6.2B**), 4) lower concentrations of lactate in V7 and V8 in comparison with the remaining vessels (**Table 6.2B**), all of which should have yielded similar concentrations as all vessels were inoculated with the same pooled sample. These variations might be due to technical errors and the picture may have been improved with more technical repeats. Unfortunately, there were limited resources and lack of time to do this. Notwithstanding the variability at time 0hr, a linear mixed model showed that there was no overall significant difference (P-value > 0.05) between SCFAs production by treatment except for lactate, which was noticed between carvacrol and combined treatment vessels at 0hr (P-value = 0.0029). However, there was significant increase (P-value < 0.05) in the production of propionate and butyrate over time, on the other hand, there was no significant increase (P-value > 0.05) in the production of succinate, acetate, and lactate over time.

Table 6.2A Effect of combined treatment of sub-lethal concentrations of carvacrol (0.2µg/ml) and ampicillin (70µg/ml) on the production of SCFAs as by-products of metabolic pathways

Vessels	Propionate				Butyrate				Succinate			
	0hr	24hr	48hr	72hr	0hr	24hr	48hr	72hr	0hr	24hr	48hr	72hr
V1 (Control)	107.177*	98.773*	142.443*	135.449*	115.074*	129.456*	155.836*	143.121*	1.900	0.000	0.000	0.344
V2 (Control)	102.016*	116.054*	139.779*	149.667*	111.548*	134.537*	148.123*	156.487	0.964	0.000	0.000	0.291
V3 (20µg/ml ampicillin)	99.064*	109.092*	130.850*	138.309*	103.861*	136.256*	159.531*	167.730*	1.487	0.000	0.335	0.479
V4 (20µg/ml ampicillin)	106.514*	124.377*	133.440*	133.379*	110.999*	117.637*	153.167*	153.065*	0.376	0.000	0.000	0.000
V5 (0.2µg/ml carvacrol)	109.932*	121.625*	134.058*	111.688*	115.031*	122.196*	148.282*	122.758*	2.269	0.000	0.000	0.000
V6 (0.2µg/ml carvacrol)	99.836*	124.081*	136.408*	140.608*	105.252*	123.336*	148.826*	149.745*	7.419	0.000	0.000	0.489
V7 (Combined treatment)	85.119*	79.447*	126.373*	126.025*	118.246*	83.386*	126.130*	110.919*	0.358	0.000	7.193	0.000
V8 (Combined treatment)	74.780*	131.478*	137.086*	118.530*	107.894*	152.086*	153.306*	132.288*	1.702	0.000	0.000	0.000

Numbers refer to a single value (in mM unit) from a single sample (n =1). * indicates significant increase (P-value < 0.05) in the production of propionate and butyrate over time.

Table 6.2B Effect of combined treatment of sub-lethal concentrations of carvacrol (0.2µg/ml) and ampicillin (70µg/ml) on the production of SCFAs as by-products of metabolic pathways

Vessels	Acetate				Lactate			
	0hr	24hr	48hr	72hr	0hr	24hr	48hr	72hr
V1 (Control)	86.169	99.948	146.511	126.322	244.151	106.310	206.695	272.827
V2 (Control)	78.197	119.755	130.556	163.512	202.243	151.162	183.400	188.822
V3 (20µg/ml ampicillin)	74.213	116.519	149.472	161.540	190.186	123.359	54.954	146.575
V4 (20µg/ml ampicillin)	84.614	94.145	143.567	173.918	236.494	126.246	60.628	33.218
V5 (0.2µg/ml carvacrol)	91.380	99.120	132.512	93.363	340.132	237.756	132.932	163.286
V6 (0.2µg/ml carvacrol)	82.233	98.965	131.282	133.468	307.912	161.106	104.100	288.945
V7 (Combined treatment)	80.914	444.217	120.646	108.867	113.412	168.457	79.537	285.180
V8 (Combined treatment)	151.518	122.419	132.131	117.617	27.192	128.784	211.592	94.676

Numbers refer to a single value (in mM unit) from a single sample (n = 1)

6.3.3 Whole population profiling using NGS technology

6.3.3.1 Alpha diversity (changes at species level within each vessel)

A total of 66 pools of DNA sequences collected from 33 samples were clustered according to their similarity and used to construct alpha rarefaction curves according to two variables: 1) treatment, and 2) time point, using two indices (chao1 and observed OTUs). Alpha diversity of the bacterial communities in different treatments was measured using chao1 (**Figure 6.3**) and observed operational taxonomic units (OTUs) (**Figure 6.4**) indices, to show bacterial diversity and richness at species level in each treatment. Kruskal-Wallis of all groups and pairwise comparisons showed insignificant difference (P-value > 0.05) in bacterial communities among different treatments. Moreover, alpha diversity of the bacterial communities at different time points was measured using chao1 (**Figure 6.5**) and OTUs (**Figure 6.6**) indices, to show bacterial diversity and richness at each time point. Kruskal-Wallis of all groups and pairwise comparisons showed significant difference (P-value < 0.05) in bacterial communities at different time points. Overall, alpha diversity results showed that changes in bacterial communities were due to the effect of time and not treatment.

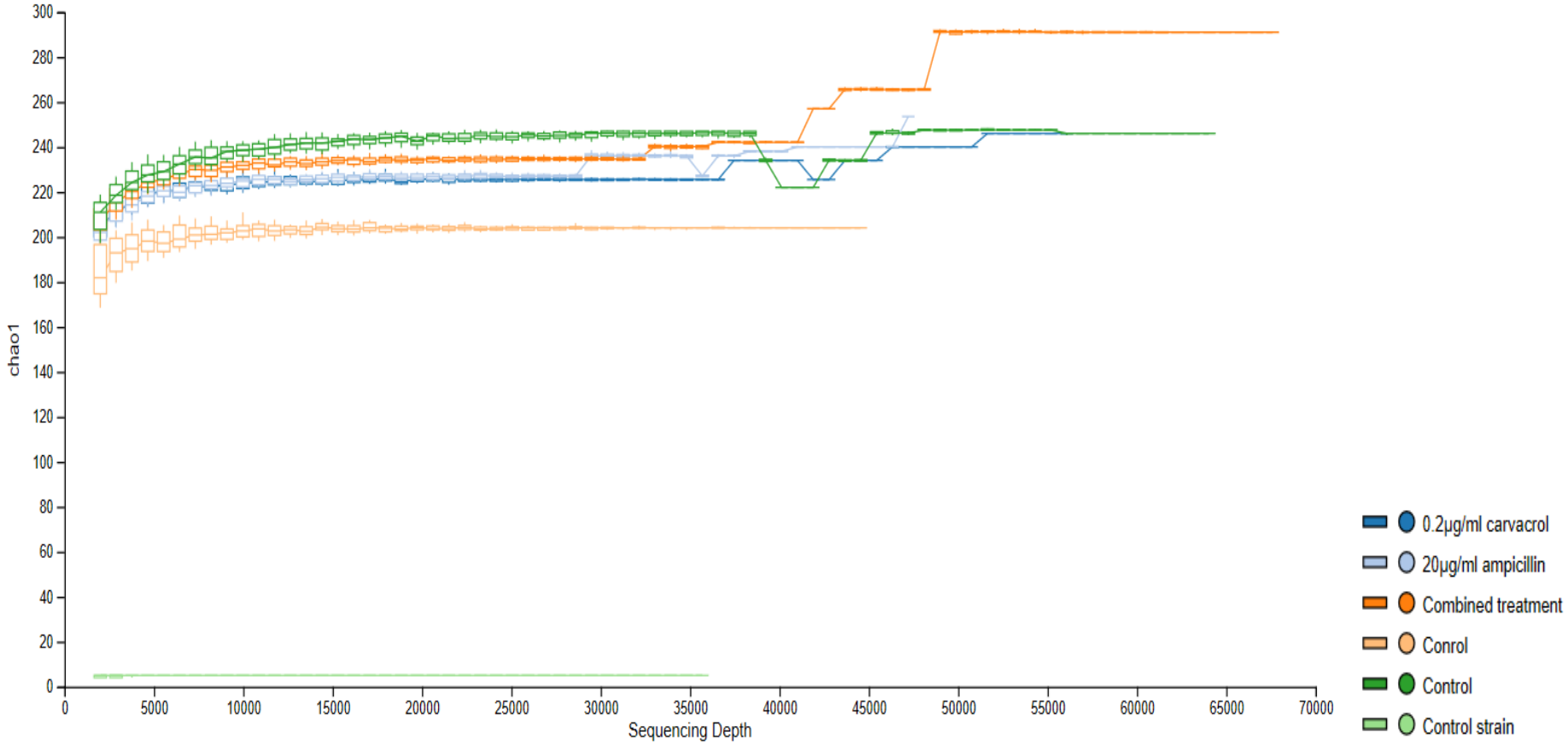

Figure 6.3 Alpha rarefaction curve of the bacterial communities in different treatments using chao1 index.

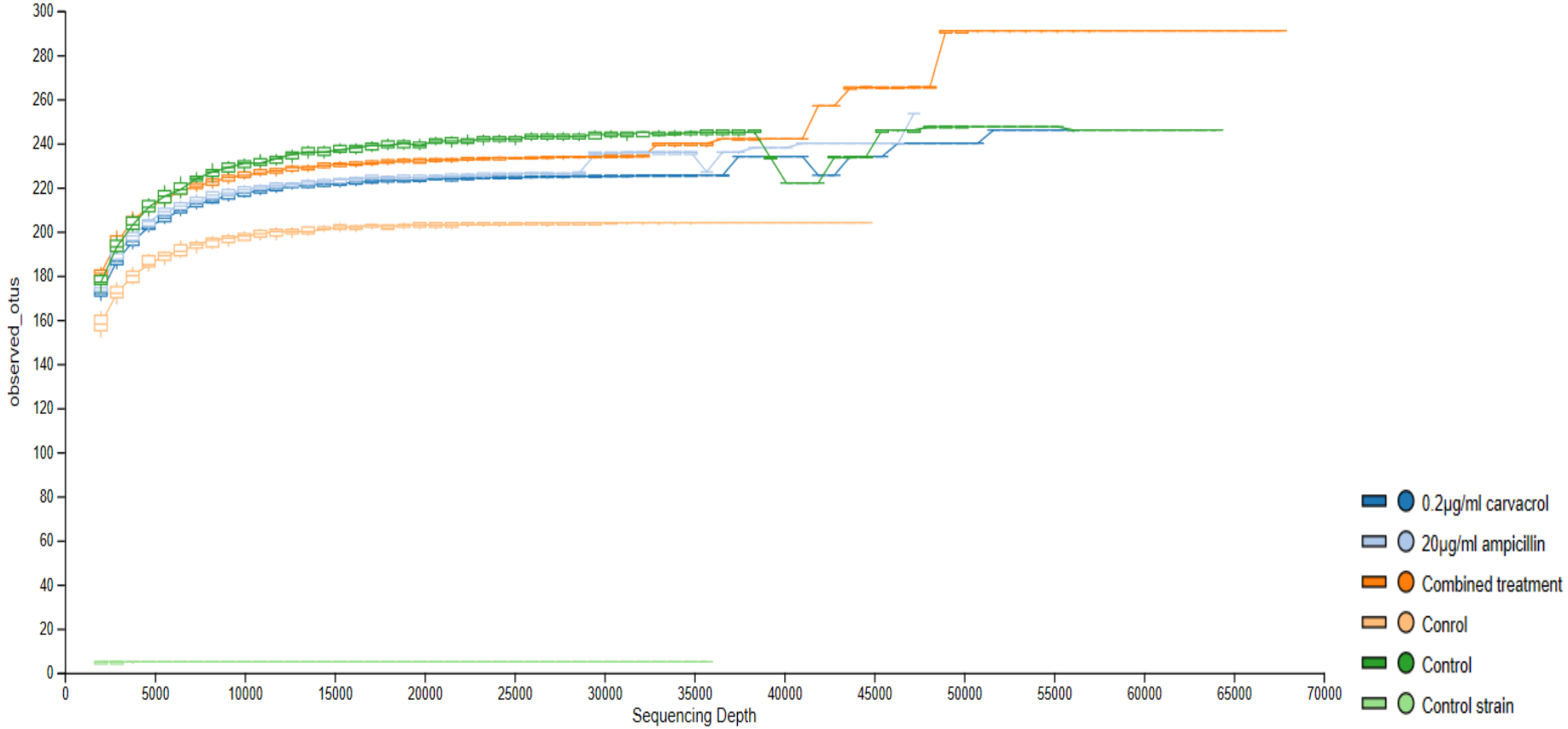

Figure 6.4 Alpha rarefaction curve of the bacterial communities in different treatments using observed OTUs index (based on 99% similarity threshold).

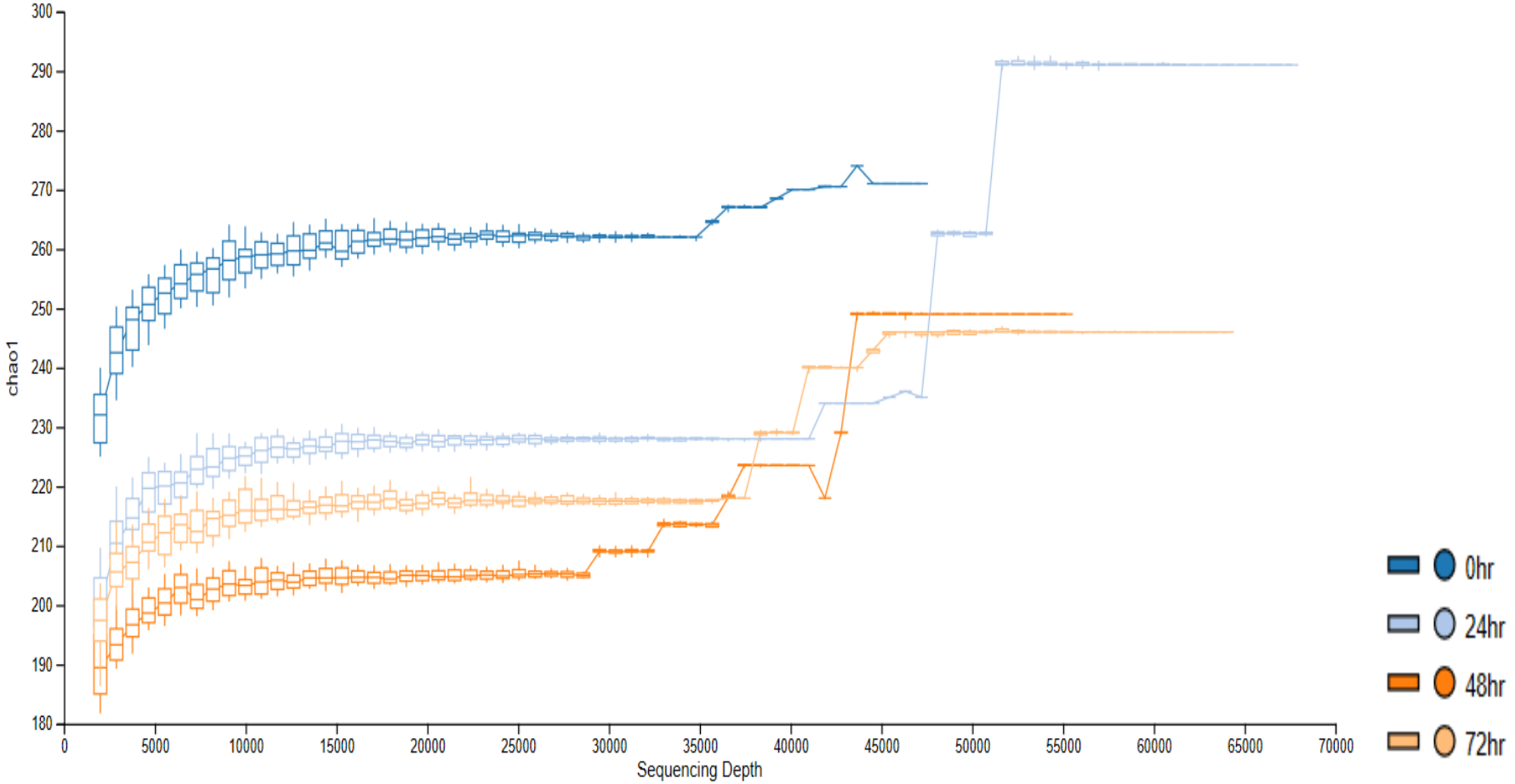

Figure 6.5 Alpha rarefaction curve of the bacterial communities at different time points using chao1 index.

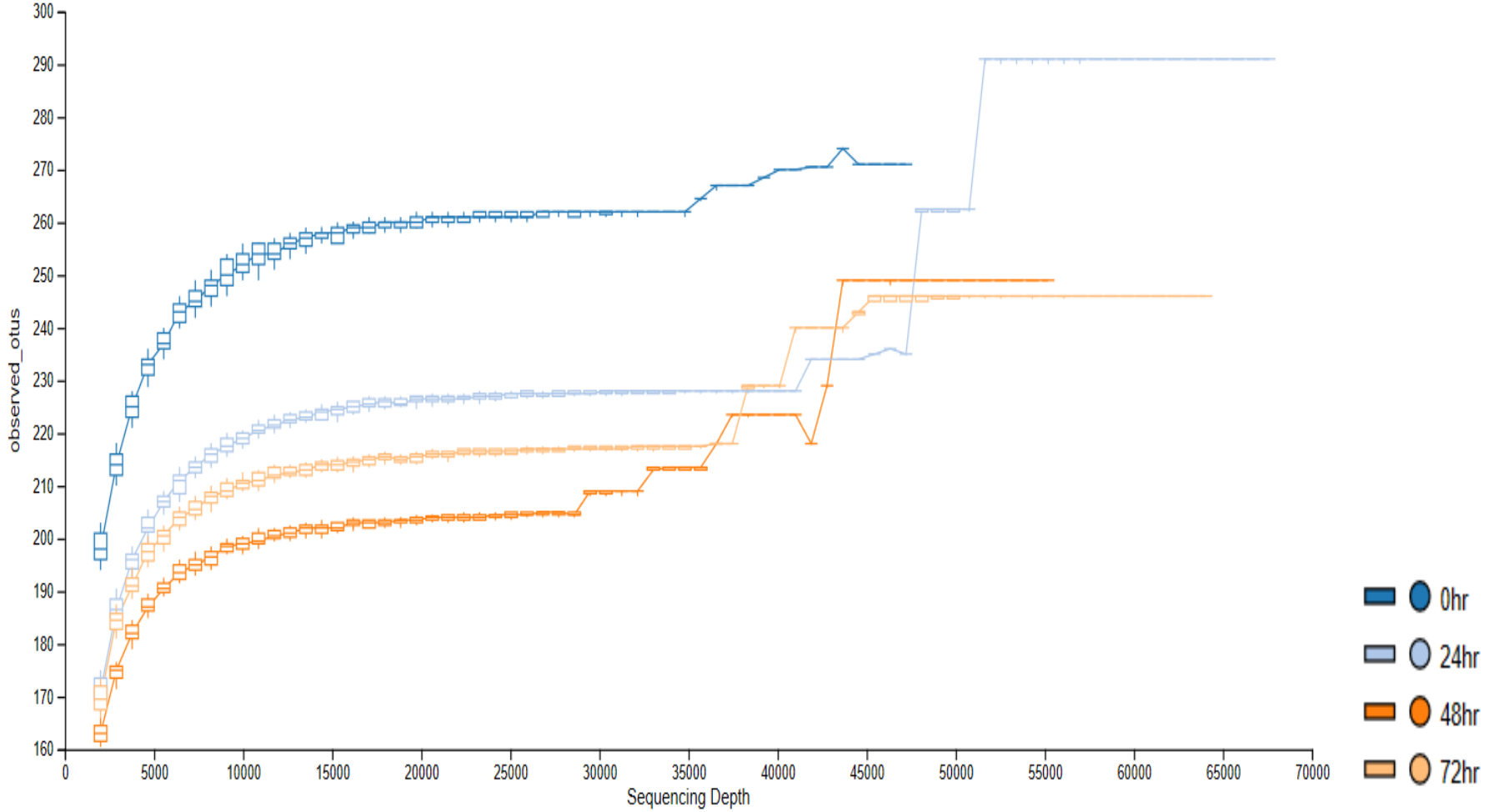

Figure 6.6 Alpha rarefaction curve of the bacterial communities at different time points using observed OTUs index (based on 99% similarity threshold).

6.3.3.2 Beta diversity (changes at species level among vessels)

Principal coordinates analysis (PCoA) profiles were constructed based on two variables: 1) treatment, and 2) time point, to show bacterial diversity among vessels. PCoA analysis profile of the bacterial communities across different treatments was measured using unweighted (qualitative) UniFrac distance metric (**Figure 6.7**) and Bray-Curtis dissimilarity (**Figure 6.8**) indices, but significance using Permutational multivariate analysis of variance (PERMANOVA) could not be calculated because of technical problem with QIIME 2 and how it reads the treatment column values. However, results in **Figure 6.7** showed that the samples clustered into four distinct groups as mentioned in the following: 1) the control strain APEC 46 clustered close to vessels containing combined treatment, 2) control and carvacrol treatment vessels were more similar to each other than the rest of the treatments, 3) ampicillin and combined treatment vessels were more similar to each other than the rest of the treatments, and 4) the remaining control, ampicillin, and combined treatment vessels. Moreover, PCoA analysis profile of the bacterial communities at different time points was measured using unweighted (qualitative) UniFrac distance metric (**Figure 6.9**) and Bray-Curtis dissimilarity (**Figure 6.10**) indices. PERMANOVA of all groups showed significant differences of the bacterial communities at different time points (overall P-value = 0.001), and significant differences (P-value < 0.05) between 0hr and all time points (24hr, 48hr, and 72hr), and between 24hr and 72hr when performing pairwise comparisons. Overall, beta diversity results showed that changes in bacterial communities across samples or vessels were due to the effect of time and not treatment.

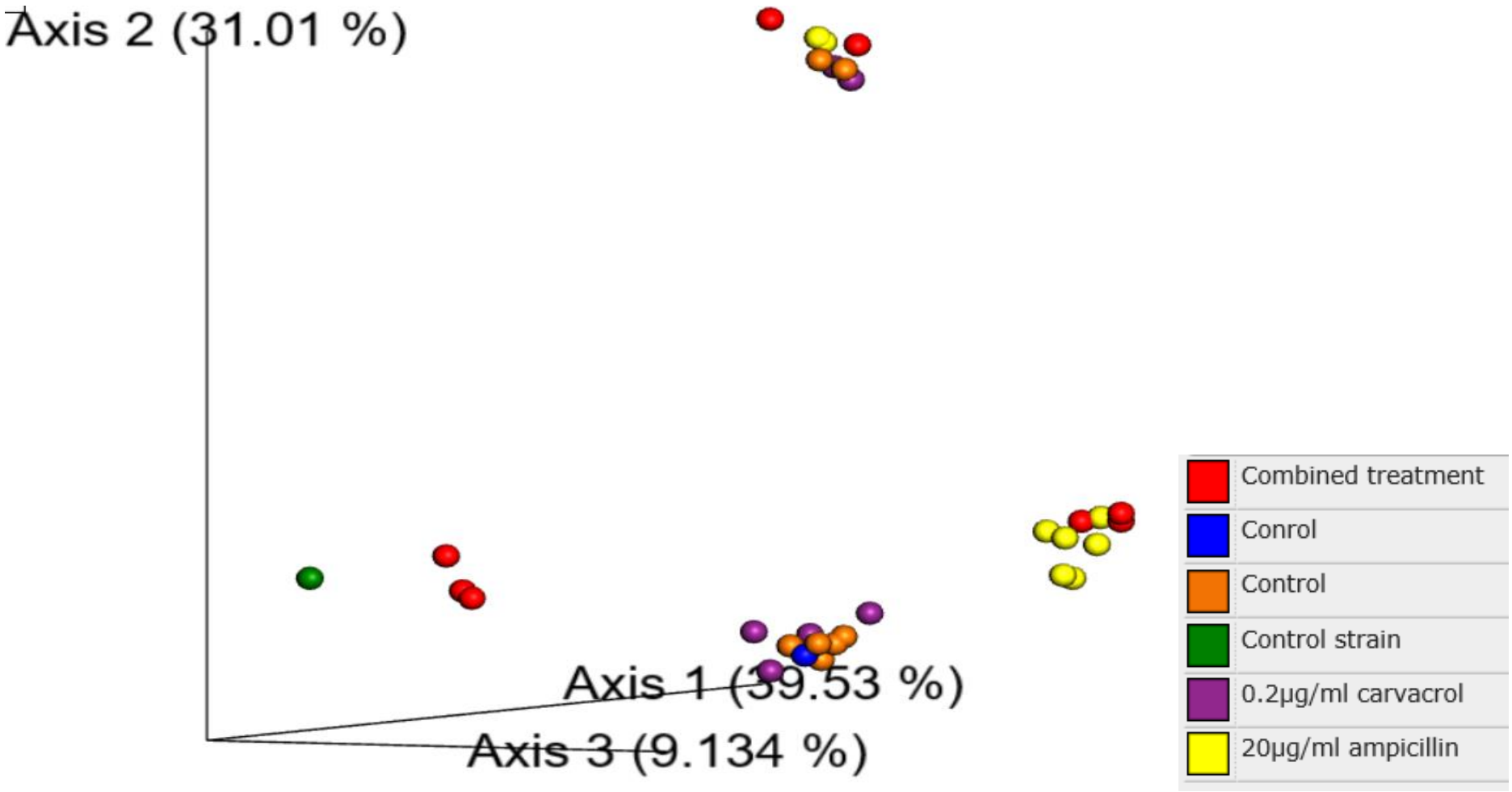

Figure 6.7 PCoA analysis profile of the bacterial communities across different treatments using unweighted (qualitative) UniFrac distance metric index.

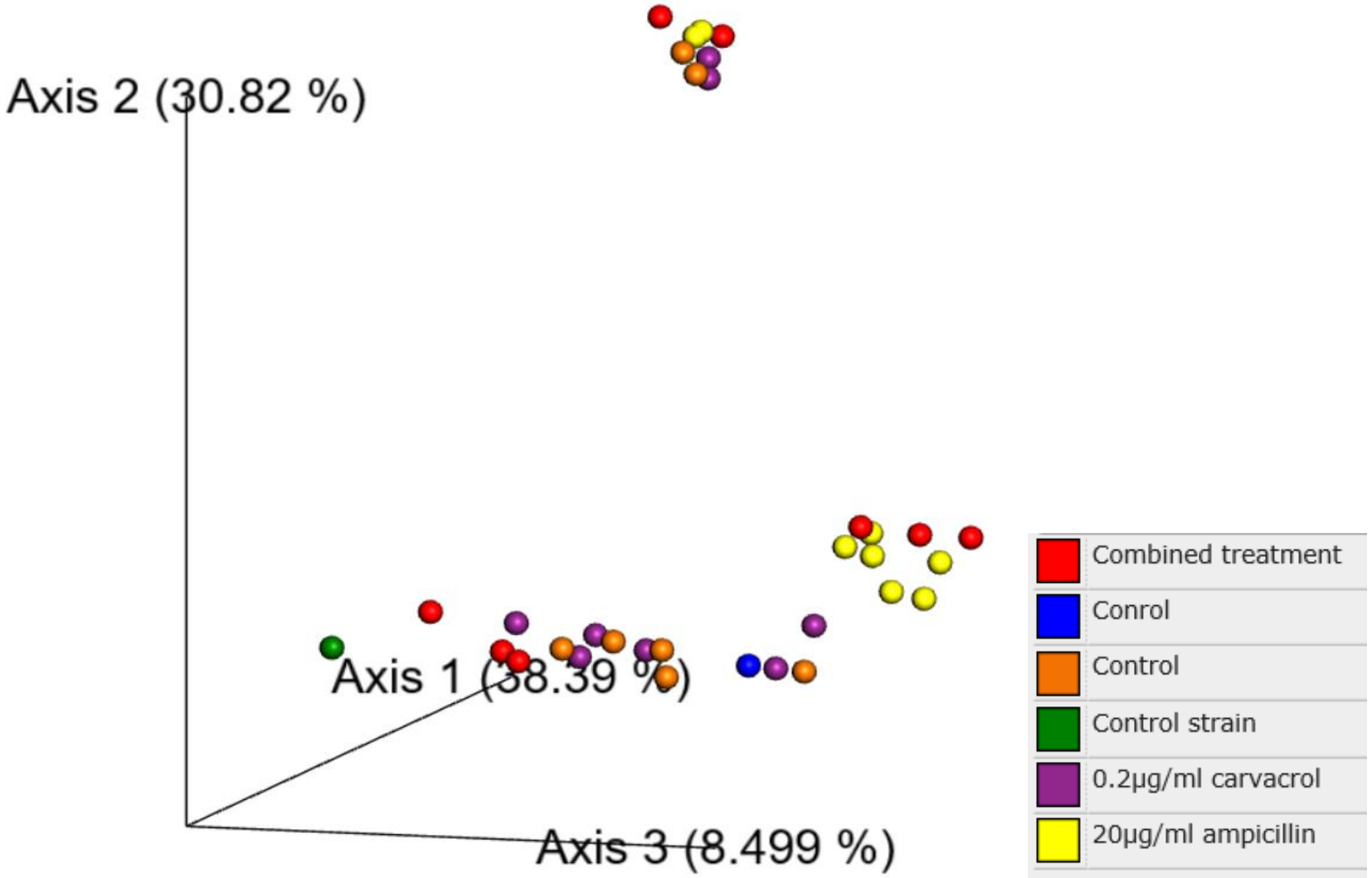

Figure 6.8 PCoA analysis profile of the bacterial communities across different treatments using Bray-Curtis dissimilarity index.

