Investigating the association between short chain fatty acid antimicrobials and *Escherichia coli* virulence

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Abstract

Antibiotic resistance in bacteria is often attributed to the excessive use of antibiotics in the agricultural and food processing sectors. Globally, antibiotics are also widely used as growth supplements in livestock, and this practice has led to an increase in multidrug-resistant microbes, raising concerns regarding the use of human-use antibiotics in livestock and food-producing animals. Due to their desirable properties, antibiotic alternatives such as organic acids have recently replaced antibiotics as antimicrobials and preventatives. As a breakdown product of non-digestible carbohydrates, some organic acids, such as propionic acid (PA) and formic acid (FA), are naturally present in the human and animal guts, and they serve crucial roles in regulating the host immune responses.

Moreover, a study revealed that long-term exposure of adherent-invasive *Escherichia coli* (AIEC), a bacterial pathotype linked with Crohn's disease, to PA significantly altered its phenotype, resulting in enhanced adherence and invasion of epithelial cells and increased persistence through biofilm formation. In addition, it remains unclear what makes AIEC pathogenic, but it was proposed that environmental factors such as organic acids have a role in altering AIEC phenotype which could make the strains more pathogenic. Therefore, organic acids and alterations of the AIEC phenotype were investigated. Since AIEC is evolutionarily and phylogenetically related to avian pathogenic *Escherichia coli* (APEC), and APEC strains are more often exposed to organic acids such as PA and FA due to their widespread use in feeds, the impact of FA and PA on APEC strains was also investigated.

In the investigation of organic acids and their association with phenotypic alteration of AIEC and APEC, several methods were used, such as Next-Generation Sequencing and an *in vitro* fermentation gut model, along with several assays to determine organic acids effects. The results revealed that PA can alter the phenotype of AIEC and increase its virulence in traits such as adhesion, invasion and biofilm formation. In addition, AIEC adapted to PA showed a significant increase in net replication within immune cells when ethanolamine is present, ethanolamine being a carbon source that becomes increasingly available during intestinal inflammation. However, FA has a different effect on AIEC and APEC strains. The gene expression of AIEC adapted to FA revealed that FA has inhibitory effects in contrast to PA. Additionally, an *in vitro* fermentation gut model indicated that more *E. coli* can be recovered from the fermentation when PA is present as opposed to FA.

In conclusion, organic acids can alter AIEC and APEC phenotypes, and some of these alterations could lead to the emergence of virulent strains of bacteria. The finding that PA increased the virulence of AIEC raises concerns about its long-term effects since it is used as an antibacterial in various food and agricultural sectors. Also, the findings show that FA is a more effective antibacterial for *E. coli*. However, APEC strains responded differently to the FA, meaning additional research is required to identify specific FA effects on APEC strains.

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Dedication

I would like to dedicate this work to my family and friends.

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Abbreviations

AFEC – Avian faecal *Escherichia coli*

AGPs – Antibiotics as growth promoters

AIEC – Adherent-invasive *Escherichia coli*

AMR – Antimicrobial resistance

ANOVA – Analysis of variance

APEC – Avian pathogenic *Escherichia coli*

APCs – Antigen presenting cells

ATG16L1– Autophagy related 16-like 1

ATG5 – Autophagy related 5

BCFA – Branched chain fatty acid

CD – Crohn's disease

CCK-8 – Cell counting kit 8

CEACAM6 – Carcinoembryonic antigen-related cell adhesion molecule 6

CFU – Colony forming unit

ColV – Colicin V

CSP – Competence-stimulating peptide

CSF – Cerebrospinal fluid

DCs – Dendritic cells

DEGs – Differentially expressed genes

E. coli – *Escherichia coli*

EEN – Exclusive enteral nutrition

ExPEC – Extraintestinal pathogenic *Escherichia coli*

EHEC – Enterohaemorrhagic *E. coli*

EAEC – Enteroaggregative *E. coli*

FBS – Foetal bovine serum

FDA – Food and Drug Administration

FHL– Formate hydrogenlyase

Fish – Fluorescent in situ hybridization

FNR – Fumarate and nitrate reduction

GIT – Gastrointestinal tract

Gp96 – Glycoprotein 96

Gp2 – Glycoprotein 2

GRAS – Generally Recognized as Safe

GWAS – Genome-wide association studies

HGT– Horizontal gene transfer

HPI – Hours post-infection

HPTT – Horizontal plasmid transfer via transformation

H_2O_2 – Hydrogen peroxide

IBD – Inflammatory bowel disease

IECs – Intestinal epithelial cells

IgA – Immunoglobulin A

IL-1 – Interleukin-1

IL-6 – Interleukin-6

IL-12 – Interleukin-12

IL-17A – Interleukin-17A

IFN-γ – Interferon-gamma

LPS – Lipopolysaccharides

LPF – Long polar fimbriae

OMVs – Outer membrane vesicles

OTUs – Operational taxonomic units

IM – Intramuscular

MCFAs – Medium chain fatty acids

MDR – Multidrug resistance

MLi-C – Membrane bound lysozyme inhibitor of C-type

MOI – Multiplicity of infection

MRSA – Methicillin-resistant *Staphylococcus aureus*

NF-κB – Nuclear factor kappa β

NMEC – Neonatal meningitis *Escherichia coli*

PBS – Phosphate buffered saline

PCA – Principal components analysis

PCoA – Principal coordinate analysis

PCR – Polymerase chain reaction

PCU – per population correction unit

pKa – acid dissociation constant

PMA – Phorbol 12-Myristate 13-Acetate

PPM – Parts per million

PPS – Peyer's patches

PT – Plasmid transformation

qPCR – Quantitative real-time PCR

RPKM – Reads Per Kilobase of transcript, per Million mapped reads

RPMI – Roswell Park Memorial Institute

SCFAs – Short chain fatty acids

SPB – Sodium phosphate buffer

STAT1 – Signal transducer and activator of transcription 1

STAT3 – Signal transducer and activator of transcription 3

STAT4 – Signal transducer and activator of transcription 4

STM – Signature-tagged transposon mutagenesis

TCAs – Tricarboxylic acids

TCS – Two component systems

Th – T helper cells

TNFα – Tumour necrosis factor α

UC – Ulcerative colitis

UTI – Urinary tract infection

USD – United States dollar

UPEC – Uropathogenic *Escherichia coli*

WGS – Whole genome sequencing

WHO – The World Health Organization

Author's Declaration

I declare that the work contained in this thesis is original and is the work of the author Ghaith Fallata. I have been solely responsible for the organisation and working of this study, laboratory analysis and data processing, unless otherwise referenced and made to the contribution of others.

Ghaith Fallata, MSc

Chapter 1

General Introduction

1.1 Antibiotics

1.1.1 Overview

Antibiotics are a class of antimicrobial agents that are effective against microorganisms. They can be produced naturally by microorganisms, semi-synthetically, or as synthetic chemicals. Antibiotics such as penicillin, tetracycline and erythromycin are produced naturally by bacteria and fungi (Guardabassi et al., 2009). Other antibiotics such as amoxicillin, clarithromycin and doxycycline are semi-synthetically produced, while sulfonamides and fluoroquinolones are chemically synthesised (Guardabassi, Jensen, & Kruse, 2009). Antibiotics are the most effective antimicrobial agent for treating bacterial infections for many reasons. They can inhibit the growth of, or kill, bacteria and are widely used in treating and preventing bacterial infections such as *Escherichia coli* (*E. coli*) infection (Glisson et al., 2004). Consequently, antibiotics are considered essential for human and animal life preservation. (Tullo et al., 2019). Moreover, antibiotics are used in several sectors, including in livestock. Statistics show that 80% of the antibiotics sold in the United States are used in livestock, while 20% are used for humans (Martin et al., 2015). Livestock production is vital worldwide; it provides food and contributes to economic success of many countries (Idamokoro et al., 2022; Thornton, 2010). As living conditions have improved, meat consumption has increased, and as a result, livestock breeding facilities have been expanded in different places to meet the expanding need (Idamokoro et al., 2022; Thornton, 2010). As a result, substantial amounts of antibiotics are used in livestock to prevent and cure animal infections and to increase growth (Lee et al., 2021).

1.1.2 Antibiotics use in livestock

Antibiotics in livestock are used in three primary ways: disease treatment, disease prevention, and growth promotion. Antibiotics such as amoxicillin, penicillin, erythromycin, quinolones, gentamicin, novobiocin, tylosin, tilmicosin, and tetracycline are utilised in meat-producing animals to cure and prevent the most prevalent diseases in livestock which include bovine pneumonia, diarrhoea, and shipping fever (Economou et al., 2015). For the treatment of pneumonia, oxytetracyclines and spectinomycin are the first-line antibiotics, followed by florfenicol and macrolides (especially tilmicosin), and finally, second-, third-, and fourth-generation cephalosporins. Nonetheless, antibiotics are given at least once through the feed for various reasons, including the prevention of liver abscesses and respiratory infections,

and to enhance growth (Economou et al., 2015). In situations of clinical mastitis, narrow-spectrum antimicrobials are preferred, with β-lactam antimicrobials used as the first choice when treating mastitis caused by *Streptococci* and penicillin used as the first choice when treating *Staphylococcus* induced mastitis. In certain circumstances, antibiotics are administered intramammary to the whole herd throughout the non-lactating phase to avoid contagious mastitis (Economou et al., 2015).

In the case of infectious diseases in livestock, disease prevention is often attained by treating the whole flock to avoid disease spread, despite clinical signs in few animals. This is referred to as metaphylaxis, and it involves the administration of large dosages of antibiotics for a short duration. Nonetheless, the red line between antibiotics used for treatment and antibiotic used for prevention is not apparent (Economou et al., 2015). In comparison, antimicrobial usage for prevention (sometimes called prophylaxis) refers to administering antimicrobials in low dosages in feed or drinking water for an extended length of time, often several weeks (Economou et al., 2015). While the animals do not exhibit clinical indications throughout this period, the possibility of infection persists (Economou et al., 2015).

Using antibiotics as growth promoters (AGPs) is common in the poultry industry. This approach has been used in agriculture since the 1950s and has provided benefits to the agricultural sector by increasing production efficiencies and maximising livestock health (Marshall et al., 2011). In 1951, the U.S. Food and Drug Administration (FDA) allowed the use of antibiotics as feed additives for livestock without a prescription. Following that, the use of AGPs in livestock has become common practice worldwide, increasing by 10- to 20-fold since the 1950s (Lees et al., 2021; Marshall et al., 2011). In poultry, antibiotics used for therapeutic purposes are often administered through water, while antibiotics used for growth promotion are added to feed (Hofacre et al., 2013). The most commonly used antibiotics in poultry are penicillins (amoxicillin), quinolones (enrofloxacin), tetracyclines (doxycycline, oxytetracycline), macrolides (erythromycin, tylosin), aminoglycosides, sulfonamide/trimethoprim combination, polymyxins (colistin), and other antimicrobials (tiamulin) (Economou et al., 2015).

AGPs are usually administered in relatively low concentrations, ranging from 2.5 mg/kg to 125 mg/ kg parts per million (ppm), depending on the drug type and animal species and (Table 1-1) shows the top AGPs (Costa et al., 2011). A study focused on worldwide antimicrobial use based on species-specific antimicrobial consumption coefficients per

population correction unit (PCU), showed that intensive chicken production has a wider range of antimicrobial use than pork production (Boeckel et al., 2015). In addition, the study indicated that the rapid growth rate and short life span of broilers had been associated with the use AGPs at high doses in animal feeds to improve the product's quality, resulting in a lower percentage of fat, with a relatively high concentration of polyunsaturated fatty acids and a higher protein content of the meat (Boeckel et al., 2015). Because of their ease of usage, low cost, and benefits, AGPs have been used in poultry production worldwide (Rafiq et al., 2022). This widespread use of AGPs in poultry production has changed intensive poultry production by increasing gut health and reducing subclinical infections, while promoting growth, production, and feed conversion efficiency.

Class	Antibiotic	Spectrum	animals
Aminoglycosides	Neomycin	Narrow spectrum	Cattle
	Gentamicin	Broad spectrum	Cattle, Swine
	Spectinomycin		Sheep
	Streptomycin		Chickens, Swine, Sheep, Cattle
Penicillins	Penicillin G potassium	Broad spectrum	Chickens
	Penicillin G procaine		Chickens, swine
Ionophores	Lasalocid sodium	Broad spectrum	Cattle
	Salinomycin		Cattle, Swine
	Narasin		Swine
	Monesin		Cattle
Macrolides	Erythromycin	Broad spectrum	Chickens
	Tylosin	Broad spectrum	Swine
	Tilmicosin	Narrow spectrum	Chickens
Streptogramins	Virginiamycin	Broad spectrum	Chickens, swine
Tetracyclines	Oxytetracycline	Broad spectrum	Chickens, swine, sheep, cattle
	Chlortetracycline		
B-lactam	Amoxicillin	Narrow spectrum	Chickens, swine, sheep, cattle
	Ampicillin	Broad spectrum	
	Penicillin V	Narrow spectrum	Swine
Bacitracin	Polypeptides	Narrow spectrum	Bovine

Table 1-1. Adapted from Brown et al., 2017; Ronquillo et al., 2017. Antibiotics employed as antibiotic growth promoters (AGPs) in livestock.

1.1.3 Methods of delivering antibiotics to livestock

Antimicrobial agents like antibiotics can be administrated to animals via various routes. They can be applied directly to the site of infection through topical or intramammary routes, or they can be supplied orally (enteral administration) for local action in the gastrointestinal tract (GIT) or for absorption and systemic effect (Page et al., 2012). They may also be given parenterally to avoid the gastrointestinal system, such as by intravenous, intramuscular (IM), or subcutaneous injection (Page et al., 2012). Further, antibiotics are frequently added to drinking water in response to a rising disease epidemic, especially in pigs and poultry. This practice is referred to as water medication, another way of administrating antimicrobial agents to livestock (Page et al., 2012). This method of medication administration is often favoured, as the water supply, particularly that of confined livestock, can be treated instantly through header tanks or proportioning equipment. In addition, it is thought that sick animals would continue to drink even if their appetite is low (Page et al., 2012). However, this assumption may not be well-founded because a study reveals that *Actinobacillus pleuropneumoniae* toxin-exposed pigs exhibited a similar reduction in feed and water consumption (Pijpers et al., 1991). When water medication is selected, it is essential to know the average water consumption to determine the inclusion rate of the antimicrobial agent. The amount of water consumed by poultry, pigs, and beef cattle is influenced by several factors, including age and stage of growth, activity level, environmental temperature and humidity, water temperature, water quality (including hardness, mineral content, and sulphate content), water palatability, feed composition, and particularly for poultry, lighting programme (Fairchild et al., 2015). In addition, there is the possibility of varying wastage based on the kind of drinkers and how they are managed (Y. Z. Li et al., 2005; Torrey et al., 2008). Failure to ensure that these factors are optimised can result in inefficient medication delivery, as demonstrated by a study of enrofloxacin in hens (Sumano et al., 2010; Sumano et al., 2004).

Because of the variety of factors that influence water intake, it is usually suggested that current water intake be measured and included in dilution rate calculations. In addition to an accurate estimate of water intake, it is essential to have an antimicrobial agent formulation that allows it to dissolve or remain adequately suspended in drinking water while preserving its chemical stability (Page et al., 2012). Medication can be administered as a pulse (a dose of drugs added to water consumed in a short period) or constantly throughout the treatment period (Page et al., 2012). In many instances, pulse dosing may be more effective, although

the higher drug concentration in the water may alter palatability, resulting in water rejection, as has been described with florfenicol (Gutiérrez et al., 2011). In some instances, even high dose rates cannot overcome the inherent low bioavailability of the drug. Tetracycline hydrochloride administered to pigs in water at concentrations up to 500 mg/L resulted in mean steady-state plasma concentrations of less than 0.8 mg/L, inadequate for treatment of even highly susceptible bacteria (Dorr et al., 2009; Mason et al., 2009). In another investigation, adding citric acid to water increased the bioavailability of chlortetracycline (Pollet et al., 1985). Moreover, the variability in the water flow rate in the animal facility is a further significant element influencing the administered antimicrobial dose. A recent study showed that water flow rates differed between farms, barns within farms, and pens within barns, as well as by drinker type (Dorr et al., 2009).

Feed medication is another method of administering antibacterial drugs to livestock. Feed medication is one of the most extensively used procedures in the world and should be administered under good animal feeding standards (FAO/WHO, 2015). The medication may be applied on top of the feed for smaller groups of animals, whereas for larger groups, the drug is usually included in the final feed. The medicated feed can be provided as a dry loose mixture, a mash, crumbles, or pellets. The physical form of antimicrobial premixes can affect carry-over, as shown with sulphamethazine, which has a high electrostatic attraction as a powder but loses this property when it is granulated (Rosenberg, 1985). For certain localised infections, antimicrobial drugs can be delivered by intra-articular, intrapleural, intratracheal, ophthalmic, subconjunctival, topical (e.g., skin lesions, wounds), interdigital, intravaginal, and intrauterine routes, as well as the in ovo route in chickens (Page et al., 2012). In general, antibiotic use in livestock is a widespread practice because antibiotics can be used to treat and prevent bacterial diseases. Additionally, they can enhance the growth and health of animals. Despite antibiotic use in livestock having several benefits, significant concern has been raised regarding the antibiotic role in creating antibiotic-resistant microbes.

1.1.4 Antimicrobial resistance

1.1.4.1 *Risk of antibiotic overuse*

Antimicrobial resistance (AMR) is widely recognised as one of the most serious worldwide public health issues. It is estimated that 700,000 people die annually because of AMR, and this number is expected to increase to 10 million by 2050 (Neill, 2014). The irrational and indiscriminate use of antibiotics in humans, livestock, and the poultry industry is the primary cause of this danger (Rafiq et al., 2022). Because of the increased consumption of meat and eggs which are now readily available, relatively inexpensive, and rich in the most vital nutrients, the poultry industry is today one of the fastest-growing subsectors of the agriculture and veterinary sectors (Rafiq et al., 2022). These factors helped drive the increase in the use of antibiotics in the poultry industry. The increasing and indiscriminate use of antibiotics may result in residues being deposited in poultry food products and microorganisms developing resistance to these antibiotic residues. As a result, several human and animal diseases which are dependent on antibiotic intervention are getting more difficult to treat (Rafiq et al., 2022).

Additionally, the use of low-dose antibiotics as a AGPs leads to antibiotic residue in food products, which can have detrimental effects on human health (Costa et al., 2017). On the other hand, several studies have shown that antibiotics given to poultry and livestock are poorly absorbed in the intestine and are often excreted without being taken up or metabolised (Chee-Sanford et al., 2009; Manyi-Loh et al., 2018; Mehdi et al., 2018; Thanner et al., 2016). These excreted antibiotics ultimately accumulate in the environment and enter the human food chain, resulting in drug residue bioaccumulation in the human body (Rafiq et al., 2022; Thanner et al., 2016). Furthermore, incorrect standards in food processing industries, such as slaughtering and processing plants, where productivity may be significant in terms of the number of processed animals (especially for poultry), raise the risk of AMR bacteria in the food chain (Caruso, 2018). There is irrefutable evidence that foods from various animal sources and all processing stages contain abundant resistant bacteria and resistance genes (Chang et al., 2015; Witte, 2000).

1.1.4.2 *Methods of acquiring antibiotic resistance genes in the environment*

The natural environment provides a favourable platform for exchanging genes among the bacterial population. Bacteria can acquire various resistance mechanisms to survive extreme environmental conditions. Environmental pressures require bacteria to develop intrinsic or extrinsic resistance mechanisms to ensure survival. Bacteria can acquire resistance through spontaneous mutations, which result from errors in the cellular and metabolic processes such as DNA replication, transcription, recombination (Schroeder et al., 2018). For instance, mutations in the target genes alter the binding site of antibiotics, or mutations at the target sites can lead to the overexpression of targets during the transcriptional step. These targets otherwise are naturally expressed at a very low level. In some instances, specific mutations can modify the drug targets. As a result, the minimum inhibitory concentration of a particular antibiotic increases beyond the therapeutic limit (Barbier et al., 2016). Altered efflux systems mediated by many different mutations can also cause up-regulation or overexpression of the efflux systems, therefore antibiotics cannot reach to the target sites of the bacterial cells (Ruppé et al., 2015). Fluoroquinolone resistant *E. coli* and *Pseudomonas aeruginosa* are the two best examples of this type of resistance (Ruppé et al., 2015).

In addition, antibiotic resistance can be acquired by horizontal gene transfer (HGT), mobile gene transfer, and recombination that provide resistance to a wide variety of antibiotics such as trimethoprim, sulphonamides, quinolones, and erythromycin (Maiden, 1998). HGT is the method through which the transmission of genetic material between two bacteria occurs without sharing hereditary material (Andam et al., 2011). The horizontally transmitted genes can range from gene fragments to complete genes, operons, and even the entire genome (Igarashi et al., 2001). HGT gives bacteria a selective advantage in adapting to a new habitat (Marri et al., 2006). The transmission of antibiotic-resistant genes by HGT has resulted in the fast appearance, mutation, and diversification of bacteria, which is the underlying cause of the escalation of multidrug resistance (MDR) (Trotta et al., 2021). It has been discovered that HGT is responsible for modifying the original genome for better adaption, ultimately leading to the formation of new species from an existing population (Welch et al., 2002). In addition, HGT can bridge the gap between the traits of two species by transferring genetic material (Fraser et al., 2005). Indeed, there are three main mechanisms in HGT which are conjugation (both plasmid and chromosomal), transformation (the uptake of DNA from the environment) and phage-mediated transduction.

Conjugation is the transfer of genetic material such as plasmid DNA from donor bacteria to recipient bacteria through direct physical cell-to-cell contact (Virolle et al., 2020). Conjugation is the most important way of horizontal transfer, and this mechanism is widely present in bacteria (Leungtongkam et al., 2018). Conjugation is a contact-dependent process where mobile genetic elements, such as plasmids and integrating and conjugation elements, are transported through a pilus or pore between bacteria close to each other (Partridge et al., 2018). Resistance genes can be transmitted through the conjugation between the same genus or different species. The spread of mobile genetic elements has been observed in commensal and opportunistic pathogens while colonising the human gut (Porse et al., 2017). Conjugation of plasmid-mediated antimicrobial resistance genes and the transmission of drug resistance pose a serious threat to human health (Wang et al., 2018). The plasmids carrying carbapenemase resistance genes (such as bla_{KPC}, bla_{NDM}, and $bla_{OXA\text{-}48}$) in Gram-negative bacteria can be rapidly transmitted to other susceptible bacteria by conjugation, which has become a major global health threat (Hasan et al., 2022). It has been reported that the plasmid encoding OXA-48, carbapenem resistance, from *Enterobacter cloacae* may be conjugally transferred to other members of the Enterobacteriaceae family in the GIT (Steed et al., 2020). Studies have demonstrated that the conjugation elements-mediated drug resistance transmission mechanisms can also be found in Gram-positive bacteria, such as *Streptococcus* spp (San Millan, 2018).

Transduction is the process by which bacteriophages can package DNA from donor bacteria which, upon injection into recipient bacteria, undergoes homologous recombination with the recipient genome. Generalised transduction involves the packaging of random chromosomal fragments by lytic bacteriophages, whereas specialised transduction is mediated by lysogenic bacteriophages that package DNA adjacent to their sites of insertion in the donor genome owing to imprecise excision (Chiang et al., 2019). Phages can coexist with antibiotic resistance genes in the same ecological environment and the same bacteria, indirectly suggesting that phages may play a role in the spread of drug resistance genes (Torres-Barceló, 2018; Voigt et al., 2021). Resistance transduction is more common in *Staphylococcus aureus* (Foster, 2017). Methicillin-resistant *Staphylococcus aureus* (MRSA) acquires resistance from other bacterial species conferring the *mecA* gene by phage-mediated transduction (Craft et al., 2019). Indeed, experiments in mouse models have demonstrated that transduction is a driving force behind genetic diversity in gut-colonising *E. coli* strains and can promote the emergence of drug resistance in gut bacteria (Frazão et al., 2019; Modi et al., 2013).

Transformation can be compared to the recombination processes that sexual organisms undertake during sexual reproduction, except that only a portion of the chromosome is involved (Maiden, 1998). It is thought that most mosaic genes known so far arose because of transformation phenomena. There are three requirements for bacterial transformation. First, exogenous DNA generated from genetically separate bacteria must be present in the host bacterium's immediate environment. Second, the quantity and quality of DNA accessible for transformation will vary depending on the environment. Third, exogenous DNA generated from genetically separate bacteria must be present in the host bacterium's immediate surroundings (Maiden, 1998). The quantity and quality of DNA accessible for transformation in a particular environment depends on the rate at which it is released (by autolysis, for instance) compared to its half-life in the environment. Numerous bacteria, including both Gram-positive and Gram-negative species, contain absorption mechanisms that are unique to DNA. Although the primary biological function of these DNA absorption systems has been the subject of debate, it is evident that they facilitate genetic exchange by permitting transformation (Lorenz et al., 1994). The most plausible method for the permanent integration of recruited DNA into the new host microbial genome is homologous recombination, which is mediated by RecA (Maiden, 1998). This method depends on the introduced DNA and requires that host DNA has at least 70% nucleotide sequence similarity leading to the replacement of chromosomal regions with homologous DNA (Maiden, 1998).

E. coli is not thought to be naturally transformable; it develops high genetic competence only under artificial conditions, such as exposure to high Ca^{2+} concentrations and temperature shock (Sambrook et al., 2006), polyethylene glycol treatment, or electrical shock (Hanahan, 1983; Mandel et al., 1970; Sambrook et al., 2006). However, *E. coli* could demonstrate moderate competence under specific conditions that are consistent with its natural environments (Hasegawa et al., 2018). Hereafter, transformation in which a plasmid was added externally is defined as plasmid transformation (PT). In contrast, transformation in which plasmid DNA is derived from dead bacterial cells from the environment is referred to as horizontal plasmid transfer via transformation (HPTT).

E. coli has multiple DNA-uptake mechanisms, including two well-known ones; one that is dependent on "competence genes," which are commonly found in Gram-negative and Gram-positive bacteria (Finkel et al., 2001; Jaskólska et al., 2015; Palchevskiy et al., 2006; Seitz et al., 2013; Sinha et al., 2012). In *E. coli,* these genes are not considered to contribute to PT because PT requires the uptake of intact double-stranded circular DNA (Johnston et al.,

2014; Sinha et al., 2012). Therefore, this mechanism is unlikely to contribute to PT in the environment. The second mechanism is dependent on external environmental factors, such as divalent metal ions, heat shock, and physical stresses (Hanahan, 1983; Mandel et al., 1970; Rodríguez-Beltrán et al., 2013; Yoshida, 2007). These stimuli are commonly considered to induce the formation of pore-like structures in the bacterial cell surface for the passing of intact double-stranded DNA, including circular plasmids, although the details remain unclear (Asif et al., 2017; Natoli et al., 1988; Reusch, 2013; Reusch et al., 1995; L. Sun et al., 2013). The most common competence-inducing factors are Ca^{2+} and Mg^{2+} ions. Environments often include several millimolar concentrations of these ions, which are sufficient to generate modest but detectable competence in *E. coli* (Hasegawa et al., 2018). As a result, this mechanism of HGT is possible outside of labs. In addition to the two mechanisms described, it has been proposed that another mechanism involves an ABC transporter and particular periplasmic and inner membrane proteins (Guo et al., 2015; Sun et al., 2016; Zhang et al., 2012) Because this mechanism is regulated by internal transcriptional regulators RpoS and CRP, it was hypothesised that it is a genetically controlled natural process.