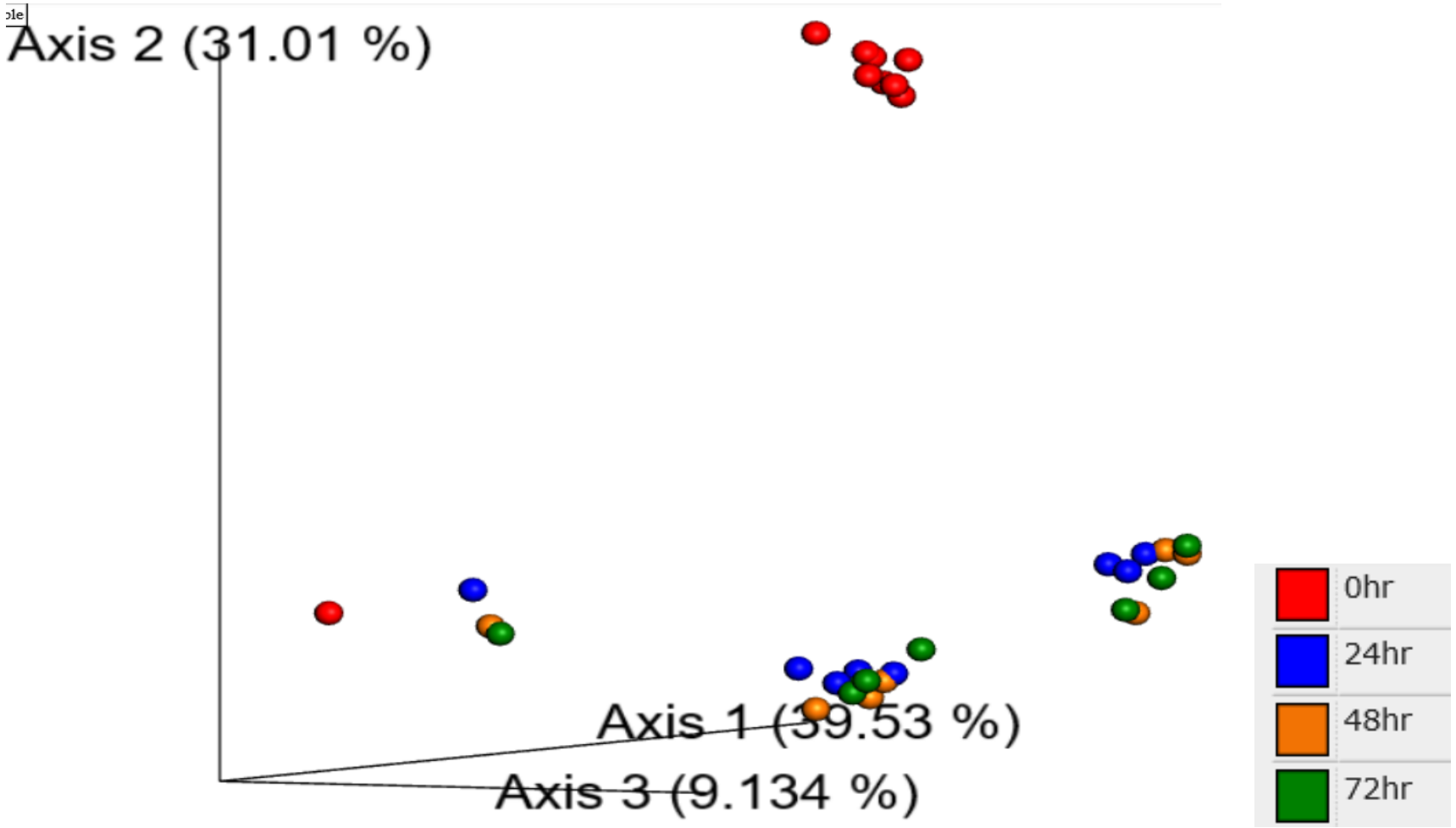

Figure 6.9 PCoA analysis profile of the bacterial communities at different time points using unweighted (qualitative) UniFrac distance metric index.

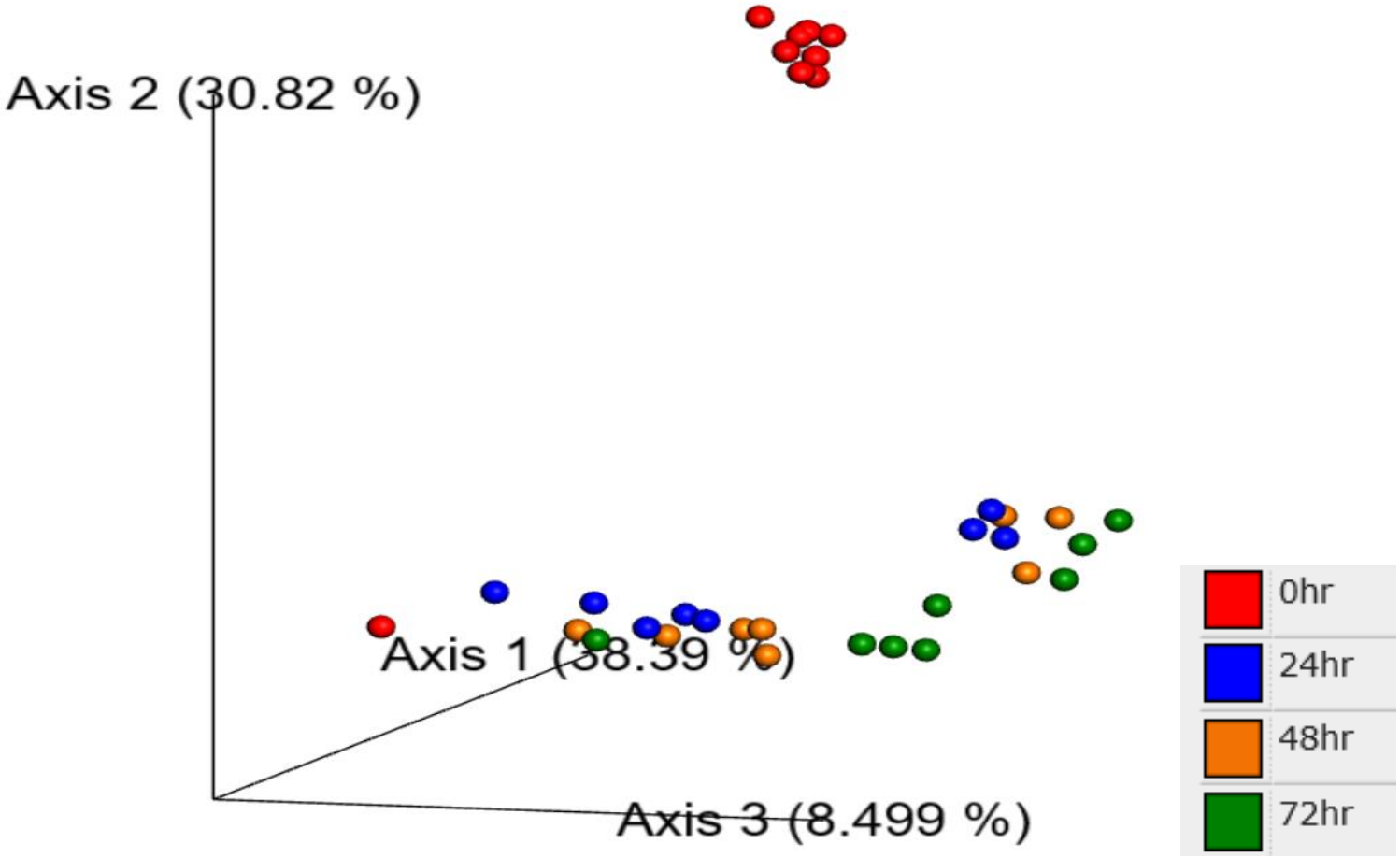

Figure 6.10 PCoA analysis profile of the bacterial communities at different time points using Bray-Curtis dissimilarity index.

6.3.3.3 Taxonomic profiling at phylum level

Taxonomic profiles were constructed based on two variables: 1) treatment, and 2) time point, to show changes in bacterial composition at phylum, class, order, family, and genus levels. **Figure 6.11** showed that there were six different phyla (Firmicutes, Proteobacteria, Tenericutes, Bacteroidetes, Cyanobacteria, and Actinobacteria) in all treatment vessels, but Firmicutes was the most abundant phylum (93% as the highest percentage in V5). **Figures 6.11** and **6.12** showed that Firmicutes was the most abundant phylum (relative frequency of ~80%) in all treatments and at different time points. The second phylum in its abundance; Proteobacteria was increased in its relative frequency to 83% in the combined treatment vessels (**Figure 6.11**) but maintained its relative frequency after 0hr at a low percentage (less than 20%) (**Figure 6.12**). Proteobacteria which includes our bacterium of interest (*E. coli*) showed its relative sensitivity to carvacrol treatment in comparison with ampicillin treatment (**Figure 6.11**), and that its number was increased to 20% after 72hr (**Figure 6.12**).

6.3.3.4 Taxonomic profiling at family level

Family-level taxonomic bar plot in **Figure 6.11** showed the presence of different families in all vessels irrespective of the treatment. The most abundant phylum; Firmicutes showed the presence of different families, and *Ruminococcaceae* was the most abundant family followed by *Lachnospiraceae* in all vessels. The third most abundant family; *Enterobacteriaceae* which includes *E. coli* showed its relative sensitivity to carvacrol treatment in comparison with other treatments (**Figure 6.11**), but it was able to maintain its growth but at low level throughout different time points (**Figure 6.12**).

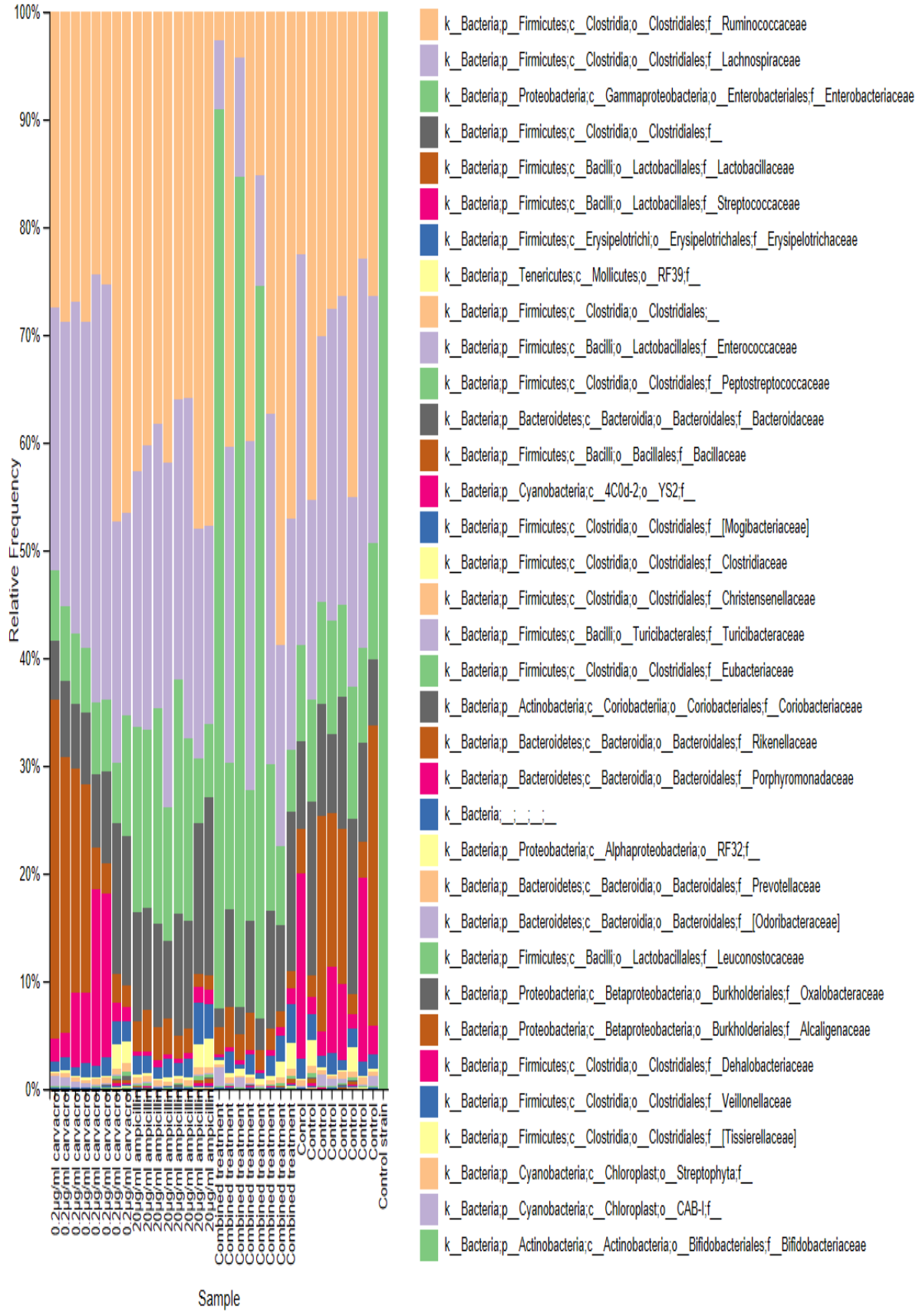

Figure 6.11 Taxonomic bar plot showing family level of the bacterial communities in different treatments.

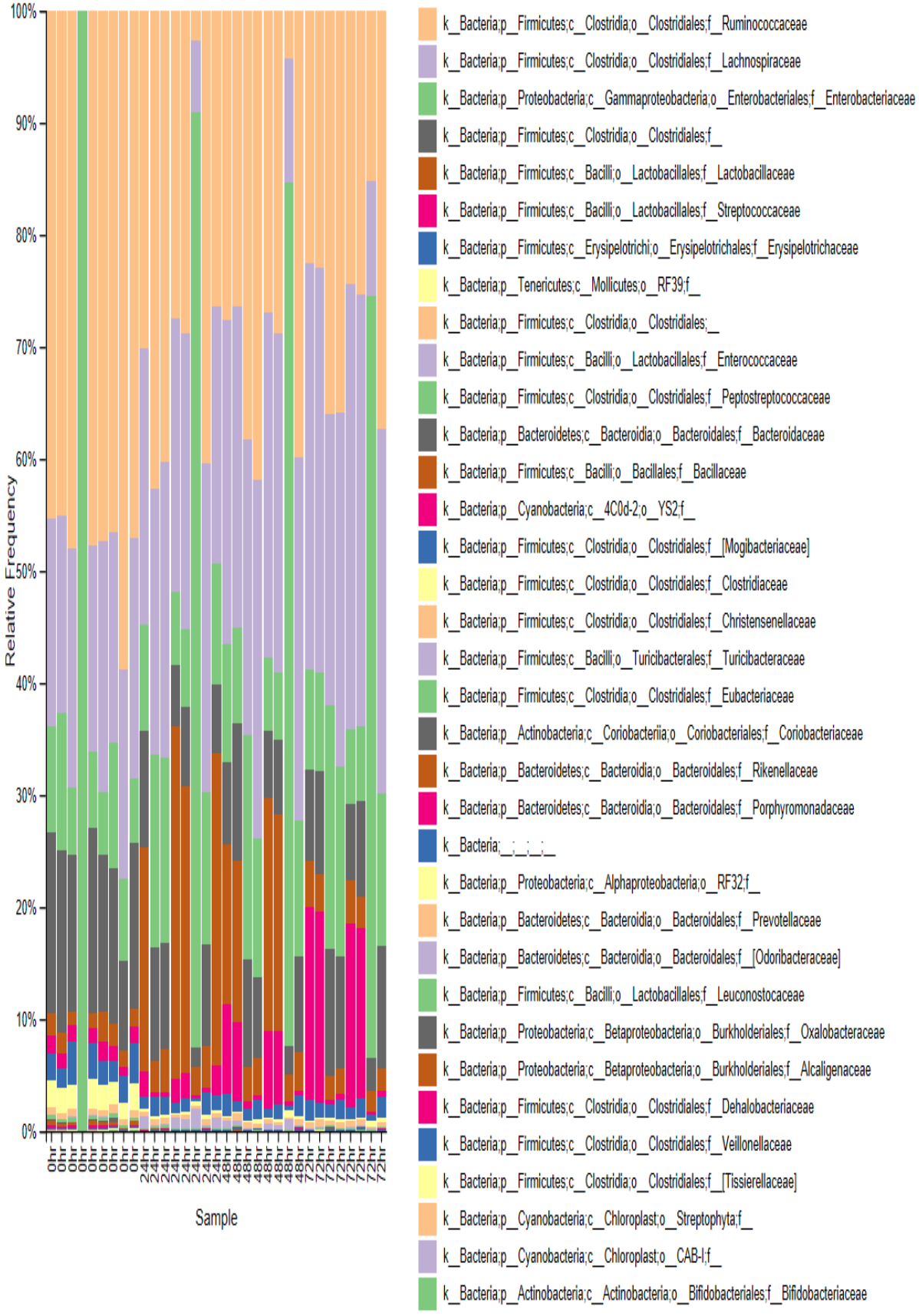

Figure 6.12 Taxonomic bar plot showing family level of the bacterial communities according to different time points.

6.3.3.5 Taxonomic profiling at genus level

Genus-level taxonomic bar plot in **Figure 6.13** showed the presence of different genera in all vessels irrespective of the treatment. The most abundant family; *Ruminococcaceae* showed the presence of different genera, and *Oscillospira* was the most abundant genus in all vessels. The added APEC 46 strain (*E. coli-Shigella* sp.) was present in all vessels, but at 24hr, its abundance was decreased in carvacrol treatment (6.5% in V5 and 6.9% in V6) in comparison with ampicillin treatment (17.3% in V3 and 16.5% in V4) and combined treatment (63.5% in V7 which was considered an outlier and 13.6% in V8) vessels (**Figure 6.13**). According to time points, it was able to survive throughout the experiment, but at low percentage (**Figure 6.14**).

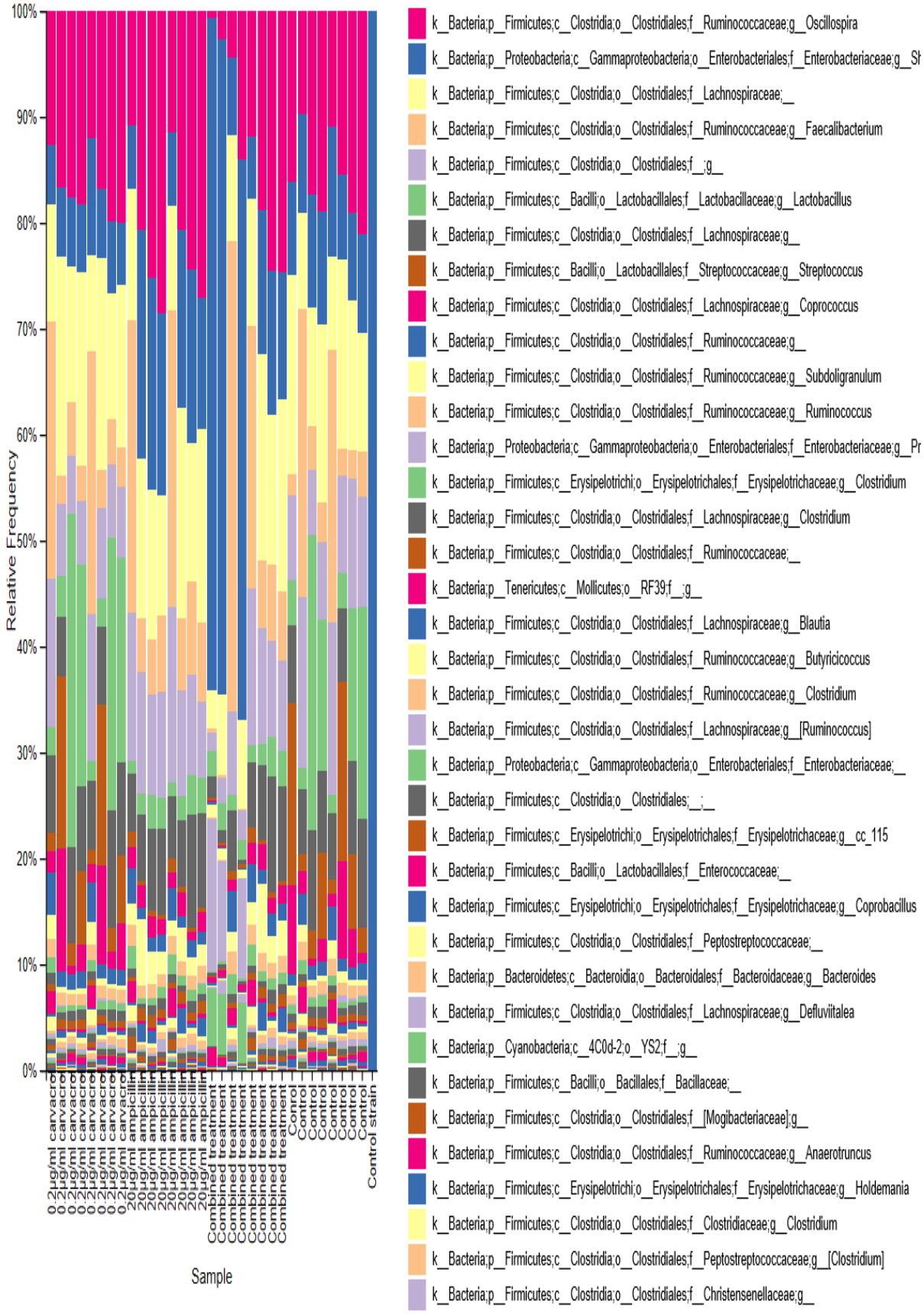

Figure 6.13 Taxonomic bar plot showing genus level of the bacterial communities in different treatments.

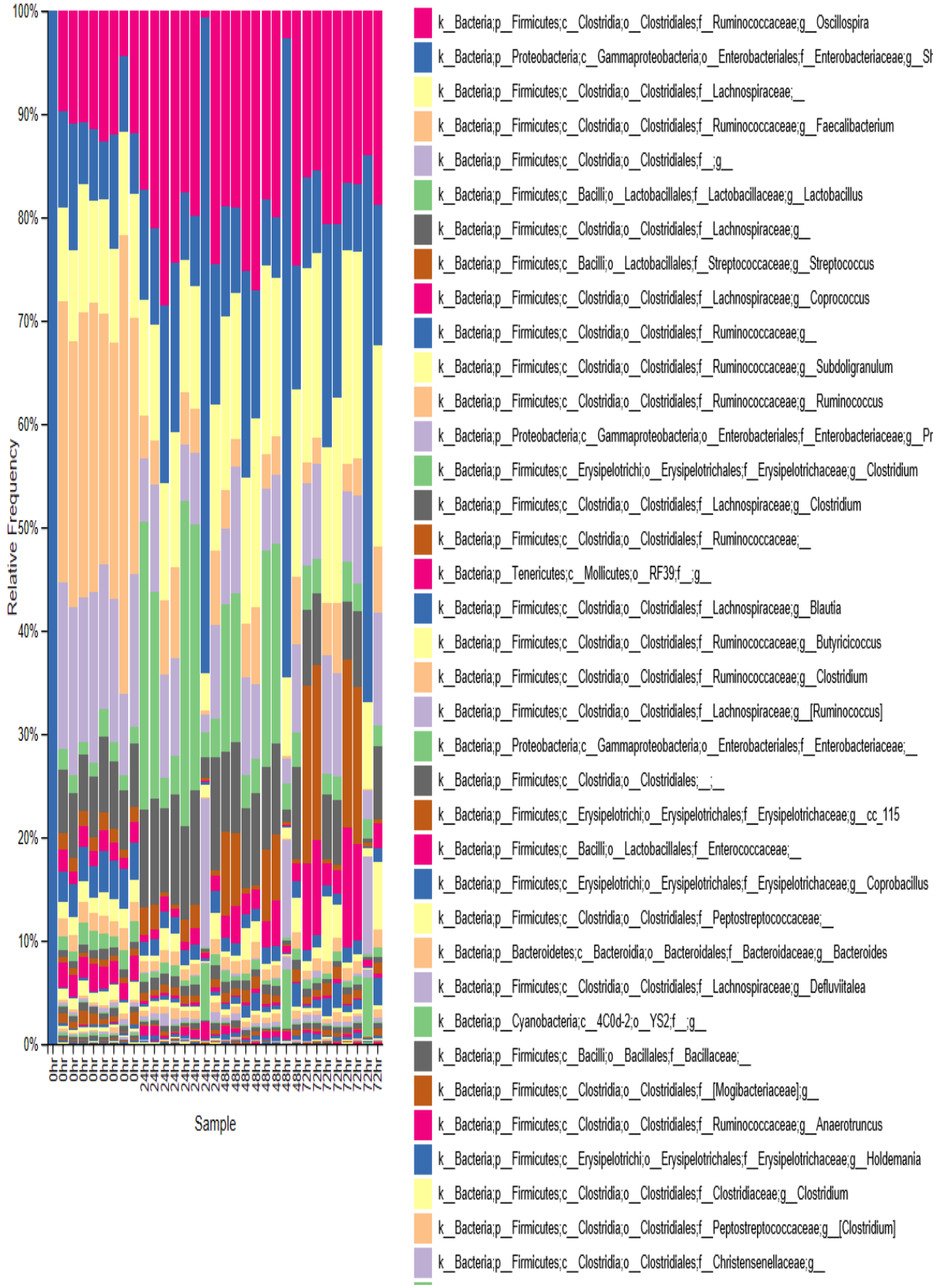

Figure 6.14 Taxonomic bar plot showing genus level of the bacterial communities according to different time points.

6.3.3.6 Taxonomic profiling at species level

Species-level taxonomic bar plot in **Figure 6.15** showed the presence of different species in all vessels irrespective of the treatment. The added APEC 46 strain (*E. coli-Shigella* sp.) was present in all vessels, but its abundance was the highest in the combined treatment vessel V7 which was considered an outlier (**Figure 6.15**). At 24hr, this strain showed its sensitivity to carvacrol treatment (6.5% in V5 and 6.9% in V6) in comparison with ampicillin treatment (17.3% in V3 and 16.5% in V4) and combined treatment vessels (63.5% in V7 and 13.6% in V8). According to time points, it was able to survive throughout the experiment, but at low percentage (**Figure 6.16**).