1.2 Zoonotic disease

1.2.1 Overview

Humans, animals, and the environment all have a role in the origin and transmission of several infectious diseases (Rahman et al., 2020). The majority of infectious diseases that affect humans originate from animals. According to the "Asia Pacific strategy for emerging diseases: 2010" study, around 60% of new human infections are zoonotic in nature, with more than 70% of these pathogens originating from wildlife species (Rahman et al., 2020). In recent decades, newly emerging human diseases were of animal origin and were directly associated with animal-origin foods (Slingenbergh, 2013). The term "zoonoses" is derived from the Greek words "zoon" (animal) and "nomos" (disease). The World Health Organization (WHO) defines zoonosis as any illness or infection naturally transmissible from vertebrate animals to humans or from humans to animals (World Health Organization | TDR Disease Reference Group on Zoonoses and Marginalized Infectious Diseases of Poverty, 2012). Approximately 61% of human pathogens are zoonotic (Rahman et al., 2020).

Therefore, zoonoses are a significant public health problem and a direct human health risk that may result in mortality (Grace et al., 2012). The 13 most prevalent zoonoses have had the greatest impact on impoverished livestock workers in low and middle-income countries, causing an estimated 2.4 billion cases of disease and 2.7 million deaths per year, in addition to their harmful influence on human health (Grace et al., 2012). Most of these diseases also impact animal health and reduce livestock production (Grace et al., 2012). The 13 most prevalent zoonoses have had the greatest impact on impoverished livestock workers in low- and middle-income countries, causing an estimated 2.4 billion cases of disease and 2.7 million deaths per year, in addition to their harmful influence on human health (Grace et al., 2012). Most of these diseases also impact animal health and reduce livestock production (Grace et al., 2012).

A wide variety of pathogens cause zoonotic diseases. Zoonoses are classified based on their aetiology: bacterial zoonoses (such as anthrax, salmonellosis, tuberculosis, Lyme disease, brucellosis, and plague), viral zoonoses (such as rabies, acquired immune deficiency syndrome-AIDS, Ebola, and avian influenza), parasitic zoonoses (such as trichinosis, toxoplasma), and fungal zoonoses (such as ringworm) (Schaechter, 2009). Bacterial zoonotic diseases can be transmitted to humans in various ways (Trevejo et al., 2005). Several zoonotic bacterial infections are food or waterborne in origin, posing a particular threat to immunocompromised persons (Trevejo et al., 2005). The most probable site for zoonotic disease transmission is in or around the household, where routine activities such as food preparation, pet care, and gardening may result in zoonotic disease transmission to the immunocompromised. For example, foodborne pathogens, primarily transmitted by the faecal-oral route, may infect meat, vegetables, and other food products (Trevejo et al., 2005). Outdoor leisure activities, including camping, swimming, and hunting, may expose you to zoonotic infections. Ingestion of river, stream, or lake water, or contact with it, might result in indirect exposure to animal urine or faeces (Trevejo et al., 2005). Hunters are also in danger of contracting zoonotic infections via direct contact with animal corpses or eating infected tissue. Particular vocations, such as veterinary medicine, agriculture, and others that involve direct animal interaction, are more likely to lead to exposure to zoonotic infections (Trevejo et al., 2005). Knowing the environments in which zoonotic diseases are most likely to spread may help develop patient prevention recommendations and methods (Trevejo et al., 2005).

Animal mortality caused by zoonotic infections may cause significant losses in any country's livestock industry. Even if animals do not die, animal health and productivity might be harmed. This may result in a significant loss, up to 70%, of animal products such as meat, milk, and eggs. Human health and nutrition are also impacted by the shortage of high-protein animal-derived foods such as milk, meat, and eggs (Rahman et al., 2020). Zoonotic infections like brucellosis and toxoplasmosis may cause infertility, abortion, and weak offspring. This may result in significant economic losses for farms and the economy. The economy is also significantly affected by zoonoses control and eradication measures such as zoonoses surveillance, diagnosis, isolation, quarantine, restrictions on animal transportation, treatment and vaccine programmes, meat and milk inspections, and biosecurity. The worldwide economic effect of zoonotic outbreaks exceeded 120 billion USD between 1995 and 2008 (Rahman et al., 2020) The economic costs of zoonotic diseases have also been significant in the United Kingdom (Rahman et al., 2020). *Campylobacter spp*., *Salmonella* (non-typhoidal), *E. coli* O157, *Listeria monocytogenes*, and norovirus caused severe economic losses in the United Kingdom in 2007 (Rahman et al., 2020). In addition, some countries suffered significant economic losses as a result of outbreaks of zoonotic food-borne pathogens. Ireland, for example, has experienced significant economic losses as a result of *Salmonella* contamination of its swine products (Rahman et al., 2020). According to the World Bank indicated that Australia's livestock industry has lost 16% of its value due to outbreaks affecting beef and sheep (Rahman et al., 2020).

Bacterial zoonoses have a significant impact on global public health. The closer contact with companion animals and rapid socioeconomic changes in the food production system has increased animal-borne bacterial zoonoses. The emergence of AMR due to the overuse or misuse of antibiotics is another significant public health issue. These diseases negatively influence global travel, business, and the economy. Antibiotic-resistant zoonotic bacterial infections are a concern in most developed countries for vulnerable groups such as infants, the elderly, pregnant women, and immune-compromised individuals (Cantas et al., 2014). There are more than 200 million food-producing animals (cattle, pigs, sheep, goats, and chickens) living on farms throughout Europe, and it has been shown that farm animals serve as reservoirs for several human zoonotic infections (Lahuerta et al., 2011; Wells et al., 2001).

1.2.2 Colibacillosis disease

1.2.2.1 *Avian Colibacillosis*

Colibacillosis is an important bacterial infection that affects poultry and other bird species and is caused by *E. coli*. Even though *E. coli* is considered a commensal inhabitant of the GIT, the disease is caused by pathogenic strains of *E. coli* referred to as avian pathogenic *Escherichia coli* (APEC) (Kathayat et al., 2021). In both mammalian and avian species, *E. coli* can cause systemic infections in a manner associated with immune-deficiency and/or co-infections (Collingwood et al., 2014; Guabiraba et al., 2015; Nolan et al., 2017). Indeed, systemic colibacillosis can occur in calves and piglets when they have hypogammaglobulinemia, a disorder caused by low serum immunoglobulin or antibody levels (Wray et al., 1985; Yong et al., 2008). Also, the disease can occur in colostrum-deprived animals' condition. Indeed, it may also occur in some colostrum-fed animals which have failed to absorb the immunoglobulins.

Factors such as infectious bursal disease, mycoplasmosis, coccidiosis, Newcastle disease or infectious bronchitis, or dietary inadequacies, make birds susceptible to this disease (Ghunaim et al., 2014). In addition, inhaled coliform-contaminated dust has been implicated as one of the most important sources for infecting air sacs of susceptible birds (Nolan et al., 2017). Exposure to chicken-house dust and ammonia results in deciliation of the upper respiratory tract of birds, permitting inhaled *E. coli* to colonize and cause respiratory infection. Maintaining good air and litter quality is fundamental to reducing the risk of a flock developing colibacillosis because bacteria preferentially can adhere to dust particles because of electrostatic charges (Nolan et al., 2017). The combination of dust and ammonia results in birds inhaling high numbers of bacteria and being unable to clear them from their respiratory tract (Nolan et al., 2017).

Colibacillosis causes several diseases in poultry, including yolk sac infection, omphalitis which is a noncontagious infection of the navel in young poultry, respiratory tract infection, swollen head syndrome, septicaemia, polyserositis, coligranuloma, enteritis, cellulitis, and salpingitis (Mellata, 2013; Nolan et al., 2019). Colibacillosis in poultry is characterised by septicaemia leading to mortality in its acute form, and by pericarditis, airsacculitis, and peri-hepatitis in its subacute form. It has been shown that avian colibacillosis is a major infectious disease affecting birds of all ages. Therefore, this disease has a significant economic effect

on global poultry production. The majority of economic losses are a result of bird mortality and decreased production (Mellata, 2013).

1.2.2.2 *Aetiology and Classification*

The causative agent of colibacillosis is *E. coli* which is a member of the family *Enterobacteriaceae*, and a Gram-negative, non-acid-fast, uniform-staining bacillus that grows aerobically and anaerobically and may vary in size and shape. Many strains are motile with peritrichous flagella. *E. coli* is considered to be a part of the commensal microbiota of the bird gut, but some strains, such as those identified as APEC, spread into different internal organs to cause colibacillosis (Agunos et al., 2012; Tivendale et al., 2010). *E. coli* isolates that are pathogenic for poultry often belong to certain serogroups, notably O78, O1, and O2, and to a lesser degree O15 and O55. Avian colibacillosis is usually linked with *E. coli* strains from serotypes O78:K80, O1:K1, and O2:K1. The prevalence of colibacillosis in avian was shown to be widespread in all age groups of hens (9.52–36.73%), with a particularly high incidence rate in adult layers (36.73%) (Lutful Kabir, 2010).

1.2.2.3 *Hosts and Distribution*

Colibacillosis has been reported in all poultry and avian species, including chickens, turkeys, ducks, quails, pheasants, pigeons, guinea fowls, waterfowl, ostriches, emus, peacocks, gulls, starlings, crows, doves, lapwings, wild turkeys, hawk, songbirds, sparrows, swan (Collingwood et al., 2014; Dominguez et al., 2018; Guabiraba and Schouler, et al., 2015; Nolan et al., 2017). Younger birds are often more susceptible to death caused by this disease than older birds (Nolan et al., 2017). The intestinal tract of poultry is the primary reservoir for *E. coli* the causative agent of the disease. There are around 10^9 CFU of bacteria per gram of chicken faeces, of which 10^6 CFU are *E. coli.* Additionally, *E. coli* has often been isolated from the upper respiratory tract. Also, it can be found on the skin and feathers of the bird (Kaikabo et al., 2017).

1.2.2.4 *Transmission of colibacillosis*

Colibacillosis is mainly spread by water or feed contaminated with faeces because *E. coli* is a commensal inhabitant of intestinal tracts. Faecal material contamination within the environment plays a significant role in disease transmission in poultry. Many *E. coli* are maintained in the environment of poultry houses due to faecal contamination. The natural route of infection for APEC is not clearly defined, although the oral and respiratory routes seem to be significant modes of entry. APEC has been reported to persist in dry environments, and dust in poultry houses can harbour up to 10^6 CFU of *E. coli* per gram (Harry et al., 1964). Inhalation of this contaminated dust is thought to cause systemic APEC infections. Additionally, cutaneous wounds, the cloaca, injured intestinal mucosa, and the navel are all possible bacterial entrance points into birds. From these entrance points, *E. coli* may spread locally or enter the blood circulation and induce coli septicaemia, which may lead to death if acute septicaemia develops. Infection may also spread to the serosal surfaces, resulting in subacute polyserositis and persistent granulomatous inflammation. Studies have shown that APEC can colonise the chicken gastrointestinal and respiratory tracts without causing disease and only translocate to extra-intestinal sites as an opportunistic pathogen in the presence of stressors (production-related stress, immunosuppression, and concurrent infections (Lionetto et al., 2020; Nolan et al., 2017).

1.2.2.5 *Clinical Signs and Pathological Features of colibacillosis*

The morbidity and mortality caused by avian colibacillosis, as well as the associated clinical symptoms, vary widely depending on the nature of the disease involved and the specific organs that are affected. Whether the infection is systemic or localised, the disease causes the birds to be underweight, with a shabby appearance, and less productive (Bryan et al., 2015; Collingwood et al., 2014; Dziva et al., 2008; Guabiraba et al., 2015; Nolan et al., 2017). The septicaemic form of the disease causes a moribund state characterised by lethargy and insensitivity to stimuli. The faeces mostly are diarrheic with a greenish to yellowish white colour. Anorexia and severe dehydration, as evidenced by dark skin tone or raised skin folds, are also reported (Bryan et al., 2015; Collingwood et al., 2014; Dziva et al., 2008; Guabiraba et al., 2015; Nolan et al., 2017). In the intestinal form of colibacillosis, the organisms enter the host after ingestion of contaminated feed or water, before colonising the intestinal tract leading to disease. In the case of localised infection, the symptoms will

change depending on the infected organ or tissue. When the joints or bones of the legs are affected, movement becomes difficult, leading to partial to total reluctance to stand or walk. Generally, birds are reluctant to move and may remain seated if an internal organ, such as the yolk sac, is infected. In cases when the visceral organs are affected, abdominal distention may also be observed (Dziva et al., 2008; Guabiraba et al., 2015; Nolan et al., 2017).

1.3 Avian pathogenic *Escherichia coli*

1.3.1 APEC pathotype

Pathogenic *E. coli* groups can be defined with traits, unlike APEC pathotypes. There is no single trait or group of features that define the APEC pathotype, but some phenotypic and genotypic characteristics are associated with this group of pathogens. Characterising *E. coli* antigens allows for serotyping, which remains an essential tool in studying avian colibacillosis. Antigens of *E. coli* are classified into three types: O antigen (comprising the lipopolysaccharides (LPS) component of the cell wall), H antigen (consisting of the flagellar protein), and K antigen, determined by the capsular proteins and the pilus antigen (F) (Gyles, 2007; Nolan et al., 2017). Specific serotypes of APEC such as O1, O2, O18, O35, O36, O78, and O111, are known to be predominantly associated with disease in avian species (Nolan et al., 2017).

Biotyping and serotyping are often performed on colibacillosis-related isolates. In the majority of countries, the predominant *E. coli* serogroups recovered from infected birds are O1, O2, and O78 (Cheville et al., 1978; Cloud et al., 1985; Dozois et al., 1992; Ewers et al., 2004; Sojka et al., 1961; Whittam et al., 1988). Therefore, representative strains from these serotypes serve as the focus for understanding APEC virulence mechanisms and developing and evaluating vaccine candidates. These prominent serogroups can also be recovered from the faeces of healthy birds (Blanco et al., 1998), supporting the hypothesis that the intestinal tract could serve as an important natural reservoir for APEC and that predisposing factors may be essential for disease to occur. Several studies have shown that there are shared characteristics, such as serogroups, between commensal *E. coli* and APEC (Blanco et al., 1998; Heller et al. 1977; McPeake et al., 2005; Rodriguez-Siek et al., 2005), indicating that APEC might arise from commensal *E. coli* after acquiring pathogenic traits. Indeed, widespread variation in phenotypic and virulence characteristics has been reported within a single APEC serotype.

1.3.2 Whole genome sequencing (WGS)

WGS has the potential to allow researchers to decipher APEC molecular pathogenesis by identifying virulence-associated genes that have previously gone undetected and assessing the phylogenetic relatedness of strains using a larger genome-scale. The more whole genomes that are available to the public, the more our understanding of APEC gone further. There are several APEC genome sequences publicly available (Huja et al., 2015; Mageiros et al., 2021) ; APEC O1:K1:H7 isolated from a turkey, APEC SCI-07 (untypable O antigen) isolated from a layer hen in Brazil, APEC O78 (chi7122) isolated from an infected turkey and another APEC O78 (Johnson et al., 2007; Dziva et al., 2013; Mangiamele et al., 2013). The first of these genomes to be made available was APEC ST-95 O1:K1:H7 (APEC O1), which was selected for sequencing, as it appeared to represent other APEC strains based on the authors' previous work showing its similarities in virulence-associated genes carriage and genetic typing to other APEC strains. APEC O1 was originally isolated from the lung of an infected chicken (Johnson et al., 2007). The same research group also published the most recently available APEC O78 genome which consists of 1 chromosome and 2 plasmids (Mangiamele et al., 2013). The full genome sequencing of APEC O78 (chi7122) and (APEC IMT2125) was used to decipher the evolutionary genetic lineages of APEC (Dziva et al., 2013). Results from this study suggest APEC strains are likely to originate from multiple different lineages and those of ST-23 (chi7122 and IMT2125) appear distinct from APEC O1. To date, there are no broiler chicken associated APEC genomes available. In addition, studies have showed a huge diversity among APEC isolated from a single farm (Kemmett et al., 2014; Kemmett et al., 2013).

APEC pathotype remains poorly defined; no single molecular typing technique is 100% discriminative for APEC and non-APEC isolates. Multiple molecular typing methods are used in conjunction, perhaps allowing strengthened discrimination of isolates. Our understanding of the APEC evolutionary background remains incomplete. Correlation between pulsed field gel electrophoresis pulsotype clusters and specific serogroups were reported by Ewers et al., 2004. Furthermore, serogroups O1, O2 and O78 mostly cluster into MLST ST-23 and ST-95 (Olsen et al., 2011; Pires-dos-Santos et al., 2013). On the other hand, these same studies report a diverse APEC phylogenetic background. WGS comparisons suggest that APEC have evolved from *E. coli* of multiple genetic lineages through the acquisition of distinct virulence-associated genes. Furthermore, distinguishing strains by their disease manifestations (i.e. extraintestinal or diarrheagenic) is not fully

supported by genetic analysis. A similar case has recently been described for EAEC and UPEC capable of causing urosepsis (Estrada et al., 2012; Mcnally et al., 2013).

1.3.3 Genetic Similarity and Commonality in Virulence Genes

The comparison of virulence and clonality of APEC from different sources indicates that APEC could be a potential health risk because it is genotypically closely related to *E. coli* types that are involved in human urinary tract infections and meningitis which are uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC), respectively (Jørgensen et al., 2019; Zhuge et al., 2019) Multiple studies have shown that APEC can cause urinary tract infections (UTI) and meningitis, similar to UPEC and NMEC. *E. coli* isolates from chicken meat, and shell eggs were lethal similar to UPEC in a mouse model of UTI, caused sepsis in a mouse sepsis model, and infected the cerebrospinal fluid (CSF) similar to NMEC in a neonatal rat meningitis model (Mitchell et al., 2015). Extraintestinal pathogenic *Escherichia coli* (ExPEC) zoonotic risk appears to be primarily attributable to their large plasmids. There is substantial evidence that APEC plasmids may serve as a source of virulence genes for other ExPEC strains. A study has shown that UPEC and APEC isolates share genes associated with APEC's large transmissible plasmids. Some virulence genes identified on APEC plasmids are also present on UPEC plasmids (Jørgensen et al., 2019). APEC and NMEC also share virulence genes of colicin V (ColV) plasmids, and APEC plasmids in *E. coli* may contribute to the pathogenicity of UTIs in mice and meningitis in rats (Jørgensen et al., 2019)

1.3.4 APEC Virulence Factors

1.3.4.1 *Adhesins*

Adhesins are bacterial appendages or cell-surface components that promote adhesion or adherence to other cells or surfaces, often in the host they inhabit or infect (Dziva et al. 2008; Sarowska et al., 2019). Adherence is required for colonising a new host and is a crucial stage in infection by bacteria (Dziva et al., 2008; Sarowska et al., 2019). Primarily type 1 fimbriae, P fimbriae, and S fimbriae enhance APEC adhesion (Dziva et al., 2008; Sarowska et al., 2019). Indeed, there are several genes associated with adhesion in APEC such as *fimH*, *fimC* (type 1 fimbriae), *papA*, *papC*, *papEF*, *papG I*, *papG II*, *papG III*, *felA* (P fimbriae), *sfa*/*sfaS*

(S fimbriae), *focGE* (F1C fimbriae), *afaIBC* (a fimbriae), *lpfA*, *lpf0141*, and *lpf*. Additionally, these adhesins influence motility, biofilm formation, and APEC survival in macrophages (Dziva et al., 2008; Sarowska et al., 2019). Moreover, the gene encoding fimbriae, *yfcO*, enhances adhesion, colonisation, and resilience to environmental stressors (Li et al., 2016), whereas *yadC* promotes adhesion, intracellular survival, and motility (Kathayat et al., 2021) Autotransporter adhesin genes (*aatA*, *aatB*, *upaB*) similarly contribute to adhesion, colonisation, and biofilm formation (Zhu Ge et al., 2013).

1.3.4.2 *Invasins*

Invasins are a class of proteins involved in pathogenesis (Dziva and Stevens, 2008; Sarowska et al., 2019). Invasins facilitate the first phase of infection (Dziva et al., 2008; Sarowska et al., 2019). Multiple genes encoding invasins, *ibeA* (also known as ibe10), *ibeB* (invasion protein), *tia* (toxigenic invasion locus), and *gimB* (genetic island linked to newborn meningitis) have been reported in APEC isolates (Wang et al., 2011; Wang et al., 2012; Maciel et al., 2017). Moreover, invasins contribute to APEC resistance to oxidative stress caused by macrophages, biofilm formation, colonisation, and host proliferation (Wang et al., 2011; Wang et al., 2012). *ibeR*, a regulator of the ibeRAT operon contributes to invasion, serum and environmental stress tolerance, and virulence gene expression (Wang et al., 2015). Similarly, *ychO*, a suspected invasion gene, is involved in motility, adhesion, invasion, biofilm formation and the production of membrane proteins, and genes involved in metabolism regulation (Pilatti et al., 2016).

1.3.4.3 *Iron Acquisition Systems*

Iron is an essential micronutrient for bacterial growth and proliferation inside the host after bacteria have effectively colonised and invaded the host (Dziva et al., 2008; Sarowska et al., 2019). APEC has numerous iron acquisition systems, including multiple siderophores (aerobactin, salmochelin, and yersiniabactin) and transporters that sequester iron from body fluids. Several genes encoding iron uptake and transport systems have been identified in APEC, including *iucCD*, *iutA*, *aerJ* (aerobactin), *iroBCDEN* (salmochelin), *fyuA* (yersiniabactin), *sitABCD*, *mntH* (iron and manganese transporter), *irp2* (iron repressible protein), *feoB* (ferrous ion transporter), *fepC* (ferric enterobactin transporter), *ireA* (iron-regulated virulence gene), *eitABCD* (putative iron transporter), *chuA* (outer membrane

hemin receptor), and *bfr* (bacterioferritin). Furthermore, these siderophores and transporters are involved in APEC adhesion, invasion, tolerance to environmental stressors, expression of additional virulence genes, colonisation, and persistence in the host (Kathayat et al., 2021). Furthermore, enterobactin production and transport genes (*entE* and *entS*) work in tandem with the gene encoding the outer membrane efflux protein (*tolC*) to promote invasion, colonisation, and persistence (Mu et al., 2020).

1.3.4.4 *Toxins*

Toxins are biological poisons that assist in the bacterial ability to invade and cause damage to the tissues (Dziva et al., 2008; Sarowska et al., 2019). Several genes encoding multiple types of toxins, *hlyF*, *hlyA, hlyE* (putative avian hemolysin), *vat* (vacuolating autotransporter toxin), *sat* (secreted autotransporter toxin), *cdtB, cdtS* (cytolethal distending factor), *astA*, *EAST-1* (heat-stable enterotoxin), *stx2f* (Shiga toxin variant), *pic* (serine protease autotransporter), *espC* (serine protease), and *ace4/35* (acetylcholine esterase) have been reported in APEC. These toxins also facilitate colonisation, motility, biofilm formation, agglutination, vacuolisation induction, and outer membrane vesicles formation (Murase et al., 2015).

1.3.4.5 *Antiphagocytic activity*

APEC must evade the avian immune system to infect extraintestinal sites. The respiratory tract lacks a resident innate cellular defence system with very few macrophages and heterophils, the chicken orthologue of mammalian polymorphonuclear neutrophils, residing in the respiratory tissue (Toth et al., 1987). Thus, the respiratory tract must rely on the infiltration of peripheral blood monocytes along with macrophages and heterophils residing in bronchus-associated lymphoid tissues (Reese et al., 2006; Toth et al., 1987). APEC may reside free in the air sac lumen or in close contact with macrophages, with some speculation over the ability of APEC to replicate intracellularly (Pourbakhsh et al., 1997). Pourbakhsh et al 1997, correlated virulence with the ability of APEC to resist killing by macrophages (Pourbakhsh et al., 1997). APEC infection studies using signature-tagged transposon mutagenesis (STM) or specific gene knockouts have been used to test molecular Koch's postulates in a number of different studies to evaluate the contribution of virulence-associated genes to APEC colonisation and persistence in respiratory tissues (Antão et al.,

2009; Li et al., 2005; Pourbakhsh et al., 1997). The K1 capsular antigen N-acetylneuraminic acid shows little immunogenic capacity. In epidemiological and mutational studies, the pathogenic association of K1 has previously been associated with NMEC and APEC, although not all studies support the beneficial effects of the K1 capsule in pathogenesis (Li et al., 2005; Pluschke et al., 1983; Wooley et al., 1993). In addition, a study showed that the absence of P-fimbriae, K1 and the O78 antigen promoted association of APEC with phagocytic cells (Mellata et al., 2003).

APEC strains are exposed to the bactericidal effects of the complement system, a component of the innate immune system present in sera once they enter the bloodstream. Several virulence-associated genes have been associated with complement resistance including *iss*, which encodes the 10-11KDa increased serum survival (Iss) outer-membrane lipoprotein (Barondess et al., 1995). Iss reportedly contributed to a 100-fold increase in *E. coli* virulence (Binns et al., 1979). High sequence homology between *iss* and the phage lambda *bor* gene, also involved in serum resistance, indicates *iss* evolved from a *bor* precursor (Barondess et al., 1995). Johnson et al. 2008, described the presence of 3 *iss* alleles associated with ColV/BM plasmids and at least 2 encoded on the *E. coli* chromosome (Johnson et al., 2006; Johnson et al., 2008). Between 38 and 82.7% of APEC possess the *iss* gene and past studies have focused on this gene as a potential vaccine target offering homologous and heterologous protection (Ewers et al., 2005; Lynne et al., 2006). Not all research concurs with the importance of *iss*, indicating *iss* may play only a subtle role in virulence (Mellata et al., 2003; Tivendale et al., 2004).

Other proteins associated with serum survival include the outer membrane proteins TraT (Binns et al. 1979; Pfaff-McDonough et al. 2000). TraT inhibits complement by preventing the deposition of C3 and the formation of the C5b6 complex in bacterial cells, which ultimately leads to bacterial lysis (Aguero et al., 1984; Pramoonjago et al., 1992). TraT has also been associated with Enteroaggregative *E. coli* (EAEC) pathogenesis (Boll et al., 2013). Lysozyme is another bactericidal component of the innate immune system, non-specifically destroying bacterial cell walls by hydrolysing the peptidoglycan layer. Two genes have been associated with APEC resistance to lysozyme and increased virulence *in vivo* using knock-out mutants; an inhibitor of vertebrate lysozyme (ivy) and a membrane bound lysozyme inhibitor (MLi-C) of C-type lysozyme (Vanderkelen et al., 2012).

1.3.4.6 *Transcriptional Regulators Multiple*

Multiple transcriptional regulators are involved in APEC pathogenesis. Two global transcriptional regulators, autA and autR, regulate the expression of K1 capsule and acid resistance systems, as well as a shift in adaptive lifestyle to promote infection (Zhuge et al., 2016). Another global transcriptional regulator, FNR (fumarate and nitrate reduction), promotes adhesion, invasion, production of type 1 fimbriae and type VI secretion system, and resistance to oxidative stress (Nielsen et al., 2018). mcbR is involved in biofilm formation and stress response (Kathayat et al., 2021). In contrast, *tyrR* (a transcriptional regulator involved in the manufacture and transport of aromatic amino acids) enhances invasion, motility, and intracellular survival (Pilatti et al., 2016). *YjjQ* (LuxR family transcriptional regulator) contributes to flagellar motility (Wiebe et al., 2015). while RfaH, a transcriptional anti-terminator, contributes to invasion, intracellular survival, and resistance to serum bactericidal action (Gao et al., 2013).

1.3.4.7 *Two-Component Systems*

Two-component systems (TCS) are essential bacterial signalling proteins that allow bacteria to adapt to changing environments by altering gene expression (Tu et al., 2016). Several TCSs are involved in APEC pathogenesis (Herren et al., 2006; Tu et al., 2016; Mu et al., 2020; Tu et al., 2016; Xue et al., 2020). PhoPQ, a membrane-associated TCS, is involved in biofilm formation, motility, adhesion, invasion, intracellular survival, systemic dissemination, and the expression of virulence genes and genes involved in flagellar assembly, ABC transporters, quorum sensing, and bacterial chemotaxis (Tu et al., 2016). BasSR, a membrane-associated TCS, is also involved in biofilm formation, APEC pathogenicity, and *in vivo* colonisation (Yu et al., 2020). KdpDE, a TCS that controls potassium transport, modulates the expression of flagella-related genes, flagellum formation, motility, and serum bactericidal activity resistance (Xue et al., 2020). Similarly, RstAB, a TCS that regulates nitrogen metabolism, contributes to iron uptake, acid tolerance, intracellular survival, and colonisation (Mu et al., 2020). BarA-UvrY has a role in adhesion, invasion, persistence, intracellular survival, resistance to serum bactericidal activity and oxidative stress, as well as control of exopolysaccharide synthesis and expression of type 1 and P fimbriae (Herren et al., 2006).

1.3.4.8 *Metabolism-Associated Genes*

Several genes associated with bacterial metabolism contribute to APEC pathogenesis. The operon encodes the acetate assimilation system, *acs-yjcH-actP*, and facilitates intracellular survival, proliferation, colonisation, and the generation of proinflammatory cytokines and nitric oxide (Zhuge et al., 2019). Similarly, *potE* (putrescine transporter) and *nirC* (nitrite transporter), respectively involved in polyamine biosynthesis and putrescine transport, nitrogen metabolism, and cytoplasmic detoxification, mediate adhesion, and colonisation (de Paiva et al., 2015; Guerra et al., 2018). *arcA* (aerobic respiratory control), which is involved in citrate transport and metabolism, contributes to motility and chemotaxis (Jiang et al., 2015). Overall, multiple APEC virulence factors are involved in causing colibacillosis in poultry. As a result of the involvement of multiple factors, there is a hindrance in developing therapeutics broadly effective against APEC infections. These virulence factors promote infection and adaptation in poultry. Therefore, understanding these factors in depth will help develop effective therapeutics against colibacillosis in poultry.

1.3.5 Treatment and Control of Colibacillosis

The control of APEC infections in poultry relies on antibiotics and vaccination, other than managing the environmental stressors, applying biosecurity measures, and vaccinating the chickens against viral and bacterial diseases (Dziva et al., 2008; Kathayat et al., 2021) Antibiotics are widely used in poultry to control APEC infections (Agunos et al., 2012). Many antibiotics from different classes are used in the poultry industry globally such as, tetracyclines (tetracycline, oxytetracycline, chlortetracycline), sulfonamides (sulfadimethoxine, trimethoprim, sulfadiazine, sulfamethazine, sulfaquinoxaline, ormethoprim), aminoglycosides (apramycin, gentamicin, neomycin, spectinomycin), penicillins (amoxicillin, ampicillin), cephalosporins (ceftiofur), quinolones (danofloxacin, sarafloxacin, enrofloxacin), polymyxins (colistin), chloramphenicols (florfenicol), macrolides (erythromycin), and lincosamides (lincomycin). However, APEC resistance to multiple antibiotics has been reported, including medically essential antibiotics such as β-lactams, colistin, and carbapenems. This indicates that using antibiotics to control APEC infections will be more challenging (Nhung et al., 2017). Currently, only two vaccines are commercially available for use in poultry (live-attenuated APEC O78 *aroA* Poulvac® *E. coli* vaccine and inactivated Nobilis® *E. coli* vaccine, including F11 fimbrial and FT flagellar

antigens) (Kathacoli vaccine021). To control APEC infections in poultry, this situation necessitates the development of new and alternative treatments.

1.4 Alternatives to antibiotics

1.4.1 Overview

Animal productivity and efficiency are increasingly important in today's livestock sector. In the past, as outlined above, high production levels were achieved by using pharmaceutical methods such as antibiotic growth promoters. However, health concerns about antibiotic-resistant pathogen outbreaks and the rise of antibiotic-resistant bacteria such as *E. coli*, *Klebsiella pneumoniae*, *Salmonella* spp., *Streptococcus pneumoniae,* and *Staphylococcus aureus* have resulted in worldwide regulatory restrictions limiting antibiotic use and encouraging the spread of new types of production (such as antibiotic-free and no antibiotic ever) whose viability requires the highest standards. To promote animal growth and health without antibiotics, diet plays a critical role beyond nutritional provision, and high-quality feed ingredients and additives are therefore needed. Alternative feed additives such as biologically produced nanoparticles, probiotics, prebiotics, symbiotics, herbal extracts, essential oils, organic acids, enzymes, essential amino acids, and so on have been widely used to replace AGPs (Abd El-Hack et al., 2022; Dittoe et al., 2018; Gadde et al., 2017). Organic acids, probiotics, prebiotics, and enzymes are common feed additives (Swelum et al., 2021; Wenk, 2000). They have many potential benefits, including improved nutrient digestion and absorption, feed conversion ratio optimisation, growth performance, immunomodulation, and improved gut function and health through pathogen exclusion and inhibition in the intestine; as a result, the level of safety of poultry products for human consumption is improved.

In general, part of the alternative feed additives has been associated with direct impact on the host gut microbiome, while others have indirect impact on the host gut microbiome. For example, enzymes that are used as alternative feed additives, they improve performance and nutrient digestibility when they are added to poultry diets (Khattak et al., 2006). Moreover, enzymes that are added to poultry diets; especially diets containing cereals rich in non-starch polysaccharides such as wheat, barley, and rye, reduce the viscosity in the diet and digestion

(Khattak et al., 2006). Reducing the viscosity in diet and digestion is associated with enhancing digestion and absorption of nutrients (Khattak et al., 2006). Enzymes such as ß-glucanases, phytases, proteases, lipases, and amylases are commonly used in poultry feeds, and these enzymes reduce the viscosity in the diet and digestion by reducing the molecular weight through hydrolysis of nutrients into smaller compounds and thus a reduction in the feed viscosity (Khattak et al., 2006). Indeed, this suggests that enzymes have no direct impact gut microbiome like other alternative feed additives such as antimicrobial peptides and organic acids (Gadde et al., 2017).

Furthermore, alternative feed additives are beneficial in poultry production and other animal products such as pig production. Indeed, the efficacy of alternative feed additives as growth promoters depends on various factors, including diet compatibility and the alternatives used, hygiene standards, and sound farm management practices (Gadde et al., 2017).

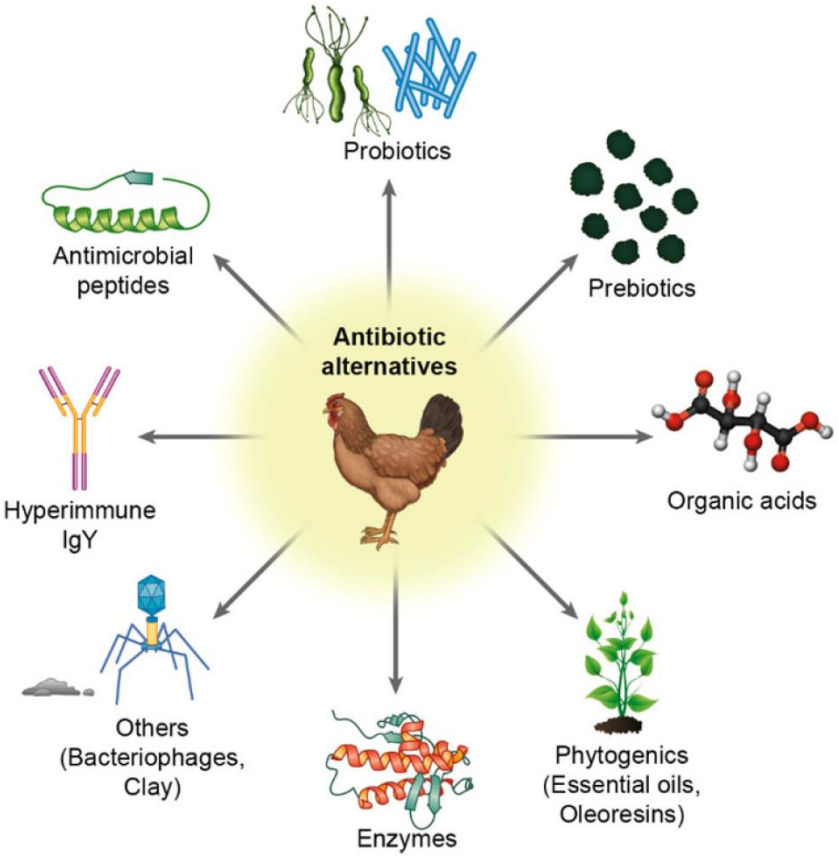


Figure 1-1. Adapted from Gadde et al., 2017. Different types of antibiotic alternatives are available for use in poultry production.

1.4.2 Organic acids

Organic acids are among the alternative feed additives that have been widely used in recent decades due to their beneficial influence on growth efficiency. They are considered critical components in poultry and pig production (Dibner et al., 2002; Papatsiros et al., 2013; Abd El-Hack et al., 2022). Organic acids are weak acids with a carboxylic acid group (R-COOH), intermediates in the degradation pathways of carbohydrates, amino acids, and fats. They are utilised in animal feed for their nutritional value and antibacterial effects (Fernández et al., 2009; Papatsiros et al., 2013; Pourabedin et al., 2015). In poultry production, organic acids are extensively employed as an alternative feed additive due to their ability to promote gut barrier cellular integrity, modulate intestinal microbiota, improve digestion and nutrient absorption rate, and contribute to improved production performance (van Immerseel et al., 2009; Papatsiros et al., 2013; Abd El-Hack et al., 2022). As organic acids are volatile and corrosive in their free forms, they are commercially produced into salt forms to promote palatability and bioavailability in the gut of birds (Donovan and Stringer 1971; Dittoe et al., 2018). Organic acids can be classified into three main functional categories, which are short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), and tricarboxylic acids (TCAs) (Dittoe et al., 2018).

SCFAs are carboxylic acids with a maximum of five carbon atoms. They are produced by the microbial fermentation of indigestible carbohydrates and amino acids in the lower intestine of mammals. Specifically, acetic, propionic (PA), and butyric acids are produced in a physiological ratio (60:25:15) in humans, and they play a crucial role in intestinal mucosa trophism and general metabolism (Tedelind et al., 2007; Tugnoli et al., 2020). These three fatty acids which are acetic, PA, and butyric acids accounted for more than 95% of the total SCFAs and, the ratio of these three fatty acids is not constant, as it relies on many factors, such as dietary components, microbiota composition, and the site of fermentation (Fredstrom et al., 1994). Acetate is most abundant in the colon, accounting for more than half of the total SCFAs detected in faces (Louis et al., 2007) and acetate can be produced through either the acetyl-CoA or Wood-Ljungdahl pathways (Ragsdale et al., 2008). These two pathways involve *Bacteroides* spp., *Bifidobacterium* spp., *Ruminococcus* spp., *Blautia hydrogenotrophica*, and *Clostridium* spp (Petra et al., 2014; Rey et al., 2010). In addition, Acetogenic bacteria which are a specialised group of strictly anaerobic bacteria, also can produce acetate from carbon dioxide and formate through the Wood-Ljungdahl pathway (Imkamp et al., 2007; Louis et al., 2014).

Propionate formation consists of two pathways: propionate can be produced via succinate, which involves the decarboxylation of methylmalonyl-CoA to propionyl-CoA (Figure 1-2). Firmicutes and Bacteroidetes participate in this pathway (Reichardt et al., 2014). Propionate can also be synthesized through the acrylate pathway, which converts lactate to propionate; however, only a few members of the family such as Veillonellaceae and Lachnospiraceae participate in this pathway (Figure 1-2) (Flint et al., 2014; Hetzel et al., 2003).

Additionally, several Firmicutes produce butyrate from acetyl-CoA (the classical pathway) (Figure 1-2) (Zhang et al., 2021). Previous studies have indicated that many gut microbiota members, such as Actinobacteria, Proteobacteria, and Thermotogae, may be potential butyrate-producing bacteria, because these microbiotas contain vital enzymes to synthesise butyrate, such as butyryl coenzyme A dehydrogenase, butyryl-CoA transferase, and butyrate kinase (Figure 1-2) (Vital et al., 2014). Furthermore, butyrate can be synthesised from proteins via the lysine pathway, which demonstrates that gut microbiota can accommodate changes in the fermentation substrate, with the aim of retaining metabolite synthesis (Figure 1-2) (Vital et al., 2014). In general, SCFAs are mainly used as feed acidifiers, silage inoculants, and preservatives in animal feed due to their liquid state (Tugnoli et al., 2020).

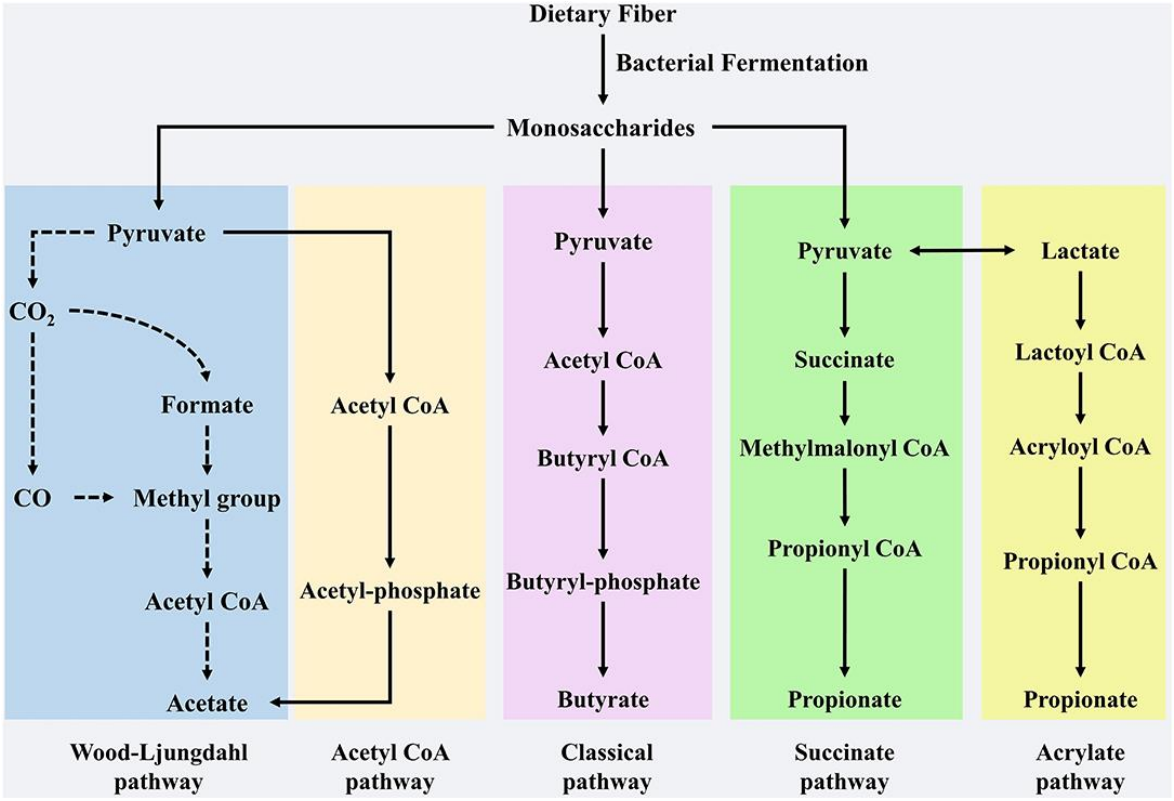


Figure 1-2. Adapted from Liu et al., 2021. The synthesis pathway of SCFAs. The primary SCFAs produced by dietary fibres of microbial fermentation in the gut are acetate, propionate, and butyrate. Acetate is made from pyruvate via the Acetyl-CoA and Wood-Ljungdahl pathways. Butyrate is made from Acetyl CoA, which is then reduced to Butyryl-CoA, which can then be converted to butyrate via the so-called classical pathway via Butyryl-phosphate. Propionate can be synthesized from succinate, which is then transformed to methylmalonyl-CoA via the succinate pathway.

The other group is MCFAs; they have aliphatic chains containing 6 to 12 carbon atoms (Dittoe et al., 2018; Ricke et al., 2020). These acids can be rapidly absorbed into the membranes of phospholipids and have a significant role in feeding young piglets, where they serve as a significant energy source (Tedelind et al., 2007). The last group are TCAs, and they are metabolic intermediates of the Krebs cycle and are therefore crucial for energy metabolism. TCAs improve gut morphology and barrier function, positively affecting the gut microbiota (Tedelind et al., 2007; Smith et al., 2013) Aside from these categories, a few organic acids, such as benzoic, sorbic, and lactic acid, are widely used in food and animal feed preservation due to their antifungal and antimicrobial effects (Fernández et al., 2009; Papatsiros et al., 2013; Dittoe et al., 2018).

The antibacterial activity of organic acids is well established, while the mechanism of action is not well defined. Several possible mechanisms could occur in poultry such as a decrease

in pH level in the upper GIT (crop proventriculus, gizzard), which leads to physiological changes in the gut mucosa (Panda et al., 2009; Samanta et al., 2008). Another mechanism is altering the gut microbiome through either direct killing through cell-wall penetration or indirect modification of the intestinal luminal pH and reducing the numbers of pathogenic bacteria, increasing acid-tolerant beneficial species such as *Lactobacillus spp*., and reducing competition for nutrients by altering microbes (Biggs et al., 2008; Boroojeni et al., 2014; Czerwiński et al., 2010; Nava et al., 2009). Organic acids also promote nutrient digestibility by increasing protein and dry matter retention, improving mineral absorption and phosphorous utilisation (Rafacz-Livingston et al., 2005); and enhancing gut health through direct effects on epithelial cells. For instance, SCFAs are a direct energy source for the growth of epithelial cells. (Dibner et al., 2002; Kim et al., 2015). Despite the proven benefits, the use of organic acids to enhance performance is inconsistent. Variables such as inclusion rates which is the ratio of different ingredients, the sources of organic acids, and the buffering capacity of other dietary ingredients, contribute to this inconsistency (Dibner et al., 2002; Kim et al., 2015).

Not all organic acids have the same antimicrobial effects. Their antibacterial activity against microbes is dependent on the carbon chain length and degree of unsaturation, but the acid's dissociation constant (pKa) overall determines its antimicrobial mechanism of action (Dittoe et al., 2018; Ricke et al., 2020). Every organic acid is identified by a pH value at which 50% of the acid is found in a dissociated form pKa. Russell and Diez-Gonzalez introduced the anion model in 1998, which states that the inhibitory impact of organic acids is significantly connected to their undissociated form (Russell et al., 1998). Organic acids in their undissociated state can pass through the bacterial cell membrane and dissociate within the cell, releasing H^+ ions and reducing intracellular pH, depending on the environmental pH and pKa values (Fernández et al., 2009; van Immerseel et al., 2009; Papatsiros et al., 2013; Abd El-Hack et al., 2022). To overcome the pH decrease, microbes activate proton pumps, which consume energy, while the anion RCOO- is toxic to DNA replication, and this eventually will affect metabolic functions and raise osmotic cell pressure (Fernández et al., 2009; van Immerseel et al., 2009; Papatsiros et al., 2013; Abd El-Hack et al., 2022). These two actions combined inhibit bacterial reproduction and growth, resulting in bacteriostatic or bactericidal effects. The anion model is widely accepted as the mode of action for all organic acids, but the efficacy of different organic acids is primarily determined by two factors: first, the lipophilic nature of the acid affects its ability to pass through the microorganism wall; second, upon dissociation inside the cell, different anions can have

different inhibitory mechanisms on cellular functions (Abd El-Hack et al., 2022; Fernández et al., 2009; Papatsiros et al., 2013; van Immerseel et al., 2009).

In addition to the pKa, several additional factors impact the antimicrobial effect of organic acids. Polar groups, number of double bonds, molecular size, and solubility in non-polar solvents are the four primary chemical and physical characteristics that can be used to predict the inhibitory effect of organic acids, according to the mathematical model of Principal Components Analysis (PCA) calculated by Hsiao and Siebert (Papatsiros et al., 2013; Diarra et al., 2014; Dittoe et al, 2018). Depending on the nature of the target organism and in particular, the complexity and structure of its outer cell wall and membrane, the effective range of organic acids may vary (Dittoe et al., 2018; Feye et al., 2020; S C Ricke, 2003). Gram-positive bacteria (e.g., *Clostridium perfringens*, *Enterococcus* spp., *Streptococcus* spp.) are more susceptible to MCFAs. In contrast, Gram-negative bacteria (e.g., *E. coli*, *Campylobacter jejuni*, *Salmonella spp*.) are more sensitive to SCFAs (Fernández et al., 2009; Dittoe et al., 2018). This may be explained by the lipophilic character of MCFAs, which enables them to exhibit a more potent antimicrobial effect against Gram-positive species. At the same time, the presence of lipopolysaccharide (LPS) in the cell wall of Gram-negative bacteria confers resistance on these microorganisms (Dittoe et al., 2018). PA and butyric acid are potent mould inhibitors, while acetic acid is often used as an antifungal and reduces the production of aflatoxins (Dittoe et al., 2018). Indeed, formic acid (FA) and PA have been examined for bactericidal activity *in vivo* in poultry due to their ability to decontaminate animal feed, and potentially reduce enteric bacteria internally in poultry (Dittoe et al., 2018).

1.4.3 Short chain fatty acids (SCFAs)

1.4.3.1 *Formic acid*

FA has a long history of utilisation as an antibacterial feed additive, but also certain insects can produce it as an antimicrobial defensive chemical (Ricke et al., 2020). The antibacterial properties associated with FA by ants indicate that it could potentially be applied as an external additive compound (Ricke et al., 2020). When synthetic FA was added to resin, it results in a significant increase in antifungal activity. As further evidence of the potency of FA and its biological utility, giant anteaters that cannot produce gastric hydrochloric acid

consume ants containing FA to provide the concentrated FA as a substitute for digestive acid (Ricke et al., 2020).

The practical agricultural application of FA has been considered and examined for several years. Specifically, FA has utility as an additive for animal feed and silage. Both solid and liquid forms of sodium formate as a source of FA have been considered safe for all animal species as well as consumers and the environment (Ricke et al., 2020). Based on their assessment (Ricke et al., 2020), a maximum concentration of 10,000 mg FA equivalents/kg of feed was deemed safe for all animal species, while 12,000 mg FA equivalents/kg of feed was considered safe for swine. The application of FA as a feed amendment for animal nutrition has been examined for several years. It has been viewed as having commercial value as a preservative in silage and as an antimicrobial for animal and poultry feeds.

The majority of the research on FA as an antimicrobial for use in food animal production has focused on the foodborne pathogen *Salmonella spp*. There have been some studies with other pathogens inhabiting the GIT. It has been shown by *in vitro* work that FA may be effective against other GIT foodborne pathogens such as *E. coli* and *C. jejuni* (Kovanda et al., 2019). Early studies suggested that organic acids, such as lactic acid and commercial blends, including FA as one of many components, might reduce *Campylobacter* levels in poultry (Kovanda et al., 2019; Ricke et al., 2020). However, employing FA as an antimicrobial agent against *Campylobacter* may need some caution exercised, as noted earlier by Beier et al., (2019). Since FA is a primary source of energy for *C. jejuni's* respiration, this may be especially problematic for supplementing poultry diets. In addition, it is hypothesised that part of its biological niche in the GIT is to cross-feed metabolically on the mixed acid fermentation products such as FA produced by GIT bacteria (Ragaa et al., 2016; Ricke et al., 2020). Some evidence demonstrated that due to the fact FA is a chemoattractant for *C. jejuni*, double mutants impaired in both formate dehydrogenase and formate hydrogenase display decreased caecal colonisation in broilers compared to the wild-type *C. jejuni* strain (Beier et al., 2019). It is unknown how much external FA supplementation could affect the establishment of *C. jejuni* in the chicken GIT. Several variables could impact this as the actual GIT FA concentration could be lower due to catabolism of FA by other GIT bacteria or absorption of FA in the upper part of the GIT. In addition, FA is a potential fermentation product produced by certain GIT bacteria, which might contribute to overall FA levels in the GIT. Quantifying FA in GIT contents and metagenomics to identify formate dehydrogenase genes might clarify FA microbial ecology.

Developing optimal antimicrobial feed additives while targeting pathogens should have minimal influence on the total GIT microbiota, especially on beneficial bacteria members of the host's microbiome. However, the presence of externally introduced organic acids may have a negative effect on the native GIT microbial community, which might partially counterbalance their pathogen prevention benefits. For instance, a study detected a reduction in crop lactic acid bacteria in birds fed a mixture of FA and PA, indicating that the presence of these external organic acids in the crop caused a decrease in the crop lactic acid bacterial population (Ricke et al., 2020). The presence of lactic acid bacteria in the crop is considered a barrier to *Salmonella spp*; hence altering this resident crop microbiota could be problematic for achieving a successful reduction in *Salmonella* GIT colonisation (Ricke et al., 2020). A study showed that differences in total intestinal bacteria such as *E. coli*, in 42-day-old broilers who consumed FA acidified water were not detected (Ricke et al., 2020). As the authors hypothesised, this might be due to the FA being metabolised in the upper part of the GIT as noted by others for externally introduced SCFAs (Ricke et al., 2020).

1.4.3.2 *Propionic acid*

PA and its calcium, sodium, and potassium salts are considered Generally Recognized as Safe (GRAS) food additives by the Food and Drug Administration. PA is one of the most important chemical intermediates that are widely used as anti-microbial (Król et al., 2011; Rivero et al., 2013), anti-inflammatory agents (Loaiza-Ambuludi et al., 2013; Turan-Zitouni et al., 2015), herbicides (Degenhardt et al., 2011), food preservatives (Nobile et al., 2016; Sabra et al., 2013), and artificial flavours (Liu et al., 2012). Indeed, PA and its salts have been used as food additives to control food contamination by microorganisms such as *Salmonella spp*, *E. coli* O157:H7, and *Listeria monocytogenes* during pre- and post-harvesting of food products. Moreover, PA is an additive in most bread that is mass produced in the UK, often in the form of calcium propionate (López et al., 2012; "Scientific Opinion on the Re-Evaluation of Propionic Acid (E 280), Sodium Propionate (E 281), Calcium Propionate (E 282) and Potassium Propionate (E 283) as Food Additives," 2014). In addition, the activity of PA against pathogens can be increased by being used in combination with other organic acids such as acetic, lactic, malic, and citric acids (Park et al., 2011). In addition, PA, like other organic acids, can pass a cell membrane into the cytoplasm in its non-dissociated form. Due to the alkaline intracellular environment, it can release protons

that cause a pH gradient across the cell membrane, which has detrimental effects on nutrition transfer and the growth of moulds, yeast, and bacteria (Zhang et al., 2009).

PA is produced as a metabolic by product of bacterial fermentation and is thus found in high concentrations within the intestinal lumen of humans and other animals (Ormsby et al., 2020). It has long been recognised that PA and other SCFAs influence the physiology and behaviour of bacteria (Abd El-Hack et al., 2022; Huyghebaert et al., 2011; Tedelind et al., 2007; van Immerseel et al., 2009). It has been shown that the primary effect of intestinal PA on *Salmonella* is the repression of invasion (Hung et al., 2013). Investigations clearly show that propionyl-CoA is the crucial metabolite for suppressing invasion generated by use of PA (Eş et al., 2017; Hung et al., 2013). In the presence of PA, deletion of the genes required for synthesising propionyl-CoA from both endogenous and exogenous sources significantly restored invasion gene expression (Eş et al., 2017; Hung et al., 2013). However, invasion gene expression was not fully restored, indicating that there may be other, unidentified pathways of propionyl-CoA metabolism preventing the mutants examined from exerting their complete impact. Therefore, further propionyl-CoA metabolism is necessary for its repressive action. In that case, it is not performed through the 2-methyl citrate cycle since inhibiting this pathway did not limit the suppression of invasion gene expression (Eş et al., 2017; Hung et al., 2013).

The invasion of the intestinal epithelium in an animal host is essential for productive infection by *Salmonella*. The fact that PA, a common chemical constituent in that organ, has such significant anti-invasion effects may first seem inconsistent. Nonetheless, *Salmonella* probably uses this SCFA as an environmental signal to differentiate intestinal regions. Although PA can be present in the small intestine, its concentration is much higher in the colon and cecum, where the presence of resident microbiota inhibits *Salmonella* invasion (Hapfelmeier et al., 2005; Stecher et al., 2005). In humans, PA concentration in the ileum is reported to be 1.5 mM, whereas its concentration in the colon ranges by region from 14 to 27mM. (Cummings et al., 1987). Thus, once *Salmonella* reaches the large intestine, PA may be an essential signal indicating to this pathogen that the possibility of productive infection has passed, allowing *Salmonella* to change its energies to those required for survival within the intestinal lumen and passage into new hosts.