According to different treatments, the other noticeable changes (**Figure 6.16**) were in the following: 1) At 24hr, *Lactobacillus* sp. was present in higher percentage in control (12.5% in V1 and 17.2% in V2) and carvacrol (19.3% in V5 and 15% in V6) in comparison with ampicillin (2.4% in V3 and 3.3% in V4) and combined (2.1% in V7 and 3.1% in V8) treatment which was proven to be significant (P-value < 0.05) by the analysis of composition of microbiome (ANCOM), 2) At 24hr, *Lactobacillus salivarius* was present in higher percentage in control (9% in V1 and 6.3% in V2) and carvacrol (10.8% in V5 and 9.2% in V6) in comparison with ampicillin (less than 0.1% in V3 and 0.1% in V4) and combined (less than 0.1% in V7 and 0.1% in V8) treatment which was proven to be significant (P-value < 0.05), 3) At 72hr, *Streptococcus* sp. was present in higher percentage in control (17% in V1 and 17% in V2) and carvacrol (16.3% in V5 and 15.3% in V6) in comparison with ampicillin (0.5% in V3 and 0.5% in V4) and combined (0.3% in V7 and 0.5% in V8) treatment which was proven to be significant (P-value < 0.05), 4) At 72hr, *Coprococcus* sp. was present in higher percentage in control (8.4% in V1 and 9.2% in V2) and carvacrol (11.6% in V5 and 9.4% in V6) in comparison with ampicillin (2.1% in V3 and 2.3% in V4) and combined (0% in V7 and 2% in

V8) treatment which was proven to be significant (P-value < 0.05), 5) At 72hr, *Proteus* sp. was present in higher percentage in combined (9.2% in V7 and 0% in V8) in comparison with control (0.7% in V1 and V2), carvacrol (0.1% in V5 and 0.2% in V6) and ampicillin (0% in V3 and 0.2% in V4) treatment.

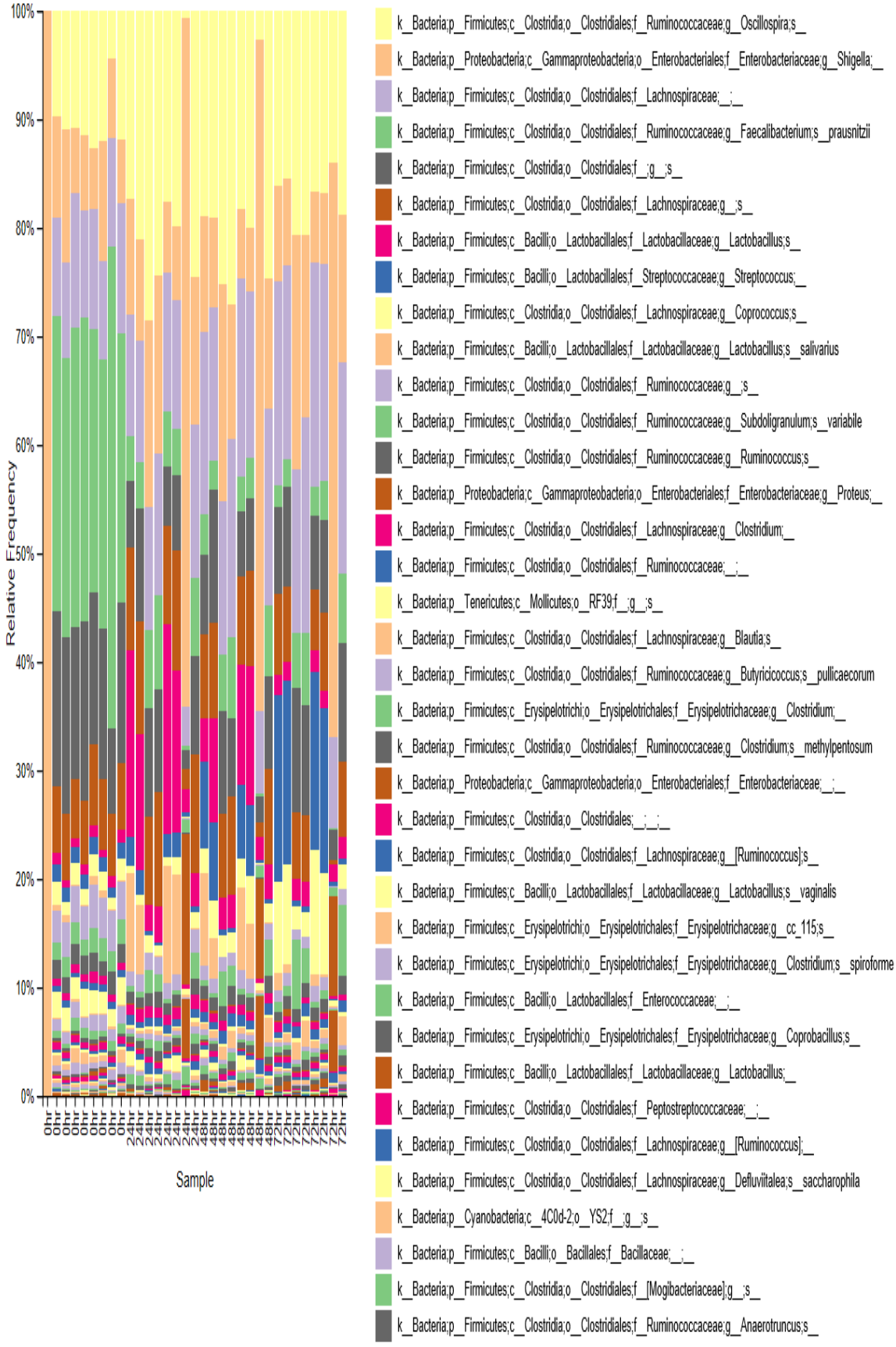

Figure 6.15 Taxonomic bar plot showing species level of the bacterial communities according to different time points.

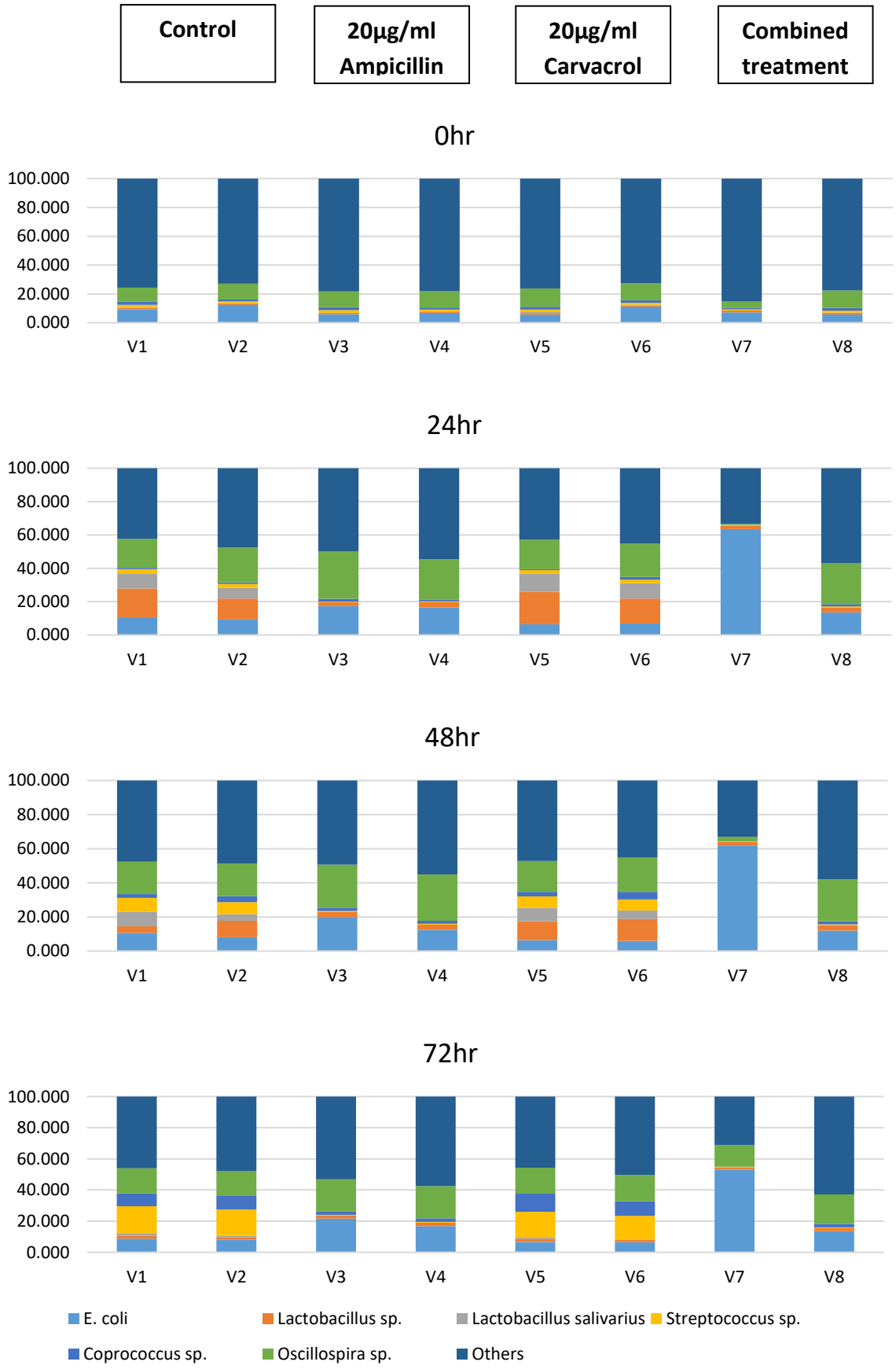

Figure 6.16 Taxonomic bar plots showing major changes in some bacterial species due to treatment effect at different time points.

6.3.4 Combining GC and NGS results

In order to link between bacterial species and their metabolic by-products, **Figure 6.17** was constructed. These were the bacterial species found in the pooled caecal samples and as identified by 16S rRNA sequencing results, and which were associated with SCFAs profile. However, at this point, it was not possible to determine which bacterial species made the greatest contribution to the produced SCFAs.

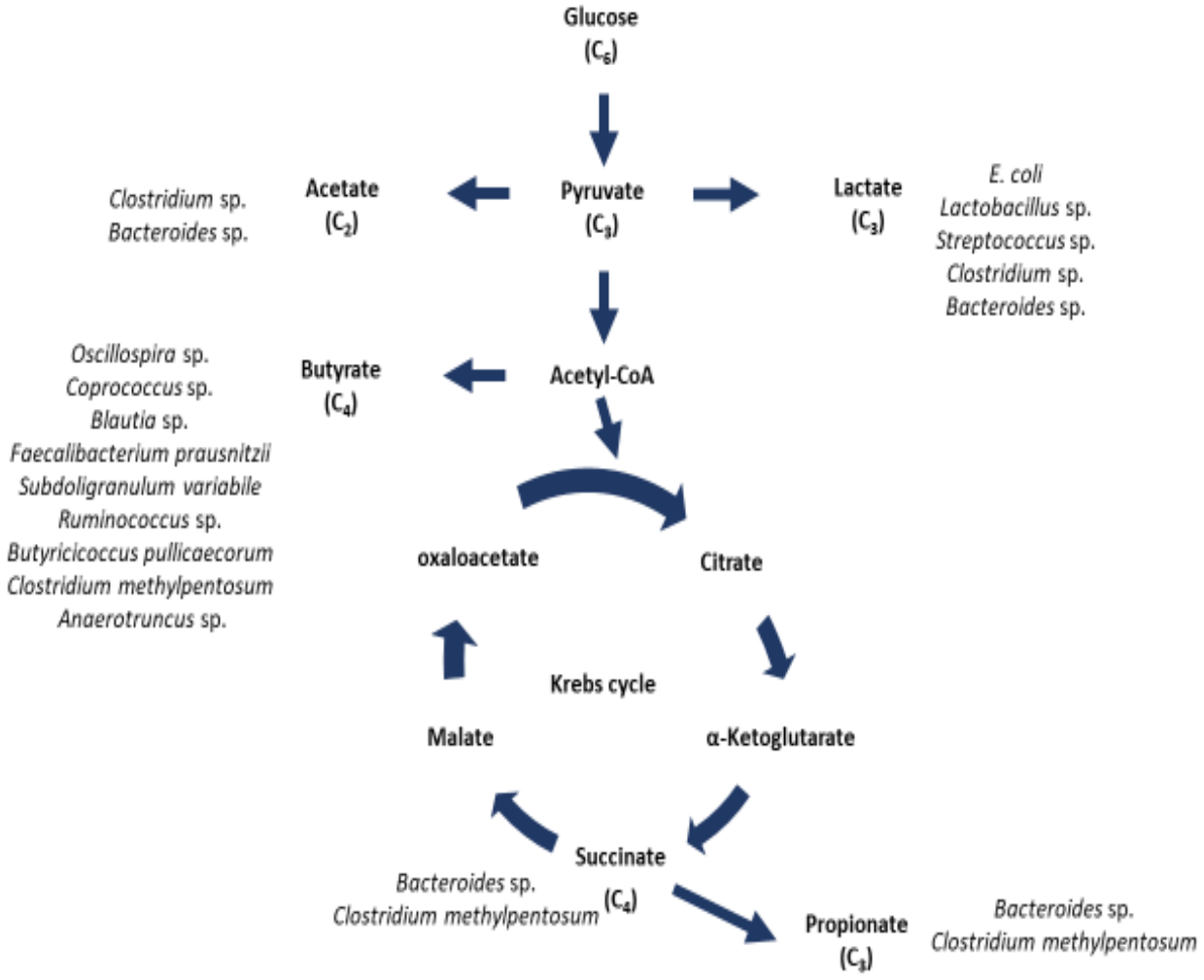

Figure 6.17 Major metabolic pathways of carbon metabolism in the chicken caeca with the major bacterial species responsible for their production. Adapted from (Józefiak et al., 2004).

6.4 Discussion

The previous chapter (**Chapter 5**) showed that three *E. coli* strains grew and survived in batch culture with sub-lethal carvacrol and/or ampicillin treatments suggesting the stresses applied were insufficient to control these three microorganisms. There are two issues to be considered: 1) the dose of the treatments was calculated to be the likely doses that would reach the caecum in the bird given current therapeutic dosing with ampicillin and oregano supplementation as used on farm, 2) the study in the previous chapter (**Chapter 5**) involved pure cultures of *E. coli* that were not exposed to the complexity of caecal contents in terms of biochemical and microbiological diversity. Thus, the study in this chapter involved exposing one APEC strain (APEC 46) to a more complex environment (pooled caecal contents) in the presence of a mixed bacterial community. Three measurement parameters were monitored in this study; total viable counts of *E. coli*, production of SCFAs, and changes of bacterial population at different taxonomical levels to assess the impact of different treatments.

To assess the modulatory effect of sub-lethal concentrations of carvacrol on caecal contents challenged with an APEC 46 strain, the total viable number of *E. coli* and ampicillin-resistant *E. coli* present was achieved by simple culturing method. Results in **Figures 6.1** and **6.2** showed that there was a significant increase (P-value < 0.05) in the total number of viable *E. coli* and ampicillin-resistant *E. coli* by time in all vessels, indicating that the *E. coli* grew over the course of the experiment and were not suppressed by treatment or by the rest of the microbial community. This increase in *E. coli* numbers does not reflect the *E. coli* numbers in the actual chicken gut given the age of the birds. While this confirms that VL medium supports the growth of *E. coli* (Lei et al., 2012), there was an insignificant difference (P-value > 0.05) by each treatment on the numbers of *E. coli*, and ampicillin-resistant *E. coli* (**Figures 6.1** and **6.2**). This suggests that the *E. coli* being counted were either APEC 46 or APEC 46 and a

resident ampicillin-resistant Gram-negative bacterial strains found in the pooled caecal contents. Given the final concentration of the added APEC 46 strain (10^5 CFU/ml in the 20ml vessel), it is likely that the numbers of the *E. coli* equated to the input APEC strain dose and not to resident *E. coli* strains. This could have been confirmed by genetic testing for similarities e.g. MLST, but it was not an essential addition to the study as the treatment failed to suppress any *E. coli*.

There were differences in the counting (two log difference) at 0hr between treatments, which should have all been the same, but these counts were significantly increased (P-value < 0.05) by time and there were no significant differences (P-value > 0.05) between treatments at 24hr and 48hr. This technical issue stems from the fact that the nature of cecal content is very condensed, so it was not be easily mixed in VL medium resulting in precipitation of big clumps, and this may explain the differences of counting at 0hr in the vessels. It may be concluded that differences in starting numbers of *E. coli* were not proven to be a contributing factor here. However, in order to validate these data sets and confirm the lack of effect of each treatment on the *E. coli* numbers, this experiment should be repeated several times with an extra vessel for each treatment. The lack of anti-bacterial effect of carvacrol on the *E. coli* numbers might be due to the use of a very low concentration of carvacrol (0.2μg/ml) which was not enough to cause *E. coli* suppression, as it was shown by a previous batch culture study suggesting the efficacy of carvacrol on gut microbiota but at higher concentrations (Lei et al., 2012). Of course, the impact of higher concentrations on other bacteria would need to be assessed as well and considering possible impacts on the host cells would also need evaluation.

The gut environment contains substrates such as simple sugars and starch which are used by the bacteria colonising epithelium layer of the intestine to provide energy for its host (Stevens and Hume, 2004). Therefore, it was important to understand the carbon metabolism of bacterial population in the chicken gut that was challenged with an APEC strain and this was done by interrogating production of SCFAs as part of bacterial metabolic profiling. Generally, the highest produced SCFAs was lactate which can be formed as a by-product from multiple pathways. Also, this reflects the actual environment they came from where anaerobic conditions stimulate fermentation processes, and that the caeca represent the major site for fermentation with the highest numbers of bacteria. Bacterial population and type and quantity of the substrates entering the caeca determine the type of fermentation (Annison et al., 1968). However, one must consider that the results in **Table 6.2A** and **6.2B** are only an estimate of the residual SCFAs in the chicken caeca, as these SCFAs can be utilised by other bacteria in different and/or distant parts of the GIT away from the caecum (Apajalahti et al., 2002), and moreover, the caecal samples were diluted in VL broth medium.

Propionate, butyrate, and acetate are the major SCFAs produced in the caeca with an important role in the establishment of gut microbiota during chicken growth (Ricke, 2003). The results in **Table 6.2A** demonstrate the presence of propionate and butyrate which was proven to be significantly increased (P-value < 0.05) over time, suggesting the abundance of propionate and butyrate-producing bacteria which may be causing an inhibitory effect on other bacteria producing other SCFAs (acetate, lactate, and succinate) or due to the effect the batch culture system. Propionate has anti-bacterial properties with the ability to inhibit colonisation of salmonellae infection in chicks (Nisbet et al., 1996). Butyrate has higher anti-bacterial properties when it is in the form of an undissociated acid (Lesson, 2007) and takes on an important part in the development of the intestinal epithelial cells (Brouns et al., 2002). Also,

the presence of very low concentrations of succinate in comparison with propionate and butyrate might be explained by the conversion of succinate to propionate, hence the decrease in succinate and in result, the increase in propionate concentration. Succinate is part of the Krebs cycle and its reduction suggests that there is a metabolic shift from respiration to fermentation (Picone et al., 2013). In terms of treatment, a linear mixed model showed that there were no significant differences (P-value > 0.05) between SCFAs production and the effect of each treatment, except for lactate, which was proven to be significantly different (P-value = 0.0029) between carvacrol and combined treatment vessels at 0hr only, which might be due to the changes from *in vivo* to *in vitro* conditions. This is a curious finding as much as it would be anticipated that every vessel should have had identical starting compositions, but it is possible that the initial exposure to the treatments even at sub-lethal concentrations for at least *E. coli* may have induced metabolic responses by the population.

Whole population profiling by 16S sequencing approach was considered an appropriate technique for use in this study to observe any changes in bacterial populations that may explain the interesting differences shown in SCFAs profile and/or show treatment effect. Previous experiments relied on culture-dependent techniques which involved laborious plating and classical or phenotypic procedures, but population profiling is a culture-independent method to qualify and quantify the bacterial population in the batch cultures derived from pooled caecal contents. Conventional methods allow only a small measurement of microbial populations with known culturing methods and rarely can determine their species (Apajalahti et al., 2003, Oliver, 2005). Therefore, it was essential to introduce effective and efficient technologies such as NGS to look at a better and clearer picture of the bacterial community in a non-biased way without worrying about the issues of classical methods (Apajalahti et al., 2004). In this chapter, the intention was to look at the changes of bacterial communities at different taxonomic levels with

a focus on the added APEC 46 strain using different treatments and at different time points. The caecal contents showed the dominance of Firmicutes as the first phylum followed by Proteobacteria, which is in agreement with many previous studies (Lan et al., 2002, Wei et al., 2013, Shaufi et al., 2015). The predominance of Firmicutes was not affected by treatment nor time, and this probably explains the significant increase of butyrate over time (**Table 6.2A**) as it is produced by Firmicutes (Eeckhaut et al., 2011), and as shown by different Firmicutes members in **Figure 6.17**. The second most abundant phylum; Proteobacteria which includes facultative anaerobic *E. coli* was present in all vessels during the course of the experiment but at low percentages, and this might be due to the chicken age (Ballou et al., 2016). This comparatively low percentage of Proteobacteria in the vessels at the start of the experiment was increased in the ampicillin and combined treatment in comparison with control and carvacrol treatment. The presence of the remaining phyla (Tenericutes, Bacteroidetes, Cyanobacteria, and Actinobacteria) was documented by a previous study (Corrigan et al., 2015), but were present in very low percentages throughout the course of the experiment.

At the family level, the cecal contents were predominated by *Ruminococcaceae* followed by *Lachnospiraceae* which are Firmicutes and this is in agreement with a previous study (Danzeisen et al., 2011), which harbour butyrate-producing anaerobic bacteria (Antharam et al., 2013). The third most abundant family; *Enterobacteriaceae* which includes *E. coli*, was present but in a low percentage, as it was noticed that the high levels of the SCFAs (propionate and butyrate in specific) decrease the total viable count of *Enterobacteriaceae* in chicken caeca (van der Wielen et al., 2000).

Generally, the caecal contents showed the presence of diverse bacterial communities which included different genera and species which confirms the finding of previous studies (Amit-Romach et al., 2004, Bjerrum et al., 2006, Gong et al., 2007). The predomination of *Oscillospira* sp. was documented in a previous study (Wang et al., 2016) which is a butyrate-producing bacterium (Gophna et al., 2017) as shown in **Figure 6.17**. Other bacterial species were present but in lower percentage such as *Lactobacillus* sp., *Streptococcus* sp., and *Clostridium* sp., which is similar to what was found in a previous study (Lu et al., 2003), and they might be responsible for the presence of high concentrations of lactate (Józefiak et al., 2004) in comparison with the remaining SCFAs as shown in **Figure 6.17**.

Overall, the added APEC 46 strain with CFU of 10^7 per ml as this is the required concentration to cause an infection (Maturana et al., 2011). As a strain, it was able to survive throughout the course of this experiment (0hr to 72hr) but at low percentage (**Figure 6.14**), but it was not possible to know its source; whether it came from the added APEC 46 strain or from the caecal sample. This might be solved by using *E. coli* specific primers with fluorescent labelling to track it. However, by looking at the species level, only two bacterial species were detected from the family *Enterobacteriaceae* which involved *E. coli-Shigella* sp. and *Proteus* sp. (**Figure 6.15**). This might explain that the source of *E. coli-Shigella* sp. came from the added APEC 46 strain and not the pooled caecal contents. The relative abundance of *E. coli-Shigella* sp. was decreased at 24hr in carvacrol treatment in comparison with ampicillin treatment and combined treatment vessels, and this may show the efficacy of sub-lethal concentration of carvacrol to control the numbers of *E. coli-Shigella* sp. present in pooled caecal contents.

Other changes were noticed at the species level in carvacrol treatment, but at different time points. At 24hr, carvacrol treatment showed a significant increase (P-value < 0.05) in *Lactobacillus* sp. and *Lactobacillus salivarius* which then decreased at 72hr. *Lactobacillus* sp. is a beneficial probiotic strain which is proven to be responsible for prevention of *Salmonella* colonisation in chickens (Pascual et al., 1999). Also, it might be responsible for prevention of APEC 46 strain (*E. coli-Shigella* sp.) to colonise and start an infection. At 72hr, carvacrol treatment showed a significant increase (P-value < 0.05) in *Streptococcus* sp. and *Coprococcus* sp. in comparison with the remaining treatments. *Streptococcus* sp. is an anaerobic bacterium present mostly in the chicken caeca (Barnes et al., 1977), increases in number as part of the mature microbiota after 40 days (Coloe et al., 1984), and might be exhibiting anti-salmonella activity in young chicks along with other anaerobic bacteria (Barnes et al., 1979). *Coprococcus* sp. is also an anaerobic bacterium found in chicken caeca with an anti-Salmonella activity in young chicks (Barnes et al., 1979). Thus, carvacrol treatment showed modulatory effect on the bacterial population inhabiting chicken caeca challenged with an APEC strain in result of an increase in beneficial probiotic bacteria and bacteria with anti-Salmonella activity. These promising results might support the idea that carvacrol as a feed additive (used at a very low concentration of 0.2µg/ml) might enhance avian gut health and might be a good substitute to antibiotics in poultry industry. However, this needs to be repeated several times to confirm these results and eventually be tested *in vivo* as well.

Ampicillin and combined treatment showed an increase in *E. coli-Shigella*, and *Oscillospira* sp. at 24hr and decrease in the previously-mentioned beneficial bacteria. This was anticipated as the administration of antibiotics including ampicillin was proven to decrease the number of *Lactobacillus* sp. (Guban et al., 2006). Also, this confirms that the use of antibiotics causes dysbiosis (Atterbury et al., 2007) which is a challenging problem in animal production. This

shift in microbiota may result in an increase in the harmful or pathogenic bacteria and a decrease in the beneficial bacteria which might be associated with intestinal diseases (Lupp et al., 2007). On the other hand, *Proteus* sp. which is a facultative anaerobic bacterium isolated from caeca of pathogen-free chickens (Coloe et al., 1984) was noticed at a higher percentage at 24hr and in the combined treatment in comparison with the remaining treatments. As a bacterial species, it can also cause deadly infection such as yolk sac infection or omphalitis in post-hatched young chicks (Iqbal et al., 2006), and one of the causative agent of UTI in animals and humans (Jones et al., 1990). The adverse effect of carvacrol when added with ampicillin is a cause of concern and does not reflect an overall synergistic effect as proven earlier (**see Chapter 5**). This might be due to the use of very low concentration of carvacrol (0.2µg/ml) in comparison with ampicillin concentration (20µg/ml). *In vivo* studies are required to confirm these findings in broiler production systems.

The previously mentioned observations reflect what happened *in vitro* and it might be different from what is happening *in vivo* for the following reasons: 1) closed batch culture system which is different from the actual chicken gut, 2) focusing on caecal contents and ignoring the remaining chicken gut system which are connected to each other, 3) transportation of samples from farm to laboratory might led to the loss of bacterial population, 4) effect of different intrinsic and extrinsic factors on avian gut health which are neglected here.

This *in vitro* study provides introductory information that can inform the design of more complex ones such as 1) effect of each treatment before and after continuous addition of sub-lethal concentrations of carvacrol and ampicillin on a daily basis, 2) *in vitro* chicken gut model, and with different combinations of sub-lethal concentrations of ampicillin and carvacrol, and

at the same pH and at different pH values to mimic different parts of the chicken GIT system, in order to determine their effect in a successional or dose-dependent manner, 3) *in vivo* study include challenging healthy chickens with an APEC strain, and then study the effect of each treatment, and what might happen throughout the life cycle of the chickens, 4) *in vivo* study to look at the chicken gut microbiota in chickens fed diet supplied with oregano herbs.

In conclusion, sub-lethal concentration of carvacrol treatment showed modulatory effect on the bacterial populations inhabiting the chicken caeca challenged with an ampicillin-resistant APEC strain. Thus, carvacrol as a feed additive might enhance avian gut microbiota and may be used in novel ways to control potentially pathogenic and antibiotic-resistant APEC strains and provide alternative strategies to the use of synthetic antibiotics.

CHAPTER 7: General discussion

Antibiotics have become less effective in supporting poultry health and productivity and in controlling bacterial pathogens responsible for current issues in the poultry industry, because over- and inappropriate-use has selected ARBs. These ARBs might disseminate and colonise the human gut eventually (Schjørring and Krogfelt, 2011). Resistance has emerged through various mechanisms including chromosomal mutations such as *gyrA* mutants giving rise to quinolone resistance and the accumulation of horizontally transmissible elements such as transposons and plasmids that encode resistance genes. Thus, the overall aim of this thesis was to establish the potential role of certain phytochemicals as plant-based dietary supplements to promote poultry health and productivity and by assessing their anti-bacterial properties to control bacterial pathogens such as APEC including antibiotic-resistant APEC in animal production. A primary aim was to assess whether certain phytochemicals may reduce the burden on antibiotic use for pathogen control with APEC as an exemplar pathogen. In order to support these aims, the general testable hypotheses of this thesis were the following: 1) whether it is possible to control APEC pathogens responsible for current issues in poultry production using phytochemicals or not? 2) whether it is possible to use phytochemicals to decrease the antibiotics residue in poultry production or not? 3) whether it is possible to mitigate antibiotic resistance in poultry using phytochemicals or not?