The anti-inflammatory effects of PA were demonstrated by (Tedelind et al., 2007). PA can inhibit secretion of a tumour necrosis factor α (TNFα) which is a pro-inflammatory cytokine

that has an essential role in controlling infection (Tedelind et al., 2007). TNFα secretion induced by LPS can be inhibited by PA from human neutrophils, but not via secretion of IL-8 cytokine. In addition, TNFα-mediated activation of the nuclear factor kappa β (NF-κB) pathway in a colon cancer cell line was suppressed by PA (Tedelind et al., 2007). These findings indicate that PA immunomodulatory effects could effectively treat inflammatory conditions such as inflammatory bowel disease (IBD).

1.5 Adherent-invasive *E. coli* infection associated with Crohn's disease

1.5.1 Crohn's disease

Crohn's disease (CD) was first recognised as a distinct type of IBD approximately 80 years ago (Reyt, 2018). In contrast to ulcerative colitis (UC), which is another type of IBD, CD is characterised by discontinuous and granulomatous inflammation that may affect any part of the GIT, from the mouth to the anus (Bandzar et al., 2013). TNFα is thought to be the main contributor to the excessive inflammation that characterises CD and is present in extremely high quantities in the CD GIT (Marcello et al., 1997). CD is debilitating and expensive to treat, affecting approximately 150-200 /100,000 in the UK (Reyt, 2018). The prevalence of CD has marked it out as a Western disease, but it is rising in incidence in both developing and developed countries (Bandzar et al., 2013). Recently, a study of paediatric Scottish patients confirmed that this increase is especially evident among paediatric patients (Henderson et al., 2012). CD is now recognised as an incurable disorder, and current treatments only help patients to achieve or maintain periods of remission, which include antibiotics, immune system suppressants, anti-inflammatory mediators, dietary modulation, and surgery as a last option.

1.5.2 Crohn's Disease is a multifactorial disorder

CD is recognised as a multifactorial disorder of complicated aetiology. CD is recognised as a condition with several causes and complicated aetiology. It is proposed that disease development is the result of abnormal immune responses to intestinal bacteria in response to an environmental trigger. This process is intimately linked to the genotype of the host. The increased concordance for CD disease in monozygotic twins (36%) and increased risk of

acquiring IBD if a first-degree relative has CD. indicate a genetic determinant in disease pathogenesis (Van Limbergen et al., 2008; Russell et al., 2004). Genome-wide association studies (GWAS) have identified 71 unique genetic susceptibility loci for CD (of 163 IBD loci examined) (Jostins et al., 2012). However, CD-associated polymorphisms are only associated with a 30-40-fold increase in disorder risk, and the expression of all polymorphisms in a single person account for less than 20% of total risk (van Limbergen et al., 2009). Consequently, GWAS cannot predict disease development and emphasises that genetics alone cannot explain an individual's tendency to develop CD. Several of the risk susceptibility genes identified have allowed us to improve our understanding of the aetiology of CD. The essential genetic susceptibility loci contain genes that code for different components involved in innate pattern recognition (NOD2/CARD15), differentiation of Th17 lymphocytes (IL-23R, JAK2, STAT3, CCR6, ICOSLG), autophagy (ATG16L1, IRGM, LRRK2), and intestinal epithelial barrier integrity (IBD5, DLG5, PTGER4, ITLN1, DMBT1, and XBP1) (Franke et al., 2010). The discovered risk susceptibility genes suggest a significant microbial component in CD and a crucial role in bacterial recognition and clearance in the pathogenesis of the disease. Indeed, several studies have shown that CD patients have an altered or dysbiosis gut microbiota composition (Baumgart et al., 2007; Fujimoto et al., 2013; Kim et al., 2019; Mukhopadhya et al., 2012). Gut dysbiosis in CD is characterised by an increase in Proteobacteria, mainly *E. coli*, and a reduction in Firmicutes bacteria (Manichanh et al., 2006; Mukhopadhya et al., 2012). It is yet to be determined if gut dysbiosis is a primary factor in the development of CD, or is a secondary factor triggered by environmental triggers or specific host factors.

Dietary variations are a possible cause of CD-associated dysbiosis. Over the last half-century, Western society has shifted to a high-fat, high-carbohydrate diet, which has significantly increased the disease prevalence in this region (Burisch et al., 2013). The mechanisms by which the Western diet promotes dysbiosis and intestinal inflammation were proven in mice to be caused by dietary fat, through alterations in bile salt metabolism, promoting intestinal blooms of *Bilophila wadsworthii* and bacterial dysbiosis (Devkota et al., 2012). *B. wadsworthia* blooms were associated with colitis and increased production of pro-inflammatory cytokines in Il-10$^{-/-}$ mice, suggesting that host genotype has a role in diet-induced intestinal inflammation (Devkota et al., 2012). It was also shown that a high-fat diet accelerated intestinal inflammation development in a murine CD-like ileitis model, independent of weight gain (Gruber et al., 2013). In this case, accelerated pathogenesis was mediated by increased intestinal permeability due to decreased occludin expression and

altered immunological responses characterised by increased Th17 responses (Gruber et al., 2013). Moreover, administration of emulsifiers to Il-10$^{-/-}$ mice were found to induce perturbation of gut microbiota characterised by a significant reduction in microbial diversity, blooms of members of the *Verrucomicrobia* phyla, and an increase in the abundance of mucosa-associated pro-inflammatory Proteobacteria (Chassaing et al., 2015). In Il-10$^{-/-}$ mice, emulsifier exposure raised faecal levels of bioactive LPS and increased the incidence of colitis (Chassaing et al., 2015). While diet is a factor in development, modulation of diet can also be used as a treatment. For example, Exclusive enteral nutrition (EEN) diet induces clinical remission in approximately 80% of CD patients and promotes gut healing (Svolos et al., 2019).

Inflammation has also been identified as a significant cause of intestinal dysbiosis, suggesting that disease is a prerequisite for a pathogenic shift in the gut microbiota. It was shown that severe inflammation produced by *Toxoplasma gondii* infection caused significant gut dysbiosis in mice, with a shift from >95% *Firmicutes* to >95% *Proteobacteria*. This included decreased bacterial diversity and increased *E. coli* mucosal invasion (Lv et al., 2022).

Anti-TNFα- antibodies administered to mice alleviated gut dysbiosis and decreased bacterial mucosal invasion. Indeed, intestinal dysbiosis in CD extends further than an alteration in microbial composition with dysregulated mucosal immune responses and impaired intestinal barrier function. A significant percentage of CD patients compared to healthy controls have been shown to have increased intestinal permeability (Jenkins and Rooney 1987, Wyatt et al. 1997). In addition, individuals with active CD have increased intestinal permeability, which reduces during remission (Sanderson et al., 1987). Anti-inflammatory therapies such as infliximab, an anti-TNFα agent, reduce intestinal permeability in CD patients (Suenaert et al., 2002; Zeissig et al., 2004). It is proposed that the adaptive immune system mediates and perpetuates intestinal inflammation in CD, although it is not recognised as the primary cause of inflammatory symptoms.

CD is characterised by an imbalance between tolerance maintaining Treg cells and inflammatory T helper cells (Th) (Hu et al., 2014). Different T-cell subpopulations are aberrantly activated in CD according to human and murine studies (Fuss et al.,1997). Initial investigations demonstrated that a T-helper Th1 immune response primarily mediates CD through activation of the Interleukin-12 (IL-12)/ Signal transducer and activator of

transcription 4 (STAT4) and interferon-gamma (IFN-γ)/ Signal transducer and activator of transcription 1 (STAT1) signalling pathways (Sperandio et al., 2014). Nevertheless, there may be a partial Th1/Th2 polarisation in CD involving additional cytokines such as TNFα, Interleukin-1 (IL-1), and Interleukin-17A (IL-17A), which predominantly activate NF-κB, Interleukin-6 (IL-6), and Interleukin-10 (IL-10), which then activate STAT3 (Z. J. Liu et al., 2009; Sanchez-Muñoz et al., 2008). In addition, CD is also characterised by defects in systems essential for microbial detection and eradication. Impaired autophagy and activation of the innate MyD88 pathway have been observed in CD monocytes (Homer et al., 2010; Kuballa et al., 2008; Lapaquette et al., 2010). Additionally, lower synthesis of host AMPs including Paneth cell produced α-defensins have been observed (Wehkamp et al., 2005).

1.5.3 Increased abundance of *E. coli* in Crohn's disease

A wide range of culture-based and molecular-based studies have demonstrated *E. coli* intestinal overgrowth in CD (Neut et al., 2002; Baumgart et al., 2007; Willing et al., 2009; Lopez-Siles et al.m 2014). In the cohort of Martinez-Medina et al., 2009. *E. coli* 16S rRNA gene copies comprised 14% and 33% of total bacterial 16S rRNA in healthy and ileal CD patients, respectively (Martinez-Medina et al., 2009). In addition, patients with active CD have been shown to have higher amounts of *E. coli* than those in remission (Baumgart et al., 2007; Schwiertz et al., 2010; Lopez-Siles et al., 2014; Ormsby et al., 2019). In prior research using fluorescent in situ hybridization (FISH), higher *E. coli* numbers were identified in the epithelium and lamina propria of individuals with active CD compared to those with inactive CD (Mylonaki et al., 2005).

Different theories have been proposed for the inflammatory condition and the increase in relative abundance of facultative anaerobes, such as *E. coli*, over other normally abundant anaerobic microbiome members. One of the theories is "food hypothesis" which proposes that the inflamed gut offers an altered nutrient environment that can be utilised only by a specific group of bacteria (Winter et al., 2010). For instance, there is an increase in the shedding of dead epithelial cells during inflammation. Ethanolamine is a product of bacterial activity upon degradation of one of the phospholipid epithelial cell membrane components, phosphatidylethanolamine. Ethanolamine can be strictly utilised as a sole carbon source by only certain bacteria, such as *E. coli*, in the presence of oxygen (Garsin, 2010; Stecher, 2015). Indeed, it has been found that there was increased metabolism of ethanolamine by *E.*

coli in paediatric CD during active disease compared to the same patients when in clinical remission and also compared against healthy controls (Ormsby et al., 2019).

Increased *E. coli* numbers have also been associated with reduced time before disease relapse (Lopez-Siles et al., 2014). This correlates with work showing that high levels of antibodies against *E. coli* outer membrane protein C (OmpC) are associated with disease progression, longer duration and need for surgery in CD patients (Cabrera-Abreu et al., 2004; Landers et al., 2002; Mow et al., 2004). DNA from *E. coli* is detected more frequently in granulomas of CD patients (80%) compared with non-CD control granulomas (10%) (Ryan et al., 2004). In addition, *E. coli* has been detected in the mucus layer close to intestinal epithelial cells (IECs) in CD patients (Swidsinski et al., 2005; Walmsley et al., 1998). It was detected that patients with CD have a higher prevalence of mucosa-associated *E. coli* than healthy controls, and some genotypes appear to be associated with the disease. Further investigation of these mucosa-associated *E. coli* strains demonstrated strong adhesive and invasive characteristics. Based on this phenotype, these mucosa-associated strains have been classified as a novel *E. coli* pathovar associated with CD, designated as AIEC for adherent-invasive *E. coli* (Darfeuille-Michaud, 2002).

1.5.4 AIEC definition

The AIEC pathotype is defined as *E. coli* strains that adhere to and invade IECs, and it involves host cell actin polymerisation and microtubule recruitment in bacterial uptake. AIEC survive and replicate within macrophages, inducing pro-inflammatory cytokine release (Darfeuille-Michaud, 2002). Since its designation, ExPEC-specific invasive determinants have been identified in several AIEC strains (Martinez-Medina et al., 2009). These virulence phenotypes identify AIEC as a potential CD-associated pathogen that has significant overlap with other pathogenic *E. coli* strains (UPEC, APEC, NMEC and Diarrheagenic *Escherichia coli*) (Nash et al., 2010).

1.5.5 Genetic classification of AIEC

AIEC strains that have been isolated from CD patients are clonally diverse and belong to different serogroups. No specific clone has been associated with the aetiology of the disease (Baumgart et al., 2007; Kleessen et al., 2002; Sepehri et al., 2011). AIEC generally belong to the B2 and D phylogroups, as is the case for ExPEC (Kotlowski et al., 2007). In addition,

AIEC possesses distinct virulence genes that are characteristic of ExPEC (Elliott et al., 2013). Furthermore, virulence genes not associated with commensal *E. coli*, such as *afaC*, *pks*, *malX*, and *lpf*, have been frequently detected in AIEC strains (Dreux et al., 2013; Rolhion et al., 2005). Moreover, analysis of sequenced AIEC genomes has failed to identify a unique genetic characteristic for this group (Miquel et al., 2010; Nash et al., 2010).

1.5.6 AIEC is associated with ileal and colonic Crohn's disease

AIEC can be described as a specialised pathogen in CD, and also it can be isolated from the gut mucosa of healthy individuals (Baumgart et al., 2007; Martinez-Medina et al., 2014). AIEC comprise approximately 3.58% and 0.9% of ileal and colonic *E. coli* in healthy subjects, however in this setting, it is not shown to translocate across the intestinal mucosa barrier (Martinez-Medina et al., 2009; Mylonaki et al., 2005). In the first research paper studying this link, Darfeuille-Michaud suggested more than 20 years ago that AIEC and CD are associated (Darfeuille-Michaud et al., 2004). In this preliminary investigation, culture-dependent approaches showed that AIEC could be isolated from individuals with ileal CD (36%) more often than those with colonic CD (3.7%). This study strongly indicated that AIEC had a significant role in ileal disease. Indeed, several independent studies have reported a high prevalence of AIEC in ileal CD patients compared to healthy controls (Baumgart et al., 2007; Darfeuille-Michaud et al., 2004; Martinez-Medina et al., 2009; Sasaki et al., 2007). According to research, patients with ileal CD have an approximately 25% higher prevalence of AIEC than healthy controls (Dogan et al., 2013).

Other research has shown a significant incidence of AIEC in colonic CD, indicating that this *E. coli* pathotype has a role in colonic and ileal disease. A high number of mucosa-associated *E. coli* that displayed the AIEC phenotype were detected in mucosa preparations isolated from CD colonic biopsy samples (Martin et al., 2004). It was shown that mucosa-associated bacteria were isolated from 71% of cases with ileal or ileocolonic CD and from 86% of cases with colonic CD (Martin et al., 2004). Another work showed that AIEC strains were detected in the ileum of 38.5% of CD patients with ileal involvement but also in the colon of 37.5% of patients with colonic CD (Baumgart et al., 2007). In addition, a study reported AIEC strains in the ileum of 54.5% of CD patients and the colon of 50% of CD patients when a collection of 95 - 150 *E. coli* colonies per patient was used (Martinez-Medina et al., 2009). FISH analysis also reported the presence of mucosa-associated *E. coli* in 42% of colonic biopsy samples obtained from CD patients (Mylonaki et al., 2005).

1.5.7 AIEC and CD pathogenesis

1.5.7.1 *Colonisation of the intestinal mucosa*

The AIEC reference strain LF82 has been used extensively in research to identify the pathogenic mechanisms of AIEC in CD (Darfeuille-Michaud et al., 2004). In 1999, AIEC LF82 was isolated from a CD chronic ileal lesion and has been subsequently utilised as the model organism for AIEC-associated CD infection by several researchers (Boudeau et al., 1999). Several *in vitro* and *in vivo* studies have shown that LF82 is an excellent biofilm-forming bacteria that can form robust biofilms on the intestinal epithelial cell surface (Chassaing et al., 2013). Biofilm formation is a common phenotype of AIEC strains. AIEC strains were classified as biofilm producers in one study compared to non-AIEC bacteria, all of which were intestinal isolates (Weiss-Muszkat et al., 2010). LF82 adheres to IECs in ileal CD and colonises the intestinal mucosa. LF82 adheres to IECs via a common type I pili adhesin FimH variant that recognises and interacts with host intestinal receptor carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) in a mannose-dependent manner (Barnich et al., 2003; Barnich et al., 2007). Interestingly, CEACAM6 expression is upregulated in ileal CD patients, and this may increase susceptibility to AIEC colonisation (Barnich et al., 2007). This demonstrates a strong link between genetic susceptibility, inflammation, and intestinal dysbiosis in disease aetiology. A recent study has shown that AIEC expresses FimH variants that promote more effective adhesion to IECs (Dreux et al., 2013). In a mechanism linked to active motility, it has also been shown that AIEC flagella facilitate colonisation of the intestinal mucosa (Barnich et al., 2003). Flagella also contribute to the persistence of intestinal inflammation by inducing the production of pro-inflammatory cytokines from polarised intestinal epithelial cells, resulting in the recruitment of macrophages and dendritic cells (DCs) to the localised region (Eaves-Pyles et al., 2008; Subramanian et al., 2008). These immune cells, which release proinflammatory cytokines such as TNFα and IFN-γ, promote the expression of CEACAM6 receptors, hence increasing AIEC colonisation (Barnich et al., 2007). AIEC will likely colonise the colonic mucosa via adhesins other than FimH, namely the afimbrial adhesin Afa-1. Increased afimbrial adhesion *afa-1* operon prevalence was detected in mucosal *E. coli* isolates from CD patients compared to healthy controls (Prorok-Hamon et al., 2014). In addition, *afa-1*

expression was associated with *E. coli* ability to adhere and invade IECs (Prorok-Hamon et al., 2014).

1.5.7.2 *Invasion of intestinal epithelial cells*

AIEC are proposed to invade IECs by a mechanism similar to macropinocytosis (Boudeau et al., 1999). Outer membrane vesicles (OMVs) also play a crucial role in the invasion of IECs by AIEC. Rolhion and colleagues demonstrated that OMVs rich in OmpA fused to the membrane of host IECs via the binding of LF82 OmpA to endoplasmic stress response chaperone glycoprotein 96 (Gp96) (Rolhion et al., 2010). This is predicted to facilitate the release of bacterial effector proteins that induce actin polymerisation and microtubule rearrangement of the epithelium, hence bacterial translocation. In addition, Gp96 is also overexpressed in the ileum of CD patients, increasing their susceptibility to AIEC infection, demonstrating the multifactorial and complex aetiology of this disease (Rolhion et al., 2010). For another AIEC prototype strain, NRG857c, it was recently revealed that deletion of the gene encoding the invasive protein *ibeA* decreased invasion but not adherence to IECs *in vitro* (Cieza et al., 2015). Deletion of *ibeA* however did not affect AIEC persistence in the murine GIT, indicating that AIEC possesses additional adhesins and invasions that facilitate interaction with the host. Following bacterial invasion, LF82 is detected within intracellular compartments within IECs including LC-3 positive autophagosomes (Lapaquette et al., 2010). A recent study has shown that LF82 reduces the level of autophagy in IECs through activating NF-κB, resulting in increased production of the microRNAs, MIR30C and MIR130A, and decreased expression of essential autophagy genes autophagy related 16-like 1 (*ATG16L1*) and autophagy related 5 (*ATG5*) (Nguyen et al., 2014). Ileal samples from CD patients revealed higher levels of these microRNAs and lower levels of ATG5 and ATG16L1 (Nguyen et al., 2014).

1.5.7.3 *AIEC translocation of the intestinal mucosa*

AIEC has an ability to translocate across the intestinal mucosa in addition to its ability to invade IECs (Chassaing et al., 2011). AIEC translocate through the gut mucosa via M cells of Peyer's patches (PPs). M cells are highly specialised cells that phagocytose and transcytose intestinal lumen macromolecules, antigens, and microorganisms. AIEC target M cells via type I pili and long polar fimbriae (LPF) cells (Chassaing et al., 2011). The

glycoprotein 2 (Gp2) cell surface protein of M cells is reported to recognise the FimH component of bacterial type I pili, promoting bacterial uptake (Hase et al., 2009). The M cell-specific receptor for AIEC LPF during bacterial translocation is currently unidentified. A recent study has shown that the GipA factor plays a role in the colonisation and translocation of PPs by AIEC (Vazeille et al., 2016). It has been shown that AIEC *gipA* deletion mutants, reduced M cell translocation in *ex vivo* murine infection models (Vazeille et al., 2016). GipA was also reported to positively regulate the expression of the *lpf* operon under bile-salt enriched conditions (Vazeille et al., 2016). Furthermore, LF82 induce the expression of the pore-forming protein, claudin-2, and displace ZO-1 and E-cadherin from apical tight junctions, resulting in increased intestinal permeability (Denizot et al., 2012; Wine et al., 2009).

1.5.7.4 *AIEC survival and persistence within host macrophages*

Once AIEC is entered into the lamina propria, it invades and survives within macrophages inducing the production of pro-inflammatory cytokines. (Bringer et al., 2006; Glasser et al., 2001). Extensive replication of LF82 within macrophages has been reported in several independent studies (Mpofu et al., 2007; Subramanian et al., 2008). Intracellular LF82 survives within phagolysosome-like compartments within macrophages, demonstrating its ability to survive and persist in environments with acidic pH, oxidative stress, and proteolytic enzymes (Bringer et al., 2006). Indeed, Bringer and colleagues showed that an acidic environment within the phagolysosome is essential for AIEC intracellular replication (Bringer et al., 2006). The specific mechanisms involved in this process have yet to be determined. It has been reported that the stress protein HtrA and the thiol-disulphide oxidoreductase DsbA are essential for survival and replication within macrophages (Bringer et al., 2007). LF82 *htrA* deletion mutants showed increased sensitivity to oxidative stress induced by hydrogen peroxide (H_2O_2) and decreased growth in phagolysosome-reproductive environments (Simonsen et al., 2011).

In addition, it is proposed that Hfq, an RNA-binding protein that acts as a global post-transcriptional regulator of gene expression, has a role in LF82 survival and replication within macrophages (Simonsen et al., 2011). Vazeille and colleagues have reported a crucial role for GipA in AIEC intramacrophagic persistence. LF82 *gipA* mutants showed

significantly reduced survival in macrophages at 7 h post-infection compared to the wild-type strain. Reduced survival of isogenic mutant within macrophages was explained by the ability of GipA to increase tolerance of LF82 to oxidative stress and acidic pH (Vazeille et al., 2016). A role for *IbeA* in AIEC persistence within macrophages has also been demonstrated whereby mutant AIEC that lack *ibeA* show significantly reduced intramacrophage survival up to 24 h post-infection (Cieza et al., 2015). AIEC persistence in macrophages increases the secretion of pro-inflammatory cytokines, such as IL-6 and TNFα, without inducing host cell death (Glasser et al., 2001). This has been linked to granuloma formation, the characteristic of inflammation associated with CD, and has been demonstrated *in vitro* (Meconi et al., 2007). Prolonged survival of AIEC-infected macrophages has been attributed to the direct role of LF82 in delaying apoptosis. It was recently demonstrated that LF82 infection of macrophages increased proteasomal degradation of caspsase-3, a crucial regulator of apoptosis, hence promoting LF82 intracellular persistence (Dunne et al., 2013). Multiple *in vitro* studies have shown that the expansion of AIEC in CD is a multifactorial process including host genetic determinants, environmental triggers, and the presence of specific *E. coli* with improved ability to interact with the host mucosa.

1.6 Hypothesis and aims.

As part of the investigation into the AIEC strains that are associated with CD, I wanted to examine how AIEC strains are affected by organic acids and whether these effects are associated with the bacterial phenotype alteration. Indeed, organic acids such as PA and FA which are commonly used as antimicrobials in different fields and can be detected in the mammalian intestines as metabolites due to food digestion, can be associated with bacterial phenotype alteration (Gadde et al., 2017; Ma et al., 2021; Zhu et al., 2021). A key fact here is that high levels of PA, in particular, are protective in the human intestine. Repeated and prolonged exposure to this SCFA rendered AIEC more virulent (Ormsby et al., 2020). Since, AIEC and APEC are evolutionarily and phylogenetically related (Nash et al., 2010) and a study indicated that APEC could infect humans and induce a similar disease to that cause by pathogenic *E. coli* that are known to infect humans only, our hypothesis is that a similar phenomenon may be occurring with APEC when exposed to PA or other SCFAs at increasing concentrations in the intestine of poultry (Nash et al., 2010; Maturana, 2011). Here my aim was to study this by comparing the response of APEC and AIEC strains to exposure to the SCFAs PA and FA; studying their virulence through a variety of *in vitro*

phenotypic assays, gene expression via transcriptomics, and their ability to survive in a complex microbial community derived from chicken intestinal contents. in response to their presence. Additionally, I wanted to understand how SCFA exposure influenced the ability of AIEC and APEC to respond to ethanolamine, a readily available carbon source released in the human intestine during times of inflammation.

Chapter 2

Materials and methods

2.1 Bacterial isolates

Avian pathogenic *Escherichia coli* (APEC) strains were included in this study; 3770 (urogenital isolate), 601 (pulmonary isolate), CCN284 (faecal isolate), APEC O1 (gastrointestinal isolate) and TW731/12 (table 2-1). These APEC strains previously isolated and used in a study aimed to explore the carriage of APEC virulence associated genes and the molecular epidemiology of faecal and systemic *E. coli* in commercial broiler chickens (Kemmett et al., 2013). Adherent invasive *E. coli* strain (AIEC) LF82 was used as well, and this strain was originally isolated from a chronic lesion of a CD patient (Ormsby et al., 2020). Other clinical AIEC isolates were included in this study; B92.4, B95, B115, B122, and B125. These clinical AIEC isolates were recovered from patients with Crohn's disease (table 2-1). The median (range) age was 13.7 (11.2 to 15.2), height z-score was −0.4 (−2.0 to 0.2), weight z-score was −0.7 (−3.4 to −0.1), and BMI z-score was −1.3 (−4.0 to 0.4). Symptom duration prior to diagnosis was median 7.5 months (5 to 12). 50% had granulomas present on initial histology. Phenotypes by Paris criteria (Levine et al., 2011) at diagnosis were: B94- colonic, non-stricturing/non-penetrating; B115- colonic, non-stricturing/non-penetrating; B122- ileocolonic, stricturing; B125- ileocolonic, non-stricturing/non-penetrating. This study is publicly registered on the United Kingdom Clinical Research Network Portfolio (9633). All the strains were stored at -80°C and grown in Luria-Bertani (LB) broth with 180 rpm shaking overnight at 37°C.

Strain	Description	Source	Reference
1- LF82	Adherent-invasive *E. coli*	Isolated from ileal biopsy of CD patient (Daniel Walker lab, the Institute of Infection, Immunity and Inflammation, University of Glasgow)	Ormsby et al., 2020
2- B92.4	Colonic, non-stricturing, and non-penetrating	Clinical isolates were recovered from patients with Crohn's disease. These strains were from the ''Bacteria in Inflammatory bowel disease in Scottish Children Undergoing Investigation before Treatment'' study.	(Hansen et al. 2013)
3- B115	Colonic, non-stricturing, and non-penetrating		
4- B122	Ileocolonic, and stricturing		
5- B125	Ileocolonic, non-stricturing, and non-penetrating		
6- APEC O1	APEC strain isolated from broiler gut, and serotyping has identified as O1	APEC strains were isolated from broilers and laying hens (Paul Wigley lab, the Institute of Infection and Global Health, University of Liverpool)	(Kirsty Kemmett et al., 2013)
7- CCN284	APEC strain isolated from broiler faeces		
8- 601	APEC strain isolated from broiler lung		
9- 3770	APEC strain isolated from a reproductive tract of laying hens		
10- TW731/12	APEC strain isolated from broiler faeces		

Table 2-1. Bacterial isolates that were used in this thesis, which include APEC and AIEC strains.