Of the five main groups of phytochemicals (Martins et al., 2016), polyphenols have been shown to have nutritional values contributing to chicken welfare (Surai, 2014) and human health (Dueñas et al., 2015), and furthermore, polyphenols in the form of essential oils, such as carvacrol and oregano, showed inhibitory effect on many micro-organisms including antibiotic-resistant *E. coli* strains, which are widespread among the guts of poultry raised for

human consumption (Linton et al., 1977b). The focus of this study was upon poultry production for several key reasons. First, this is the most rapidly growing animal production sector, particularly white meat production that is used extensively and increasingly as a source of protein in human diets (Speedy, 2003). Second, poultry is a known source of major zoonotic pathogens including *Salmonella enterica* and *Campylobacter jejuni*, for which control measures are urgently needed (Hong et al., 2003). Third, the incidence of antibiotic resistance in poultry production is very high (Apata, 2009, Fuh et al., 2018, Amador et al., 2019), and considered to be a potential source of resistant micro-organisms entering human via poultry products (Smith et al., 2002). Fourth, poultry production suffers from several common bacterial diseases such as avian colibacillosis caused by APEC strains (Sojka and Carnaghan, 1961), necrotic enteritis caused by *Clostridium perfringens* Type A or C (Keyburn et al., 2008), ulcerative enteritis caused by *Clostridium colinum* (Berkhoff, 1985), and swollen head syndrome caused by the combination of two agents; rhinotracheitis virus and *E. coli* (Morley and Thomson, 1984) which need to be controlled by means other than antibiotics. Fifth, *E. coli* which is one of the first bacteria to colonise the gut (Hudault et al., 2001), is the most common carrier of ESBLs-producing antibiotic resistance (Donachie and Begg, 1970). Sixth, *E. coli* is one of the best studied bacteria in laboratories, have commensal and pathogenic types (Hobman et al., 2007), and is responsible for most cases of UTIs in humans (Stamm and Hooton, 1993) with suggestion that UTI are caused by zoonotic APEC.

Chapter 2 aimed at studying the behaviour and setting differential criteria different APEC and commensal *E. coli* strains using different biochemical and genetic markers in order to use them as representatives of the poultry gut to study the anti-bacterial roles of phytochemicals. The reasons behind this is because: 1) there is no clear definition that separates APEC from commensal *E. coli*, as there are two definitions for APEC strains according to Nolan laboratory

(Johnson et al., 2008) and Woodward laboratory (Cordoni et al., 2016), 2) poultry gut usually harbours both commensal and pathogenic strains which some of them might be zoonotic (Stanley et al., 2014), and under the state of normobiosis, commensal strains outnumber pathogenic strains (Floch, 2011), 3) healthy broiler chickens gut can serve as a reservoir for APEC strains harbouring virulence genes similar to commensal *E. coli* strains isolated from chickens of the same flock or of faecal sources (Kemmett et al., 2013), 4) commensal *E. coli* strains might be opportunistic pathogens with the possibility of causing colibacillosis disease under certain stresses (Wigley, 2015), 5) commensal bacteria or commensal *E. coli* strains of AMR nature might facilitate the transfer of AMR genes to the pathogenic strains (Andremont, 2003, Blake et al., 2003). Most importantly what came out from this chapter was that APEC strains were able to utilise more sets of sugars, possess more set of virulence and antimicrobial resistance genes, and thereby most of the APEC were able to cluster separately from the commensal chicken and turkey strains. Also, chicken strains clustered in between APEC and turkey strains which as discussed are likely to be a mixture of commensal and pathogenic types, and that turkey strains seemed to be distinct and using the challengeable criteria of this study maybe considered the "most commensal" types of the three groups. This might give them advantages to colonise the gut and compete with the commensal strains, and therefore higher chance of survival in the presence of sub-lethal concentrations of phytochemicals. These studies were only initial studies which included limited number of strains (APEC = 12 strains, commensal chicken strains = 9 strains and commensal turkey strains = 10 strains) and therefore future studies should include more in-depth studies such as WGS approach in order to fully understand the genetic make-up of each criterion, and also more strains should be included to establish powerful set of data.

Chapter 3 looked at the ability of the two phytochemicals (carvacrol and oregano) to act as anti-bacterial agents by looking at their ability to inhibit *E. coli* growth, to form biofilm, and to produce SCFAs through aerobic and anaerobic pathways. Results showed that these phytochemicals were able to inhibit their growth at very low concentrations in comparison with antibiotics. However, the effect of sub-lethal concentrations of phytochemicals did not show any changes in affecting aerobic and anaerobic pathways of *E. coli* strains by looking at the GC results. Also, these phytochemicals were effective in inhibiting biofilm formation that is associated with virulence and survival mechanisms, provides a protective layer against penetration of antibiotics (Fux et al., 2005) and facilitates transfer of ARGs through conjugation (Hausner and Wuertz, 1999). Thus, future experiments should include the following: 1) transcriptomic studies by looking at the mRNA profile of the genes encoding for production of acetate and lactate to understand the genetic basis behind it, 2) competitive index studies to demonstrate whether chicken or turkey isolates will be selected by chicken or turkey hosts. More studies are needed to support these observations, as to our knowledge, this is the first study to include the effect of sub-lethal concentrations of phytochemicals on the production of SCFAs as part of the carbon metabolism of the *E. coli* strains of commensal and pathogenic types. This might be achieved by considering a more holistic approach such as nuclear magnetic resonance (NMR) metabolomics which is needed in order to understand the effect of these phytochemicals on other metabolic pathways occurring at the *E. coli* cell level.

Chapter 4 investigated the possible mechanisms of actions of these phytochemicals, as there is limited knowledge in literature. Using WGS approach to link between phenotype and genotype, the trained strains showed changed phenotypic behaviours featured by an increase in MIC value. This was associated with genetic changes due to the presence of missense mutations in genes responsible for antibiotic resistance (*marR*) and survival mechanisms under

unfavourable acidic conditions (*cadC*). These behavioural changes were detected after long period of training (60 days) which might indicate that *E. coli* strains cannot easily develop resistance against these phytochemicals which is contrary to the case of antibiotics. This needs to be investigated again using the same experiment set-up to confirm these findings including higher number of strains. Collectively, these mutations did arise in genes associated with different roles; antibiotic resistance and defence or survival mechanisms in *E. coli* strains. Also, this needs to be investigated *in vivo* to confirm these results, as oregano is now used commercially as a feed additive and yet no studies have been reported that cover the possible shift in microbiota or emergence of adapted strains.

Growth experiments were performed using *E. coli* mutants obtained from Keio library which were limited in the first instance to mutants in efflux pumps and PBPs genes. Results showed that carvacrol might have specific target sites in the cell wall to inactivate certain antibiotic resistance mechanisms: 1) affecting *acrA* and *tolC* of the *acrAB* of the efflux pump system, 2) affecting *ampC*, *ampH*, *pbpC*, and *pbpG* of the β-lactamase enzyme responsible for ampicillin resistance. The anti-bacterial activity of carvacrol on the efflux pump system of *E. coli* bacteria was demonstrated in a previous study but without investigating which efflux system was affected (Miladi et al., 2016). On the other hand, the role of carvacrol as a cell wall synthesis inhibitor was investigated here for the first time. Though, at this level of investigation, it was not possible to know exactly what happened to the either efflux pump system or the β-lactamase enzyme. Therefore, in order to know the exact binding site of carvacrol, it might be tackled by using radioactive tracer approaches to identify its exact binding site or by using mutant *E. coli* strains with certain mutations.

Chapter 5 investigated the in vitro effect of sub-lethal concentrations of carvacrol and ampicillin, separately and combined, on single pure cultures of ampicillin-resistant *E. coli* strains and on a mixed *E. coli* culture composed of an APEC strain and two commensal *E. coli* strains. This combination approach was considered, as it might help in increasing the efficacy of ampicillin when administered with carvacrol to solve the issue of ampicillin resistance, as this was supported by a previous study that demonstrated the synergistic interactions of different phytochemicals and antibiotics to control plasmid-mediated antibiotic resistance in *E. coli* strains (Yap et al., 2013). The focus here was on ampicillin, because plasmid-mediated ESBL resistance is widely spread among *E. coli* bacteria of avian sources (Ewers et al., 2012, Kluytmans et al., 2012). This study showed an overall synergistic effect of the combination therapy on growth experiments, but AST and GC-MS were not successful in detecting changes in antibiotic resistance profiling and metabolic activities. These issues might be tackled in the future by using more sophisticated approaches such as real-time PCR and NMR. The second experiment was not successful in showing the inhibitory effect of carvacrol on a mixed *E. coli* culture and very limited as it included a single vessel for each treatment. Thus, it did not give us good data to decide whether carvacrol was a good growth inhibitor of APEC versus commensal *E. coli* strains, and whether the combined treatment of carvacrol and ampicillin was effective in controlling ampicillin-resistant *E. coli* strains, as there was a general increase in the total viable numbers of *E. coli* over time in all treatments. Therefore, this study should be improved in the future by including more vessels for each treatment and by including more sets of different combinations of the combined treatment, to establish good dataset and evaluate the efficacy of each treatment. Negating the limitations of this study, it was an important experiment to be included, in order to set bases to perform future complex experiments to support further *in vitro* studies (**see Chapter 6**).

Chapter 6 explored the *in vitro* effect of sub-lethal concentrations of carvacrol and ampicillin, separately and combined, on chicken caecal content challenged with an ampicillin-resistant APEC strain which to our knowledge has not been investigated before. This study which involved duplicate vessels for each treatment also showed that *E. coli* population grew over time in all treatments, and GC did not explain any treatment effect but only a time effect. In terms of the techniques used which was a weakness of this study was that counting was done using simple plating technique, and this can be improved by using a more robust technique such as fluorescent *in situ* hybridisation (FISH). Also, GC can be replaced with NMR in order to screen more metabolic outputs. However, 16S sequencing results showed some beneficial carvacrol treatment effects based on an increase in probiotic strains (*Lactobacillus* sp. and *Lactobacillus salivarius*) and other beneficial bacteria with anti-Salmonella activity (*Streptococcus* sp. and *Coprococcus* sp.) and decrease in opportunistic bacteria (*E. coli-Shigella*, *Proteus* sp., and *Oscillospira* sp.), which ampicillin and combined treatment showed the opposite. This study was performed one time and on a small sample size number of caecal contents collected from five broiler chickens only. Therefore, this study should be repeated using triplicate vessels for each treatment and using different combinations of sub-lethal concentrations of carvacrol and ampicillin.

In conclusion, these collective set of studies aimed at setting preliminary findings which should be taken further in order to justify the use of oregano in the poultry industry. Oregano in the form of fresh or dried leaves or essential oil is being currently used in different doses as a feed additive in the poultry industry, but with minimal or limited information available on its beneficial role which was only observed in terms of an increased chickens' weight (Bozkurt et al., 2009), improved FCR (Alp et al., 2012), increased in intestinal villi height (Fonseca-García et al., 2017), enhanced immune responses (Silva-Vázquez et al., 2018), and improved meat

quality (Botsoglou et al., 2002), but knowledge is lacked on its effect on the chicken gut microbiota or microbiome level.

Future studies

Future studies should include *in vivo* studies to expand our knowledge in evaluating the effect of different sub-lethal concentrations of carvacrol (to determine the optimum dose of carvacrol) and by collectively involving comprehensive carvacrol evaluation on:

1) Chicken's health and performance in terms of weight, feed consumption, FCR, body fat, percentages of mortality and liveability, and carcass characteristics.
2) Chicken tissues to study the safety of carvacrol and to identify any changes in the physiology of the GIT in terms of villus height and crypt depth. This can be performed by collecting samples of intestinal epithelial tissues supported by electron microscopy examination.
3) Chicken gut microbiome to include eukaryotic and prokaryotic microorganisms rather than focusing on prokaryotic bacteria only. This can be achieved by performing 18S and16S whole population sequencing.
4) Bacterial populations inhabiting the chicken gut by including samples from other parts of the chicken GIT such as ileum, gizzard, and duodenum rather relying on caecal part only, given the differences in their condition.
5) Chicken faecal microbiome to identify any fluctuations in the gut microbiome. This can be investigated using 18S and 16S whole population sequencing.
6) Prevalence of antibiotic resistance genes found in the chicken faeces. This can be explored using real-time PCR.
7) Colonisation of gut bacteria by performing 16S pyrosequencing and WGS.

REFERENCES

AARESTRUP, F. M. 2003. Effects of termination of AGP use on antimicrobial resistance in food animals. *World Health Organization, Geneva, Switzerland*, 6-11.

AFRC, R. F. 1989. Probiotics in man and animals. *Journal of Applied Bacteriology,* 66, 365-378.

ALAEDDINOGLU, N. G. & CHARLES, H. 1979. Transfer of a Gene for Sucrose Utilization into Escherichia colik12, and Consequent Failure of Expression of Genes for d-Serine Utilization. *Microbiology,* 110, 47-59.

ALEKSHUN, M. N. & LEVY, S. B. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. *Antimicrobial agents and chemotherapy,* 41, 2067.

ALEKSHUN, M. N. & LEVY, S. B. 2004. The Escherichia coli mar locus-Antibiotic resistance and more. *ASM News-American Society for Microbiology,* 70, 451-456.

ALEKSHUN, M. N. & LEVY, S. B. 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell,* 128, 1037-1050.

ALP, M., MIDILLI, M., KOCABAĞLI, N., YILMAZ, H., TURAN, N., GARGILI, A. & ACAR, N. 2012. The effects of dietary oregano essential oil on live performance, carcass yield, serum immunoglobulin G level, and oocyst count in broilers. *Journal of Applied Poultry Research,* 21, 630-636.

AMÁBILE-CUEVAS, C. F. & CHICUREL, M. E. 1992. Bacterial plasmids and gene flux. *Cell,* 70, 189-199.

AMADOR, P., FERNANDES, R., PRUDÊNCIO, C. & DUARTE, I. 2019. Prevalence of Antibiotic Resistance Genes in Multidrug-Resistant Enterobacteriaceae on Portuguese Livestock Manure. *Antibiotics,* 8, 23.

AMIT-ROMACH, E., SKLAN, D. & UNI, Z. 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poultry Science,* 83, 1093-1098.

ANDREMONT, A. 2003. Commensal flora may play key role in spreading antibiotic resistance. *ASM news,* 69, 601-607.

ANNISON, E., HILL, K. & KENWORTHY, R. 1968. Volatile fatty acids in the digestive tract of the fowl. *British journal of nutrition,* 22, 207-216.

ANNISON, G. 1991. Relationship between the levels of soluble nonstarch polysaccharides and the apparent metabolizable energy of wheats assayed in broiler chickens. *Journal of Agricultural and Food Chemistry,* 39, 1252-1256.

ANTHARAM, V. C., LI, E., ISHMAEL, A., SHARMA, A., MAI, V., RAND, K. H. & WANG, G. P. 2013. Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea. *Journal of clinical microbiology*, JCM. 00845-13.

ANUKAM, K. C. & REID, G. 2007. Probiotics: 100 years (1907-2007) after Elie Metchnikoff's observation. *Communicating current research and educational topics and trends in applied microbiology,* 1, 466-474.

APAJALAHTI, J. 2005. Comparative gut microflora, metabolic challenges, and potential opportunities. *Journal of Applied Poultry Research,* 14, 444-453.

APAJALAHTI, J., KETTUNEN, A. & GRAHAM, H. 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal,* 60, 223-232.

APAJALAHTI, J. & VIENOLA, K. 2016. Interaction between chicken intestinal microbiota and protein digestion. *Animal Feed Science and Technology,* 221, 323-330.

APAJALAHTI, J. H., KETTUNEN, A., NURMINEN, P. H., JATILA, H. & HOLBEN, W. E. 2003. Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. *Applied and environmental microbiology,* 69, 5731-5735.

APAJALAHTI, J. H., KETTUNEN, H., KETTUNEN, A., HOLBEN, W. E., NURMINEN, P. H., RAUTONEN, N. & MUTANEN, M. 2002. Culture-independent microbial community analysis reveals that inulin in

the diet primarily affects previously unknown bacteria in the mouse cecum. *Applied and environmental microbiology,* 68**,** 4986-4995.

APATA, D. 2009. Antibiotic resistance in poultry. *International Journal of Poultry Science,* 8**,** 404-408.

ARIZA, R., COHEN, S., BACHHAWAT, N., LEVY, S. & DEMPLE, B. 1994. Repressor mutations in the marRAB operon that activate oxidative stress genes and multiple antibiotic resistance in Escherichia coli. *Journal of bacteriology,* 176**,** 143-148.

ASFAR, S., ABDEEN, S., DASHTI, H., KHOURSHEED, M., AL-SAYER, H., MATHEW, T. & AL-BADER, A. 2003. Effect of green tea in the prevention and reversal of fasting-induced intestinal mucosal damage. *Nutrition,* 19**,** 536-540.

ATTERBURY, R. J., VAN BERGEN, M., ORTIZ, F., LOVELL, M., HARRIS, J., DE BOER, A., WAGENAAR, J., ALLEN, V. & BARROW, P. 2007. Bacteriophage therapy to reduce Salmonella colonization of broiler chickens. *Applied and environmental microbiology,* 73**,** 4543-4549.

BABAI, R., BLUM-OEHLER, G., STERN, B. E., HACKER, J. & RON, E. Z. 1997. Virulence patterns from septicemic Escherichia coli O78 strains. *FEMS microbiology letters,* 149**,** 99-105.

BAHRANI-MOUGEOT, F. K., BUCKLES, E. L., LOCKATELL, C., HEBEL, J., JOHNSON, D., TANG, C. & DONNENBERG, M. 2002. Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic Escherichia coli virulence determinants in the murine urinary tract. *Molecular microbiology,* 45**,** 1079-1093.

BALLOU, A. L., ALI, R. A., MENDOZA, M. A., ELLIS, J., HASSAN, H. M., CROOM, W. & KOCI, M. D. 2016. Development of the chick microbiome: how early exposure influences future microbial diversity. *Frontiers in veterinary science,* 3**,** 2.

BANDYOPADHYAY, B. & MANDAL, N. C. 2014. Probiotics, Prebiotics and Synbiotics-In Health Improvement by Modulating Gut Microbiota: The Concept Revisited. *Int. J. Curr. Microbiol. App. Sci,* 3**,** 410-420.

BARICEVIC, D. & BARTOL, T. 2002. 8 The biological/pharmacological activity of the Origanum Genus.

BARNES, E. M. 1979. The intestinal microflora of poultry and game birds during life and after storage. *Journal of Applied Microbiology,* 46**,** 407-419.

BARNES, E. M. & IMPEY, C. 1972. Some properties of the nonsporing anaerobes from poultry caeca. *Journal of Applied Microbiology,* 35**,** 241-251.

BARNES, E. M., IMPEY, C. & STEVENS, B. 1979. Factors affecting the incidence and anti-salmonella activity of the anaerobic caecal flora of the young chick. *Epidemiology & Infection,* 82**,** 263-283.

BARNES, E. M., IMPEY, C., STEVENS, B. & PEEL, J. 1977. Streptococcus pleomorphus sp. nov.: an anaerobic streptococcus isolated mainly from the caeca of birds. *Microbiology,* 102**,** 45-53.

BARNES, E. M., MEAD, G., BARNUML, D. & HARRY, E. 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. *British poultry science,* 13**,** 311-326.

BARNES, H., VAILLANCOURT, J. & GROSS, W. 2003. Colibacillosis, p 631–652. *Diseases of poultry, 11th ed. Iowa State University Press, Ames, IA*.

BARROW, P. 1992. Probiotic for chickens in Probiotics The Scientific Basis, Edited by Roy Fuller. Chapman and Hall. London.

BARRY, A., AMSTERDAM, D., COYLE, M., GERLACH, E., THORNSBERRY, C. & HAWKINSON, R. 1979. Simple inoculum standardizing system for antimicrobial disk susceptibility tests. *Journal of clinical microbiology,* 10**,** 910-918.

BARTSCH, H., OHSHIMA, H. & PIGNATELLI, B. 1988. Inhibitors of endogenous nitrosation mechanisms and implications in human cancer prevention. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 202**,** 307-324.

BASMACIOĞLU MALAYOĞLU, H., BAYSAL, Ş., MISIRLIOĞLU, Z., POLAT, M., YILMAZ, H. & TURAN, N. 2010. Effects of oregano essential oil with or without feed enzymes on growth performance, digestive enzyme, nutrient digestibility, lipid metabolism and immune response of broilers fed on wheat–soybean meal diets. *British Poultry Science,* 51**,** 67-80.

BASS, L., LIEBERT, C. A., LEE, M. D., SUMMERS, A. O., WHITE, D. G., THAYER, S. G. & MAURER, J. J. 1999. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avianEscherichia coli. *Antimicrobial Agents and Chemotherapy,* 43**,** 2925-2929.

BEAUREGARD, P. B., CHAI, Y., VLAMAKIS, H., LOSICK, R. & KOLTER, R. 2013. Bacillus subtilis biofilm induction by plant polysaccharides. *Proceedings of the National Academy of Sciences,* 110**,** E1621-E1630.

BELOIN, C., ROUX, A. & GHIGO, J.-M. 2008. Escherichia coli biofilms. *Bacterial Biofilms.* Springer.

BEN ARFA, A., COMBES, S., PREZIOSI-BELLOY, L., GONTARD, N. & CHALIER, P. 2006. Antimicrobial activity of carvacrol related to its chemical structure. *Letters in applied microbiology,* 43**,** 149-154.

BENNETT, J. W. & CHUNG, K.-T. 2001. Alexander Fleming and the discovery of penicillin. *Advances in applied microbiology,* 49**,** 163-184.

BERKHOFF, H. A. 1985. Clostridium colinum sp. nov., nom. rev., the causative agent of ulcerative enteritis (quail disease) in quail, chickens, and pheasants. *International Journal of Systematic and Evolutionary Microbiology,* 35**,** 155-159.

BERNER, L. A. & O'DONNELL, J. A. 1998. Functional foods and health claims legislation: applications to dairy foods. *International Dairy Journal,* 8**,** 355-362.

BETTS, M. J. & RUSSELL, R. B. 2003. Amino acid properties and consequences of substitutions. *Bioinformatics for geneticists***,** 289-316.

BINO, R. J., HALL, R. D., FIEHN, O., KOPKA, J., SAITO, K., DRAPER, J., NIKOLAU, B. J., MENDES, P., ROESSNER-TUNALI, U. & BEALE, M. H. 2004. Potential of metabolomics as a functional genomics tool. *Trends in plant science,* 9**,** 418-425.

BJERRUM, L., ENGBERG, R., LESER, T. D., JENSEN, B. B., FINSTER, K. & PEDERSEN, K. 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poultry science,* 85**,** 1151-1164.

BLAKE, D., HILLMAN, K., FENLON, D. & LOW, J. 2003. Transfer of antibiotic resistance between commensal and pathogenic members of the Enterobacteriaceae under ileal conditions. *Journal of Applied Microbiology,* 95**,** 428-436.

BORDA-MOLINA, D., SEIFERT, J. & CAMARINHA-SILVA, A. 2018. Current Perspectives of the Chicken Gastrointestinal Tract and Its Microbiome. *Computational and Structural Biotechnology Journal,* 16**,** 131-139.

BORTOLAIA, V., BISGAARD, M. & BOJESEN, A. M. 2010. Distribution and possible transmission of ampicillin-and nalidixic acid-resistant Escherichia coli within the broiler industry. *Veterinary microbiology,* 142**,** 379-386.

BOTSOGLOU, N., FLOROU-PANERI, P., CHRISTAKI, E., FLETOURIS, D. & SPAIS, A. 2002. Effect of dietary oregano essential oil on performance of chickens and on iron-induced lipid oxidation of breast, thigh and abdominal fat tissues. *British poultry science,* 43**,** 223-230.

BOZKURT, M., KÜÇÜKYILMAZ, K., ÇATLI, A. & ÇINAR, M. 2009. Effect of dietary mannan oligosaccharide with or without oregano essential oil and hop extract supplementation on the performance and slaughter characteristics of male broilers. *South African Journal of Animal Science,* 39.

BRINAS, L., MORENO, M. A., TESHAGER, T., SÁENZ, Y., PORRERO, M. C., DOMÍNGUEZ, L. & TORRES, C. 2005. Monitoring and characterization of extended-spectrum β-lactamases in Escherichia coli strains from healthy and sick animals in Spain in 2003. *Antimicrobial agents and chemotherapy,* 49**,** 1262-1264.

BRISBIN, J. T., GONG, J. & SHARIF, S. 2008. Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Animal Health Research Reviews,* 9**,** 101-110.

BROUNS, F., KETTLITZ, B. & ARRIGONI, E. 2002. Resistant starch and "the butyrate revolution". *Trends in Food Science & Technology,* 13**,** 251-261.

BRUNKARD, J. M., AILES, E., ROBERTS, V. A., HILL, V., HILBORN, E. D., CRAUN, G. F., RAJASINGHAM, A., KAHLER, A., GARRISON, L. & HICKS, L. 2011. Surveillance for waterborne disease outbreaks associated with drinking water—United States, 2007–2008. *MMWR Surveill Summ,* 60**,** 38-68.

BRYANT, J., CHEWAPREECHA, C. & BENTLEY, S. D. 2012. Developing insights into the mechanisms of evolution of bacterial pathogens from whole-genome sequences. *Future microbiology,* 7**,** 1283-1296.

BUNYAN, J., JEFFRIES, L., SAYERS, J. R., GULLIVER, A. & COLEMAN, K. 1977. Antimicrobial substances and chick growth promotion: The growth-promoting activities of antimicrobial substances, including fifty-two used either in therapy or as dietary additives. *British Poultry Science,* 18**,** 283-294.

BURT, S. A., OJO-FAKUNLE, V. T., WOERTMAN, J. & VELDHUIZEN, E. J. 2014. The natural antimicrobial carvacrol inhibits quorum sensing in Chromobacterium violaceum and reduces bacterial biofilm formation at sub-lethal concentrations. *PLoS One,* 9**,** e93414.

BUSH, K. & JACOBY, G. A. 2010. Updated functional classification of β-lactamases. *Antimicrobial agents and chemotherapy,* 54**,** 969-976.

CANNATELLI, A., GIANI, T., ANTONELLI, A., PRINCIPE, L., LUZZARO, F. & ROSSOLINI, G. M. 2016. First detection of the mcr-1 colistin resistance gene in Escherichia coli in Italy. *Antimicrobial agents and chemotherapy,* 60**,** 3257-3258.

CARATTOLI, A. 2008. Animal reservoirs for extended spectrum β-lactamase producers. *Clinical Microbiology and Infection,* 14**,** 117-123.

CARATTOLI, A., BERTINI, A., VILLA, L., FALBO, V., HOPKINS, K. L. & THRELFALL, E. J. 2005. Identification of plasmids by PCR-based replicon typing. *Journal of microbiological methods,* 63**,** 219-228.

CARATTOLI, A., VILLA, L., FEUDI, C., CURCIO, L., ORSINI, S., LUPPI, A., PEZZOTTI, G. & MAGISTRALI, C. F. 2017. Novel plasmid-mediated colistin resistance mcr-4 gene in Salmonella and Escherichia coli, Italy 2013, Spain and Belgium, 2015 to 2016. *Eurosurveillance,* 22.

CARNIEL, E. 1999. The Yersinia high-pathogenicity island. *International Microbiology,* 2**,** 161-167.

CARON, P., CRAUFURD, P., MARTIN, A., MCDONALD, A., ABEDINI, W., AFIFF, S., BAKURIN, N., BASS, S., HILBECK, A. & JANSEN, T. 2009. Impacts of AKST on development and sustainability goals. *Embrapa Pecuária Sudeste-Capítulo em livro científico (ALICE)*.

CASEWELL, M., FRIIS, C., MARCO, E., MCMULLIN, P. & PHILLIPS, I. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of antimicrobial chemotherapy,* 52**,** 159-161.

CASTANON, J. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry science,* 86**,** 2466-2471.

CATTOIR, V., POIREL, L., ROTIMI, V., SOUSSY, C.-J. & NORDMANN, P. 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. *Journal of antimicrobial chemotherapy,* 60**,** 394-397.