2.2 Adaptation of bacterial strains to organic acids

Bacterial isolates were exposed to PA or FA by streaking each strain onto M9 minimal agar media plates supplemented with 20 mM PA or FA. Plates were then incubated for 48 hours at 37^{o}C. The bacterial colonies from each strain were re-cultured over a series of five successive re-cultures to adapt the strains to organic acids. The ability of bacteria strains to grow in media that contain organic acids overnight is the criterion for considering bacteria to be adapted, noting that bacteria cannot grow rapidly in the presence of organic acids in the media. A similar approach has been used in a study that aimed to investigate PA impact on AIEC (Ormsby et al., 2020).

2.3 Characterization of the bacterial isolates

2.3.1 Biofilm formation

Strains both exposed and non-exposed to either PA and FA, were serially diluted in Roswell Park Memorial Institute (RPMI) 1640 media, and microtitre plates were inoculated with 180 µl of these dilutions. The plates were then wrapped in parafilm and incubated aerobically in a 5% CO_2 incubator at 37^{o}C for 7 days or anaerobically in the anaerobic chamber for 7 days. The plates were then inverted and shaken to remove all non-adherent cells. The plates were washed twice with distilled water then 200 µl of 0.1 X crystal violet solution was added to each well and left for 15 minutes. The crystal violet was discarded out of the wells and left inverted for 1 hour. To quantify the biofilm, 125 µl of 30% acetic acid in water to each well of the microtiter plate was added to solubilize crystal violet. The microtiter plate was incubated at room temperature for 10-15 min. The absorbance was quantified in a plate reader at 550 nm using 30% acetic acid as the blank (O'Toole, 2010).

2.3.2 Bacterial motility

Twitching motility was measured by stab-inoculating 1.5% LB agar plates with a single bacterial colony. Plates were incubated at 37^{o}C for 18–20 h, and the diameter of the twitching zone at the plastic–agar interface was noted. Any bacterial strain that had a detectable twitching zone upon visible inspection was scored as positive (Murray et al., 2010).

2.3.3 Cell viability

Cell counting kit 8 (CCK-8) assay that detects cell viability was used to measure survival rate of RAW 264.7 macrophages (Abcam). Approximately 1 X 10^4 cells were seeded in 96-well plates with 100 µl medium for each well. After 24 h cultivation, *E. coli* strains, exposed or unexposed to organic acids PA and FA, were used to infect the cells for 4 h. Each well was incubated with 10 µl of CCK-8 solution for 2 h away from light before measuring the absorbance at 450 nm using a FluoStar Optima fluorescent plate reader (BMG Biotech). The relative viability was expressed by the formula: % viability = $((A_{exp} – A_{Blank})/A_{control} - A_{Blank}))$ X 100%.

2.3.4 Acid tolerance

Cultures of bacteria were grown overnight at 37°C in LB. The pH of these cultures was lowered to pH 3 using 1 M HCl. Culture samples were taken every 20 min for 1 h and serially diluted in LB. Dilutions were plated in triplicate onto LB agar and incubated overnight at 37°C. Colonies were counted to determine the number of surviving cells. (Ormsby et al., 2020).

2.4 Gentamicin invasion (protection) assays

2.4.1 Cell line seeding and preparation protocols

RAW 264.7 cells are a macrophage-like, Abelson leukaemia virus transformed cell line derived from BALB/c mice which is American Type Culture Collection (ATCC TIB71). RAW 264.7 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine at 37°C in 5% CO_2. Other cell lines such as THP-1 and Caco-2 were cultured using a similar approach to RAW 264.7. THP-1 is a human monocytic cell line derived from an acute monocytic leukaemia patient (ATCC TIB-202), while the Caco-2 cell line is an immortalized cell line of human colorectal adenocarcinoma cells (ATCC HTB-37).

2.4.2 Bacterial preparation for culture assays

Bacterial strains were grown overnight in RPMI containing 3% FBS and 1% L-glutamine and incubated at 37°C in 5% CO_2. Then, bacterial strains were back diluted (1:10), and grown

for 1.5-2 hours until an OD_{600} of 0.6 was reached. Bacterial strains were diluted after the OD_{600} reached 0.6 to get the required multiplicity of infection (MOI) of 10 or 100 bacteria per cell depending on the assay.

2.4.3 Intestinal epithelial cell assays

2.4.3.1 Adhesion and invasion to Caco-2 intestinal cell line

Prior to infection Caco-2 cells were seeded into 24-well plates at a density of 2 x 10^6 cells per well and the medium was replaced with 1 ml of RPMI-1640 supplemented with 3% FBS and 1% L-glutamine. The cells were incubated at 37ºC, 5% CO_2 for two days until they become polarised. Caco-2 cells were infected at MOI of 10 bacteria per cell. The infection was left to progress for 4 hours at 37°C with 5% CO_2.

For the invasion assay, Caco-2 cells were then washed three times to remove excess bacteria with 1 ml RPMI containing 50 μg/ml gentamicin (dilute 1:1000; 50 μl in 49.95ml RPMI). The cells were left for 40 min after the wash, then the cells washed again after 40 min, but they were washed only with RPMI before plating. For the adhesion assay, the cells were washed only with RPMI three times to remove excess bacteria. The cell harvesting process is the same for the adhesion and invasion assays. Following gentamicin treatment, supernatants were discarded, cells were lysed for 5 minutes in 200μl PBS containing 2% Triton X-100 (Sigma). For invasion or adhesion assays bacterial numbers were counted by doing serial dilutions before being plated onto LB agar plates to determine the number of colony forming units (CFU) per ml recovered from cell lysates.

2.4.4 Net replication assays

2.4.4.1 RAW 264.7 macrophages

Before infection RAW 264.7 cells were seeded into 24-well plates at a density of 2 x 10^5 cells per well and the medium was replaced with 1 ml of RPMI-1640 supplemented with 3% FBS and 1% L-glutamine, and RAW 264.7 cells were then activated by adding LPS (1 μg/ml) overnight. RAW 264.7 cells were infected at an MOI of 100. After one hour incubation at 37°C with 5% CO_2, infected macrophages were washed twice with fresh cell culture medium, and fresh cell culture medium containing 50 μg/ml of gentamicin was added to kill extracellular bacteria. The infected macrophages were incubated for different time

points such as 4, 6, 24, and 48 hours. To measure intracellular survival, infected macrophages were washed with medium and lysed using 0.2 ml of 2% Triton X-100 (Sigma) in phosphate buffered saline (PBS) for 5 min at room temperature. Samples were removed, diluted, and plated onto LB agar plates to determine the number of CFU per ml recovered from cell lysate.

2.4.4.2 Monocytic cell (THP-1)

The net replication assay for THP-1 is similar to RAW 264.7 cell line as described above but there are a number of differences because in contrast to RAW 264.7 cells, THP-1 cells are a floating cell line. In the seeding process of THP-1 cells, Phorbol 12-Myristate 13-Acetate (PMA) was added to cells at a concentration of 1.62 mM to promote the cell differentiation and the adherence. The cells were ready for infection 2 days after seeding them with PMA. THP-1 cells don't need LPS to be activated, PMA exerts a similar effect.

2.5 RNA sequencing (RNA-Seq)

2.5.1 Bacterial growth curves

Growth curves were carried out to characterise growth of the bacterial isolates LF82 (AIEC), 601 (APEC), and TW731/12 (APEC) in the presence of FA. Overnight cultures were grown in RPMI 1640 at 37°C and 150 revolutions per minute (rpm) in a shaking incubator. Following that, cultures were divided in two groups, one supplemented with FA (20 mM). Both groups were diluted 1:100 into 30 ml of fresh RPMI 1640 in flasks and returned for incubation under the same conditions. Every hour, 1ml of culture was collected into a cuvette for measurement of optical density at 600 nm (OD_{600}) using a Multiskan FC Microplate reader (Thermo Scientific, UK). Sterile RPMI 1640 media was used to blank the instrument.

2.5.2 Preparation of RNA-seq

2.5.2.1 RNA extraction

Preparation of bacterial RNA for next-generation sequencing requires several steps to ensure bacterial RNA suitable for sequencing. Two types of groups were selected in this study for RNA sequencing. A group of bacterial strains (LF82, 601, and TW731/12) was grown in

RPMI medium that contains 20 mM of FA, while the other group was grown without FA and three biological replicates were used for each strain. After growing an overnight culture in RPMI media and incubating until the OD_{600} reached 0.6; RNA extraction and purification steps were applied by using different kits as follows (table 2-2). All protocols were used as per the manufacturer's instructions.

RNA sequencing steps	Kit name	Company name
1-RNA extraction	PureLink™ RNA Mini Kit	Thermo Fisher Scientific (12183018A)
2-DNA removal and RNA Purify	MEGAclear™ Transcription Clean-Up Kit	Thermo Fisher Scientific (AM1908)
3-mRNA enrichment	MICROB*Express*™ Bacterial mRNA Enrichment Kit	Thermo Fisher Scientific (AM1905)

Table 2-2 RNA sequencing process and used Kits for *E. coli.*

2.5.2.2 Bioinformatics and analysis

RNA-seq transcriptome generation, data analysis, cDNA synthesis and sequencing were performed at the University of Glasgow Polyomics Facility by the author and the facility members, essentially as described by (Connolly and Roe 2016). Briefly, sequencing was preformed using an Illumina NextSeq 500 platform obtaining 75 bp single end reads. Samples were prepared and sequenced in triplicate. Raw reads were QC checked using FastQC (Babraham Bioinformatics, Cambridge, UK) and trimmed accordingly using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). Trimmed reads were mapped to the LF82 reference genome (NCBI accession number: CU651637) allowing for 3 mismatches per read. Analysis of differential expression was performed using the Empirical analysis of DGE tool, which implements the EdgeR Bioconductor tool (Robinson et al., 2010). Differentially expressed genes were identified by absolute fold change (cutoffs log2) and a P value of % 0.05. Volcano plots were generated in CLC Genomics Workbench. A training for data analysis was provided by Prof Thomas Otto, Dr Graham Hamilton, and Mr John Cole at Glasgow Polyomics.

2.6 *In vitro* fermentation gut model

2.6.1 Batch fermentations

2.6.1.1 Faecal sample collection and preparation

Faecal samples were collected from Rhode Island Red chickens in disposable containers and processed within two hours of chicken defecation. The chickens' age during the sample collection process was 13 months, and they were on Layers Pellets diet. Moreover, these chickens are considered home-reared chickens. The collected fresh samples from the chickens were mixed and homogenised with Sorensen's buffer (which is a phosphate mixed sodium salts) pH 7. Every gram of faecal sample was mixed with a buffer, and the ratio was (1:10). Samples were vortexed thoroughly using beads, and then 0.5 ml of this faecal slurry was aliquoted into a microfuge tube with 0.5 ml of 50% glycerol. All aliquots were stored at -80°C.

2.6.1.2 Fermentation media

The fermentation media consists of several solutions including bicarbonate buffer, macromineral solution, micromineral solution, and sodium phosphate buffer and each solution were prepared as per the following:

1. **Bicarbonate Buffer (500ml)**
    a. 2 g of NH_4HCO_3 (Ammonium bicarbonate)
    b. 17.5 g of $NaHCO_3$ (Sodium bicarbonate) - (Sigma: 401676)
    c. Sterile distilled water was added to a final volume of 500 ml and stored at 4°C until use.
2. **Macromineral solution (500ml)**
    a. 3.57 g $Na_2HPO_4.2H_2O$ (Sodium phosphate dibasic dihydrate) - (VWR: 102494C)
    b. 3.92 g of $KH_2PO_4.H_2O$ (Potassium dihydrogen phosphate) - (Fisher: P/4800/53)
    c. 0.3 g of $MgSO_4.7H_2O$ (Magnesium Sulfate Heptahydrate) - (BDH: 101514Y)
    d. Sterile distilled water was added to a final volume of 500 ml and stored at 4°C until use.
3. **Micromineral solution (100ml)**
    a. 13.2 g of $CaCl_2.2H_2O$ (Calcium chloride dehydrate) –(Sigma: C-3881)
    b. 10 g of $MnCl_2.4H_2O$ (Manganese (II) Chloride Tetrahydrate) - (Acros: 205895000)

c. 1 g of $CoCl_2.6H_2O$ (Cobalt (II) Chloride Hexahydrate) - (BDH: 27790)
d. 8 g of $FeCl_3.6H_2O$ (Iron (III) Chloride Hexahydrate) - (Sigma: 236489)
e. Sterile distilled water was added to a final volume of 500 ml and stored at 4°C until use.

4. **Sodium Phosphate buffer (500ml to be split into 4 x 125ml bottles)**
   a. 0.066 M KH_2PO_4 (9.078g/L): 4.55 g in 500ml
   b. 0.066 M Na_2HPO_4 (11.867g/L): 5.95 g in 500ml
   c. 195 ml of (a) into 30 ml of (b)
   d. The pH was adjusted to 7 using 1 M NaOH and stored at 4°C

After preparing the solutions, the fermentation medium was made up in 1 litre volumes. It consisted of 225 ml of macromineral, 225 ml bicarbonate buffer, 112.5 µl of micromineral, 1.125 ml of 0.1% resazurin. Once the solution was made, it was boiled, degassed under oxygen-free nitrogen, and adjusted to pH 7 to mimic the distal intestinal environment. Reducing solution (50 ml) was made up of 2 ml of 1 M NaOH, 312.5 mg of cysteine hydrochloride and 312.5 mg of $Na_2S{\cdot}9H_2O$. The fibre substrate that was used was Fancy Feeds Layers Pellets (Fancy Feed Company), made up of wheat, wheat feed, hi-pro soya, full fat soya, limestone, grass meal, maize, dicalcium phosphate, soya oil, paprika pigment, vitamins and minerals, lutein and zeoxanthin from marigold (Natural Yolk Pigment). We chose this fibre as indicative of chicken feed that is consumed in the UK poultry. In addition, this chicken feed doesn't contain organic acid additives or preservatives. This feed is considered essential to promote good health and well-being in the bird as well as supporting egg production. The pellets were grinded and added to media in each of the fermentation's bottles (100 mg).

The fermentation media that was used in this study consists of several solutions such as bicarbonate buffer, macromineral solution, micromineral solution, sodium phosphate buffer, and other compounds and these are detailed in table 2-3.

Fermentation medium	Volume of solutions in (1 L)
Tryptone (Sigma: T7293)	1.125 g in 225 ml of SDW
Micromineral solution	112.5 µl in 1000 ml of total media
Bicarbonate buffer	225 ml
Macromineral solution	225 ml
0.1% Resazurin solution	1.125 ml
H_2O	Used to adjust the final volume to 1000 ml

Table 2-3. Fermentation media composition for *in vitro* fermentation.

2.6.1.3 Fermentation method

Fermentation medium was boiled until the colour changed from blue to pink, then the sodium phosphate buffer (SPB) was boiled in the microwave. Both SPB and fermentation media was cooled down after the boiling step, under oxygen free nitrogen (OFN) until the temperature dropped to 40°C. Once the medium reached 40°C the pH of the medium was adjusted to pH 7 using 6 M HCl and the medium was placed in a water bath to maintain it at 40°C. Each fermentation bottle contained 8.6 ml of fermentation solution, 0.4 ml reducing solution, 1 ml of the faecal slurry that was mixed with 5 ml of SPB, and 100 mg of chicken feed. Anaerobic conditions were induced by degassing under oxygen-free nitrogen streams. Fermentation bottles were incubated vertically in the shaking water bath at 40°C with 60 strokes/min for 24 hours. Under some conditions, 100µl of *E. coli* 601 which is APEC strain was spiked in the samples (1.9×10^8 to represent the 100% of 0.1% *E. coli* in the gut). In addition, 200 µl of each of FA or PA (to a concentration of 20 mM) were added. Aliquots of fermentation slurry for SCFA analysis were collected and stored in 3:1 ratio with 1 M NaOH at − 20°C until analysis. Fermentation slurry aliquots were stored at -80°C and total DNA was extracted later. In this fermentation experiment, seven fermentation conditions were applied that are detailed below;

1. A control sample (chicken faecal sample)
2. A control sample (chicken faecal sample) + *E. coli* strain spiked.
3. Faecal sample + FA + *E. coli* strain spiked.
4. Faecal sample + PA + *E. coli* strain spiked.
5. Faecal sample + chicken Feed + *E. coli* strain spiked.
6. Faecal sample + chicken Feed+ FA + *E. coli* strain spiked.
7. Faecal sample + chicken Feed+ PA + *E. coli* strain spiked.

2.6.2 Short chain fatty acid (SCFA) analysis

2.6.2.1 SCFA extraction

Faecal samples and standards that are used in measuring SCFAs were prepared before the SCFA extractions. Six external standards of SCFA that includes acetic acid (185.8 mM), PA (144.5 mM), butyric acid (114.2 mM), valeric acid (83.4 mM), caproic acid (52.6 mM), and isovaleric acid (87.0 mM) were set up (10, 25, 50, 100, 200 and 300 µl) for quantification of the compounds. Eight hundred microlitres of distilled water was added to each external standard with 100 µl of internal standard used to account for any losses in the sample

(Gerasimidis et al., 2020). In addition, 100 µl of orthophosphoric acid (16 M) was added to each standard and 3 ml of diethyl ether followed by vertexing the tubes on IKA shaker for 1 min at a speed of 1500 rpm. Addition of diethyl ether was repeated three times with vortexing. Along with the external standards, two controls were prepared by weighing 100 mg of freeze-dried faecal samples that are used as a control and placed in tubes containing 300 µl distilled water, 100 µl of internal standard, and 100 µl of orthophosphoric acid. Following this, diethyl ether was added as previously described above for the external standards. Preparation of samples for SCFA extraction was similar to preparation of external standards. Eight hundred microlitres of supernatant of fermentation sample (faecal slurry) was placed in the tube and then all procedures were applied starting from adding the internal standard (table 2-4). The only difference was that distilled water was not included in the preparation of the samples as it was in the controls.

Std No.	**Vol. Ext Std**	**Vol. of water to be added**	**Vol. of Int. Std**	**Vol. of Orthophosphoric acid (16M)**	**Final Volume**
1	10 µl	790 µl	100 µl	100 µl	1000 µl
2	25 µl	775 µl	100 µl	100 µl	1000 µl
3	50 µl	750 µl	100 µl	100 µl	1000 µl
4	100 µl	700 µl	100 µl	100 µl	1000 µl
5	200 µl	600 µl	100 µl	100 µl	1000 µl
6	300 µl	500 µl	100 µl	100 µl	1000 µl
Vol. of Sample.	**Vol. of Int. Std**	**Vol. of Orthophosphoric acid (16M)**			**Final Volume**
800 µl	100 µl	100 µl			1000 µl

Table 2-4 Preparation of SCFAs standards and the samples. Details of the preparation of the standards and samples prior to SCFA analysis via gas chromatography.

2.6.2.2 Measurement of SCFAs by gas chromatography.

SCFAs (acetic acid, PA, butyric acid, valeric acid, caproic acid, heptanoic acid, and caprylic acid) and BCFAs (iso-butyric and iso-valeric acid) were extracted three times using diethyl ether from acidified slurries. Extracts were analysed using gas chromatography (Agilent 7890A) with a flame ionisation detector, as previously reported (Svolos et al., 2019; Gerasimidis et al., 2020). Each of the SCFAs was quantified against calibration curves plotted using authentic external standards acetic acid (185.8 mM), PA (144.5 mM), butyric acid (114.2 mM), valeric acid (83.4 mM), caproic acid (52.6 mM), heptanoic acid (65.8 mM), caprylic acid (53.2 mM), isobutyric acid (97.3 mM), and isovaleric acid (87.0 mM) all stored in 2 M NaOH and using 2-ethylbutyric acid (74.0 mM) as an internal standard. Each sample was measured twice, and in all cases the average concentration was calculated unless the % co-efficient of variation was greater than 10% in which case a third replicate was analysed. Concentration of SCFA (μmol) is reported per volume (ml) of fermentation slurry.

2.6.3 Quantitative real-time PCR (qPCR) analysis

2.6.3.1 DNA extraction

Prior to bacterial DNA extraction from the faecal materials, 1.5 ml of the faecal slurry samples collected from the fermentation batches were weighed and distributed in tubes then frozen at -80ºC. Two hundred μl of mixed bacterial cultures for each time point and fermentation condition were centrifuged for 5 min at 10,000 x g. After discarding the supernatant, the pelleted cells were used for DNA extraction. Genomic DNA was extracted using the DNeasy® PowerSoil Kit (250) as per the manufacturer's protocol. The relative purity and integrity of DNA were assessed visually by Qubit Fluorometric Quantification that uses fluorescent dyes to determine the concentration of nucleic acids in a sample. The protocol of Qubit™ dsDNA HS (High Sensitivity) and (Broad Range) BR Assay Kits was used as per the manufacturers' protocols.

2.6.3.2 Quantification of *E. coli* and total bacteria by quantitative real-time PCR (qPCR)

This technique was used to detect the absolute quantity and relative abundance of *E. coli* and total bacteria (16S rRNA gene copy number/ml slurry) under different fermentation conditions that mimic the chicken gut. Absolute quantification and relative abundance were performed against serial dilutions of external standards (DNA of *E. coli* was extracted from pure bacterial culture by Prof. Konstantinos Gerasimidis's lab at the University of Glasgow, and these extracted DNA used for total bacteria standard). The relative proportion of *E. coli* in relation to the detected total bacteria amount (%Total) was calculated to assess changes in relative abundance. The stock standard was serially diluted 10-fold five times and then the six different standard dilutions were used to plot a standard calibration curve. Under sterile conditions all qPCR assays were prepared in triplicate using a 7500 Real-Time PCR System (Applied Biosystems, UK). Each 15 µl reaction was prepared in MicroAmp™ optical 96-well reaction plates. TaqMan® chemistry was used in assays and each contained 12.5 µl TaqMan® gene expression master mix (Catalog no. 4370074), 1 µl of appropriately diluted DNA 1:50 (template or standard), 0.5 µl Bovine serum albumin (BSA), and 2.25 µl nuclease free water. All primers and probes for total bacteria and *E. coli* were purchased from Applied Biosystems (UK). Cycles were performed with 95ºC for 10 min, 45 cycles of 95ºC for 15 s, and 60ºC for 1 min. Oligonucleotide sequences, and concentrations, are listed in Table 4. A negative control with water instead of DNA template was run on every plate in duplicate. The plate was sealed with MicroAmp® 96-well optical adhesive film (Applied Biosystem, UK).

Bacterial isolates	Oligonucleotide	Sequence 5' – 3'	Reference
E. coli	Probe: 2.5 µM	TAT TAA CTT TAC TCC CTT CCT CCC CGC TGAA	(Penders et al., 2005)
	Forward primer: 9 µM	CAT GCC GCG TGT ATG AAGA A	
	Reverse primer: 9 µM	CGG GTA ACG TCA ATG AGC AAA	
Total bacteria	Probe: 2.5 µM	CTT GTA CAC ACC GCC CGT C	(Furet et al., 2009)
	Forward primer: 9 µM	CGG TGA ATA CGT TCC CGG	
	Reverse primer: 9 µM	TAC GGC TAC CTT GTT ACG ACT T	

Table 2-5. Oligonucleotide sequences that were used to quantify the *E. coli* and the total bacteria.

2.6.4 Microbiome analysis

2.6.4.1 DNA Sequencing and data processing

Extraction of genomic DNA was carried out using faecal slurry samples collected from the fermentation batches were weighed and distributed in tubes then frozen at -80ºC. DNA extraction applied to samples similar to the process that were described in section 2.6.3.1. Furthermore, sample preparation for Polymerase chain reaction (PCR) was performed similarly to that described by (Costello et al., 2013). Each sample was amplified in triplicate, combined, and cleaned using the MoBio 96 HTP PCR clean up kit. PCR reactions contained 13 µl MoBio PCR water, 10 µl 5 Prime ot Master Mix, 0.5 µl each of the forward and reverse primers (10 µM final concentration), and 1.0 µl genomic DNA. Reactions were held at 94°C for 3 min to denature the DNA, with amplification proceeding for 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; a final extension of 10 min at 72 °C was added to ensure complete amplification. Cleaned amplicons were quantified using Picogreen dsDNA reagent in 10 mM Tris buffer (pH 8.0). A composite sample for sequencing was created by

combining equimolar ratios of amplicons from the individual samples, followed by gel purification and ethanol precipitation to remove any remaining contaminants and PCR artifacts. Moreover, purified DNA preparations were used to create libraries of 16S rRNA gene fragments by PCR using universal primers (F515/R806) for the V4 variable region: The primers sequence of (F515/R806) : (GTGCCAGCMGCCGCGGTAA/GGACTACVSGGGTATCTAAT). This primer pair amplifies the region 533–786 in the *Escherichia coli* strain 83972 sequence (greengenes accession no. prokMSA_id:470367). The samples were sent to Illumina for sequencing.

2.6.4.2 Paired end reads assembly and quality control.

PCR amplification of targeted regions was performed by using specific primers connecting with barcodes. The PCR products with proper size were selected by 2% agarose gel electrophoresis. Same amount of PCR products from each sample was pooled, end-repaired, A-tailed and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform to generate 250bp paired-end raw reads. The experimental procedures of DNA library preparation are shown as follows: Paired end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Then, paired end reads were merged using FLASH (V1.2.7) for sequence assembly. Further, data analysis was performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME (V1.7.0) quality-controlled process (Youssef et al., 2009; Hess et al., 2011). The tags were compared with the reference database (Gold database) using (Uchime algorithm) to identify chimeric sequences, and then to delete the chimeric sequences (DeSantis et al., 2006; Hess et al., 2011). Finally, the Effective Tags were acquired.

2.6.4.3 Operational Taxonomic Units and species annotation

Operational Taxonomic Units (OTUs) of each sample were obtained by clustering with 97% identity on the effective tags of all samples, and then identified. Sequence analysis was performed by Uparse software (Uparse v7.0.1001). Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For Species annotation, each representative sequence of the GreenGene Database (Bulgarelli et al., 2015) was used based on the RDP classifier (Version 2.2) (Li et al., 2013) algorithm to annotate taxonomic information. Phylogenetic relationships constructed for different OTUs, and the difference of the dominant species in different samples(groups), and multiple sequence alignment were conducted using the MUSCLE software (Version 3.8.31) (Lundberg et al., 2013). The abundance of OTUs was normalised using a sequence number standard corresponding to the sample with the least sequences. Based on this output normalised data, further analyses of alpha and beta diversity. The analysis was established by Novogene, and the author continued analysing data.

2.6.4.4 Alpha Diversity

Alpha diversity is applied in analysing complexity of species diversity for a sample through 6 indices, including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage. All these indices in the samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

2.6.4.5 Beta Diversity

Beta diversity was performed to evaluate differences of samples in species complexity analysis by QIIME software (Version 1.7.0). Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package in R software (Version 2.15.3). Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data, and the analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software (Version 1.7.0).

2.7 Statistical analysis

Values are represented as means and standard deviation. All statistical tests were performed with GraphPad Prism software, version 7.0c. All replicates in this study were biological; that is, repeat experiments were performed with freshly grown bacterial cultures and immortalized cells, as appropriate. Technical replicates of individual biological replicates were also conducted and averaged. Significance was determined using t-tests (multiple and individual as indicated in the figure legends) and one-way ANOVA corrected for multiple comparisons (as indicated in the figure legends). Values were considered statistically significant when P values were *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Chapter 3

Characterization of the effects of the organic formic and propionic acids on the phenotype of adherent-invasive and avian pathogen *E. coli*

3.1 Introduction

IBD which includes CD and UC, is a long-term inflammatory disorder of the GIT that cause pain and damage. In some cases, long-term inflammation with IBD can lead to bowel complications such as intestinal obstruction, fistulas, and abscesses. For instance, CD may cause intestinal wall thickening. Over time, the thicker portions of the intestine might contract, resulting in intestinal obstruction. A bowel obstruction occurs when a partial or total intestinal obstruction obstructs the movement of food or faeces through the intestines (Burisch et al., 2013; Lundberg et al., 2013). Extensive prior studies have indicated IBD is caused by environmental or genetic variables that disrupt the epithelial barrier, resulting in dysregulation of the mucosal immune system and responses to gut microbiota (Burisch et al., 2013; Lundberg et al., 2013; Nguyen et al., 2014). It has been established that patients with IBD exhibit intestinal dysbiosis, with a decrease in the number of potentially beneficial bacteria such as *Bifidobacteria*, *Lactobacilli*, and *Firmicute*s, and an increase in the number of putative pathogenic bacteria such as *Bacteroides* and *Escherichia coli* (Nguyen et al., 2014). Gut dysbiosis may lead to inflammation because beneficial commensal bacteria can promote intestinal homeostasis by blocking the harmful effects of pathogenic bacteria, stimulating innate immune responses, balancing the production of pro-and anti-inflammatory cytokines, and enhancing the physical integrity of the epithelium (Artis, 2008; Chassaing et al., 2011; Sartor, 2008).