CAVALIERI, S. J., BOHACH, G. A. & SNYDER, I. 1984. Escherichia coli alpha-hemolysin: characteristics and probable role in pathogenicity. *Microbiological reviews,* 48**,** 326.

CENCIC, A. & CHINGWARU, W. 2010. The role of functional foods, nutraceuticals, and food supplements in intestinal health. *Nutrients,* 2**,** 611-625.

CHADWICK, D. & GOODE, J. 1997. *Antibiotic resistance: origins, evolution, selection and spread*, John Wiley & Sons.

CHANT, E. L. & SUMMERS, D. K. 2007. Indole signalling contributes to the stable maintenance of Escherichia coli multicopy plasmids. *Molecular microbiology,* 63**,** 35-43.

CHOI, K., NAMKUNG, H. & PAIK, I. 1994. Effects of dietary fructooligosaccharides on the suppression of intestinal colonization of Salmonella typhimurium in broiler chickens. *Korean Journal of Animal Science (Korea Republic)*.

CHOWDHURY, S., MANDAL, G. P., PATRA, A. K., KUMAR, P., SAMANTA, I., PRADHAN, S. & SAMANTA, A. K. 2018. Different essential oils in diets of broiler chickens: 2. Gut microbes and morphology, immune response, and some blood profile and antioxidant enzymes. *Animal Feed Science and Technology,* 236**,** 39-47.

CHUECA, B., RENZONI, A., BERDEJO, D., PAGÁN, R., KELLEY, W. L. & GARCÍA-GONZALO, D. 2018. Whole-genome sequencing and genetic analysis reveals novel stress responses against individual

constituents of essential oils in Escherichia coli. *Applied and environmental microbiology*, AEM. 02538-17.

CLANCY, M., GRAEPLER, A., WILSON, M., DOUGLAS, I., JOHNSON, J. & PRICE, C. S. 2006. Active screening in high-risk units is an effective and cost-avoidant method to reduce the rate of methicillin-resistant Staphylococcus aureus infection in the hospital. *Infection Control,* 27, 1009-1017.

CLENCH, M. H. & MATHIAS, J. R. 1995. The avian cecum: a review. *The Wilson Bulletin*, 93-121.

CLOWES, R. 1972. Molecular structure of bacterial plasmids. *Bacteriological reviews,* 36, 361.

COATES, M. E., FORD, J. & HARRISON, G. 1968. Intestinal synthesis of vitamins of the B complex in chicks. *Brit. J. Nutrition,* 22, 493-500.

COHEN, S., MCMURRY, L. & LEVY, S. 1988. marA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of Escherichia coli. *Journal of bacteriology,* 170, 5416-5422.

COHEN, S. P., HÄCHLER, H. & LEVY, S. 1993. Genetic and functional analysis of the multiple antibiotic resistance (mar) locus in Escherichia coli. *Journal of bacteriology,* 175, 1484-1492.

COLLIGNON, P. 2009. Resistant Escherichia coli—we are what we eat. *Clinical infectious diseases,* 49, 202-204.

COLOE, P., BAGUST, T. & IRELAND, L. 1984. Development of the normal gastrointestinal microflora of specific pathogen-free chickens. *Epidemiology & Infection,* 92, 79-87.

CONNELL, I., AGACE, W., KLEMM, P., SCHEMBRI, M., MĂRILD, S. & SVANBORG, C. 1996. Type 1 fimbrial expression enhances Escherichia coli virulence for the urinary tract. *Proceedings of the National Academy of Sciences,* 93, 9827-9832.

CONWAY, T. & COHEN, P. S. 2015. Commensal and pathogenic Escherichia coli metabolism in the gut. *Microbiology spectrum,* 3.

COOKSON, A. L., COOLEY, W. A. & WOODWARD, M. J. 2002. The role of type 1 and curli fimbriae of Shiga toxin-producing Escherichia coli in adherence to abiotic surfaces. *International Journal of Medical Microbiology,* 292, 195-205.

CORDONI, G., WOODWARD, M. J., WU, H., ALANAZI, M., WALLIS, T. & LA RAGIONE, R. M. 2016. Comparative genomics of European avian pathogenic E. Coli (APEC). *BMC genomics,* 17, 960.

CORRIGAN, A., DE LEEUW, M., PENAUD-FRÉZET, S., DIMOVA, D. & MURPHY, R. 2015. Phylogenetic and functional alterations of bacterial community composition in the broiler caecum as a result of mannan oligosaccharide supplementation. *Applied and environmental microbiology*, AEM. 04194-14.

COSENTINO, S., TUBEROSO, C. I. G., PISANO, B., SATTA, M., MASCIA, V., ARZEDI, E. & PALMAS, F. 1999. In-vitro antimicrobial activity and chemical composition of Sardinian thymus essential oils. *Letters in applied microbiology,* 29, 130-135.

COSGROVE, S. E. 2006. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. *Clinical Infectious Diseases,* 42, S82-S89.

COSTERTON, J. W., CHENG, K., GEESEY, G. G., LADD, T. I., NICKEL, J. C., DASGUPTA, M. & MARRIE, T. J. 1987. Bacterial biofilms in nature and disease. *Annual Reviews in Microbiology,* 41, 435-464.

COSTERTON, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. & LAPPIN-SCOTT, H. M. 1995. Microbial biofilms. *Annual Reviews in Microbiology,* 49, 711-745.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science,* 284, 1318-1322.

CRISTANI, M., D'ARRIGO, M., MANDALARI, G., CASTELLI, F., SARPIETRO, M. G., MICIELI, D., VENUTI, V., BISIGNANO, G., SAIJA, A. & TROMBETTA, D. 2007. Interaction of four monoterpenes contained in essential oils with model membranes: implications for their antibacterial activity. *Journal of Agricultural and Food Chemistry,* 55, 6300-6308.

DANZEISEN, J. L., KIM, H. B., ISAACSON, R. E., TU, Z. J. & JOHNSON, T. J. 2011. Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PloS one,* 6**,** e27949.

DAVE, M., HIGGINS, P. D., MIDDHA, S. & RIOUX, K. P. 2012. The human gut microbiome: current knowledge, challenges, and future directions. *Translational Research,* 160**,** 246-257.

DAVEY, M. E. & O'TOOLE, G. A. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and molecular biology reviews,* 64**,** 847-867.

DAVIES, D. G., CHAKRABARTY, A. M. & GEESEY, G. G. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by Pseudomonas aeruginosa. *Applied and environmental microbiology,* 59**,** 1181-1186.

DAVIES, J. & DAVIES, D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and molecular biology reviews: MMBR,* 74**,** 417-433.

DEFELICE, S. L. 1995. The nutraceutical revolution: its impact on food industry R&D. *Trends in Food Science & Technology,* 6**,** 59-61.

DEFLAUN, M. & LEVY, S. 1989. Genes and their varied hosts. *Gene Transfer in the Environment***,** 1-32.

DELGADO, C. 2005. Rising demand for meat and milk in developing countries: implications for grasslands-based livestock production.

DELICATO, E. R., DE BRITO, B. G., GAZIRI, L. C. J. & VIDOTTO, M. C. 2003. Virulence-associated genes in Escherichia coli isolates from poultry with colibacillosis. *Veterinary microbiology,* 94**,** 97-103.

DELL, C. L., NEELY, M. N. & OLSON, E. R. 1994. Altered pH lysine signalling mutants of cadC, a gene encoding a membrane-bound transcriptional activator of the Escherichia coli cadBA operon. *Molecular microbiology,* 14**,** 7-16.

DEMIGNÉ, C. & RÉMÉSY, C. 1985. Stimulation of absorption of volatile fatty acids and minerals in the cecum of rats adapted to a very high fiber diet. *The Journal of nutrition,* 115**,** 53-60.

DEMIGNE, C., REMESY, C. & MORAND, C. 1999. Short chain fatty acids. *Colonic Microbiota, Nutrition and Health.* Springer.

DETHLEFSEN, L., HUSE, S., SOGIN, M. L. & RELMAN, D. A. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS biol,* 6**,** e280.

DETHLEFSEN, L. & RELMAN, D. A. 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proceedings of the National Academy of Sciences,* 108**,** 4554-4561.

DEWEY, C. E., COX, B. D., STRAW, B. E., BUSH, E. J. & HURD, H. S. 1997. Associations between off-label feed additives and farm size, veterinary consultant use, and animal age. *Preventive veterinary medicine,* 31**,** 133-146.

DHO-MOULIN, M. & FAIRBROTHER, J. M. 1999. Avian pathogenic Escherichia coli (APEC). *Veterinary research,* 30**,** 299-316.

DI MARTINO, P., FURSY, R., BRET, L., SUNDARARAJU, B. & PHILLIPS, R. 2003. Indole can act as an extracellular signal to regulate biofilm formation of Escherichia coli and other indole-producing bacteria. *Canadian journal of microbiology,* 49**,** 443-449.

DI PASQUA, R., HOSKINS, N., BETTS, G. & MAURIELLO, G. 2006. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *Journal of agricultural and food chemistry,* 54**,** 2745-2749.

DI PASQUA, R., MAURIELLO, G., MAMONE, G. & ERCOLINI, D. 2013. Expression of DnaK, HtpG, GroEL and Tf chaperones and the corresponding encoding genes during growth of Salmonella Thompson in presence of thymol alone or in combination with salt and cold stress. *Food research international,* 52**,** 153-159.

DIARRASSOUBA, F., DIARRA, M. S., BACH, S., DELAQUIS, P., PRITCHARD, J., TOPP, E. & SKURA, B. J. 2007. Antibiotic resistance and virulence genes in commensal Escherichia coli and Salmonella isolates from commercial broiler chicken farms. *Journal of food protection,* 70**,** 1316-1327.

DIERIKX, C., VAN ESSEN-ZANDBERGEN, A., VELDMAN, K., SMITH, H. & MEVIUS, D. 2010. Increased detection of extended spectrum beta-lactamase producing Salmonella enterica and Escherichia coli isolates from poultry. *Veterinary microbiology,* 145**,** 273-278.

DIERIKX, C. M., VAN DER GOOT, J. A., SMITH, H. E., KANT, A. & MEVIUS, D. J. 2013. Presence of ESBL/AmpC-producing Escherichia coli in the broiler production pyramid: a descriptive study. *PloS one,* 8**,** e79005.

DOCQUIER, J.-D. & MANGANI, S. 2017. An update on β-lactamase inhibitor discovery and development. *Drug Resistance Updates*.

DOLARA, P., LUCERI, C., DE FILIPPO, C., FEMIA, A. P., GIOVANNELLI, L., CADERNI, G., CECCHINI, C., SILVI, S., ORPIANESI, C. & CRESCI, A. 2005. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 591**,** 237-246.

DONACHIE, W. & BEGG, K. 1970. Growth of the bacterial cell. *Nature,* 227**,** 1220.

DORMAN, H. & DEANS, S. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology,* 88**,** 308-316.

DOYLE, M. P., STEENSON, L. R. & MENG, J. 2013. Bacteria in food and beverage production. *The prokaryotes.* Springer.

DUEHOLM, M. S., NIELSEN, S. B., HEIN, K. L., NISSEN, P., CHAPMAN, M., CHRISTIANSEN, G., NIELSEN, P. H. & OTZEN, D. E. 2011. Fibrillation of the major curli subunit CsgA under a wide range of conditions implies a robust design of aggregation. *Biochemistry,* 50**,** 8281-8290.

DUEÑAS, M., MUÑOZ-GONZÁLEZ, I., CUEVA, C., JIMÉNEZ-GIRÓN, A., SÁNCHEZ-PATÁN, F., SANTOS-BUELGA, C., MORENO-ARRIBAS, M. & BARTOLOMÉ, B. 2015. A survey of modulation of gut microbiota by dietary polyphenols. *BioMed research international,* 2015.

DUNKLEY, K., CALLAWAY, T., CHALOVA, V., MCREYNOLDS, J., HUME, M., DUNKLEY, C., KUBENA, L., NISBET, D. & RICKE, S. 2009. Foodborne Salmonella ecology in the avian gastrointestinal tract. *Anaerobe,* 15**,** 26-35.

ECKERT, C., GAUTIER, V., SALADIN-ALLARD, M., HIDRI, N., VERDET, C., OULD-HOCINE, Z., BARNAUD, G., DELISLE, F., ROSSIER, A. & LAMBERT, T. 2004. Dissemination of CTX-M-type β-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. *Antimicrobial agents and chemotherapy,* 48**,** 1249-1255.

EDWARDS, P. R. & EWING, W. H. 1962. Identification of enterobacteriaceae. *Identification of Enterobacteriaceae.*

EDWARDS, P. R. & EWING, W. H. 1972. Identification of enterobacteriaceae. *Identification of Enterobacteriaceae.*

EECKHAUT, V., VAN IMMERSEEL, F., CROUBELS, S., DE BAERE, S., HAESEBROUCK, F., DUCATELLE, R., LOUIS, P. & VANDAMME, P. 2011. Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. *Microbial biotechnology,* 4**,** 503-512.

ELIOPOULUS, G. & MOELLERING, R. 1996. Antimicrobial combinations. Antibiotics in Laboratory Medicine. Edited by: Lorian V. 1996. Baltimore, MD: The Williams & Wilkins Co.

ELOFF, J. N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta medica,* 64**,** 711-713.

ERTAS, O. N., GULER, T., ÇIFTÇI, M., DALKILIÇ, B. & SIMSEK, U. G. 2005. The effect of an essential oil mix derived from oregano, clove and anise on broiler performance. *International Journal of Poultry Science,* 4**,** 879-884.

ESCHERICH, T. 1886. *Die darmbakterien des säuglings und ihre beziehungen zur physiologie der Verdauung*, F. Enke.

ESIMONE, C., IROHA, I., IBEZIM, E., OKEH, C. & OKPANA, E. 2006. In vitro evaluation of the interaction between tea extracts and penicillin G against Staphylococcus aureus. *African Journal of Biotechnology,* 5.

ESTRADA, A., WILKIE, D. & DREW, M. 2001. Administration of Bifidobacterium bifidum to chicken broilers reduces the number of carcass condemnations for cellulitis at the abattoir. *Journal of Applied Poultry Research,* 10**,** 329-334.

EWERS, C., BETHE, A., SEMMLER, T., GUENTHER, S. & WIELER, L. 2012. Extended-spectrum β-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. *Clinical Microbiology and Infection,* 18**,** 646-655.

EWERS, C., JANßEN, T., KIEßLING, S., PHILIPP, H.-C. & WIELER, L. H. 2005. Rapid detection of virulence-associated genes in avian pathogenic Escherichia coli by multiplex polymerase chain reaction. *Avian diseases,* 49**,** 269-273.

EWERS, C., JANßEN, T. & WIELER, L. 2003. Avian pathogenic Escherichia coli (APEC). *Berliner und Munchener tierarztliche Wochenschrift,* 116**,** 381-395.

EWERS, C., LI, G., WILKING, H., KIEBLING, S., ALT, K., ANTÁO, E.-M., LATURNUS, C., DIEHL, I., GLODDE, S. & HOMEIER, T. 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing Escherichia coli: how closely related are they? *International Journal of Medical Microbiology,* 297**,** 163-176.

EWING, W. H. 1986. *Edwards and Ewing's identification of Enterobacteriaceae*, Elsevier Science Publishing Co. Inc.

FAIR, R. J. & TOR, Y. 2014. Antibiotics and bacterial resistance in the 21st century. *Perspectives in medicinal chemistry,* 6**,** 25.

FARAG, R., BADEI, A., HEWEDI, F. & EL-BAROTY, G. 1989. Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. *Journal of the American Oil Chemists' Society,* 66**,** 792-799.

FEIL, E. J. 2004. Small change: keeping pace with microevolution. *Nature Reviews Microbiology,* 2**,** 483.

FINEGOLD, S., SUTTER, V. & MATHISEN, G. 1983a. Human intestinal microflora in health and disease. *Academic, London***,** 3731.

FINEGOLD, S. M., SUTTER, V. L. & MATHISEN, G. E. 1983b. Normal indigenous intestinal flora. *Human intestinal microflora in health and disease,* 1**,** 3-31.

FISCHBACH, M. A. 2011. Combination therapies for combating antimicrobial resistance. *Current opinion in microbiology,* 14**,** 519-523.

FLOCH, M. H. 2011. Intestinal microecology in health and wellness. *Journal of clinical gastroenterology,* 45**,** S108-S110.

FONSECA-GARCÍA, I., ESCALERA-VALENTE, F., MARTÍNEZ-GONZÁLEZ, S., CARMONA-GASCA, C. A., GUTIÉRREZ-ARENAS, D. A. & ÁVILA-RAMOS, F. 2017. Effect of oregano oil dietary supplementation on production parameters, height of intestinal villi and the antioxidant capacity in the breast of broiler. *Austral Journal of Veterinary Sciences,* 49**,** 83-89.

FOXMAN, B., BARLOW, R., D'ARCY, H., GILLESPIE, B. & SOBEL, J. D. 2000. Urinary tract infection: self-reported incidence and associated costs. *Annals of epidemiology,* 10**,** 509-515.

FRIEDMAN, M., HENIKA, P. R. & MANDRELL, R. E. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica. *Journal of food protection,* 65**,** 1545-1560.

FROST, L. S., LEPLAE, R., SUMMERS, A. O. & TOUSSAINT, A. 2005. Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology,* 3**,** 722.

FUH, N. J., CHRISTIANA, O. M., YAMI, A. L., UTEH, U. P., EKPIKEN, E. S. & OGECHI, U. M. 2018. Prevalence and Antibiotic Resistance of Escherichia coli O157: H7 Serotype from Chicken Droppings Produced by Free-Ranged and Poultry Birds in Cross River, Nigeria. *American Journal of Biomedical and Life Sciences,* 6**,** 51-55.

FUKAI, K., ISHIGAMI, T. & HARA, Y. 1991. Antibacterial activity of tea polyphenols against phytopathogenic bacteria. *Agricultural and Biological Chemistry,* 55**,** 1895-1897.

FULLER, R. & TURVEY, A. 1971. Bacteria associated with the intestinal wall of the fowl (Gallus domesticus). *Journal of Applied Bacteriology,* 34**,** 617-622.

FUX, C., COSTERTON, J. W., STEWART, P. S. & STOODLEY, P. 2005. Survival strategies of infectious biofilms. *Trends in microbiology,* 13**,** 34-40.

GALE, E. F. & EPPS, H. M. 1942. The effect of the pH of the medium during growth on the enzymic activities of bacteria (Escherichia coli and Micrococcus lysodeikticus) and the biological significance of the changes produced. *Biochemical journal,* 36**,** 600.

GALLUCCI, N., CASERO, C., OLIVA, M., ZYGADLO, J. & DEMO, M. 2006. Interaction between terpenes and penicillin on bacterial strains resistant to beta-lactam antibiotics. *Mol Med Chem,* 10**,** 30-2.

GARRIGA, M., PASCUAL, M., MONFORT, J. & HUGAS, M. 1998. Selection of lactobacilli for chicken probiotic adjuncts. *Journal of applied microbiology,* 84**,** 125-132.

GASAWAY, W. C. 1976. Volatile fatty acids and metabolizable energy derived from cecal fermentation in the willow ptarmigan. *Comparative Biochemistry and Physiology Part A: Physiology,* 53**,** 115-121.

GEORGE, A. M. & LEVY, S. B. 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in Escherichia coli: involvement of a non-plasmid-determined efflux of tetracycline. *Journal of Bacteriology,* 155**,** 531-540.

GIANNENAS, I., FLOROU-PANERI, P., BOTSOGLOU, N., CHRISTAKI, E. & SPAIS, A. 2005. Effect of supplementing feed with oregano and/or alpha-tocopheryl acetate on growth of broiler chickens and oxidative stability of meat. *Journal of Animal and Feed Sciences,* 14**,** 521.

GIANNENAS, I., FLOROU-PANERI, P., PAPAZAHARIADOU, M., CHRISTAKI, E., BOTSOGLOU, N. & SPAIS, A. 2003. Effect of dietary supplementation with oregano essential oil on performance of broilers after experimental infection with Eimeria tenella. *Archives of Animal Nutrition,* 57**,** 99-106.

GIBSON, G. R. & ROBERFROID, M. B. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of nutrition,* 125**,** 1401-1412.

GIBSON, P., MOELLER, I., KAGELARI, O., FOLINO, M. & YOUNG, G. 1992. Contrasting effects of butyrate on the expression of phenotypic markers of differentiation in neoplastic and non-neoplastic colonic epithelial cells in vitro. *Journal of gastroenterology and hepatology,* 7**,** 165.

GILL, A. & HOLLEY, R. 2006. Disruption of Escherichia coli, Listeria monocytogenes and Lactobacillus sakei cellular membranes by plant oil aromatics. *International journal of food microbiology,* 108**,** 1-9.

GÓMEZ-ESTACA, J., BRAVO, L., GÓMEZ-GUILLÉN, M., ALEMÁN, A. & MONTERO, P. 2009. Antioxidant properties of tuna-skin and bovine-hide gelatin films induced by the addition of oregano and rosemary extracts. *Food Chemistry,* 112**,** 18-25.

GONG, J., SI, W., FORSTER, R. J., HUANG, R., YU, H., YIN, Y., YANG, C. & HAN, Y. 2007. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS microbiology ecology,* 59**,** 147-157.

GONZÁLEZ-PARAMÁS, A. M., ESTEBAN-RUANO, S., SANTOS-BUELGA, C., DE PASCUAL-TERESA, S. & RIVAS-GONZALO, J. C. 2004. Flavanol content and antioxidant activity in winery byproducts. *Journal of Agricultural and Food Chemistry,* 52**,** 234-238.

GOOSSENS, H., FERECH, M., VANDER STICHELE, R., ELSEVIERS, M. & GROUP, E. P. 2005. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *The Lancet,* 365**,** 579-587.

GOPHNA, U., KONIKOFF, T. & NIELSEN, H. B. 2017. Oscillospira and related bacteria–From metagenomic species to metabolic features. *Environmental microbiology,* 19**,** 835-841.

GORBACH, S. 1996. Microbiology of the gastrointestinal tract.

GOULD, L. H., WALSH, K. A., VIEIRA, A. R., HERMAN, K., WILLIAMS, I. T., HALL, A. J. & COLE, D. 2013. Surveillance for foodborne disease outbreaks—United States, 1998–2008. *MMWR Surveill Summ,* 62**,** 1-34.

GOUNARIS, Y., SKOULA, M., FOURNARAKI, C., DRAKAKAKI, G. & MAKRIS, A. 2002. Comparison of essential oils and genetic relationship of Origanum× intercedens to its parental taxa in the island of Crete. *Biochemical Systematics and Ecology,* 30**,** 249-258.

GREKO, C. 2001. Safety aspects on non-use of antimicrobials as growth promoters. *Gut Environment of Pigs. A. Piva, KE Bach Knudsen and JE Lindberg, ed. Nottingham University Press, Nottingham, UK***,** 219-230.

GRILLI, E., ALBONETTI, S. & PIVA, A. Role of carvacrol and cinnamaldehyde in broiler cecal fermentations. EPC 2006-12th European Poultry Conference, Verona, Italy, 10-14 September, 2006, 2006. World's Poultry Science Association (WPSA).

GROSS, W. 1994. Diseases due to Escherichia coli in poultry. *Escherichia coli in domestic animals and man***,** 237-259.

GROUP, F. W. W. 2001. Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. *Córdoba, Argentina (October 1–4, 2001)*.

GUALDI, L., TAGLIABUE, L., BERTAGNOLI, S., IERANO, T., DE CASTRO, C. & LANDINI, P. 2008. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in Escherichia coli. *Microbiology,* 154**,** 2017-2024.

GUBAN, J., KORVER, D., ALLISON, G. & TANNOCK, G. 2006. Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of Lactobacillus salivarius, and reduced bile salt deconjugation in the ileum of broiler chickens. *Poultry science,* 85**,** 2186-2194.

GURSOY, U. K., GURSOY, M., GURSOY, O. V., CAKMAKCI, L., KÖNÖNEN, E. & UITTO, V.-J. 2009. Anti-biofilm properties of Satureja hortensis L. essential oil against periodontal pathogens. *Anaerobe,* 15**,** 164-167.

GUYNOT, M., RAMOS, A., SETÓ, L., PURROY, P., SANCHIS, V. & MARÍN, S. 2003. Antifungal activity of volatile compounds generated by essential oils against fungi commonly causing deterioration of bakery products. *Journal of applied microbiology,* 94**,** 893-899.

HAMER, H. M., JONKERS, D., VENEMA, K., VANHOUTVIN, S., TROOST, F. & BRUMMER, R. J. 2008. The role of butyrate on colonic function. *Alimentary pharmacology & therapeutics,* 27**,** 104-119.

HAMMER, K. A., CARSON, C. & RILEY, T. 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of applied microbiology,* 86**,** 985-990.

HAN, X., SHEN, T. & LOU, H. 2007. Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences,* 8**,** 950-988.

HANBERGER, H., ARMAN, D., GILL, H., JINDRÁK, V., KALENIC, S., KURCZ, A., LICKER, M., NAABER, P., SCICLUNA, E. A. & VANIS, V. 2009. Surveillance of microbial resistance in European Intensive Care Units: a first report from the Care-ICU programme for improved infection control. *Intensive care medicine,* 35**,** 91-100.

HARSHEY, R. M. 2003. Bacterial motility on a surface: many ways to a common goal. *Annual Reviews in Microbiology,* 57**,** 249-273.

HARTL, D. L. & DYKHUIZEN, D. E. 1984. The population genetics of Escherichia coli. *Annual review of genetics,* 18**,** 31-68.

HASHEMI, S. & DAVOODI, H. 2010. Phytogenics as new class of feed additive in poultry industry. *Journal of Animal and Veterinary Advances,* 9**,** 2295-2304.

HASHEMI, S., ZULKIFLI, I., FARIDA, A. & SOMCHIT, M. 2008. Acute toxicity study and phytochemical screening of selected herbal aqueous extract in broiler chickens.

HASHEMI, S., ZULKIFLI, I., ZUNITA, Z., HAIR-BEJO, M., LOH, T., SOMCHIT, M., KOK, P. & DAVOODI, H. Effects of dietary supplementation with Euphorbia hirta and acidifier on performance and Salmonella colonization in broiler chickens. Proceedings of the 30th Malaysia Society of Animal Production Annual Conference, 2009.

HASHEMIPOUR, H., KERMANSHAHI, H., GOLIAN, A. & VELDKAMP, T. 2013. Effect of thymol and carvacrol feed supplementation on performance, antioxidant enzyme activities, fatty acid

composition, digestive enzyme activities, and immune response in broiler chickens. *Poultry Science,* 92**,** 2059-2069.

HAUSNER, M. & WUERTZ, S. 1999. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied and Environmental Microbiology,* 65**,** 3710-3713.

HAYES, J. R., ENGLISH, L. L., CARR, L. E., WAGNER, D. D. & JOSEPH, S. W. 2004. Multiple-antibiotic resistance of Enterococcus spp. isolated from commercial poultry production environments. *Applied and Environmental Microbiology,* 70**,** 6005-6011.

HAZAN, R., QUE, Y.-A., MAURA, D. & RAHME, L. G. 2012. A method for high throughput determination of viable bacteria cell counts in 96-well plates. *BMC microbiology,* 12**,** 259.

HERNANDEZ, F., MADRID, J., GARCIA, V., ORENGO, J. & MEGIAS, M. 2004. Influence of two plant extracts on broilers performance, digestibility, and digestive organ size. *Poultry science,* 83**,** 169-174.

HERREN, C. D., MITRA, A., PALANIYANDI, S. K., COLEMAN, A., ELANKUMARAN, S. & MUKHOPADHYAY, S. 2006. The BarA-UvrY two-component system regulates virulence in avian pathogenic Escherichia coli O78: K80: H9. *Infection and immunity,* 74**,** 4900-4909.

HERZER, P. J., INOUYE, S., INOUYE, M. & WHITTAM, T. S. 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of Escherichia coli. *Journal of bacteriology,* 172**,** 6175-6181.

HOBMAN, J. L., PENN, C. W. & PALLEN, M. J. 2007. Laboratory strains of Escherichia coli: model citizens or deceitful delinquents growing old disgracefully? *Molecular microbiology,* 64**,** 881-885.

HØIBY, N., BJARNSHOLT, T., GIVSKOV, M., MOLIN, S. & CIOFU, O. 2010. Antibiotic resistance of bacterial biofilms. *International journal of antimicrobial agents,* 35**,** 322-332.