Adherent invasive *Escherichia coli* (AIEC) strains are classified as pathobionts since they induce inflammatory diseases because of their genome's adaptive evolution in a particular and vulnerable human (Chervy et al., 2020; Palmela et al., 2018). In contrast to opportunistic infections, pathobionts exert an effect on the host indirectly via immune system activation. AIEC phenotype is associated with the ability to adhere to IEC, the ability to invade IEC via actin polymerisation and microtubule recruitment in the host cell, the ability to survive and replicate within macrophages without causing cell death, and the absence of any known invasive determinants (Chervy et al., 2020). Until now, the only method for effectively identifying AIEC strains has been to examine the interaction between bacteria and host cells. While molecular methods and genomic sequencing have discovered genes involved in AIEC invasion of IEC and replication in macrophages, a particular molecular marker related to the AIEC pathotype remains unidentified. AIEC strains have genetic similarities with extraintestinal pathogenic *E. coli* (ExPEC) in terms of phylogenetic origin and pathogenicity genotype (Palmela et al., 2018). According to their phenotypic characteristics, only 6.3

percent of ExPEC strains are AIEC (O'Brien et al., 2017). AIEC has been isolated from a variety of populations, including adults and children, healthy subjects, patients with IBD or colorectal cancer, and animals, including boxers with granulomatous colitis and animals with bovine mastitis, indicating that the AIEC pathotype is disease-specific rather than host-specific (Palmela et al., 2018).

Intestinal pathogens such as *E. coli* use a variety of mechanisms to outcompete the host microbiota, hence increasing their ability to persist and cause an infection. These mechanisms include inducing inflammation, killing commensals directly or indirectly, and utilising alternative carbon sources (Bertin et al., 2011; Dogan et al., 2014; Faber et al., 2017). During infection, intestinal pathogens utilise a variety of carbon sources: *E. coli* and *Clostridium perfringens* utilise sialic acid; enterohaemorrhagic *E. coli* (EHEC) utilise galactose, hexuronates, and ribose; and *Yersinia enterocolita* and *Salmonella enterica* serovar *Typhimurium* utilise adenosyl-cobalamin and 1,2-Propanediol degradation (1,2-PD) (Ormsby et al., 2019). Recent evidence has indicated a role for 1,2-PD metabolism during AIEC colonisation, with the *pdu* operon being overrepresented in this CD-associated pathotype and perhaps contributing to systemic inflammation (Ormsby et al., 2019). Along with 1,2-PD, phosphatidylethanolamine is a ubiquitous component of host cell membranes and is readily hydrolysed into ethanolamine and glycerol in the inflamed gut (Ormsby et al., 2019). Ethanolamine can be utilised as a carbon and nitrogen source for a variety of intestinal pathogens, including *Salmonella Typhimurium*, EHEC, *Enterococcus faecalis*, *Listeria monocytogenes*, and *Clostridium difficile* (Thiennimitr et al., 2011). Although these carbon sources are necessary for the growth of intestinal infections during inflammation, many bacteria cannot readily use them. Indeed, it is unclear whether AIEC cause intestinal inflammation that results in IBD or whether they operate as an exacerbating factor by colonising the mucosa of patients that already have the inflammatory disease.

3.2 Results

3.2.1 PA alters virulence pheotype in AIEC

An investigation was carried out previously to examine the ability of PA to alter the phenotype of *E. coli* strains and it has shown that PA can alter AIEC virulence traits such as biofilm formation and acid tolerance (Ormsby et al., 2020). To understand further how PA alters AIEC phenotype, multiple assays were conducted to investigate the effect of PA on AIEC. First, we wanted to understand how continuous PA exposure, that potentially leads to adaptation to PA, affect AIEC and whether the previously observed alterations in AIEC are confined to a specific strain. AIEC strains, which include the type of strain LF82, are associated with an ability to survive and replicate within macrophages without inducing death in these immune cells. (Nguyen et al., 2014; Palmela et al., 2018; Kathayat et al., 2021). Therefore, the net replication rate of LF82 was examined after PA exposure Along with LF82, clinical isolates from CD patients were examined to determine PA impact on other AIEC strains (Figure 3-1). The results indicate that PA has significantly increased the net replication rate of AIEC and the other two clinical isolates, indicating PA effects are not limited to the type of strain.

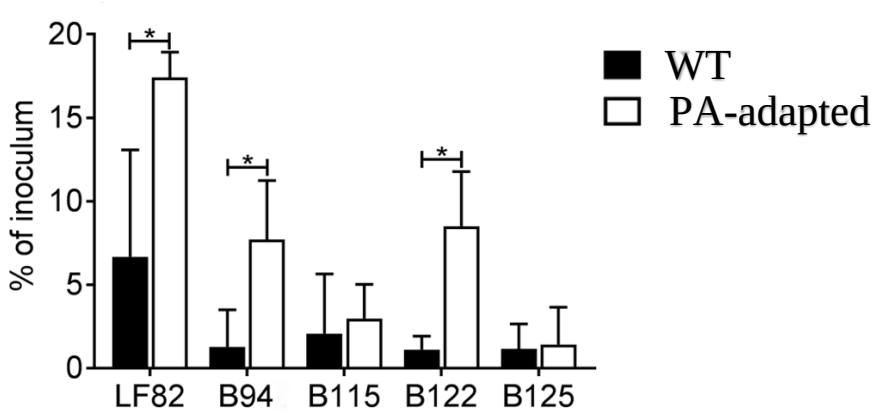


Figure 3-1 PA adaption increases the net replication of AIEC strains. RAW 264.7 macrophages cell lines were used to measure the net replication and survival of wild type, and PA-adapted AIEC strains at 24 hours post-infection (hpi). The data are displayed as the mean ± SD of three independent biological replicates. Data are expressed as mean ± SD; data were analysed using a one-way ANOVA ($p<0.05$ *).

PA as an abundant intestinal SCFA was also hypothesized to act as a signal for AIEC to alter their metabolism and increase their utilisation of ethanolamine, an intestinal metabolite utilised by pathogens during inflammation (Ormsby et al., 2019). Therefore, the net replication rate of LF82 was examined in the presence of increasing concentrations of ethanolamine. RAW264.7 macrophages were infected with LF82 and LF82 that had been adapted to PA through repeated exposure (Figure 3-2). The replication of LF82-PA within macrophages increased significantly in a dose-dependent manner in response to ethanolamine addition to the well. The response was directly as a result of ethanolamine supplementation in the media as the LF82Δ*eutR* or LF82-PAΔ*eutR* strains, which are unable to metabolise ethanolamine due to the removal of the ethanolamine utilisation operon regulator *eutR*, did not increase their replication. To confirm that the observed effects were not specific to murine macrophages, the experiment was performed in the human monocyte THP-1 cell line, where ethanolamine increased net replication in a dose and *eut*-dependent manner (Figure 3-2). These findings indicated that adaptation to PA increases replication of LF82 within macrophages in the presence of ethanolamine concentrations found in the human gut.

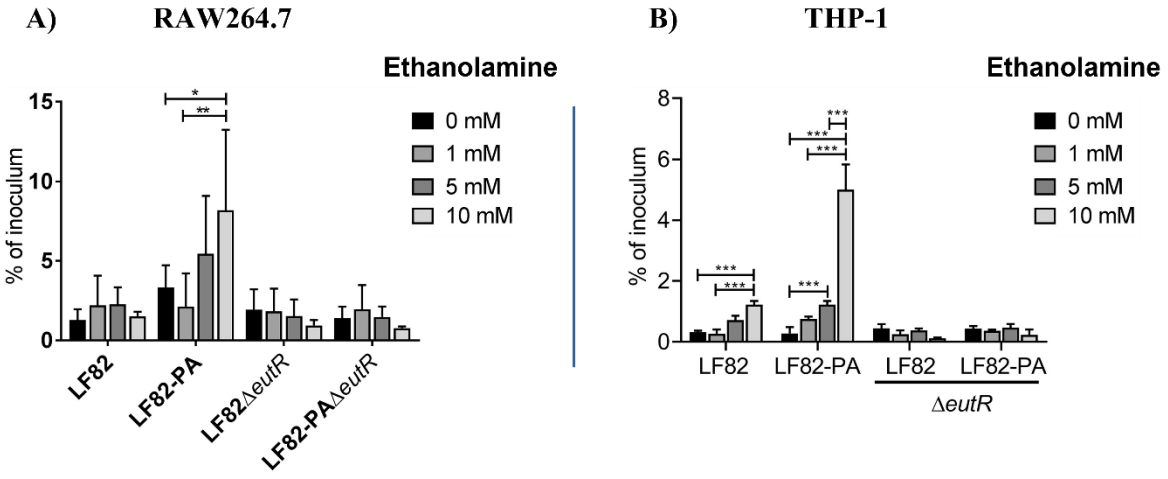


Figure 3-2 Extracellular ethanolamine increases net replication of LF82-PA. Intra macrophage (RAW264.7) and intra monocyte (THP-1) net survival and replication of wild type, PA-adapted, and LF82Δ*eutR* at 24 hpi with or without ethanolamine supplementation. For all values, the mean ± SD of three independent biological replicates is shown. Statistical analyses were preformed using GraphPad Prism, with data analysed by two-way ANOVA ($p < .05$ *; $p < .01$ **; $p < .001$ ***).

In addition, the net replication of AIEC clinical isolates was examined in THP-1 cells. However, PA adaptation and ethanolamine presence did not increase the intracellular net replication rate across all strains. Strain B94.2 was an exception, its net replication rate increased significantly when exposed to PA (B94.2-PA) when the ethanolamine dose was up to 5mM, but bacterial survival dropped significantly, likely due to toxicity of ethanolamine to the strain, at 10 mM (Figure 3-3).

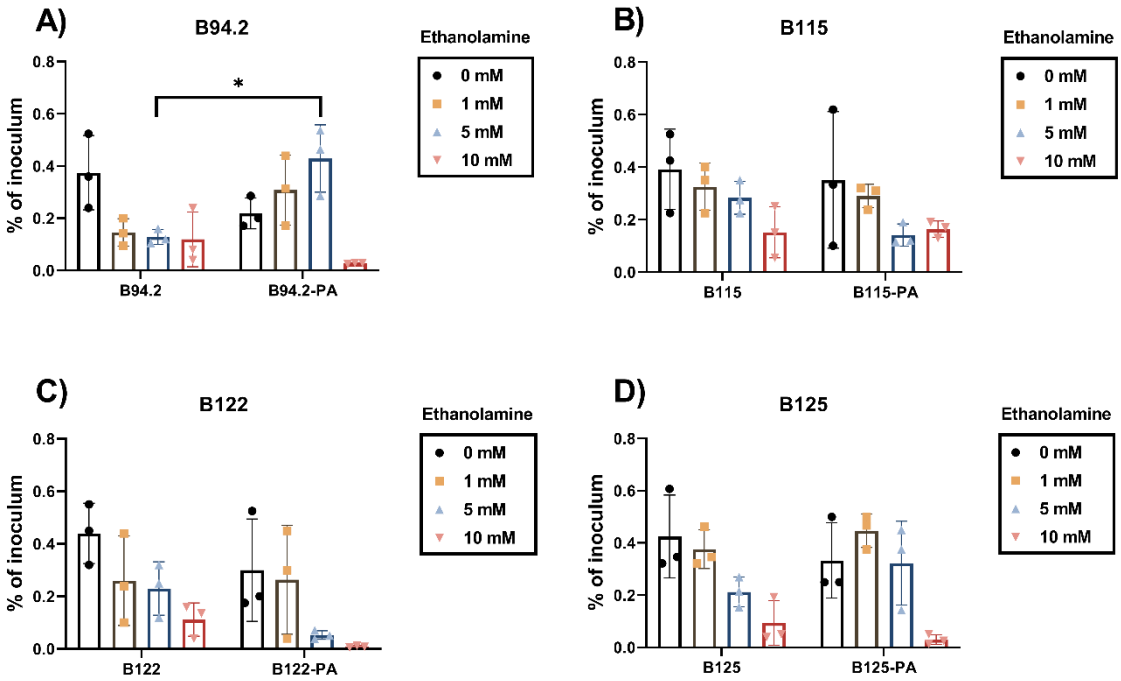


Figure 3-3 Net replication of clinical isolates in monocytes (THP-1). Survival and replication of wild type, PA-adapted, and LF82Δ*eutR* at 24 hpi with or without ethanolamine supplementation. For all values, the mean ± SD of three independent biological replicates is shown. Statistical analyses were preformed using GraphPad Prism, with data analysed by two-way ANOVA ($p < .05$ *).

The significant alteration in phenotype that LF82 demonstrated is due to PA adaptation with a concentration of 20 mM, and while this concentration is considered high, it can be found in environments such as animal water, feed, and silage. So, with the widespread usage of PA as an antimicrobial in the environment and agriculture, it is not implausible that bacteria such as AIEC come into contact with PA concentrations as high as 20 mM. Therefore, the effect of PA on another *E. coli* pathotype, APEC, that may be exposed to PA in high concentrations, was investigated. Again, the net replication rate of APEC was examined in presence ethanolamine (Figure 3-4). Similar to clinical isolates, PA adaptation and ethanolamine dosages had no significant influence on APEC strains, with the 601 strain having the lowest net replication rate in both its adapted and non-adapted forms (Figure 3-4).

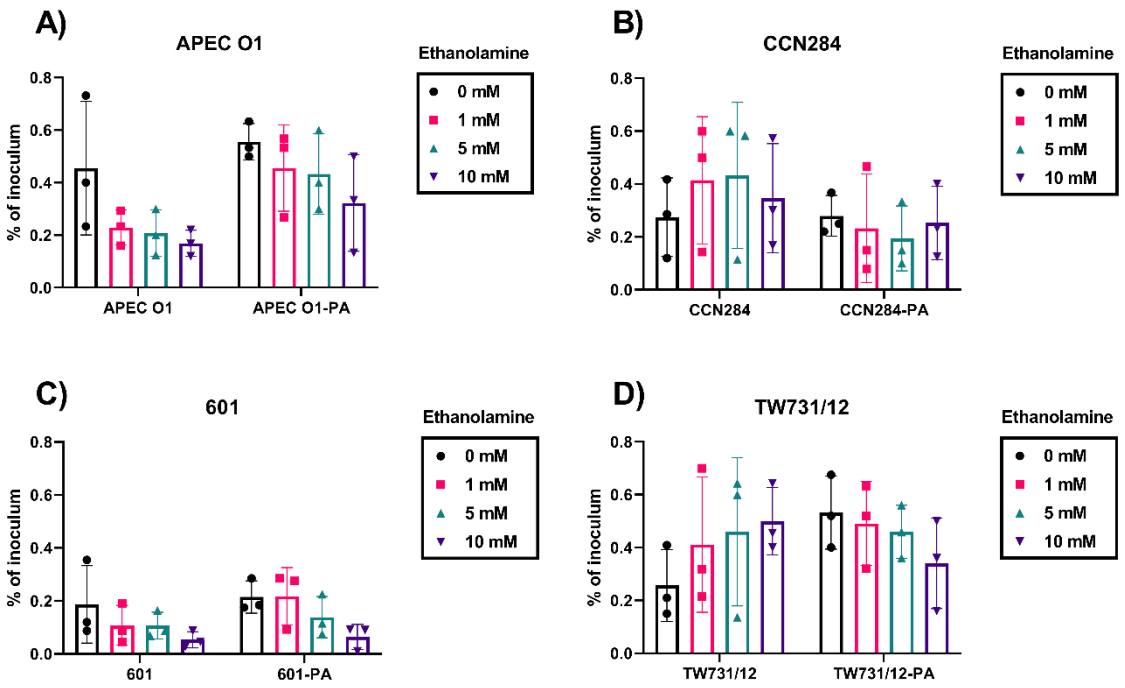


Figure 3-4 Net replication of APEC strains within monocytes (THP-1). Net survival and replication of wild-type and PA-adapted APEC strains at 24 hpi with or without ethanolamine supplementation. The data are displayed as the mean ± SD of three independent biological replicates and they were analysed using a two-way ANOVA.

Next the adhesion and invasion of AIEC and APEC was examined with the Caco-2 human intestinal epithelial cell line. These virulence traits are used to differentiate AIEC from other *E. coli* strains. The results indicate that PA adaptation significantly increased the adhesion and invasion of LF82 (Figure 3-5) However, the adhesion and invasion of APEC did not increase in response to PA, although the strains overall exhibited poor interaction with this human intestinal epithelial cell line. Additionally, it was intended to examine the adhesion and invasion of APEC on an avian intestinal epithelial cell line, however due to unavailability and other considerations, we could not accomplish this task. These findings along with previous studies indicate the PA can alter the AIEC phenotype even though is used as antimicrobial (Beier et al., 2019; Gadde et al., 2017; Mani-López et al., 2012; Quitmann et al., 2014). Given that FA is among the most commonly used SCFAs and is an effective antibacterial in the environment and agriculture, along with PA, we next examined its ability to alter virulence of AIEC and APEC.

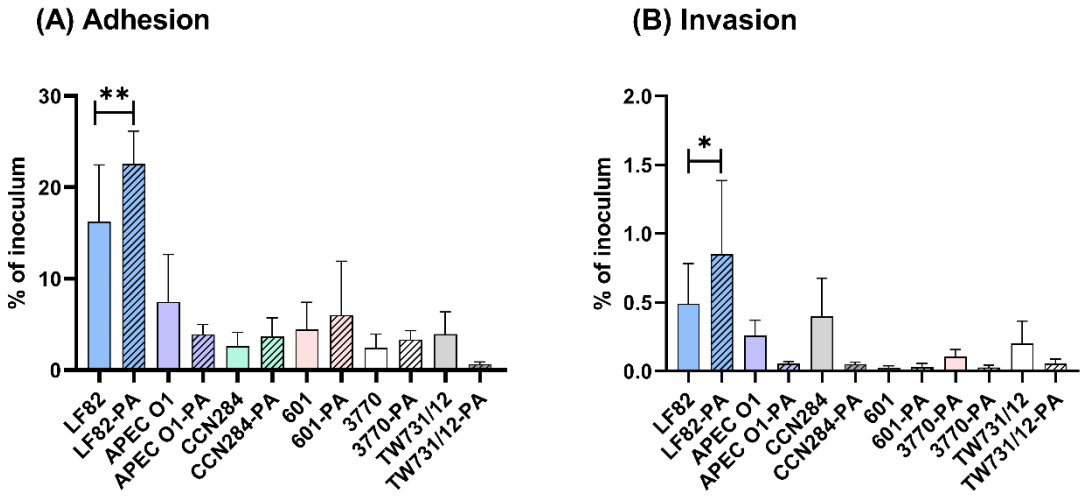


Figure 3-5 PA-adaptation increases the adhesion and invasion of AIEC. Two types of *E. coli* strains are displayed which are non-adapted strains to PA and adapted strains to PA such as LF82-PA. Adhesion and invasion are examined on the *Caco-2* cell line and the data are displayed of three independent biological replicates. The post infection duration for both adhesion and invasion were 4h. Adherence and invasion data are expressed as mean ± SD; data were analysed using a one-way ANOVA ($p<0.05$ *; $p<0.01$ **).

3.2.2 FA impact on the net replication of AIEC and APEC

Net replication within the immune cells is one of the important factors that pathogenic bacteria use. AIEC pathogenicity is correlated with their ability to survive and replicate within macrophages. Despite the acidic pH and oxidative stress, the LF82 strain does not escape into the cytoplasm but instead replicates in mature phagolysosomes (Palmela et al., 2018). This mechanism mainly leads to an increase in the host immune responses which eventually will lead to an increase the inflammation. Previously, it was shown that PA can alter the phenotype of AIEC and significantly increase net replication within macrophages. Thus, we sought to determine whether other SCFA such as FA has similar effects on AIEC and APEC. Initially several net replication assays were performed in RAW 264.7 cell to determine the effect of FA adaptation on virulence.

The findings demonstrate that FA adaptation does not have a significant effect on net replication of the *E. coli* strains, unlike PA adaptation, when measured at various time points (Figure 3-6). Furthermore, in contrast to PA, the net replication of all *E. coli* strains gradually decreased with time with the exception of CCN284. The effect of FA adaptation was also seen to vary between *E. coli* strains. For example, the net replication of CCN284-FA is lower than the non-exposed strain 4 hpi (Figure 3-6). However, as the infection progressed this trend was reversed with net replication of the non-adapted CCN284 lower than CCN284-FA at the other time points (Figure-A). A similar effect to that seen with CCN284 was observed in strain 3770 when it was compared to the strain adapted to FA (3770-FA), although again differences in net replication were not significant. In the AIEC type strain LF82, which is well known for its ability to replicate intracellularly, both the strain adapted to FA (LF82-FA) and the wild type strain showed a similar net replication pattern at all the time points. Moreover, in the 601 strain, both the adapted and non-adapted strains had the lowest net replication rate compared to other *E. coli* strains in all the time points (Figure 3-6). In fact, the gradual dropping in the net replication suggests killing and not replication. In addition, cell death could allow ingress of gentamicin at later time points accounting for the reduction over time. Overall, FA adaptation did not show significant effects on the net replication of *E. coli* strains similar to PA adaptation. Even, the AIEC type strain LF82 did not demonstrate any changes in net replication after repeated adaptation to FA.

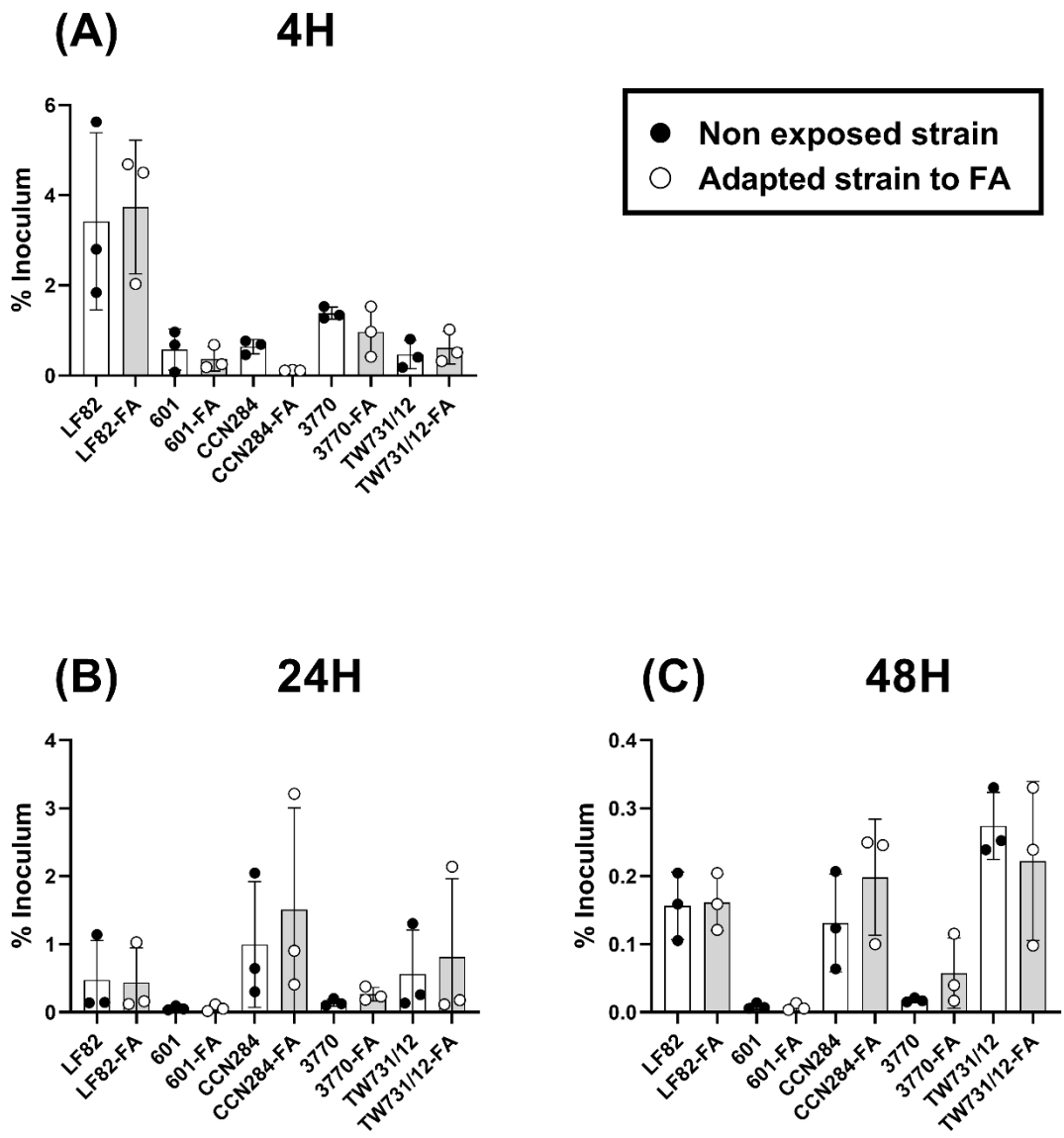


Figure 3-6 Net replication of adapted *E. coli* strains to FA. RAW 264.7 macrophages were used to assay net replication. The post infection duration at these net replication assays were 4h, 24h, and 48h. The data are displayed as the mean ± SD of three independent biological replicates and they were analysed using a one-way ANOVA.

3.2.3 FA adaptation influence the adhesion and invasion of *E. coli* strains

Adhesion and invasion are the major features that differentiate AIEC from other *E. coli* strains. In addition to AIEC, APEC strains are recognised for their potent adherence and invasion (Dziva et al., 2008; Palmela et al., 2018). These features are essential for the persistence of the bacterial strain. Some strains can adhere strongly and invade efficiently increasing their virulence due to their prolonged contact with host cells increasing their ability to invade the intestinal epithelium and induce inflammation. For instance, Type 1 pili (FimH) that are expressed on the surface of AIEC isolates interact with cell adhesion molecule 6 (CEACAM6) receptors on ileal enterocytes to facilitate adherence to the intestinal epithelium (Palmela et al., 2018). Adhesion and invasion are some of the key virulence traits that enable APEC to cause disease in poultry (Dziva et al., 2008). In addition, some studies indicated that some organic acids could potentially bacterial phenotype (Gadde et al., 2017; López et al., 2012). To investigate this further, adhesion and invasion assays were conducted on Caco-2 cells, a human intestinal epithelial cell line, to investigate the impact of FA adaptation on this aspect of AIEC and APEC infection. In addition, in this experiment the effect of a single exposure to FA was also compared to multiple (X5) exposures to determine any difference in effect.