HONG, Y., BERRANG, M. E., LIU, T., HOFACRE, C. L., SANCHEZ, S., WANG, L. & MAURER, J. J. 2003. Rapid detection of Campylobacter coli, C. jejuni, and Salmonella enterica on poultry carcasses by using PCR-enzyme-linked immunosorbent assay. *Applied and environmental microbiology,* 69**,** 3492-3499.

HUDAULT, S., GUIGNOT, J. & SERVIN, A. 2001. Escherichia coli strains colonising the gastrointestinal tract protect germfree mice againstSalmonella typhimuriuminfection. *Gut,* 49**,** 47-55.

HUGHES, V. M. & DATTA, N. 1983. Conjugative plasmids in bacteria of the 'pre-antibiotic'era. *Nature,* 302**,** 725.

HULTON, C., HIGGINS, C. & SHARP, P. 1991. ERIC sequences: a novel family of repetitive elements in the genomes of Escherichia coli, Salmonella typhimurium and other enterobacteria. *Molecular microbiology,* 5**,** 825-834.

IBEKWE, A. M., MURINDA, S. E. & GRAVES, A. K. 2011. Genetic diversity and antimicrobial resistance of Escherichia coli from human and animal sources uncovers multiple resistances from human sources. *PLoS One,* 6**,** e20819.

IQBAL, M., SHAH, I., ALI, A., KHAN, M. & JAN, S. 2006. Prevalence and in vitro antibiogram of bacteria associated with omphalitis in chicks. *Proteus,* 13**,** 8.

JAMES, J. 2006. *Microbial hazard identification in fresh fruits and vegetables*, John Wiley & Sons.

JAMROZ, D., ORDA, J., KAMEL, C., WILICZKIEWICZ, A., WERTELECKI, T. & SKORUPINSKA, J. 2003. The influence of phytogenic extracts on performance, nutrient digestibility, carcass characteristics, and gut microbial status in broiler chickens. *Journal of Animal and Feed Sciences,* 12**,** 583-596.

JANG, I., KO, Y., KANG, S. & LEE, C. 2007. Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. *Animal Feed Science and Technology,* 134**,** 304-315.

JANßEN, T., SCHWARZ, C., PREIKSCHAT, P., VOSS, M., PHILIPP, H.-C. & WIELER, L. H. 2001. Virulence-associated genes in avian pathogenic Escherichia coli (APEC) isolated from internal organs of poultry having died from colibacillosis. *International Journal of Medical Microbiology,* 291**,** 371-378.

JOENSEN, K. G., TETZSCHNER, A. M., IGUCHI, A., AARESTRUP, F. M. & SCHEUTZ, F. 2015. Rapid and easy in silico serotyping of Escherichia coli using whole genome sequencing (WGS) data. *Journal of clinical microbiology***,** JCM. 00008-15.

JOHNSON, J. R. & STELL, A. L. 2000. Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise. *The Journal of infectious diseases,* 181**,** 261-272.

JOHNSON, T. J., GIDDINGS, C. W., HORNE, S. M., GIBBS, P. S., WOOLEY, R. E., SKYBERG, J., OLAH, P., KERCHER, R., SHERWOOD, J. S. & FOLEY, S. L. 2002. Location of Increased Serum Survival Gene and Selected Virulence Traits on a Conjugative R Plasmid in an Avian Escherichia coli Isolate. *AVIAN DISEASES,* 46**,** 342-352.

JOHNSON, T. J., SIEK, K. E., JOHNSON, S. J. & NOLAN, L. K. 2006. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian Escherichia coli strains. *Journal of bacteriology,* 188**,** 745-758.

JOHNSON, T. J., WANNEMUEHLER, Y., DOETKOTT, C., JOHNSON, S. J., ROSENBERGER, S. C. & NOLAN, L. K. 2008. Identification of minimal predictors of avian pathogenic Escherichia coli virulence for use as a rapid diagnostic tool. *Journal of clinical microbiology,* 46**,** 3987-3996.

JOHNY, A. K., HOAGLAND, T. & VENKITANARAYANAN, K. 2010. Effect of subinhibitory concentrations of plant-derived molecules in increasing the sensitivity of multidrug-resistant Salmonella enterica serovar Typhimurium DT104 to antibiotics. *Foodborne Pathogens and Disease,* 7**,** 1165-1170.

JONES, B., LOCKATELL, C., JOHNSON, D., WARREN, J. & MOBLEY, H. 1990. Construction of a urease-negative mutant of Proteus mirabilis: analysis of virulence in a mouse model of ascending urinary tract infection. *Infection and immunity,* 58**,** 1120-1123.

JØRGENSEN, H., ZHAO, X.-Q., KNUDSEN, K. E. B. & EGGUM, B. O. 1996. The influence of dietary fibre source and level on the development of the gastrointestinal tract, digestibility and energy metabolism in broiler chickens. *British Journal of Nutrition,* 75**,** 379-395.

JOZEFIAK, D., RUTKOWSKI, A., FRATCZAK, M. & BOROS, D. 2004. The effect of dietary fibre fractions from different cereals and microbial enzymes supplementation on performance, ileal viscosity and short-chain fatty acids concentration in caeca of broiler chickens. *J. Anim. Feed Sci,* 13**,** 487-496.

JÓZEFIAK, D., RUTKOWSKI, A. & MARTIN, S. 2004. Carbohydrate fermentation in the avian ceca: a review. *Animal Feed Science and Technology,* 113**,** 1-15.

JUNG, S., HOUDE, R., BAURHOO, B., ZHAO, X. & LEE, B. 2008. Effects of galacto-oligosaccharides and a Bifidobacteria lactis-based probiotic strain on the growth performance and fecal microflora of broiler chickens. *Poultry science,* 87**,** 1694-1699.

KALEMBA, D. & KUNICKA, A. 2003. Antibacterial and Antifungal Properties of Essential Oils. *Current Medicinal Chemistry,* 10**,** 813-829.

KALMOKOFF, M., LANTHIER, P., TREMBLAY, T.-L., FOSS, M., LAU, P. C., SANDERS, G., AUSTIN, J., KELLY, J. & SZYMANSKI, C. M. 2006. Proteomic analysis of Campylobacter jejuni 11168 biofilms reveals a role for the motility complex in biofilm formation. *Journal of bacteriology,* 188**,** 4312-4320.

KAPER, J. B., NATARO, J. P. & MOBLEY, H. L. 2004. Pathogenic escherichia coli. *Nature Reviews Microbiology,* 2**,** 123-140.

KARIMI, A., YAN, F., COTO, C., PARK, J., MIN, Y., LU, C., GIDDEN, J., LAY JR, J. & WALDROUP, P. 2010. Effects of level and source of oregano leaf in starter diets for broiler chicks. *Journal of Applied Poultry Research,* 19**,** 137-145.

KELLY, C. J., ZHENG, L., CAMPBELL, E. L., SAEEDI, B., SCHOLZ, C. C., BAYLESS, A. J., WILSON, K. E., GLOVER, L. E., KOMINSKY, D. J. & MAGNUSON, A. 2015. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell host & microbe,* 17**,** 662-671.

KELLY, J. A., DIDEBERG, O., CHARLIER, P., WERY, J.-P., LIBERT, M., MOEWS, P., DUEZ, C., FRAIPONT, C. & JORIS, B. 1986. On the origin of bacterial resistance to penicillin: comparison of a beta-lactamase and a penicillin target. *Science,* 231**,** 1429-1431.

KEMMETT, K., HUMPHREY, T., RUSHTON, S., CLOSE, A., WIGLEY, P. & WILLIAMS, N. J. 2013. A longitudinal study simultaneously exploring the carriage of APEC virulence associated genes and the molecular epidemiology of faecal and systemic E. coli in commercial broiler chickens. *PLoS One,* 8**,** e67749.

KEYBURN, A. L., BOYCE, J. D., VAZ, P., BANNAM, T. L., FORD, M. E., PARKER, D., DI RUBBO, A., ROOD, J. I. & MOORE, R. J. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. *PLoS pathogens,* 4**,** e26.

KHACHATOURIANS, G. G. 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Canadian Medical Association Journal,* 159**,** 1129-1136.

KHASHEII, B., ANVARI, S. & JAMALLI, A. 2016. Frequency evaluation of genes encoding siderophores and the effects of different concentrations of Fe ions on growth rate of uropathogenic Escherichia coli. *Iranian journal of microbiology,* 8**,** 359.

KILIC, A., MUZ, A., ERTAS, H. & OZBEY, G. 2009. Random amplified polymorphic DNA (RAPD) analysis of Escherichia coli isolated from chickens. *FU Sag Bil Vet Derg,* 23**,** 1-4.

KIM, Y.-G., UDAYANGA, K. G. S., TOTSUKA, N., WEINBERG, J. B., NÚÑEZ, G. & SHIBUYA, A. 2014. Gut dysbiosis promotes M2 macrophage polarization and allergic airway inflammation via fungi-induced PGE 2. *Cell host & microbe,* 15**,** 95-102.

KIMURA, N., MIMURA, F., NISHIDA, S., KOBAYASHI, A. & MITSUOKA, T. 1976. Studies on the relationship between intestinal flora and cecal coccidiosis in chicken. *Poultry Science,* 55**,** 1375-1383.

KINTZIOS, S. E. 2002. Oregano: the genera Origanum and Lippia. New York, US: Taylor & Francis.

KLUYTMANS, J. A., OVERDEVEST, I. T., WILLEMSEN, I., KLUYTMANS-VAN DEN BERGH, M. F., VAN DER ZWALUW, K., HECK, M., RIJNSBURGER, M., VANDENBROUCKE-GRAULS, C. M., SAVELKOUL, P. H. & JOHNSTON, B. D. 2012. Extended-spectrum β-lactamase–producing Escherichia coli from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. *Clinical Infectious Diseases,* 56**,** 478-487.

KNARREBORG, A., SIMON, M. A., ENGBERG, R. M., JENSEN, B. B. & TANNOCK, G. W. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Applied and environmental microbiology,* 68**,** 5918-5924.

KNÖBL, T., MICKE MORENO, A., PAIXAO, R., GOMES, T. A. T., VIEIRA, M. A. M., DA SILVA LEITE, D., BLANCO, J. E. & FERREIRA, A. J. P. 2012. Prevalence of avian pathogenic Escherichia coli (APEC) clone harboring sfa gene in Brazil. *The Scientific World Journal,* 2012.

KOKKINI, S. Taxonomy, diversity and distribution of Origanum. Oregano: proceedings of the IPGRI international workshop on oregano, 1996. 8-12.

KOOLS, S. A., MOLTMANN, J. F. & KNACKER, T. 2008. Estimating the use of veterinary medicines in the European union. *Regulatory Toxicology and Pharmacology,* 50**,** 59-65.

KRISHNAN, S., CHANG, A. C., HODGES, J., COURAUD, P.-O., ROMERO, I. A., WEKSLER, B., NICHOLSON, B. A., NOLAN, L. K. & PRASADARAO, N. V. 2015. Serotype O18 avian pathogenic and neonatal meningitis Escherichia coli strains employ similar pathogenic strategies for the onset of meningitis. *Virulence,* 6**,** 777-786.

KÜPER, C. & JUNG, K. 2005. CadC-mediated activation of the cadBA promoter in Escherichia coli. *Journal of molecular microbiology and biotechnology,* 10**,** 26-39.

LAMBERT, R., SKANDAMIS, P., COOTE, P. & NYCHAS, G.-J. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology,* 91**,** 453-462.

LAN, P. T. N., HAYASHI, H., SAKAMOTO, M. & BENNO, Y. 2002. Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. *Microbiology and immunology,* 46**,** 371-382.

LAPARRA, J. M. & SANZ, Y. 2010. Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research,* 61**,** 219-225.

LARRONDO, J., AGUT, M. & CALVO-TORRAS, M. 1995. Antimicrobial activity of essences from labiates. *Microbios,* 82**,** 171-172.

LEE, J. H., BAE, I. K. & HEE LEE, S. 2012. New definitions of extended-spectrum β-lactamase conferring worldwide emerging antibiotic resistance. *Medicinal research reviews,* 32**,** 216-232.

LEE, K.-W., EVERTS, H., KAPPERT, H., FREHNER, M., LOSA, R. & BEYNEN, A. 2003a. Effects of dietary essential oil components on growth performance, digestive enzymes and lipid metabolism in female broiler chickens. *British poultry science,* 44**,** 450-457.

LEE, K.-W., EVERTS, H., KAPPERT, H., YEOM, K.-H. & BEYNEN, A. 2003b. Dietary carvacrol lowers body weight gain but improves feed conversion in female broiler chickens. *Journal of Applied Poultry Research,* 12**,** 394-399.

LEE, S. J., LEE, D.-Y., KIM, T. Y., KIM, B. H., LEE, J. & LEE, S. Y. 2005. Metabolic engineering of Escherichia coli for enhanced production of succinic acid, based on genome comparison and in silico gene knockout simulation. *Applied and environmental microbiology,* 71**,** 7880-7887.

LEE, Y.-K. & SALMINEN, S. 1995. The coming of age of probiotics. *Trends in Food Science & Technology,* 6**,** 241-245.

LEI, F., YIN, Y., WANG, Y., DENG, B., YU, H. D., LI, L., XIANG, C., WANG, S., ZHU, B. & WANG, X. 2012. Higher-level production of volatile fatty acids in vitro by chicken gut microbiotas than by human gut microbiotas as determined by functional analyses. *Applied and environmental microbiology,* 78**,** 5763-5772.

LEITNER, G. & HELLER, E. 1992. Colonization of Escherichia coli in young turkeys and chickens. *Avian diseases***,** 211-220.

LESSON, S. Butyrate lancing science versus societal issues in poultry nutrition. Nutr Abstr Rev, 2007. 1-5.

LEVY, S. 1985. Ecology of plasmids and unique DNA sequences.

LEVY, S. B. & MARSHALL, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine,* 10**,** S122-S129.

LINTON, A., HOWE, K., BENNETT, P., RICHMOND, M. & WHITESIDE, E. 1977a. The colonization of the human gut by antibiotic resistant Escherichia coli from chickens. *Journal of Applied Microbiology,* 43**,** 465-469.

LINTON, A., HOWE, K., HARTLEY, C. L., CLEMENTS, H. M., RICHMOND, M. & OSBORNE, A. 1977b. Antibiotic Resistance among Escherichia coli O-serotypes from the Gut and Carcasses of Commercially Slaughtered Broiler Chickens: a Potential Public Health Hazard. *Journal of Applied Microbiology,* 42**,** 365-378.

LIU, Y.-Y., WANG, Y., WALSH, T. R., YI, L.-X., ZHANG, R., SPENCER, J., DOI, Y., TIAN, G., DONG, B. & HUANG, X. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet infectious diseases,* 16**,** 161-168.

LÓPEZ-OLIVA, M. E., AGIS-TORRES, A., GOÑI, I. & MUÑOZ-MARTÍNEZ, E. 2010. Grape antioxidant dietary fibre reduced apoptosis and induced a pro-reducing shift in the glutathione redox state of the rat proximal colonic mucosa. *British journal of nutrition,* 103**,** 1110-1117.

LOPEZ-ROMERO, J. C., GONZÁLEZ-RÍOS, H., BORGES, A. & SIMÕES, M. 2015. Antibacterial effects and mode of action of selected essential oils components against Escherichia coli and Staphylococcus aureus. *Evidence-Based Complementary and Alternative Medicine,* 2015.

LU, J., IDRIS, U., HARMON, B., HOFACRE, C., MAURER, J. J. & LEE, M. D. 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and environmental microbiology,* 69**,** 6816-6824.

LUPP, C., ROBERTSON, M. L., WICKHAM, M. E., SEKIROV, I., CHAMPION, O. L., GAYNOR, E. C. & FINLAY, B. B. 2007. Host-Mediated Inflammation Disrupts the Intestinal Microbiota and Promotes the Overgrowth of Enterobacteriaceae. *Cell Host & Microbe,* 2**,** 119-129.

LUZ, I. D. S., DE MELO, A. N. F., BEZERRA, T. K. A., MADRUGA, M. S., MAGNANI, M. & DE SOUZA, E. L. 2014. Sublethal amounts of Origanum vulgare L. essential oil and carvacrol cause injury and changes in membrane fatty acid of Salmonella Typhimurium cultivated in a meat broth. *Foodborne pathogens and disease,* 11**,** 357-361.

MA, D., COOK, D., ALBERTI, M., PON, N., NIKAIDO, H. & HEARST, J. 1993. Molecular cloning and characterization of acrA and acrE genes of Escherichia coli. *Journal of bacteriology,* 175**,** 6299-6313.

MACFARLANE, G. 1984. *Alexander Fleming, the man and the myth*, Harvard Univ Pr.

MACFARLANE, G., STEED, H. & MACFARLANE, S. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of applied microbiology,* 104**,** 305.

MACFARLANE, G. T. & MACFARLANE, S. 2007. Models for intestinal fermentation: association between food components, delivery systems, bioavailability and functional interactions in the gut. *Current opinion in biotechnology,* 18**,** 156-162.

MACFARLANE, S. & MACFARLANE, G. T. 2003. Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society,* 62**,** 67-72.

MACHADO, E., COQUE, T. M., CANTON, R., SOUSA, J. C. & PEIXE, L. 2008. Antibiotic resistance integrons and extended-spectrum β-lactamases among Enterobacteriaceae isolates recovered from chickens and swine in Portugal. *Journal of Antimicrobial Chemotherapy,* 62**,** 296-302.

MAGRAY, S., WANI, S., KASHOO, Z., BHAT, M., ADIL, S., FAROOQ, S., RATHER, M., KABLI, Z., BANDAY, M. & NISHIKAWA, Y. 2018. Serological diversity, molecular characterisation and antimicrobial sensitivity of avian pathogenic Escherichia coli (APEC) isolates from broiler chickens in Kashmir, India. *Animal Production Science*.

MARCHESI, J. R., ADAMS, D. H., FAVA, F., HERMES, G. D., HIRSCHFIELD, G. M., HOLD, G., QURAISHI, M. N., KINROSS, J., SMIDT, H. & TUOHY, K. M. 2016. The gut microbiota and host health: a new clinical frontier. *Gut,* 65**,** 330-339.

MARTINS, N., BARROS, L. & FERREIRA, I. C. 2016. In vivo antioxidant activity of phenolic compounds: Facts and gaps. *Trends in Food Science & Technology,* 48**,** 1-12.

MATURANA, V. G., DE PACE, F., CARLOS, C., PIRES, M. M., DE CAMPOS, T. A., NAKAZATO, G., STHELING, E. G., LOGUE, C. M., NOLAN, L. K. & DA SILVEIRA, W. D. 2011. Suppl 1: Subpathotypes of Avian Pathogenic Escherichia coli (APEC) Exist as Defined by their Syndromes and Virulence Traits. *The open microbiology journal,* 5**,** 55.

MAURER, J. J., BROWN, T. P., STEFFENS, W. & THAYER, S. G. 1998. The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin tsh among avian Escherichia coli. *Avian diseases***,** 106-118.

MCARTHUR, A. G., WAGLECHNER, N., NIZAM, F., YAN, A., AZAD, M. A., BAYLAY, A. J., BHULLAR, K., CANOVA, M. J., DE PASCALE, G. & EJIM, L. 2013. The comprehensive antibiotic resistance database. *Antimicrobial agents and chemotherapy,* 57**,** 3348-3357.

MCLELLAND, J. 1989. Anatomy of the avian cecum. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology,* 252**,** 2-9.

MCNAUGHT, A. & WILKINSON, A. 1997. Compendium of Chemical Terminology. 2n d edition. *The Gold Book*.

MEIMANDIPOUR, A., SHUHAIMI, M., SOLEIMANI, A., AZHAR, K., HAIR-BEJO, M., KABEIR, B., JAVANMARD, A., MUHAMMAD ANAS, O. & YAZID, A. 2010. Selected microbial groups and short-chain fatty acids profile in a simulated chicken cecum supplemented with two strains of Lactobacillus. *Poultry Science,* 89**,** 470-476.

MENESTRINA, G., MOSER, C., PELLET, S. & WELCH, R. 1994. Pore-formation by Escherichia coli hemolysin (HlyA) and other members of the RTX toxins family. *Toxicology,* 87**,** 249-267.

METABOLHTES, N.-C. S. 2004. Phytochemical diversity of secondary metabolites.
MICHEL, J.-B., YEH, P. J., CHAIT, R., MOELLERING, R. C. & KISHONY, R. 2008. Drug interactions modulate the potential for evolution of resistance. *Proceedings of the National Academy of Sciences*.
MILADI, H., ZMANTAR, T., CHAABOUNI, Y., FEDHILA, K., BAKHROUF, A., MAHDOUANI, K. & CHAIEB, K. 2016. Antibacterial and efflux pump inhibitors of thymol and carvacrol against food-borne pathogens. *Microbial pathogenesis,* 99**,** 95-100.
MILANOV, D. S., PRUNIĆ, B. Z., VELHNER, M. J., PAJIĆ, M. L. & ČABARKAPA, I. S. 2015. RDAR morphotype: A resting stage of some Enteroba cteriaceae. *Food and Feed Research,* 42**,** 43-50.
MILLEMANN, Y., LESAGE-DESCAUSES, M.-C., LAFONT, J.-P. & CHASLUS-DANCLA, E. 1996. Comparison of random amplified polymorphic DNA analysis and enterobacterial repetitive intergenic consensus-PCR for epidemiological studies of Salmonella. *FEMS Immunology and Medical Microbiology,* 14**,** 129-134.
MILLER, J. 1972. Determination of viable cell counts: bacterial growth curves. *Experiments in Molecular Genetics,* 31**,** 36.
MILLET, S. & MAERTENS, L. 2011. The European ban on antibiotic growth promoters in animal feed: from challenges to opportunities. *Veterinary journal (London, England: 1997),* 187**,** 143-144.
MITSCH, P., ZITTERL-EGLSEER, K., KÖHLER, B., GABLER, C., LOSA, R. & ZIMPERNIK, I. 2004. The effect of two different blends of essential oil components on the proliferation of Clostridium perfringens in the intestines of broiler chickens. *Poultry Science,* 83**,** 669-675.
MITSCHER, L. A., DRAKE, S., GOLLAPUDI, S. R. & OKWUTE, S. K. 1987. A modern look at folkloric use of anti-infective agents. *Journal of natural products,* 50**,** 1025-1040.
MITSUOKA, T. 1973. The fecal flora in man. I. Composition of the fecal flora of various age group. *Zbl. Bakt. Hyg. I. Abt. Orig.,* 223**,** 333-342.
MOHAMED, L., GE, Z., YUEHUA, L., YUBIN, G., RACHID, K., MUSTAPHA, O., JUNWEI, W. & KARINE, O. 2018. Virulence traits of avian pathogenic (APEC) and fecal (AFEC) E. coli isolated from broiler chickens in Algeria. *Tropical animal health and production,* 50**,** 547-553.
MOHAPATRA, B. R., BROERSMA, K. & MAZUMDER, A. 2007. Comparison of five rep-PCR genomic fingerprinting methods for differentiation of fecal Escherichia coli from humans, poultry and wild birds. *FEMS microbiology letters,* 277**,** 98-106.
MONTAGNE, L., PIEL, C. & LALLES, J. 2004. Effect of diet on mucin kinetics and composition: nutrition and health implications. *Nutrition Reviews,* 62**,** 105-114.
MORLEY, A. & THOMSON, D. 1984. Swollen-head syndrome in broiler chickens. *Avian diseases***,** 238-243.
NAMKUNG, H., LI J. GONG, M., YU, H., COTTRILL, M. & DE LANGE, C. 2004. Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs. *Canadian journal of animal science,* 84**,** 697-704.
NASRIN, M., SIDDIQI, M., MASUM, M. & WARES, M. 2012. Gross and histological studies of digestive tract of broilers during postnatal growth and development. *Journal of the Bangladesh Agricultural University,* 10**,** 69-77.
NEILANDS, J. 1992. Mechanism and regulation of synthesis of aerobactin in Escherichia coli K12 (pColV-K30). *Canadian Journal of Microbiology,* 38**,** 728-733.
NISBET, D., CORRIER, D., RICKE, S., HUME, M., BYRD II, J. & DELOACH, J. 1996. Cecal Propionic Acid as a Biological Indicator of the Early Establishment of a Microbial Ecosystem Inhibitory toSalmonellain Chicks. *Anaerobe,* 2**,** 345-350.
NOLAN, L. K., BARNES, H. J., VAILLANCOURT, J.-P., ABDUL-AZIZ, T. & LOGUE, C. M. 2013. Colibacillosis. *Diseases of poultry***,** 751-805.
NOLAN, L. K., HORNE, S., GIDDINGS, C., FOLEY, S., JOHNSON, T., LYNNE, A. & SKYBERG, J. 2003. Resistance to serum complement, iss, and virulence of avian Escherichia coli. *Veterinary research communications,* 27**,** 101-110.

NORMAN, A., HANSEN, L. H. & SØRENSEN, S. J. 2009. Conjugative plasmids: vessels of the communal gene pool. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 364**,** 2275-2289.

NOSTRO, A., ROCCARO, A. S., BISIGNANO, G., MARINO, A., CANNATELLI, M. A., PIZZIMENTI, F. C., CIONI, P. L., PROCOPIO, F. & BLANCO, A. R. 2007. Effects of oregano, carvacrol and thymol on Staphylococcus aureus and Staphylococcus epidermidis biofilms. *Journal of medical microbiology,* 56**,** 519-523.

NOWROUZIAN, F., WOLD, A. & ADLERBERTH, I. 2001. P fimbriae and aerobactin as intestinal colonization factors for Escherichia coli in Pakistani infants. *Epidemiology & Infection,* 126**,** 19-23.

NURMI, E. & RANTALA, M. 1973. New aspects of Salmonella infection in broiler production. *Nature,* 241**,** 210-211.

NURMI, E., SCHNEITZ, C. & MAKELA, P. 1983. Process for the production of a bacterial preparation. *Canadian patent*.

O'TOOLE, G. A. & KOLTER, R. 1998. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular microbiology,* 28**,** 449-461.

OH, J., KANG, M., YOON, H., CHOI, H., AN, B., SHIN, E., KIM, Y., KIM, M., KWON, J. & KWON, Y. 2012. The embryo lethality of Escherichia coli isolates and its relationship to the presence of virulence-associated genes. *Poultry science,* 91**,** 370-375.

OKUSU, H., MA, D. & NIKAIDO, H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. *Journal of bacteriology,* 178**,** 306-308.

OLIVER, J. D. 2005. The viable but nonculturable state in bacteria. *The Journal of Microbiology,* 43**,** 93-100.

OLTRAMARI, K., CARDOSO, R. F., PATUSSI, E. V., SANTOS, A. C. B. & MIKCHA, J. M. G. 2014. Genetic heterogeneity of Escherichia coli isolated from pasteurized milk in State of Paraná, Brazil. *Brazilian Journal of Pharmaceutical Sciences,* 50**,** 337-343.

ØRSKOV, F. & ØRSKOV, I. 1992. Escherichia coli serotyping and disease in man and animals. *Canadian Journal of Microbiology,* 38**,** 699-704.

OTAKI, Y. 1995. Poultry disease control programme in Japan. *Asian Livestock (FAO)*.

OYETUNDE, O., THOMSON, R. & CARLSON, H. 1978. Aerosol exposure of ammonia, dust and Escherichia coli in broiler chickens. *The Canadian Veterinary Journal,* 19**,** 187.

PAGÈS, J.-M. & AMARAL, L. 2009. Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics,* 1794**,** 826-833.

PAJIĆ, M., ALEKSIĆ, N., VEJNOVIĆ, B. & POLAČEK, V. In press. Influence of Anticoccidials on Oxidative Stress, Production Performance and Faecal Oocyst Counts in Broiler Chickens Infected with Eimeria Species.

PALANIAPPAN, K. & HOLLEY, R. A. 2010. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. *International journal of food microbiology,* 140**,** 164-168.

PALMER, J., FLINT, S. & BROOKS, J. 2007. Bacterial cell attachment, the beginning of a biofilm. *Journal of industrial microbiology & biotechnology,* 34**,** 577-588.

PAN, D. & YU, Z. 2014. Intestinal microbiome of poultry and its interaction with host and diet. *Gut microbes,* 5**,** 108-119.

PARK, J. & FLOCH, M. H. 2007. Prebiotics, probiotics, and dietary fiber in gastrointestinal disease. *Gastroenterology Clinics of North America,* 36**,** 47-63.