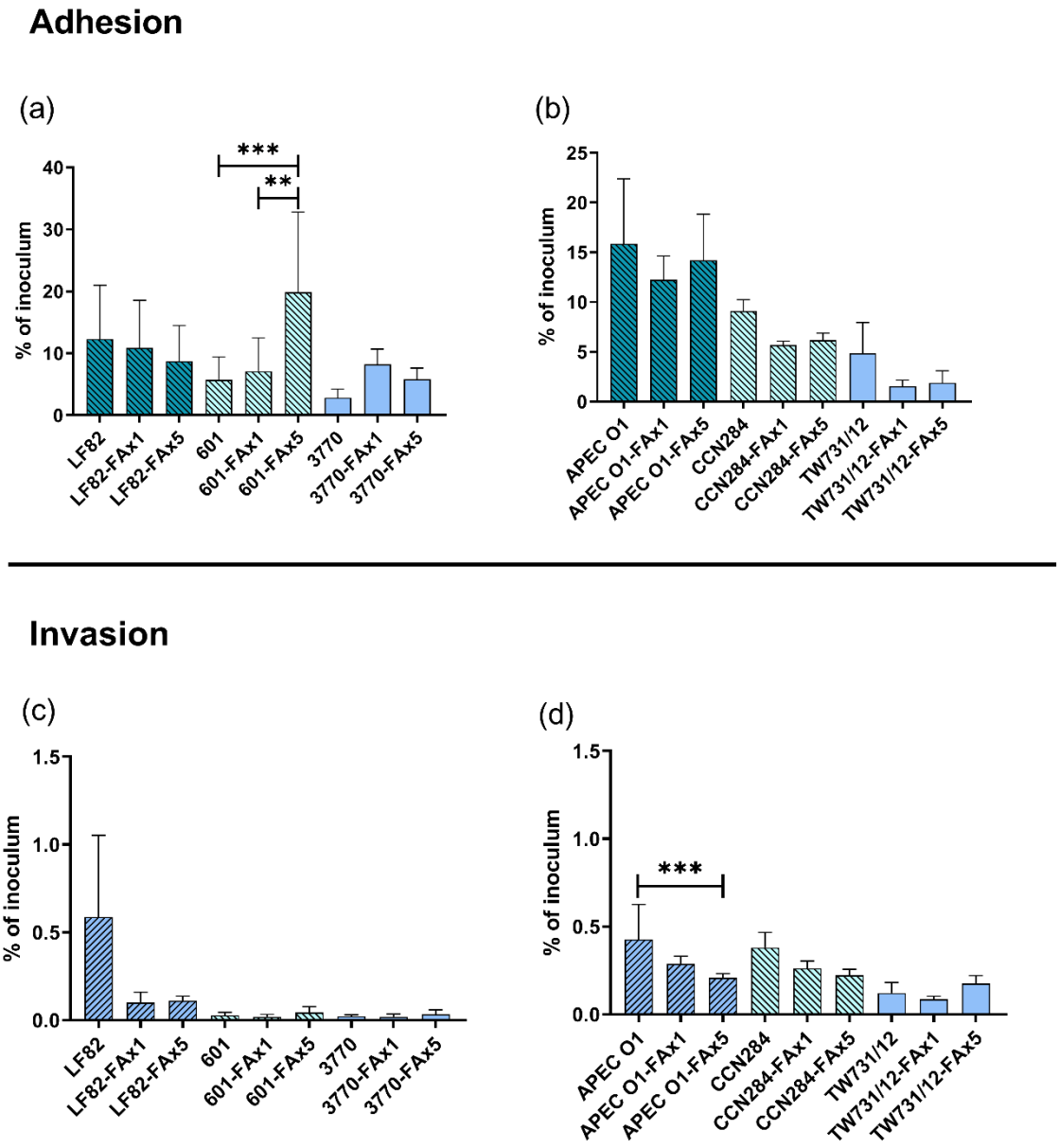


Figure 3-7 Adhesion and invasion of *E. coli* strains adapted to FA. Three types of *E. coli* strains are displayed which are non-exposed strains to FA, strains exposed to FA once (FAx1), and strains exposed to FA five times (FAx5). The strains are shown in two groups as due to the large number of replicates they were run in two independent groups. The post infection duration in this assay was 4h. Adhesion and invasion were examined using the Caco-2 cell line and the data are displayed of three independent biological replicates. Adherence and invasion data are expressed as mean ± SD; data were analysed using a one-way ANOVA ($p<0.01$ **; $p<0.001$ ***).

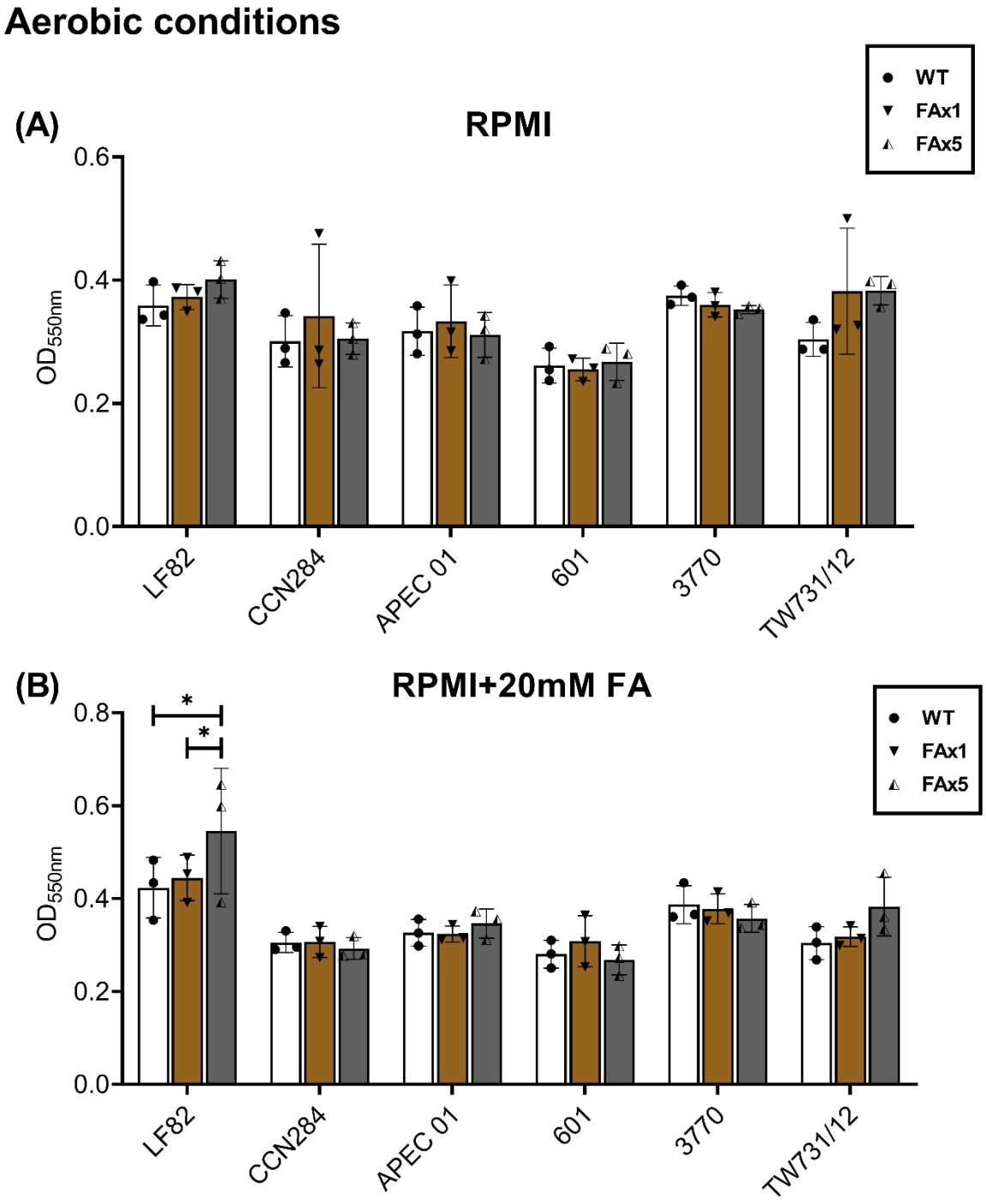
The results show that the adhesion in APEC strain 601 significantly increased after FA adaptation (Figure 3-7), but there is no associated increase in invasion (Figure 3-7). However, it is clear that 601 is ineffective at invading these cells. Therefore, although it has improved its ability to adhere, it has not become capable of invasion of this human cell line.

Again, unfortunately due to issues with obtaining a chicken intestinal epithelial cell line I could not explore if this invasion deficiency was specific to these cells. In contrast, FA adaptation significantly reduced the invasion of APEC O1, but had no effect on the adhesion of APEC O1 (Figure 3-7). No significant change was observed in any other *E. coli* strains adapted to FA in either adhesion or invasion assays. Indeed, in the case of LF82, recognised for its strong adhesion and invasion, a decrease in both adhesion and invasion were noted (Figure 3-7). In addition, unlike PA adaptation, FA adaptation has increased the adherence of 601, but reduced the invasion of APEC O1, while also having adverse effects on the adhesion and invasion of LF82 in comparison to PA. These results show that *E. coli* strains respond differentially to FA and PA, thus more investigation is required to determine the effects of FA and PA, which are employed as antimicrobials.

3.2.1 Biofilm formation of *E. coli* strains adapted to FA

The role of biofilms in bacterial colonisation of many environments has received considerable attention. However, until recently, little was known about biofilms in the intestine. Moreover, AIEC is associated with the formation of strong biofilms (Palmela et al., 2018). AIEC use biofilm formation as a virulence factor to increase their persistence. AIEC produces biofilms when interacting with the epithelial barrier, unlike non-motile strains. Those with H1 flagellar antigen are the most effective biofilm producers (Palmela et al., 2018). Moreover, it has been indicated that APEC strains are strong biofilm producers, and they can form biofilms in environments with low nutrients (Rodrigues et al., 2019). The ability of APEC to grow and survive in environments such as poultry production facilities and food processing plants is attributed to its capability to produce biofilms (Rodrigues et al., 2019).

To examine the impact of FA on biofilm formation, biofilm assays were performed in aerobic and anaerobic conditions. APEC strains (APEC O1, CCN284, 601, 3770, TW731/12) and AIEC strain (LF82) were adapted to FA by growing them in minimal media supplemented with 20 mM FA over a series of five successive re-cultures. AIEC or APEC adapted to FA once is represented by FAx1, while FAx5 means the strain was adapted to FA over five successive growth cycles. Following that the first group of strains was incubated both aerobically and anaerobically in minimal media, while the second group was incubated both aerobically and anaerobically in minimal media containing FA (Figure 3-8).(Rodrigues et al., 2019).


Aerobic conditions
(A)
RPMI
WT
FAx1
FAx5
OD550nm
0.0
0.2
0.4
0.6
LF82
CCN284
APEC 01
601
3770
TW731/12
(B)
RPMI+20mM FA
*
*
0.0
0.2
0.4
0.6
0.8

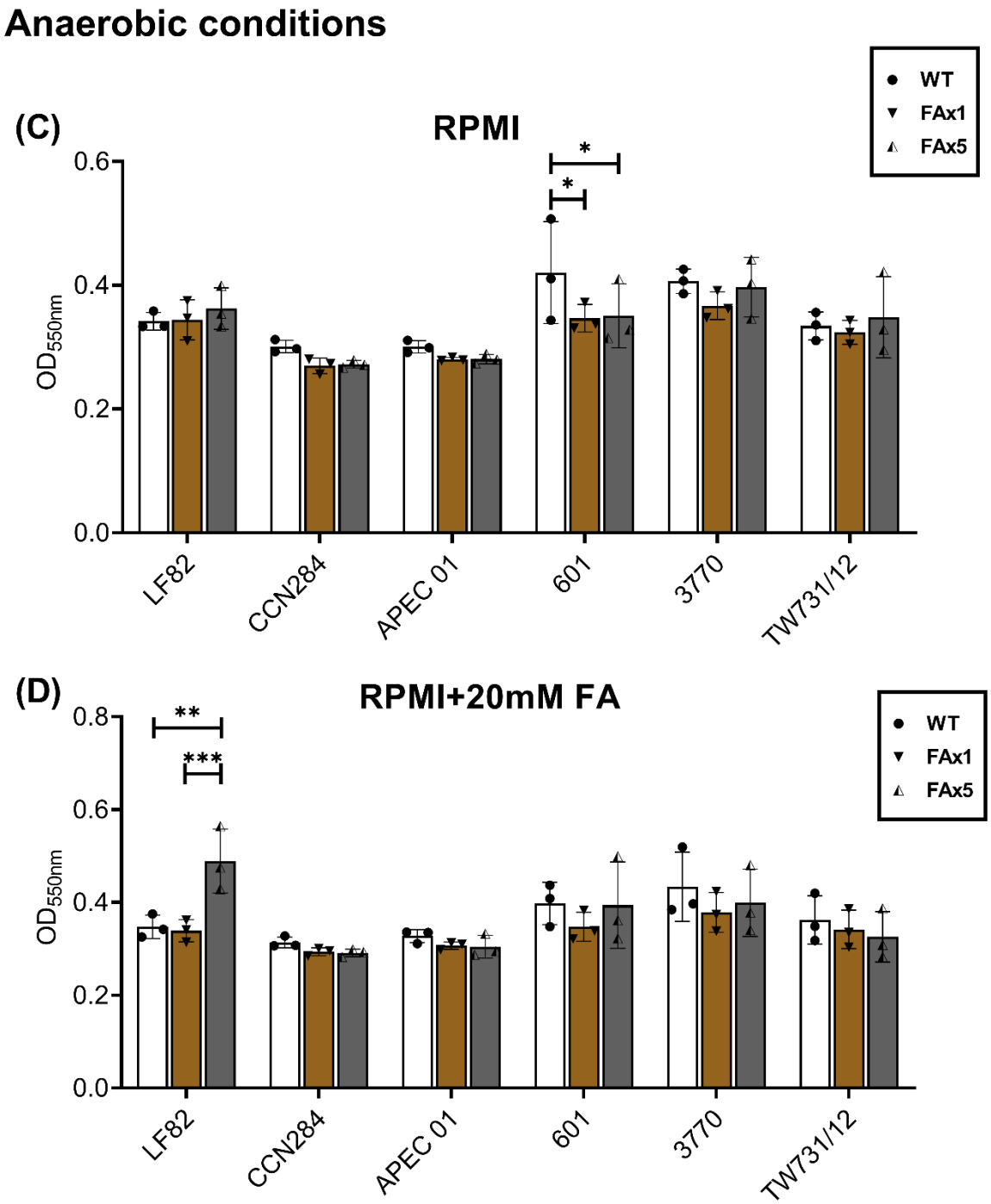


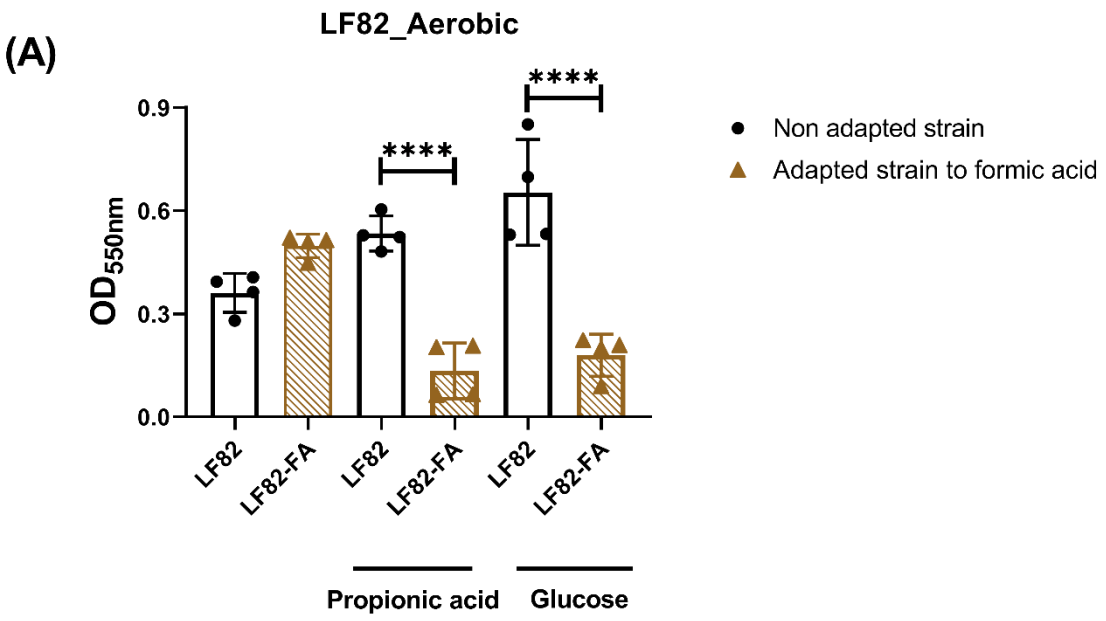
Figure 3-8 Biofilm formation by AIEC and APEC strains adapted to FA under aerobic and anaerobic conditions. To adapt *E. coli* strains to FA they were grown in minimal media supplemented with 20 mM FA over a series of five successive re-cultures. FAx1 denotes a strain isolated after 1 growth cycle, FAx5 denotes a strain isolated after 5 growth cycles, while WT denotes wild type strains not exposed to FA. The strains were incubated aerobically (A&B) or anaerobically (C&D) for 7 days in the presence of 20 mM FA as a carbon source (B&D) and in the absence of FA in RPMI media (A&C). The data are displayed of three independents biological replicates, and they were analysed using a two-way ANOVA ($p<0.05$ *; $p<0.01$ **; $p<0.001$ ***).

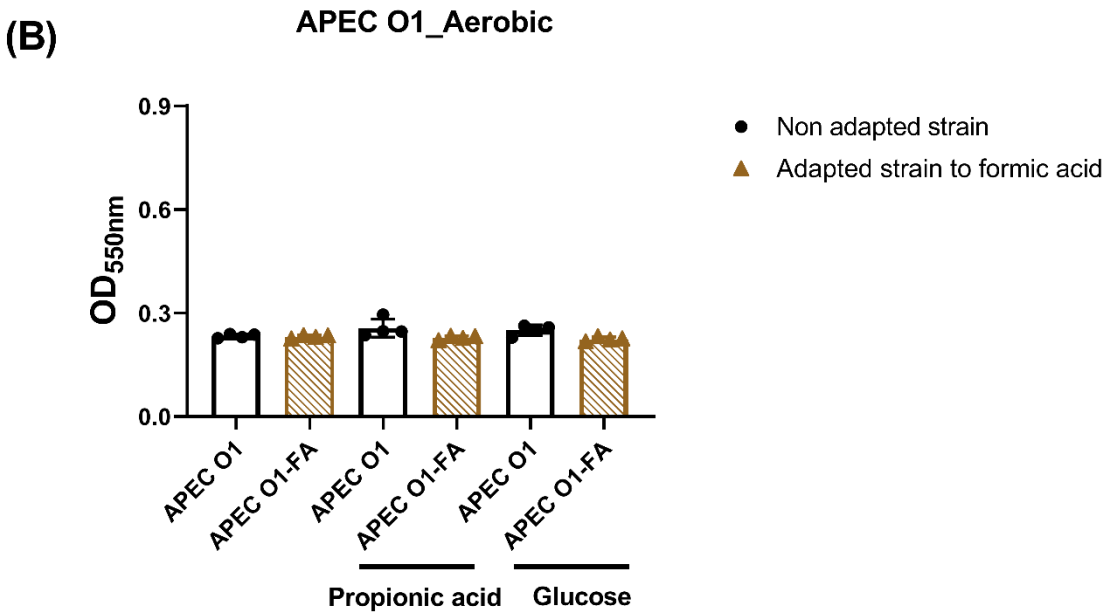
Under both aerobic and anaerobic conditions, the adapted form of LF82 formed more biofilms when FA is present as a carbon source in the media. In specific, the adapted form of LF82 which is FAx5 has a P value of 0.0118 when compared to the non-adapted form which is WT in the aerobic condition, and a P value of 0.0018 in the anaerobic condition. However, when the FA is not present, the adapted form FAx5 has a P value of 0.5186 in the aerobic condition, and a P value of 0.7473 in the anaerobic condition (Figure 3-8).

In addition, the impact of FA presences in the medium can be observed in the FAx5 when compared to FAx1 as well. In the aerobic condition, the P value of FAx5 is 0.0422, and it is 0.0010 in the anaerobic condition. While in the absent of FA, the P value of FAx5 when compared to FAx1 is 0.739 in aerobic condition and 0.7930 in the anaerobic condition. These data suggest that FA presence has impact on biofilm formation of adapted form of LF82 which is FAx5 (Figure 3-8). However, LF82 didn't exhibit any significant increase in biofilm formation compared to the non-adapted strain and FAx1 when FA was not present.

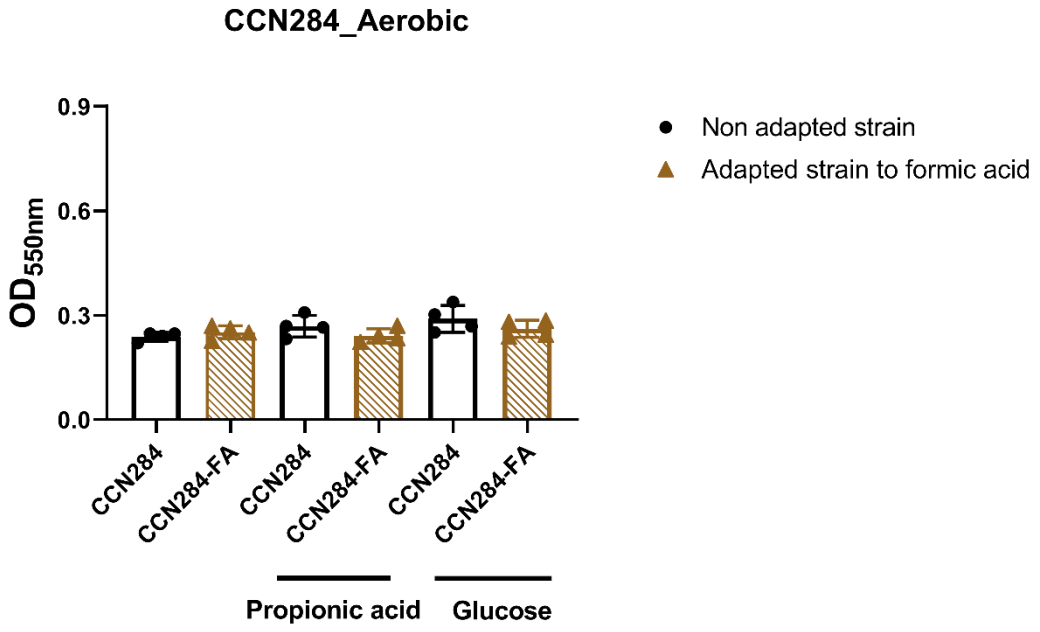
Another strain that showed a significant change is 601 which is an APEC strain. In the anaerobic and carbon source absent conditions, 601 strain adapted to FA formed significantly less biofilms compared to the non-adapted to FA (Figure 3-8). Other strains did not show any significant changes in biofilm formation after exposure to FA. Overall, this further added to the conclusion that exposure to FA was not having a positive effect on APEC biofilm formation that could help in bacterial persistence. Further investigation was done to assess the FA adaptation on biofilms formation of *E. coli* strains when different carbon sources such as PA and glucose are present in the media (Figure 3-9). A study indicated that the biofilm formation of *E. coli* is affected by carbon sources present in the environment, like glucose (Patel et al., 2021). Also, another study indicated that glucose is a preferable carbon source during the growth of *E. coli* (Wang et al., 2019). Therefore, glucose was selected as a carbon source to study its impact on biofilm formation. Besides glucose, PA was selected as s second carbon source that will be examined because it has been shown that PA could increase the biofilm formation of AIEC strain (Ormsby et al., 2020).

Aerobic conditions

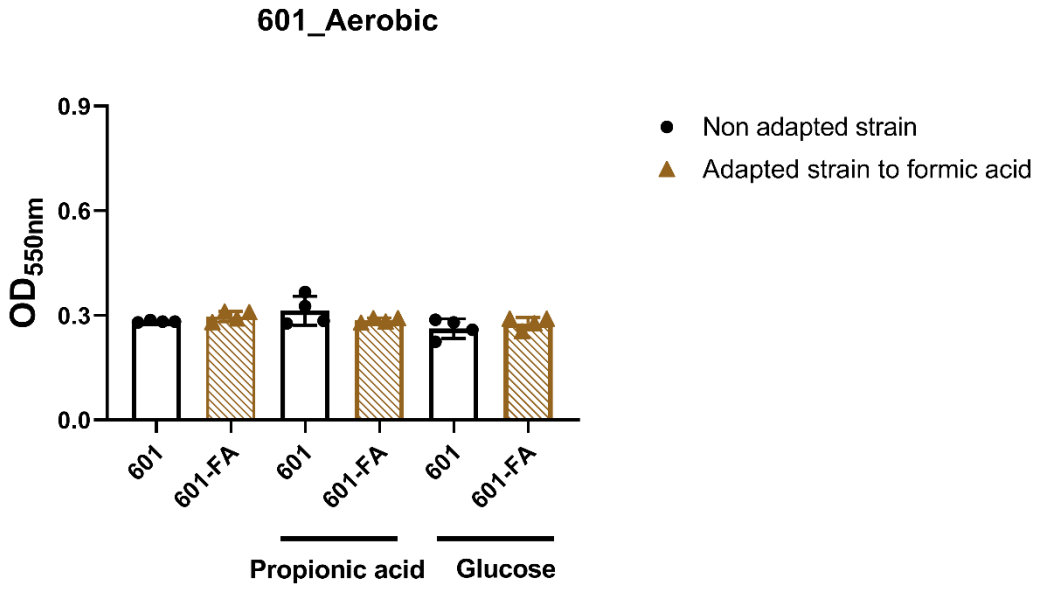




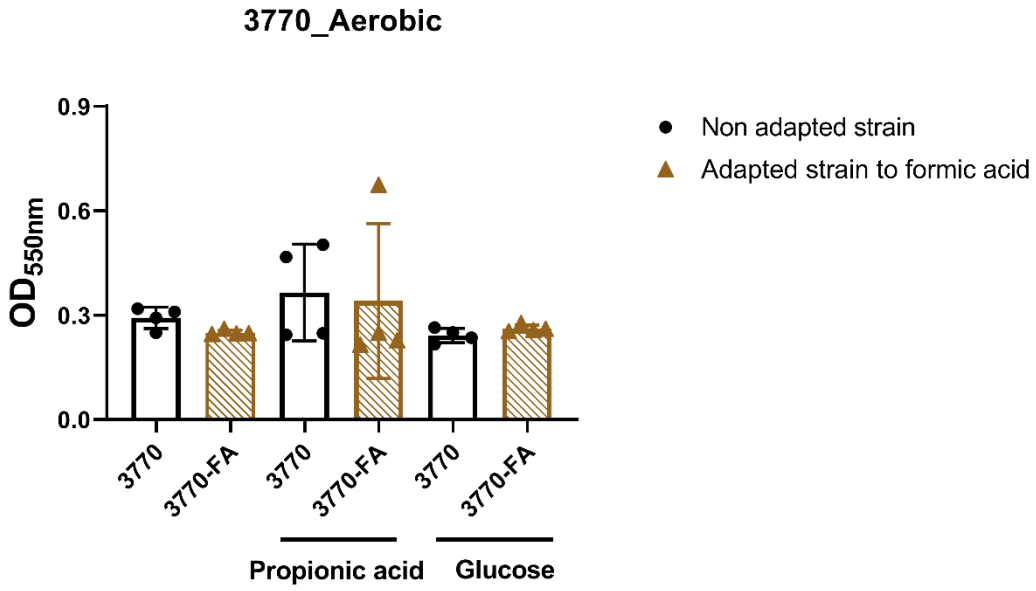
(C)


CCN284_Aerobic
0.9
0.6
0.3
0.0
OD550nm
CCN284
CCN284-FA
CCN284
CCN284-FA
CCN284
CCN284-FA
Propionic acid
Glucose
Non adapted strain
Adapted strain to formic acid