PASCUAL, M., HUGAS, M., BADIOLA, J. I., MONFORT, J. M. & GARRIGA, M. 1999. Lactobacillus salivarius CTC2197 prevents Salmonella enteritidis colonization in chickens. *Applied and environmental microbiology,* 65**,** 4981-4986.

PASTER, N., JUVEN, B., SHAAYA, E., MENASHEROV, M., NITZAN, R., WEISSLOWICZ, H. & RAVID, U. 1990. Inhibitory effect of oregano and thyme essential oils on moulds and foodborne bacteria. *Letters in Applied Microbiology,* 11**,** 33-37.

PENG, Q., LI, J., LI, Z., DUAN, Z. & WU, Y. 2016. Effects of dietary supplementation with oregano essential oil on growth performance, carcass traits and jejunal morphology in broiler chickens. *Animal Feed Science and Technology,* 214**,** 148-153.

PHAM, T. A. N. & LAWLEY, T. D. 2014. Emerging insights on intestinal dysbiosis during bacterial infections. *Current opinion in microbiology,* 17**,** 67-74.

PICONE, G., LAGHI, L., GARDINI, F., LANCIOTTI, R., SIROLI, L. & CAPOZZI, F. 2013. Evaluation of the effect of carvacrol on the Escherichia coli 555 metabolome by using 1H-NMR spectroscopy. *Food chemistry,* 141**,** 4367-4374.

PITOUT, J., THOMSON, K., HANSON, N., EHRHARDT, A., MOLAND, E. & SANDERS, C. 1998. β-Lactamases responsible for resistance to expanded-spectrum cephalosporins in Klebsiella pneumoniae, Escherichia coli, and Proteus mirabilis isolates recovered in South Africa. *Antimicrobial agents and chemotherapy,* 42**,** 1350-1354.

PRATT, L. A. & SILHAVY, T. J. 1998. Crl stimulates RpoS activity during stationary phase. *Molecular microbiology,* 29**,** 1225-1236.

PULLIAINEN, E. & TUNKKARI, P. Seasonal changes in the gut length of the willow grouse (Lagopus lagopus) in Finnish Lapland. Annales Zoologici Fennici, 1983. JSTOR, 53-56.

PUUPPONEN-PIMIA, R., AURA, A.-M., OKSMAN-CALDENTEY, K.-M., MYLLARINEN, P., SAARELA, M., MATTILA-SANDHOLM, T. & POUTANEN, K. 2002. Development of functional ingredients for gut health. *Trends in Food Science & Technology,* 13**,** 3-11.

RAINEY, P. B., REMIGI, P., FARR, A. D. & LIND, P. A. 2017. Darwin was right: where now for experimental evolution? *Current opinion in genetics & development,* 47**,** 102-109.

RAND, K., HOUCK, H., BROWN, P. & BENNETT, D. 1993. Reproducibility of the microdilution checkerboard method for antibiotic synergy. *Antimicrobial agents and chemotherapy,* 37**,** 613-615.

REDIG, P. T. 1989. The avian ceca: obligate combustion chambers or facultative afterburners?—The conditioning influence of diet. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology,* 252**,** 66-69.

REDMOND, E. C., GRIFFITH, C. J., SLADER, J. & HUMPHREY, T. J. 2004. Microbiological and observational analysis of cross contamination risks during domestic food preparation. *British Food Journal,* 106**,** 581-597.

REID, S. & ABRATT, V. 2005. Sucrose utilisation in bacteria: genetic organisation and regulation. *Applied Microbiology and Biotechnology,* 67**,** 312-321.

REILLY, K. & ROMBEAU, J. 1993. Metabolism and potential clinical applications of short-chain fatty acids. *Clinical Nutrition,* 12**,** S97-S105.

RICHARDSON, A., DELBRIDGE, A., BROWN, N., RUMSEY, R. & READ, N. 1991. Short chain fatty acids in the terminal ileum accelerate stomach to caecum transit time in the rat. *Gut,* 32**,** 266-269.

RICHTER, K., HASLBECK, M. & BUCHNER, J. 2010. The heat shock response: life on the verge of death. *Molecular cell,* 40**,** 253-266.

RICKE, S. 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry science,* 82**,** 632-639.

RODRIGUEZ-SIEK, K. E., GIDDINGS, C. W., DOETKOTT, C., JOHNSON, T. J. & NOLAN, L. K. 2005. Characterizing the APEC pathotype. *Veterinary research,* 36**,** 241-256.

RONALD, A. 2002. The etiology of urinary tract infection: traditional and emerging pathogens. *The American journal of medicine,* 113**,** 14-19.

ROOFCHAEE, A., IRANI, M., EBRAHIMZADEH, M. A. & AKBARI, M. R. 2011. Effect of dietary oregano (Origanum vulgare L.) essential oil on growth performance, cecal microflora and serum antioxidant activity of broiler chickens. *African Journal of Biotechnology,* 10**,** 6177-6183.

RUSSO, M., GALLETTI, G. C., BOCCHINI, P. & CARNACINI, A. 1998. Essential Oil Chemical Composition of Wild Populations of Italian Oregano Spice (Origanum v ulgare ssp. h irtum (Link) Ietswaart): A Preliminary Evaluation of Their Use in Chemotaxonomy by Cluster Analysis. 1. Inflorescences. *Journal of Agricultural and Food Chemistry,* 46**,** 3741-3746.

RUSSO, T. A. & JOHNSON, J. R. 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of Escherichia coli: ExPEC. *Journal of Infectious Diseases,* 181**,** 1753-1754.

RYOO, N. H., KIM, E.-C., HONG, S. G., PARK, Y. J., LEE, K., BAE, I. K., SONG, E. H. & JEONG, S. H. 2005. Dissemination of SHV-12 and CTX-M-type extended-spectrum β-lactamases among clinical isolates of Escherichia coli and Klebsiella pneumoniae and emergence of GES-3 in Korea. *Journal of Antimicrobial Chemotherapy,* 56**,** 698-702.

SAURA-CALIXTO, F., GARCÍA-ALONSO, A., GONI, I. & BRAVO, L. 2000. In vitro determination of the indigestible fraction in foods: an alternative to dietary fiber analysis. *Journal of Agricultural and food Chemistry,* 48**,** 3342-3347.

SAURA-CALIXTO, F., SERRANO, J. & GOÑI, I. 2007. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chemistry,* 101**,** 492-501.

SAVAGE, D. C. 1977. Microbial ecology of the gastrointestinal tract. *Annual Reviews in Microbiology,* 31**,** 107-133.

SAVAGEAU, M. A. 1983. Escherichia coli habitats, cell types, and molecular mechanisms of gene control. *The american naturalist,* 122**,** 732-744.

SAVARINO, S. J., FASANO, A., WATSON, J., MARTIN, B. M., LEVINE, M. M., GUANDALINI, S. & GUERRY, P. 1993. Enteroaggregative Escherichia coli heat-stable enterotoxin 1 represents another subfamily of E. coli heat-stable toxin. *Proceedings of the National Academy of Sciences,* 90**,** 3093-3097.

SCHEUTZ, F. & STROCKBINE, N. A. 2005. Escherichia. *Bergey's Manual of Systematics of Archaea and Bacteria*.

SCHJØRRING, S. & KROGFELT, K. A. 2011. Assessment of bacterial antibiotic resistance transfer in the gut. *International journal of microbiology,* 2011.

SCHUPPAN, D., JUNKER, Y. & BARISANI, D. 2009. Reviews in basic and clinical gastroenterology. *Gastroenterology,* 137**,** 1912-1933.

SCIENCE, C. F. A. 1999. *Animal agriculture and global food supply*, Council for Agricultural.

SELANDER, R., CAUGANT, D. & WHITTAM, T. 1987. Genetic structure and variation in natural populations of Escherichia coli.

SEPEHRI, S., KOTLOWSKI, R., BERNSTEIN, C. N. & KRAUSE, D. O. 2009. Phylogenetic analysis of inflammatory bowel disease associated Escherichia coli and the fimH virulence determinant. *Inflammatory bowel diseases,* 15**,** 1737-1745.

SHARPLES, G. & LLOYD, R. 1990. ERIC an intergene sequence distributed in family enterobacteriacae. *Nucleic Acid Res,* 18**,** 6503-6508.

SHAUFI, M. A. M., SIEO, C. C., CHONG, C. W., GAN, H. M. & HO, Y. W. 2015. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. *Gut pathogens,* 7**,** 4.

SI, W., GONG, J., TSAO, R., ZHOU, T., YU, H., POPPE, C., JOHNSON, R. & DU, Z. 2006. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. *Journal of Applied Microbiology,* 100**,** 296-305.

SIKKEMA, J., DE BONT, J. & POOLMAN, B. 1995a. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological reviews,* 59**,** 201-222.

SIKKEMA, J., DE BONT, J. A. & POOLMAN, B. 1995b. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological reviews,* 59**,** 201-222.

SILVA-VÁZQUEZ, R., DURAN-MELÉNDEZ, L. A., HERNÁNDEZ-MARTÍNEZ, C. A., GUTIÉRREZ-SOTO, J. G., HUME, M. E. & MÉNDEZ-ZAMORA, G. 2018. Effects of two sources of Mexican oregano oil on performance, blood profile, carcass variables, and meat of broilers. *Revista Brasileira de Zootecnia,* 47.

SILVA, F., QUEIROZ, J. A. & DOMINGUES, F. C. 2012. Evaluating metabolic stress and plasmid stability in plasmid DNA production by Escherichia coli. *Biotechnology advances,* 30**,** 691-708.

SIMÕES, M., SIMOES, L. C. & VIEIRA, M. J. 2010. A review of current and emergent biofilm control strategies. *LWT-Food Science and Technology,* 43**,** 573-583.

SIMON, O., JADAMUS, A. & VAHJEN, W. 2001. Probiotic feed additives-effectiveness and expected modes of action. *Journal of Animal and feed Sciences,* 10**,** 51-68.

SKYBERG, J., SIEK, K., DOETKOTT, C. & NOLAN, L. 2007. Biofilm formation by avian Escherichia coli in relation to media, source and phylogeny. *Journal of applied microbiology,* 102**,** 548-554.

SMITH, A., BOULNOIS, G. & ROBERTS, I. 1990. Molecular analysis of the Escherichia coli K5 kps locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. *Molecular microbiology,* 4**,** 1863-1869.

SMITH, D. L., HARRIS, A. D., JOHNSON, J. A., SILBERGELD, E. K. & MORRIS, J. G. 2002. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proceedings of the National Academy of Sciences,* 99**,** 6434-6439.

SOERGEL, K. 1994. Colonic fermentation: metabolic and clinical implications. *Journal of Molecular Medicine,* 72**,** 742-748.

SOJKA, W. & CARNAGHAN, R. 1961. Escherichia coli infection in poultry. *Res. Vet. Sci,* 2**,** 340-352.

SOMANI, S. J., MODI, K. P., MAJUMDAR, A. S. & SADARANI, B. N. 2015. Phytochemicals and their potential usefulness in inflammatory bowel disease. *Phytotherapy Research,* 29**,** 339-350.

SOMMER, M. O., CHURCH, G. M. & DANTAS, G. 2010. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. *Virulence,* 1**,** 299-303.

SOMMER, M. O., DANTAS, G. & CHURCH, G. M. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *science,* 325**,** 1128-1131.

SPEEDY, A. W. 2003. Global production and consumption of animal source foods. *The Journal of nutrition,* 133**,** 4048S-4053S.

SPELLBERG, B., POWERS, J. H., BRASS, E. P., MILLER, L. G. & EDWARDS, J. E. 2004. Trends in antimicrobial drug development: implications for the future. *Clinical Infectious Diseases,* 38**,** 1279-1286.

SPRING, P., WENK, C., DAWSON, K. & NEWMAN, K. 2000. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. *Poultry Science,* 79**,** 205-211.

STAMM, W. E. & HOOTON, T. M. 1993. Management of urinary tract infections in adults. *New England journal of medicine,* 329**,** 1328-1334.

STANLEY, D., HUGHES, R. J. & MOORE, R. J. 2014. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Applied Microbiology and Biotechnology,* 98**,** 4301-4310.

STEVENS, C. E. & HUME, I. D. 2004. *Comparative physiology of the vertebrate digestive system*, Cambridge University Press.

STEWART, P. S. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology,* 292**,** 107-113.

STOODLEY, P., WILSON, S., HALL-STOODLEY, L., BOYLE, J. D., LAPPIN-SCOTT, H. M. & COSTERTON, J. W. 2001. Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl. Environ. Microbiol.,* 67**,** 5608-5613.

STORDEUR, P., MARLIER, D., BLANCO, J., OSWALD, E., BIET, F., DHO-MOULIN, M. & MAINIL, J. 2002a. Examination of Escherichia coli from poultry for selected adhesin genes important in disease caused by mammalian pathogenic E. coli. *Veterinary Microbiology,* 84**,** 231-241.

STORDEUR, P., MARLIER, D., BLANCO, J., OSWALD, E., BIET, F., DHO-MOULIN, M. & MAINIL, J. 2002b. Examination of Escherichia coli from poultry for selected adhesin genes important in disease caused by mammalian pathogenic E. coli. *Veterinary microbiology,* 84**,** 231-241.

SUBEDI, M., LUITEL, H., DEVKOTA, B., BHATTARAI, R. K., PHUYAL, S., PANTHI, P., SHRESTHA, A. & CHAUDHARY, D. K. 2018. Antibiotic resistance pattern and virulence genes content in avian

pathogenic Escherichia coli (APEC) from broiler chickens in Chitwan, Nepal. *BMC veterinary research,* 14**,** 113.

SULAVIK, M., GAMBINO, L. & MILLER, P. 1995. The MarR repressor of the multiple antibiotic resistance (mar) operon in Escherichia coli: prototypic member of a family of bacterial regulatory proteins involved in sensing phenolic compounds. *Molecular Medicine,* 1**,** 436.

SURAI, P. 2014. Polyphenol compounds in the chicken/animal diet: from the past to the future. *Journal of Animal Physiology and Animal Nutrition,* 98**,** 19-31.

SZABÓ, E., SKEDSMO, A., SONNEVEND, A., AL-DHAHERI, K., EMÖDY, L., USMANI, A. & PAL, T. 2005. Curli expression of enterotoxigenicEscherichia coli. *Folia microbiologica,* 50**,** 40.

TACCONELLI, E., CATALDO, M., DANCER, S., ANGELIS, G., FALCONE, M., FRANK, U., KAHLMETER, G., PAN, A., PETROSILLO, N. & RODRÍGUEZ-BAÑO, J. 2014. ESCMID guidelines for the management of the infection control measures to reduce transmission of multidrug-resistant Gram-negative bacteria in hospitalized patients. *Clinical Microbiology and Infection,* 20**,** 1-55.

TALARICO, T., CASAS, I., CHUNG, T. C. & DOBROGOSZ, W. 1988. Production and isolation of reuterin, a growth inhibitor produced by Lactobacillus reuteri. *Antimicrobial agents and chemotherapy,* 32**,** 1854-1858.

TENAILLON, O., TADDEI, F., RADMAN, M. & MATIC, I. 2001. Second-order selection in bacterial evolution: selection acting on mutation and recombination rates in the course of adaptation. *Research in microbiology,* 152**,** 11-16.

TERADA, A., HARA, H., NAKAJYO, S., ICHIKAWA, H., HARA, Y., FUKAI, K., KOBAYASHI, Y. & MITSUOKA, T. 1993. Effect of supplements of tea polyphenols on the caeeal flora and caeeal metabolites of chicks. *Microbial Ecology in Health and Disease,* 6**,** 3-9.

THOMAS, D. H. & SKADHAUGE, E. 1989. Water and electrolyte transport by the avian ceca. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology,* 252**,** 95-102.

THOMKE, S. & ELWINGER, K. Growth promotants in feeding pigs and poultry. I. Growth and feed efficiency responses to antibiotic growth promotants. Annales de zootechnie, 1998. 85-97.

THORNTON, P. K. 2010. Livestock production: recent trends, future prospects. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 365**,** 2853-2867.

TIVENDALE, K. A., LOGUE, C. M., KARIYAWASAM, S., JORDAN, D., HUSSEIN, A., LI, G., WANNEMUEHLER, Y. & NOLAN, L. K. 2010. Avian-pathogenic Escherichia coli strains are similar to neonatal meningitis E. coli strains and are able to cause meningitis in the rat model of human disease. *Infection and immunity,* 78**,** 3412-3419.

TRIEU-CUOT, P., CARLIER, C., MARTIN, P. & COURVALIN, P. 1987. Plasmid transfer by conjugation from Escherichia coli to Gram-positive bacteria. *FEMS Microbiology Letters,* 48**,** 289-294.

ULTEE, A., BENNIK, M. & MOEZELAAR, R. 2002a. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. *Applied and environmental microbiology,* 68**,** 1561-1568.

ULTEE, A., BENNIK, M. & MOEZELAAR, R. 2002b. The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus cereus. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY***,** 1561-1568.

UMARU, I. J., BADRUDDIN, F. A. & UMARU, H. A. 2019. Phytochemical Screening of Essential Oils and Antibacterial Activity and Antioxidant Properties of Barringtonia asiatica (L) Leaf Extract. *Biochemistry Research International,* 2019.

VALCESCHINI, E. Poultry meat trends and consumer attitudes. Proceedings of the XII European Poultry Conference, 2006. 1-10.

VALENZUELA, A. Natural antioxidants: a new perspective for the problem of oxidative rancidity of lipids. Biotechnology in the Feed Industry. Proc. 11th Ann. Symp. Nottingham University Press, Loughborough, Leicester, RU, 1995. 207-220.

VAN DEN ABBEELE, P., GROOTAERT, C., MARZORATI, M., POSSEMIERS, S., VERSTRAETE, W., GÉRARD, P., RABOT, S., BRUNEAU, A., EL AIDY, S. & DERRIEN, M. 2010. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for

Bacteroidetes and Clostridium cluster IX. *Applied and environmental microbiology,* 76**,** 5237-5246.

VAN DEN BOGAARD, A., LONDON, N., DRIESSEN, C. & STOBBERINGH, E. 2001. Antibiotic resistance of faecal Escherichia coli in poultry, poultry farmers and poultry slaughterers. *Journal of Antimicrobial Chemotherapy,* 47**,** 763-771.

VAN DER WIELEN, P. W., BIESTERVELD, S., NOTERMANS, S., HOFSTRA, H., URLINGS, B. A. & VAN KNAPEN, F. 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. *Applied and Environmental Microbiology,* 66**,** 2536-2540.

VAN ELSAS, J. D., SEMENOV, A. V., COSTA, R. & TREVORS, J. T. 2011. Survival of Escherichia coli in the environment: fundamental and public health aspects. *The ISME journal,* 5**,** 173.

VAREL, V. H. 2002. Carvacrol and thymol reduce swine waste odor and pathogens: stability of oils. *Current microbiology,* 44**,** 38-43.

VIJAYAN, S., MALLICK, S., DUTTA, M., NARAYANI, M. & GHOSH, A. S. 2014. PBP deletion mutants of Escherichia coli exhibit irregular distribution of MreB at the deformed zones. *Current microbiology,* 68**,** 174-179.

VITETTA, L., BRISKEY, D., HAYES, E., SHING, C. & PEAKE, J. 2012. A review of the pharmacobiotic regulation of gastrointestinal inflammation by probiotics, commensal bacteria and prebiotics. *Inflammopharmacology,* 20**,** 251-266.

VYAS, U. & RANGANATHAN, N. 2012. Probiotics, prebiotics, and synbiotics: gut and beyond. *Gastroenterology research and practice,* 2012.

WANG, L., LILBURN, M. & YU, Z. 2016. Intestinal microbiota of broiler chickens as affected by litter management regimens. *Frontiers in microbiology,* 7**,** 593.

WANG, R., LI, D. & BOURNE, S. Can 2000 years of herbal medicine history help us solve problems in the year 2000. ALLTECHS ANNUAL SYMPOSIUM, 1998. 168-184.

WANG, Y. & QIAN, P.-Y. 2009. Conservative Fragments in Bacterial 16S rRNA Genes and Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies. *Studies,* 4**,** e7401.

WATSON, N., DUNYAK, D., ROSEY, E., SLONCZEWSKI, J. & OLSON, E. R. 1992. Identification of elements involved in transcriptional regulation of the Escherichia coli cad operon by external pH. *Journal of bacteriology,* 174**,** 530-540.

WEGRZYN, G. 2005. What does "plasmid biology" currently mean?: summary of the Plasmid Biology 2004 meeting. *Plasmid,* 53**,** 14-22.

WEHKAMP, J., HARDER, J., WEHKAMP, K., WEHKAMP-VON MEISSNER, B., SCHLEE, M., ENDERS, C., SONNENBORN, U., NUDING, S., BENGMARK, S. & FELLERMANN, K. 2004. NF-κB-and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. *Infection and immunity,* 72**,** 5750-5758.

WEI, S., MORRISON, M. & YU, Z. 2013. Bacterial census of poultry intestinal microbiome. *Poultry science,* 92**,** 671-683.

WEINACK, O. M., SNOEYENBOS, G., SMYSER, C. & SOERJADI-LIEM, A. 1984. Influence of Mycoplasma gallisepticum, infectious bronchitis, and cyclophosphamide on chickens protected by native intestinal microflora against Salmonella typhimurium or Escherichia coli. *Avian diseases***,** 416-425.

WIGLEY, P. 2015. Blurred lines: pathogens, commensals, and the healthy gut. *Frontiers in veterinary science,* 2**,** 40.

WINGENDER, J., NEU, T. R. & FLEMMING, H.-C. 2012. *Microbial Extracellular Polymeric Substances: Characterization, Structure and Function*, Springer Science & Business Media.

WISE, E. M. & PARK, J. T. 1965. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. *Proceedings of the National Academy of Sciences,* 54**,** 75-81.

WOLFE, A. J. 2005. The acetate switch. *Microbiology and molecular biology reviews,* 69**,** 12-50.

WONG, J. M., DE SOUZA, R., KENDALL, C. W., EMAM, A. & JENKINS, D. J. 2006. Colonic health: fermentation and short chain fatty acids. *Journal of clinical gastroenterology,* 40**,** 235-243.

WOODWARD, M. & CHARLES, H. 1982. Genes for l-sorbose utilization in Escherichia coli. *Microbiology,* 128**,** 1969-1980.

WRAY, C. & DAVIES, R. 2000. Competitive exclusion--an alternative to antibiotics. *Veterinary journal (London, England: 1997),* 159**,** 107.

XU, Z., HU, C., XIA, M., ZHAN, X. & WANG, M. 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poultry science,* 82**,** 1030-1036.

YAMAMOTO, S., TERAI, A., YURI, K., KURAZONO, H., TAKEDA, Y. & YOSHIDA, O. 1995. Detection of urovirulence factors in Escherichia coli by multiplex polymerase chain reaction. *FEMS Immunology and Medical Microbiology,* 12**,** 85-90.

YAP, P. S. X., LIM, S. H. E., HU, C. P. & YIAP, B. C. 2013. Combination of essential oils and antibiotics reduce antibiotic resistance in plasmid-conferred multidrug resistant bacteria. *Phytomedicine,* 20**,** 710-713.

YUAN, W., TEO, C. H. M. & YUK, H.-G. 2019. Combined antibacterial activities of essential oil compounds against Escherichia coli O157: H7 and their application potential on fresh-cut lettuce. *Food Control,* 96**,** 112-118.

ZAHRL, D., WAGNER, A., TSCHERNER, M. & KORAIMANN, G. 2007. GroEL plays a central role in stress-induced negative regulation of bacterial conjugation by promoting proteolytic degradation of the activator protein TraJ. *Journal of bacteriology,* 189**,** 5885-5894.

ZENG, X. & LIN, J. 2013. Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Frontiers in microbiology,* 4**,** 128.

ZHAO, L., GAO, S., HUAN, H., XU, X., ZHU, X., YANG, W., GAO, Q. & LIU, X. 2009. Comparison of virulence factors and expression of specific genes between uropathogenic Escherichia coli and avian pathogenic E. coli in a murine urinary tract infection model and a chicken challenge model. *Microbiology,* 155**,** 1634-1644.

ZHAO, L., WANG, G., SIEGEL, P., HE, C., WANG, H., ZHAO, W., ZHAI, Z., TIAN, F., ZHAO, J. & ZHANG, H. 2013. Quantitative genetic background of the host influences gut microbiomes in chickens. *Scientific reports,* 3**,** 1163.

ZHAO, X., ZHAO, F., WANG, J. & ZHONG, N. 2017. Biofilm formation and control strategies of foodborne pathogens: food safety perspectives. *RSC Advances,* 7**,** 36670-36683.

ZHU, X. Y., ZHONG, T., PANDYA, Y. & JOERGER, R. D. 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Applied and Environmental Microbiology,* 68**,** 124-137.

APPENDIX

Media recipes

Nutrient agar (NA) / broth (NB)

Ingredients	Gram per litre
Peptic digest of animal tissue	5.0g
Beef extract	3.0g
Agar (if agar)	15.0g
Final pH at 25°C	6.8 +/- 0.2

MacConkey agar / broth

Ingredients	Gram per litre
Peptone	20.00g
Lactose	10.0g
Bile salts	5.0g
Sodium chloride	5.0g
Neutral red	0.075g
Agar (if agar)	15.0g
Final pH at 25°C	7.4 +/- 0.2

Eosin-methylene blue (EMB) agar

Ingredients	Gram per litre
Peptone	10.0g
Lactose	10.0g
Dipotassium mono-hydrogen phosophate	2.0g
Methylene blue	0.065g
Eosine Y	0.4g
Agar	15.0g
Final pH at 25°C	7.1 +/- 0.2

M9 minimal medium

Ingredients	Gram per litre
Potassium phosphate	15.0g
Sodium phosphate	64.0g
Sodium chloride	2.5g
Ammonium chloride	5.0g
1M Magnesium sulphate	0.24g
1M Calcium chloride	0.02g
Agar	15.0g
Added after autoclaving	
Sugar (e.g. sucrose)	2.0g
Thiamine (if *E. coli* K12)	0.025g

Luria-Bertani (LB) agar / broth

Ingredients	Gram per litre
Tryptone (pancreatic digest of casein)	10.0g
Yeast extract	5.0g
Sodium chloride	5.0g
Agar (if agar)	15.0g
Final pH at 25°C	7.0 +/- 0.2

MacConkey agar containing 50µg/ml ampicillin

Ingredients	Gram per litre
Peptone	20.00g
Lactose	10.0g
Bile salts	5.0g
Sodium chloride	5.0g
Neutral red	0.075g
Agar	15.0g
Final pH at 25°C	7.4 +/- 0.2
Added after autoclaving	
100µg/ml Ampicillin	500µl

Modified Congo Red agar

Ingredients	Gram per litre
Congo Red	0.04g
Coomassie brilliant blue	0.02g
Yeast extract	5.0g
Tryptone (casein)	10.0g
Agar	7.50g

LB broth without salt

Ingredients	Gram per litre
Tryptone (casein)	10.0g
Yeast extract	5.0g
Final pH at 25°C	7.0 +/- 0.2

Viande-Leuvre (VL) medium

Ingredients	Gram/micro-litre/millitre per litre
Meat extract	2.5g
Glucose	2.5g
Tryptose	10.0g
Yeast extract	5.0g
Sodium chloride	5.0g
Haemin	0.05g
Vitamin K	10µl
L-Cysteine hydrochloric acid (HCl)	0.6g
Resazurin solution (0.025g in 100ml)	4.0ml

Chemicals recipes

Aqueous carvacrol /oregano in LB broth (5µg/ml)

Ingredients	Micro-litre/millilitre per 10ml
Carvacrol/ oregano	50µl
LB broth	9.95ml
Final pH at 25°C	7.0 +/- 0.2

Ethanol-based carvacrol /oregano (5µg/ml)

Ingredients	Micro-litre/ milli-litre per 10ml
Carvacrol/ oregano	50µl
LB broth	5ml
70% ethanol	4.95ml

100mg/ml Ampicillin

Ingredients	Gram per 10ml
Ampicillin/ cefotaxime	1g
Sterile-distilled water	10ml

0.5X Tris-borate EDTA (TBE) buffer

Ingredients	per 500ml
10X TBE buffer	25ml
Distilled water	475ml

GC external standard

Ingredients	per 10ml
1M Acetate (57.3µl in 942.7µl H_2O)	1ml
1M Butyrate (91.4µl in 908.6µl H_2O)	1ml
1M Formate (39.73µl in 960.3µl H_2O)	1ml
1M Propionate (74.6µl in 925.4µl H_2O)	1ml
1M Lactate (87.7µl in 912.3µl H_2O)	1ml
Sterile-distilled water	5ml

Conference: Microbiology Research Day at the University of Reading, August 2016.

Effect of carvacrol on *Escherichia coli* (*E. coli*) isolated from infected birds

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Abstract

Food intake necessitates food monitoring and screening to minimize food-borne infections. The current study analyzed *E. coli* strains isolated from infected birds. A total of 12 Gram-negative bacteria were tested for their tolerance to the phytochemical; carvacrol. All of the *E. coli* strains' growth were inhibited by higher concentrations of carvacrol. Notably, the isolated bacteria possessed pathogenicity factors such as biofilm formation potential which could be the basis for bacterial pathogenicity associated with consumption of birds. Nevertheless, the current study suggested phytochemicals may be used as natural antimicrobial agents.

I. Objectives

•To assess the microbiological and chemical characterization of *E. coli* strains isolated from infected birds, using traditional and molecular microbiological methods.

• To study the effect of the isolated bacteria to carvacrol by studying its effects of on their growth and biofilm formation.

• To evaluate the potential of using phytochemicals such as carvacrol in fighting bacteria.

II. Introduction

Plant-based natural therapies are being increased in popularity by consumers, because consumers are becoming aware of concerns of the synthetic additives (Hammer et al., 1999). Phytochemicals is a broad term that assembles non-digestible carbohydrates and compounds such as lignin, resistant protein, polyphenols and carotenoids, and some of them are considered antioxidants (Saura-Calixto et al., 2000). Carvacrol is a phenolic compound constituting the main ingredients of thyme oil. It has so many beneficial values in the field of pharmacology, as being an anti-microbial and anti-oxidant agent (Kalemba and Kunicka, 2003). It works on the cell wall / cell membrane structures and function, leading to them being disturbed resulting the rupture of bacteria (Sikkema et al., 1995).

Bacteria that form biofilms are present in food and are one of many food contamination sources due to their higher ability in resisting antimicrobial compounds and other stressors, in comparison with non-biofilm formers (Høiby et al., 2010). The consequences of food contamination are economic losses, product rejection, and diseases, if food-borne pathogens are associated (Serra, 2003). The main bacterial species which are associated with this behavior are *Listeria monocytogenes*, *Salmonella* sp., *Escherichia coli*, *Pseudomonas* sp., *Campylobacter jejuni*, and *Bacillus* sp. ((Hall-Stoodley and Stoodley, 2002; (Kalmokoff et al., 2006; Morikawa, 2006).

III. Methods

• Effect of carvacrol on *E. coli* growth

An inoculum of overnight bacterial culture grown on a nutrient agar plate was suspended in Luria broth (LB), to prepare bacterial suspensions which were adjusted to OD 0.01 at 600nm. The following were added to the wells of the Greiner CELLSTAR® 96 well plate (sterile, F-bottom, with lid), 225µl of LB minimal medium containing test phytochemical (Carvacrol) of differing concentrations, and 75µl of bacterial suspension. Then, the optical density of the growing bacterial cultures was taken spectrophotometrically using Fluostar Omega system, to determine the minimum inhibitory concentrations (MIC) of each phytochemicals against the *E. coli* strains.

• Biofilm formation potential

An inoculum of overnight culture grown on nutrient agar was suspended in LB and optical density was adjusted to an OD of 0.01 at 600nm. Then, 225µl of LB minimal medium with different dilutions of the previously-mentioned phytochemicals, and 75µl of the bacterial suspension were loaded in a polystyrene Greiner CELLSTAR® 96 well plate (sterile, F-bottom, with lid). The plate was incubated for 5 days at 25°C, and then wells were first washed with 300µl of sterile-distilled water twice, stained with 300µl of 0.1% crystal violet for 25 min for biofilm detection. After that, the wells were washed with 300µl of sterile-distilled water twice, dried, and for quantification of the formed biofilm, 300µl of (90:10) ethanol/acetone was added into each well, and then the absorbance was measured at 590nm Fluostar Omega system.

References

HALL-STOODLEY, L. & STOODLEY, P. 2002. Developmental regulation of microbial biofilms. *Current opinion in biotechnology*, 13, 228-233.
HAMMER, K. A., CARSON, C. & RILEY, T. 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of applied microbiology*, 86, 985-990.
HØIBY, N., BJARNSHOLT, T., GIVSKOV, M., MOLIN, S. & CIOFU, O. 2010. Antibiotic resistance of bacterial biofilms. *International journal of antimicrobial agents*, 35, 322-332.
KALEMBA, D. & KUNICKA, A. 2003. Antibacterial and Antifungal Properties of Essential Oils. *Current Medicinal Chemistry*, 10, 813-829.
KALMOKOFF, M., LANTHIER, P., TREMBLAY, T.-L., FOSS, M., LAU, P. C., SANDERS, G., AUSTIN, J., KELLY, J. & SZYMANSKI, C. M. 2006. Proteomic analysis of Campylobacter jejuni 11168 biofilms reveals a role for the motility complex in biofilm formation. *Journal of bacteriology*, 188, 4312-4320.
MORIKAWA, M. 2006. Beneficial biofilm formation by industrial bacteria Bacillus subtilis and related species. *Journal of bioscience and bioengineering*, 101, 1-8.
SAURA-CALIXTO, F., GARCÍA-ALONSO, A., GONI, I. & BRAVO, L. 2000. In vitro determination of the indigestible fraction in foods: an alternative to dietary fiber analysis. *Journal of Agricultural and food Chemistry*, 48, 3342-3347.
SERRA, G. P. 2003. Estudio del Biofilm: formación y consecuencias. *On line] http://www. seguretatintegral. cat/noucat/recerca/linies/biorisc/alimentaria/biofilm. pdf (23 nov. 2008)*.
SIKKEMA, J., DE BONT, J. & POOLMAN, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological reviews*, 59, 201-222.

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- Many thanks for University of Surrey who generously provided us with *E. coli* strains.

IV. Results

• Effect of carvacrol on the growth rate of *E. coli* isolate 45

• Table 1. Effect of different concentrations of carvacrol on the growth rate of the *E. coli* 45 isolated from infected birds samples

Time	Start	End	Growth rate
	4	8	
Control (0 µg/ml)	0.666	1.463	0.199
0.1 µg/ml	0.544	1.298	0.188
0.15 µg/ml	0.533	1.253	0.180
0.2 µg/ml	0.444	1.106	0.165
0.25 µg/ml	0.089	0.364	0.069
0.3 µg/ml	0.083	0.082	0.000
0.35 µg/ml	0.082	0.081	0.000
0.4 µg/ml	0.087	0.085	0.000
0.45 µg/ml	0.079	0.077	-0.001
0.5 µg/ml	0.080	0.078	0.000

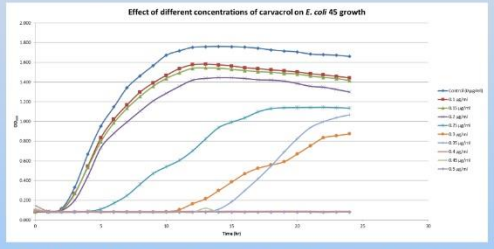
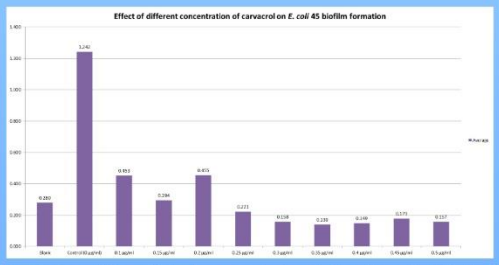


Figure 1. Effect of different concentrations of carvacrol on the growth rate of the *E. coli* 45 isolated from infected birds samples (p-value = 0.000)

In general, one main type of effect was detected in all of the isolates. The results showed that the addition of lower concentrations of carvacrol to the reaction vessels decreased the growth rate of the tested bacteria. On the other hand, the addition of higher concentrations of carvacrol inhibited the bacterial growth rate. The MIC of carvacrol for *E. coli* 45 is 0.4 µg/ml (Table 1).

•Biofilm formation

• Figure 2. Effect of different concentrations of carvacrol on the potential of the isolated bacteria to form biofilm



Examination of the effect of carvacrol showed that the potential of all examined bacteria to form biofilm were inhibited with increasing concentrations of carvacrol (p-value = 0.000) (Figure 2).

V. Discussion

In general, carvacrol as a phytochemical showed the ability to inhibit the growth and biofilm formation of the isolated bacteria at higher concentrations, possibly due to its antibacterial activity (Kalemba and Kunicka, 2003).

VI. Conclusions

There is a promising future to using natural phytochemicals such as carvacrol to fight bacteria and stop their growth instead of using synthetic chemicals.

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Oregano extract as an alternative to antibiotics in poultry feed

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Abstract
The current study focused on assessing the effects of phytochemicals, specifically one of their five main groups, polyphenols, which have been shown to have nutritional value contributing to human health. Polyphenols in the form of essential oils, oregano extract, are bacteriostatic (inhibitory or restrictor effect) at sub-lethal concentrations and bactericidal (killing effect) at or above the minimum inhibitory concentration (MIC) level on many microorganisms. In this study, the focus is upon the utility of oregano extract to suppress antibiotic-resistant avian pathogenic *Escherichia coli* (APEC) strains, which are responsible for causing colibacillosis in poultry reared for human consumption. A total of 12 APEC strains isolated from infected birds have been characterised microbiologically and biochemically, using standard laboratory methods. The phytochemical, aqueous oregano, inhibited and killed the 12 APEC strains including those that were antibiotic resistant (MIC range 0.3 – 0.5 µg/ml), and inhibited their biofilm formation. Future work will take the observed results into more in-depth dimensions in justifying the use of oregano extract in the poultry industry. The current data generated provides very promising evidence that oregano extract may be used in novel ways to control potentially pathogenic and antibiotic resistant APEC strains and provide alternative strategies to the use of antibiotics.

II. Introduction

Animal production systems have relied upon antibiotics for healthy animal development, but this comes with the known outcome of selection of resistant bacteria. (Alekshun and Levy, 2007), and resulting in an increase in the reservoir of resistance genes that may enter the food chain and ultimately the human microbiome (Sommer et al., 2010). In poultry, E. coli are often a natural component of the chicken gut flora inhabiting their mucosal surfaces (Dho-Moulin and Fairbrother, 1999), but some pathogenic types such as APEC are associated with localised or systemic diseases collectively named colibacillosis (Stordeur and Mainil, 2002). As a result of APEC infections, the poultry industry suffers from the loss of chickens and birds, which affects food economy (Dho-Moulin and Fairbrother, 1999). These APEC infections were first controlled using anti-bacterial drugs, but unfortunately, the misuse of the antibiotics led to the rise of antibiotic-resistant strains instead (Bass et al., 1999). Therefore, the usage of antibiotics should be strictly controlled and limited in the medical and veterinary areas (Khachatourians, 1998). In the European Union (EU), there is a ban on the use of prophylactic antibiotics in animal feed, and this was imposed in 2006 (Millet and Maertens, 2011). There is an urgent need to find alternatives to antibiotics.

III. Materials & Methods

- **Bacterial strains**

Twelve avian pathogenic *E. coli* (APEC) strains provided by University of Surrey, and one *E. coli* K12 strain used as a reference strain which was provided by University of Birmingham.

- **Antibiotic susceptibility testing (AST)**

The antibiotic susceptibility of the isolated bacteria to antibiotics in the form of disks (cefotaxime 30µg, nalidixic acid 30µg, chloramphenicol 30µg, colistin 10µg, ampicillin 25µg, streptomycin 25µg, and tetracycline 30µg) was determined using Kirby-Bauer method (Barry et al., 1979).

- **Effect of aqueous oregano on the growth of APEC strains**

An overnight bacterial culture grown on a nutrient agar plate was suspended in Luria broth (LB), to prepare bacterial suspensions which were adjusted to OD 0.01 at 600nm. The following were added to the wells of the Greiner CELLSTAR® 96 well plate (sterile, F-bottom, with lid), 225µl of LB minimal medium containing aqueous oregano of differing concentrations, and 75µl of bacterial suspension. Then, the optical density of the growing bacterial cultures was taken spectrophotometrically using Fluostar Omega system, to determine the minimum inhibitory concentrations (MIC) of each phytochemicals against the *E. coli* strains.

- **Effect of ethanol-based oregano on APEC biofilm**

The method of O'Toole and Kolter was adapted for the determination of the potential of APEC strains to form biofilms (O'Toole and Kolter, 1998).

V. Discussion & Conclusion

Oregano extract as a phytochemical showed its bactericidal and anti-biofilm activities against antibiotic resistant, and at a very low concentration in comparison with antibiotics. This is possibly due to its antibacterial activities resorting to its phenolic content (Rhayour et al., 2003). In conclusion, the current findings open the door to using phytochemicals against APEC strains, which are responsible for huge poultry industry loss.

References
ALEKSHUN, M. N. & LEVY, S. B. 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell,* 128, 1037-1050.
DHO-MOULIN, M. & FAIRBROTHER, J. M. 1999. Avian pathogenic Escherichia coli (APEC). *Veterinary research,* 30, 299-316.
KHACHATOURIANS, G. G. 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Canadian Medical Association Journal,* 159, 1129-1136.
MILLET, S. & MAERTENS, L. 2011. The European ban on antibiotic growth promoters in animal feed: from challenges to opportunities. *Veterinary journal (London, England: 1997),* 187, 143-144.
O'TOOLE, G. A. & KOLTER, R. 1998. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular microbiology,* 28, 449-461.
SOMMER, M. O., CHURCH, G. M. & DANTAS, G. 2010. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. *Virulence,* 1, 299-303.
STORDEUR, P. & MAINIL, J. 2002. Colibacillosis in poultry. *Annales de Medecine Veterinaire ISSN 0003-4118.*

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- Many thanks for University of Surrey who generously provided us with APEC strains.

IV. Results

Table 1. AST results using antibiotic disks and MIC values of aqueous oregano tested *in vitro* against APEC strains

Code	CTX 30µg	NA 30µg	C 30µg	CT 10µg	SAM 25µg	S 25µg	TE 30µg	Aqueous Oregano MIC (µg/ml)
APEC 7	R	R	R	R	S	S	R	0.3
APEC 14	I	R	S	R	S	S	S	0.3
APEC 43	R	S	S	R	S	I	S	0.3
APEC 45	R	S	S	R	S	S	S	0.4
APEC 46	S	R	R	R	R	R	R	0.3
APEC 47	R	S	S	R	S	S	S	0.3
APEC 48	I	R	I	R	I	R	R	0.5
APEC 49	S	S	S	R	S	S	S	0.5
APEC 51	R	S	S	R	I	S	S	0.5
APEC 52	R	R	S	R	R	R	R	0.3
APEC 53	R	R	S	R	S	S	S	0.3
APEC 54	R	S	S	R	S	I	S	0.3
E. coli K12	S	R	I	R	I	R	S	0.3

Shaded cells indicate resistance. CTX, Cefotaxime; NA, Nalidixic acid; C, Chloramphenicol; CT, Colistin; SAM, Ampicillin; S, Streptomycin; TE, Tetracycline; R, resistant; S, sensitive; I, intermediate. Diameters of inhibition zones were interpreted according to the guidelines of Clinical and Laboratory Standards Institute (CLSI), 2016.

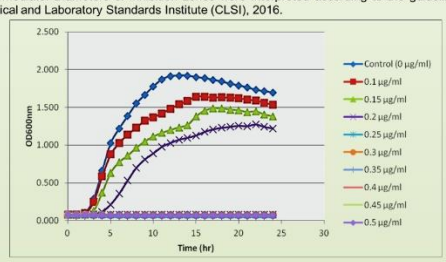

Figure 1. Representative growth curve showing the effect of different concentrations of aqueous carvacrol against APEC 7 with MIC value = 0.3µg/ml (P-value < 0.05).

Aqueous oregano, showed bactericidal effects on all the antibiotic resistant APEC strains at a maximum of 0.5µg/ml (25%). On the contrary, the most sensitive APEC strains were APEC 7, 14, 43, 52, 53 & 54 with MIC value of 0.3µg/ml, which is similar to the reference strain (*E. coli* K12) (**Table 1**).

•Table 2. Effect of different concentrations of ethanol-based oregano on APEC biofilm

Code	Ethanol –based oregano (µg/ml) 0	0.1	0.2	0.3	0.4	0.5
	Biofilm formation = corrected mean value (Mean – LB broth)					
APEC 7	3.263	1.970	-0.006	-0.089	0.018	-0.168
APEC 14	1.755	-0.145	-0.120	-0.098	-0.145	-0.161
APEC 43	3.168	2.680	-0.252	-0.310	-0.306	-0.331
APEC 45	2.535	-0.296	-0.241	-0.264	-0.313	-0.288
APEC 46	1.782	1.465	-0.035	-0.096	-0.082	-0.104
APEC 47	2.091	0.096	-0.054	-0.067	-0.096	-0.095
APEC 48	1.347	-0.004	0.020	-0.080	-0.070	-0.082
APEC 49	1.472	-0.072	-0.026	-0.055	-0.062	-0.073
APEC 51	2.529	1.894	0.034	-0.120	-0.092	-0.120
APEC 52	1.095	-0.101	-0.051	-0.076	-0.074	-0.102
APEC 53	2.170	1.487	-0.045	-0.124	-0.139	-0.163
APEC 54	1.287	-0.028	-0.102	-0.113	-0.158	-0.130
E. coli K12	0.001	-0.030	-0.048	-0.040	-0.053	-0.030

Shaded cells refer to strong biofilm formers (OD590 > 1.500). Based on the OD590 readings values, the ability of bacterial strains to form biofilm was grouped into: no biofilm formation (OD590 <0.100), weak biofilm formation (0.100≤ OD590 ≥1.500), and strong biofilm formation (OD590 > 1.500). (P-value < 0.05)

Oregano reduced biofilm formation dramatically (**Table 2**) Accordingly, APEC strains were fitted into the categories of weak biofilm formers (33.33%), but mostly of strong biofilm formers (66.67%). Since oregano was prepared in 70% ethanol first and then diluted in broth, the ethanol factor was considered. Thus, a positive control study was done, but in the presence of the same dilutions of ethanol but without oregano biofilms were formed. The final conclusion is that the ethanol dissolved phytochemicals and not ethanol was the inhibitor of biofilm formation.

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Batch culture to study the effect of carvacrol treatment on bacterial population inhabiting the chicken gut

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I. Abstract

The current *in vitro* study focused on the utility of carvacrol as a phytochemical to suppress antibiotic-resistant avian pathogenic *Escherichia coli* (APEC) strain which is responsible for causing colibacillosis in poultry reared for human consumption. A sub-lethal concentration of carvacrol showed positive impact on the bacterial population inhabiting chicken gut by nourishing the growth of beneficial probiotic bacteria (*Lactobacillus* sp., and *Lactobacillus salivarius*) and bacteria exhibiting anti-Salmonella activity in young chicks (*Streptococcus* sp. and *Coprococcus* sp.) in comparison with ampicillin and combined treatments. These bacteria might be responsible for preventing the colonisation of APEC including antibiotic-resistant strains which is the first stage of infection. The current data generated provides promising evidence that carvacrol as a feed additive might enhance avian gut health and may be used in novel ways to control potentially pathogenic and antibiotic-resistant APEC strains and provide alternative strategies to the use of synthetic antibiotics. Future work will involve *in vivo* studies to justify the use of carvacrol in the poultry industry.

II. Introduction

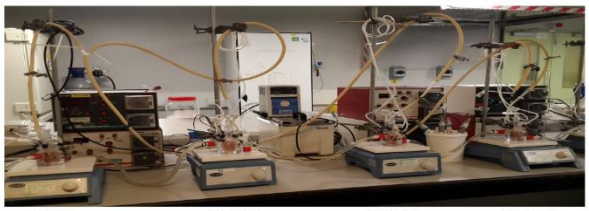
In poultry production, the rising issue of bacterial antibiotic resistance made us shift our perspective to seek alternatives to control diseases (Si et al., 2006). In order to investigate the application of phytochemicals as a potential alternative to antibiotics on the gut bacteria, *in vitro* gut models in the form of batch cultures were established to help in the study of gut bacteria in terms of their interaction and metabolism (Macfarlane and Macfarlane, 2007).

Phytochemicals come from natural sources and generally recognized as safe (GRAS) which make them good candidates to be used as feed additives in poultry production in comparison with antibiotics (Hashemi et al., 2008). They can improve animal health and performance because of their anti-microbial, anti-stress (Wang et al., 1998) and anti-oxidant properties (Valenzuela, 1995), and their ability to modulate gut microbiota (Hashemi et al., 2009) and enhance immune responses (Chowdhury et al., 2018). The phytochemical used in this study was carvacrol which is of polyphenol nature.

III. Materials & Methods

Batch cultures were inoculated with a pooled caecal slurry of (1:10) dilution in VL medium and challenged with an ampicillin-resistant APEC strain (10^7 CFU/ml) in the presence of different treatments (sub-lethal concentrations of carvacrol and ampicillin, separately and combined).

Conditions of the 20ml vessels were set as anaerobic by gas pumps, pH value (5.8 - 6.0), and a temperature of 41°C. Samples were collected at four different time points of 0, 24, 48, and 72hr for whole population profiling using NGS approach.



V1 & V2: Control	V3 & V4: 20µg/ml ampicillin	V5 & V6: 0.2µg/ml carvacrol	V7 & V8: Combined treatment

IV. Results

- **Alpha diversity**

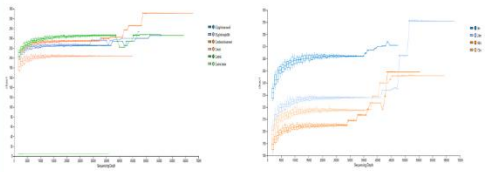


Figure 1. Alpha rarefaction curve of the bacterial communities using chao1 index according to treatment (left) and time points (right).

- **Beta diversity**

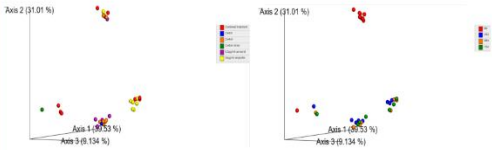


Figure 2. PCoA analysis profile of the bacterial communities using unweighted (qualitative) UniFrac distance metric index according to treatment (left) and time points (right).

Changes of the bacterial communities among each vessel (**Figure 1**) and across all vessels (**Figure 2**) were time dependent but trends were observed by treatment.

- **Taxonomic profiling at species level**

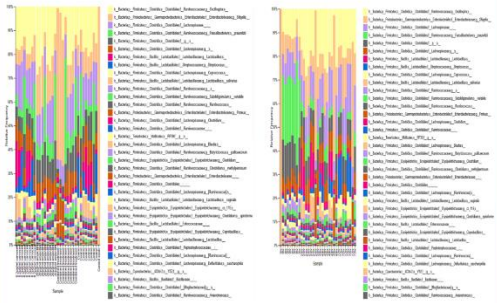


Figure 3. Taxonomic bar plot showing species level of the bacterial communities according to treatment (left) and time points (right).

The added APEC strain (*E. coli-Shigella* sp.) was present in all vessels and present in at low percentages at different time points, but its abundance was the highest in the combined treatment vessels (**Fig 3**).

A) Carvacrol treatment alone showed an increase in the following bacterial species all of which are considered beneficial….

- *Lactobacillus* sp.
- *Lactobacillus salivarius*
- *Streptococcus* sp.
- *Coprococcus* sp.

and associated with the lowest numbers of *Enterobacteriaceae*.

B) Ampicillin and combined treatment showed a decrease in those beneficial bacteria cited above but increase in the following species….

- *Oscillospira* sp.
- *E. coli-Shigella* sp.
- *Proteus* sp.

which is a cause for concern as the perceived benefits of carvacrol are dramatically reversed by ampicillin. *In vivo* studies are required to confirm these findings in broiler production systems.

V. Discussion & Conclusion

Carvacrol treatment showed modulatory effect on the bacterial population inhabiting chicken caeca challenged with an APEC strain in result of an increase in probiotic bacteria and bacteria with anti-Salmonella activity.

This *in vitro* study provides preliminary results on the use of carvacrol as a feed additive to control ampicillin-resistant APEC strains which are responsible for economical loss in poultry industry before going to *in vivo* studies.

References

CHOWDHURY, S., MANDAL, G. P., PATRA, A. K., KUMAR, P., SAMANTA, I., PRADHAN, S. & SAMANTA, A. K. 2018. Different essential oils in diets of broiler chickens: 2. Gut microbes and morphology, immune response, and some blood profile and antioxidant enzymes. Animal Feed Science and Technology, 236, 39-47.

HASHEMI, S., ZULKIFLI, I., FARIDA, A. & SOMCHIT, M. 2008. Acute toxicity study and phytochemical screening of selected herbal aqueous extract in broiler chickens.

HASHEMI, S., ZULKIFLI, I., ZUNITA, Z., HAIR-BEJO, M., LOH, T., SOMCHIT, M., KOK, P. & DAVOODI, H. Effects of dietary supplementation with Euphorbia hirta and acidifier on performance and Salmonella colonization in broiler chickens. Proceedings of the 30th Malaysia Society of Animal Production Annual Conference, 2009.

MACFARLANE, G. T. & MACFARLANE, S. 2007. Models for intestinal fermentation: association between food components, delivery systems, bioavailability and functional interactions in the gut. Current opinion in biotechnology, 18, 156-162.

SI, W., GONG, J., TSAO, R., ZHOU, T., YU, H., POPPE, C., JOHNSON, R. & DU, Z. 2006. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. Journal of Applied Microbiology, 100, 296-305.

WANG, R., LI, D. & BOURNE, S. Can 2000 years of herbal medicine history help us solve problems in the year 2000. ALLTECHS ANNUAL SYMPOSIUM, 1998. 168-184.

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```
treat     number         timepoint
 V1:3   Min.   :1.000e+05   0hr :4
 V2:3   1st Qu.:2.500e+06   24hr:4
 V3:3   Median :2.500e+08   48hr:4
 V4:3   Mean   :4.754e+08
        3rd Qu.:7.250e+08
        Max.   :2.000e+09

Analysis of Variance Table
Response: lognum
          Df  Sum Sq Mean Sq F value Pr(>F)
treat      3  0.2871 0.09569  0.0306 0.9922
Residuals  8 25.0125 3.12656
> mt<-lm(lognum~timepoint)
> anova(mt)
Analysis of Variance Table

Response: lognum
          Df  Sum Sq Mean Sq F value    Pr(>F)
timepoint  2 23.6839 11.8419  65.964 4.203e-06 ***
Residuals  9  1.6157  0.1795
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> pairs(lsmeans(mt,~timepoint))
 contrast      estimate       SE df t.ratio p.value
 0hr - 24hr  -2.6192803 0.2996004  9  -8.743  <.0001
 0hr - 48hr  -3.2425092 0.2996004  9 -10.823  <.0001
```

24hr - 48hr -0.6232289 0.2996004 9 -2.080 0.1491

Analysis of Variance Table

Response: number

Df Sum Sq Mean Sq F value Pr(>F)

treat 3 655133 218378 0.5354 0.6709

Residuals 8 3263100 407888

> mt<-lm(number~timepoint)

> anova(mt)

Analysis of Variance Table

Response: number

Df Sum Sq Mean Sq F value Pr(>F)

timepoint 2 2710728 1355364 10.102 0.005007 **

Residuals 9 1207505 134167

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> pairs(lsmeans(mt,~timepoint))

contrast estimate SE df t.ratio p.value

0hr - 24hr -298.875 259.0051 9 -1.154 0.5075

0hr - 48hr -1123.875 259.0051 9 -4.339 0.0048

24hr - 48hr -825.000 259.0051 9 -3.185 0.0271

P value adjustment: tukey method for comparing a family of 3 estimates

> pairs(lsmeans(mt,~timepoint))

contrast estimate SE df t.ratio p.value

0hr - 24hr -298.875 259.0051 9 -1.154 0.5075

0hr - 48hr -1123.875 259.0051 9 -4.339 0.0048

```
 24hr - 48hr  -825.000 259.0051  9  -3.185  0.0271


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 24hr - 48hr  -825.000 259.0051  9  -3.185  0.0271


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 0hr - 48hr  -1123.875 259.0051  9  -4.339  0.0048
 24hr - 48hr  -825.000 259.0051  9  -3.185  0.0271
```