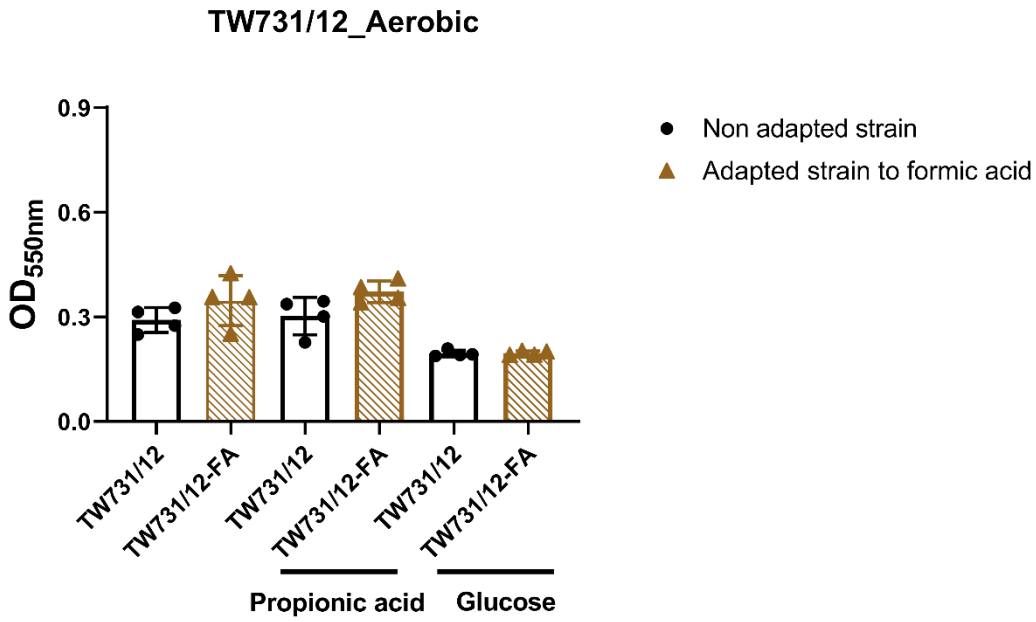
(D)


601_Aerobic
0.9
0.6
0.3
0.0
OD550nm
601
601-FA
601
601-FA
601
601-FA
Propionic acid
Glucose
Non adapted strain
Adapted strain to formic acid

(E)

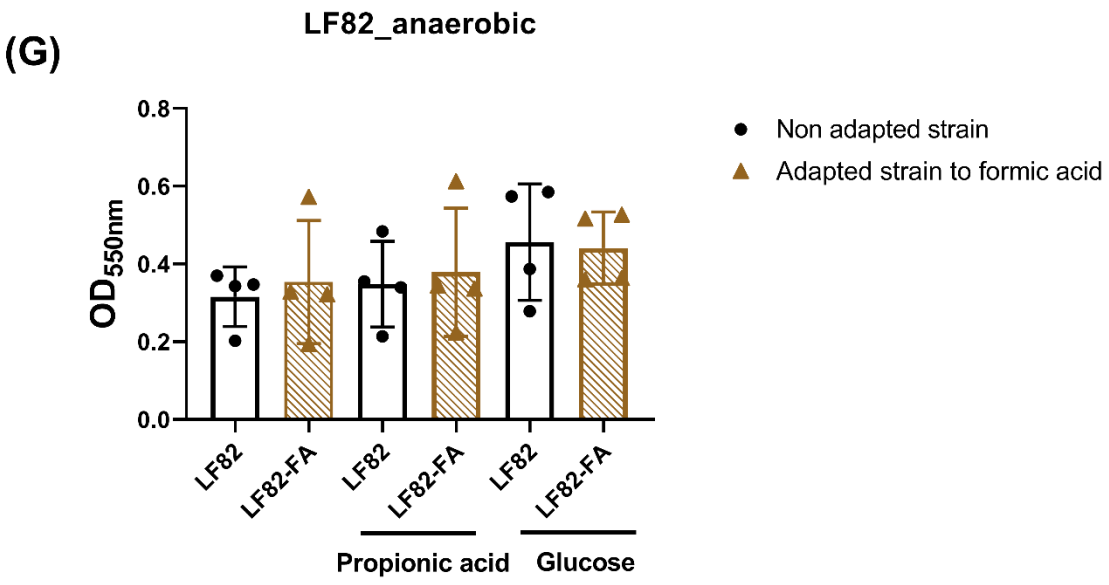

3770_Aerobic
0.9
0.6
0.3
0.0
OD550nm
3770
3770-FA
3770
3770-FA
3770
3770-FA
Propionic acid
Glucose
Non adapted strain
Adapted strain to formic acid

(F)

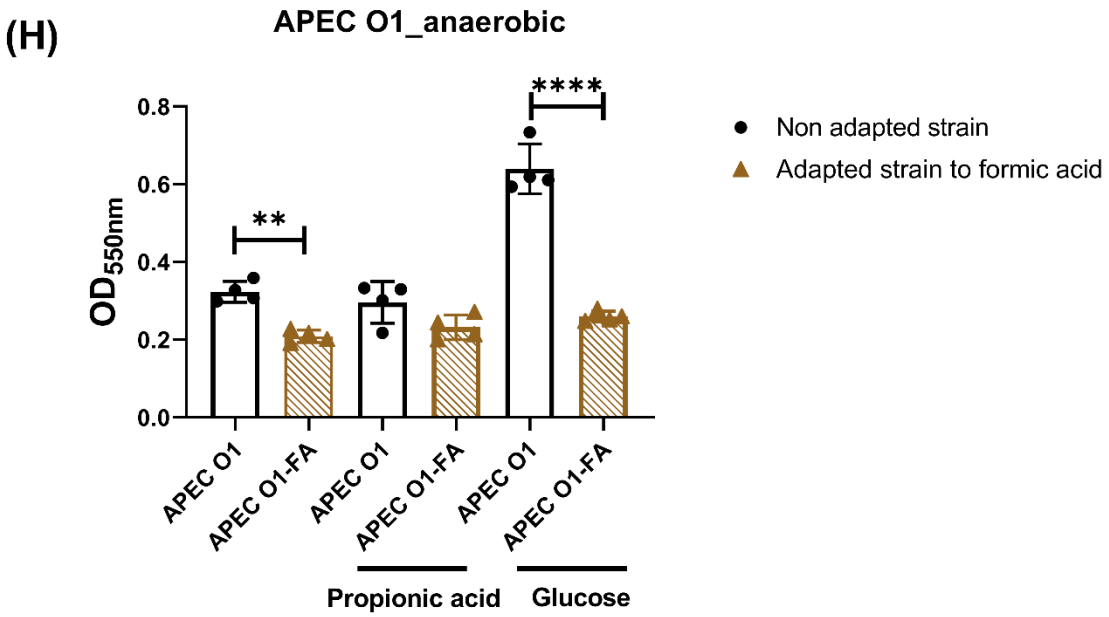

TW731/12_Aerobic
0.9
0.6
0.3
0.0
OD550nm
TW731/12
TW731/12-FA
TW731/12
TW731/12-FA
TW731/12
TW731/12-FA
Propionic acid
Glucose
Non adapted strain
Adapted strain to formic acid

Anaerobic conditions

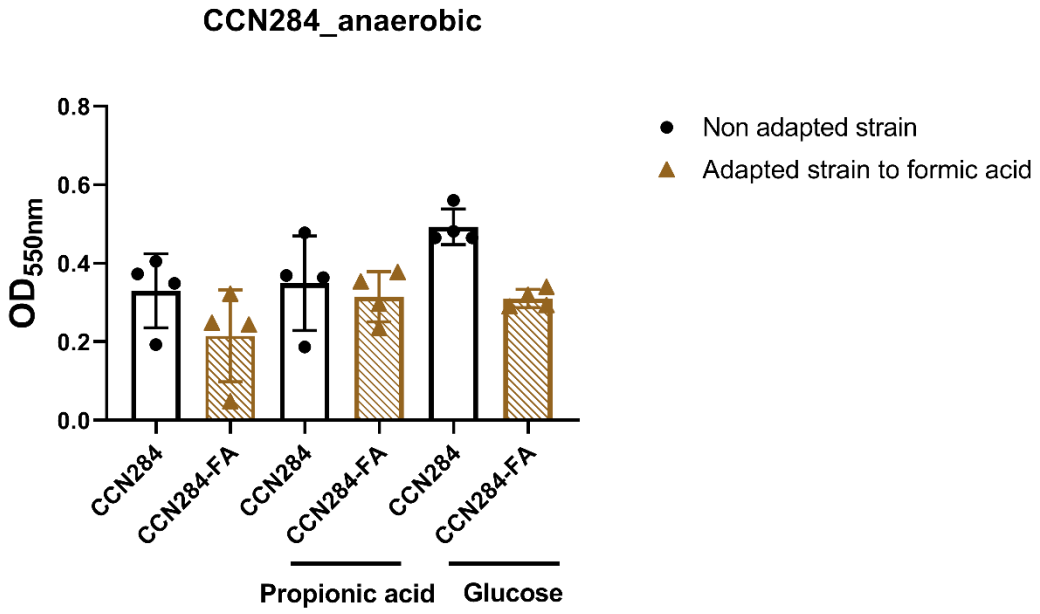
(G)



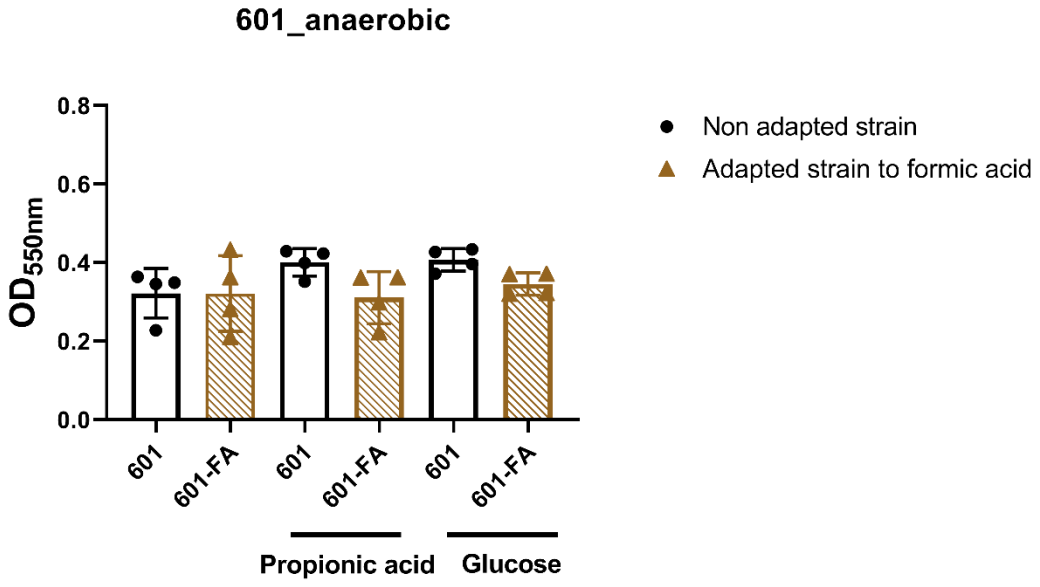
(H)



(I)


CCN284_anaerobic
OD550nm
0.0
0.2
0.4
0.6
0.8
CCN284
CCN284-FA
CCN284
CCN284-FA
CCN284
CCN284-FA
Propionic acid
Glucose
Non adapted strain
Adapted strain to formic acid

(J)


601_anaerobic
OD550nm
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Glucose
Non adapted strain
Adapted strain to formic acid

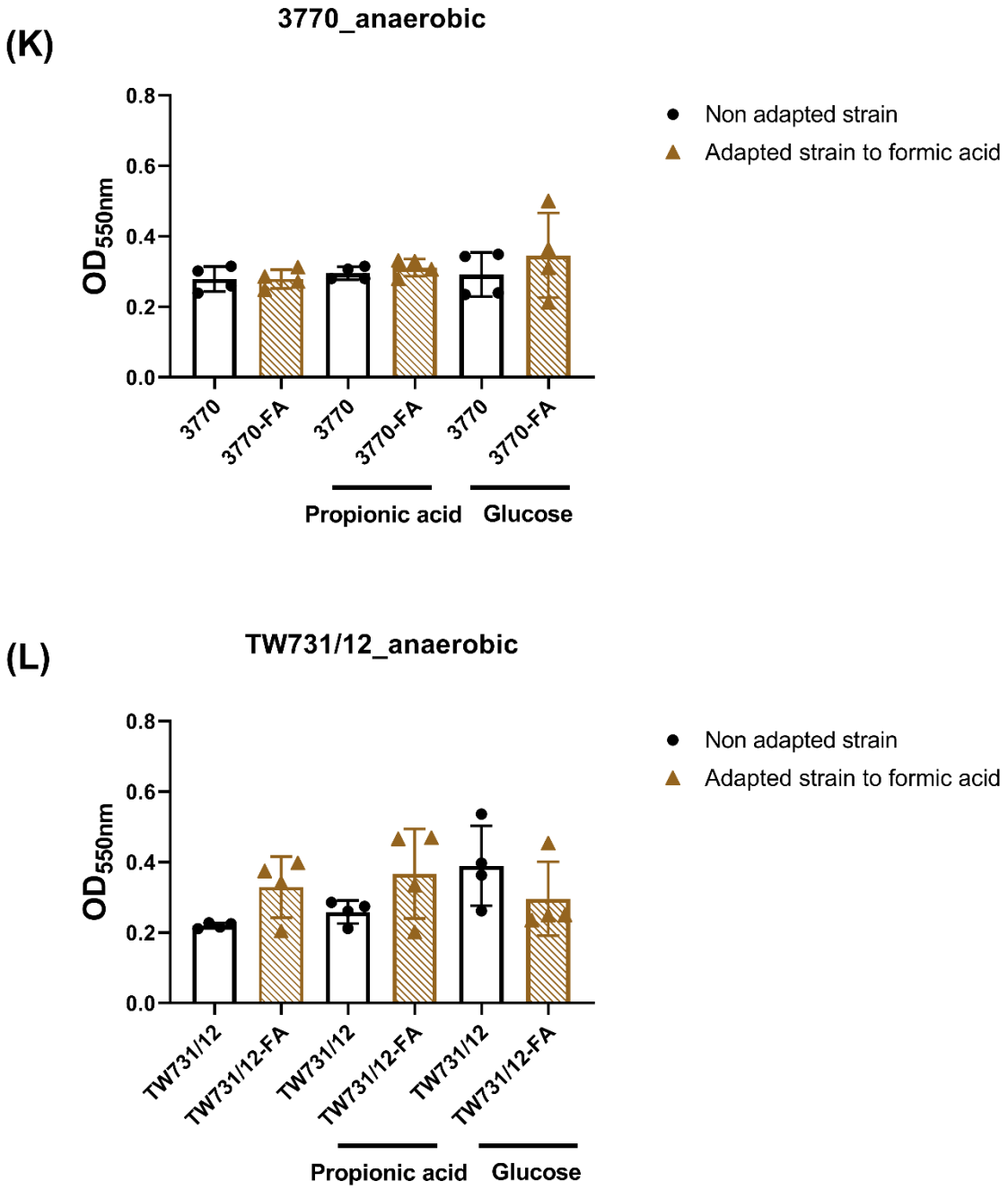

Figure 3-9 Biofilm formation of *E. coli* strains exposed to FA in an environment with alternative carbon sources. *E. coli* strains adapted to FA were incubated aerobically (A-F) and anaerobically (G-L) for 7 days in the presence of PA or glucose as a carbon source. The data are displayed as the mean ± SD of four independent biological replicates and they were analysed using a one-way ANOVA ($p<0.01$ **; $p<0.0001$ ****).

The results show that some strains were significantly negatively affected by pre-exposure to FA. The biofilm formation of LF82-FA was significantly decreased even though glucose and PA are present in the media as a carbon source. The bacterial strain did not utilise the carbon sources to promote biofilm formation under aerobic conditions. In a similar manner, the APEC O1 strain did not utilise glucose to promote biofilm formation under anaerobic conditions, even though glucose is the preferred carbon source for *E. coli* it's rapidly utilised (Martínez-Gómez et al., 2012). However, it could be seen that FA adaptation, even in the absence of an alternative carbon source had a significant negative impact on APEC O1 biofilm formation under anaerobic conditions. In contrast, PA did not influence the biofilm formation of the APEC O1 strain under either aerobic and anaerobic conditions. Biofilm formation of the other *E. coli* strains did not show a significant change due to FA adaptation or the available carbon sources. Overall, FA adaptation has an inhibitory impact on biofilm formation of some *E. coli* strains when they grow on alternative carbon sources.

3.2.2 The impact of FA adaptation on AIEC and APEC acid tolerance

Acid tolerance is one of the important mechanisms that pathogenic bacteria use to survive in harsh environments. Phenotypic alteration of *E. coli* strains could occur by adaptation to organic acids which might lead to acid tolerance. To examine the impact of FA adaption on *E. coli* strains, the media pH was measured then acid tolerance assays were performed under aerobic conditions. The results indicate that FA adaption did not have a significant impact as the bacterial strains did not become more acid-tolerant with no significant increase in bacterial recovery at the end of the assay (Figure 3-10).

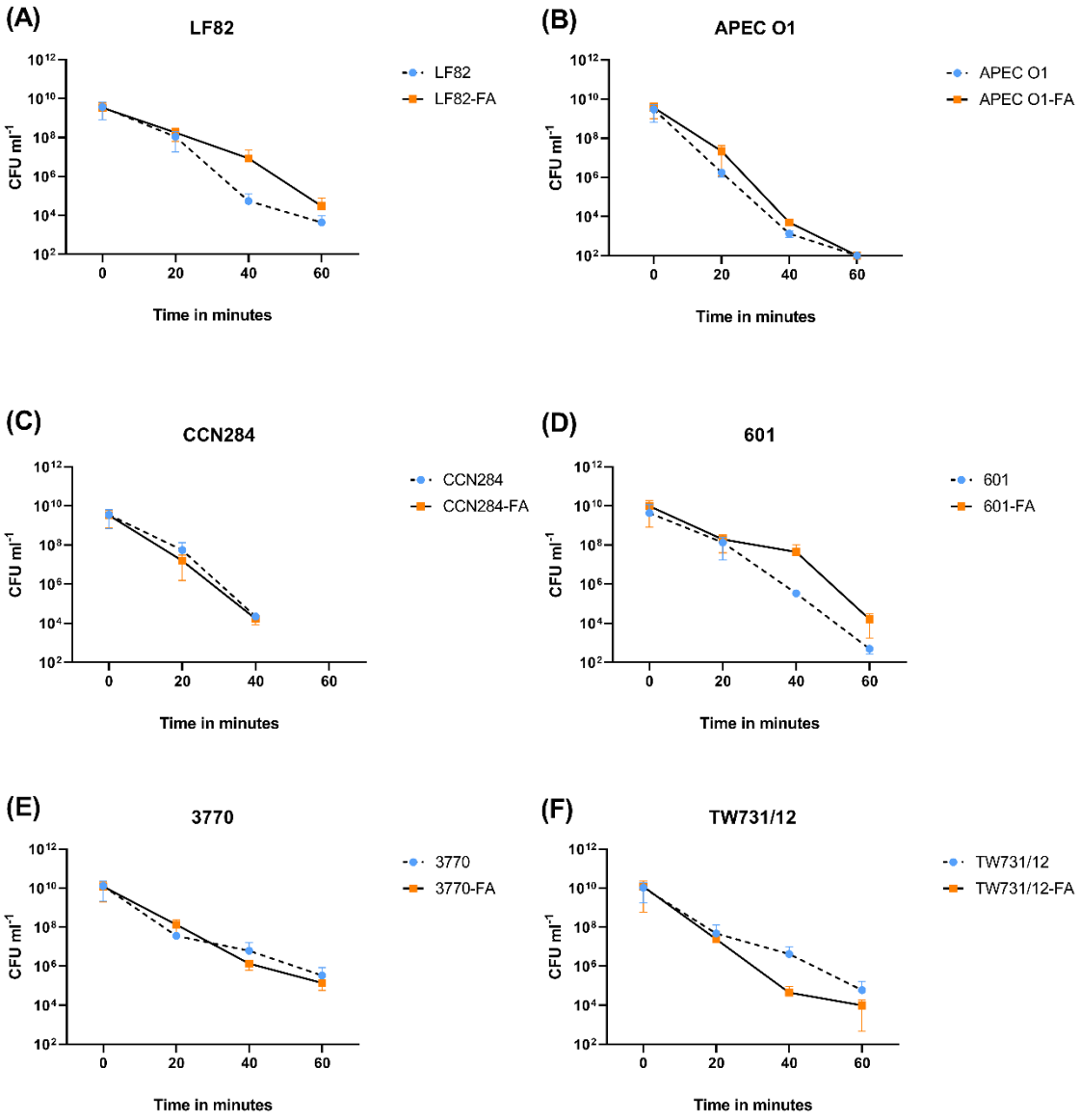

Figure 3-10. Acid tolerance of *E. coli* strains adapted to FA. Ability of *E. coli* strains to tolerate acidic pH (pH 3) over time was determined under aerobic conditions by a CFU count. AIEC strain LF82 (A) with APEC strains (B-F). The data are displayed as the mean ± SD of three independent biological replicates and they were analysed using a two-way ANOVA.

3.2.3 Formic acid effect on *E. coli* motility

The mobility of AIEC and APEC was investigated as a possible phenotypic change in the context of our study that investigates the phenotypic alteration of AIEC and APEC by organic acids. Moreover, motility is an essential survival trait for bacteria because increasing motility will improve the chances of finding nutrients, the avoidance of harmful substances, and potentially increase transmission between hosts for pathogens (Swiecickiet al., 2013). As a result, a twitching assay used to measure motility was performed to investigate the impact of FA adaption on AIEC and APEC. Indeed, twitching motility is a flagella-independent form of bacterial translocation over moist surfaces. It occurs by the extension, tethering, and then retraction of polar type IV pili, which operate in a manner like a grappling hook (Mattick, 2002).

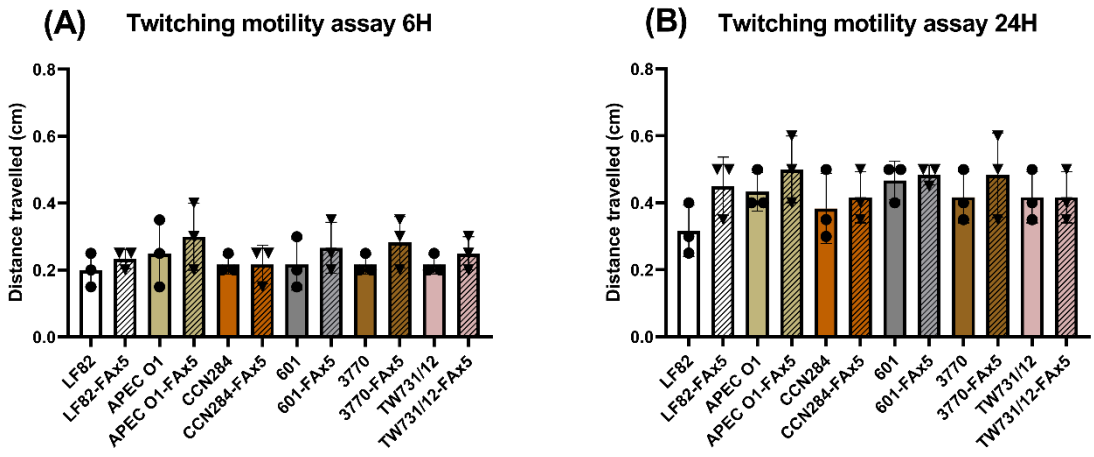

Figure 3-11. Motility of adapted *E. coli strains* to FA. Motility was determined by twitching motility assay for adapted and non-adapted *E. coli* strains at two-time points 6 and 24 h *(*A-B). The data are displayed as the mean ± SD of three independent biological replicates. The data are displayed as the mean ± SD of three independent biological replicates and they were analysed using a two-way ANOVA.

In the comparison between non-adapted strains and the strains adapted to FA, there was no significant increase in motility in any strain. Again, unlike PA adaptation, this indicated that FA adaptation does not have a positive impact on APEC and APEC traits associated with virulence (Figure 3-11).

3.2.4 The impact of FA adaptation on virulence towards RAW 264.7 cells

Cell viability for RAW 264.7 cells infected with APEC and AIEC strains adapted to FA was determined by Cell Counting Kit-8 assay. The infection duration by *E. coli* strains was 4 hours. The result shows that FA adaptation of the *E. coli* strains did not make them more virulent to RAW 264.7 macrophages, with increased cell viability in macrophages infected with FA-adapted strains, although this was not significant for any strain (Figure 3-12). Indeed, strains such as CCN284 and 3770 have a low percentage of viable infected cells, and a high percentage of cells were dead. This may indicate a factor such as gentamicin is affecting the infected cells, and this may affect the interpretations of the other data. However, the effect of FA adaptation was not observed could be due the avian strain factor or the ability of AIEC to replicate within macrophages without inducing death.

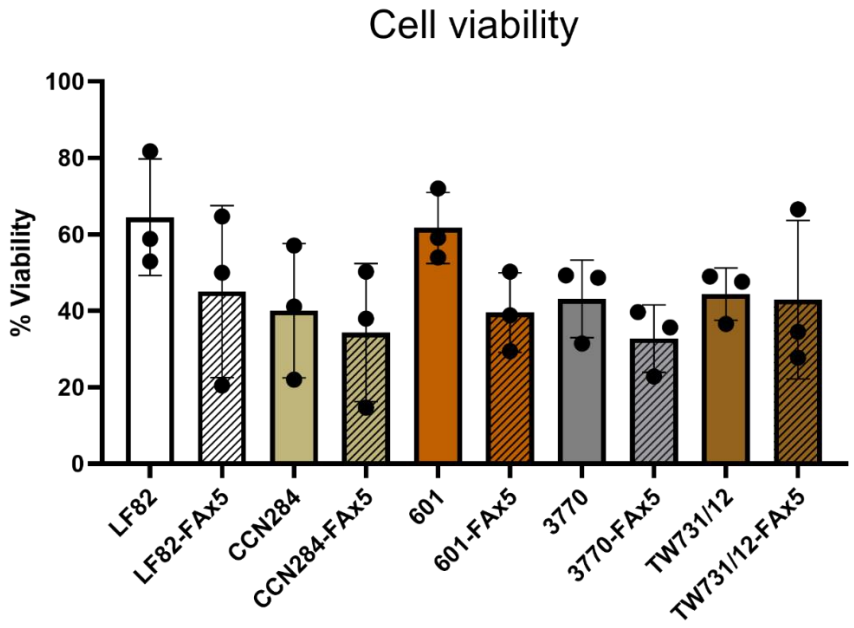

Figure 3-12 Viability of RAW 264.7 macrophages after infection by *E. coli* strains for 4 hours. The cells were treated with 10 µl of CCK-8 solution for 2 hours away from light before measuring the absorbance at 450 nm by FluoStar Optima fluorescent plate reader (BMG Biotech). The data are displayed as the mean ± SD of three independent biological replicates and they were analysed using a one-way ANOVA.

3.3 Discussion

The prevalence of certain bacteria such as AIEC within the CD bowel remains poorly understood, with little evidence to suggest a selection pressure underpinning their existence. Many studies have aimed to identify the factors that make AIEC strains more prevalent and associated with IBD whether they are environmental, genetic, immune response, or microbial factors. Recent studies indicate that intestinal microbiome dysbiosis and *E. coli*, in particular the AIEC pathotype, have been implicated in the pathogenesis of IBD (Palmela et al., 2018). Indeed, the intestinal microbiome can be affected by several factors such as organic acids which are considered a primary influence because they can alter the microbiome composition. Several studies indicated that organic acids, such as SCFAs play an important role in maintaining the health of the colonic mucosa (di Sabatino et al., 2005; Palmela et al., 2018; Seyferth et al., 1967). For example, butyric acid is one of the SCFAs beneficial for promoting colonic health and effective in stimulating the proliferation of intestinal mucosal cells as well as being a primary energy substrate for colonocytes. In addition, a study showed that a low level of SCFAs in faecal samples has been shown to be related to some diseases such as IBD, irritable bowel syndrome (IBS), and cardiovascular disease diarrhoea (Floch et al., 2001; Huda-Faujan et al., 2010; Venter et al., 1990). This indicates the importance of SCFAs in the gut (Seyferth et al., 1967).

The importance of SCFAs is not limited to the gut since they are also used as antimicrobials and preservatives. In the poultry industry the use of SCFAs such as FA, PA, lactic acid and sorbic acid as antimicrobials have resulted in unprecedented success in lowering the carriage of pathogens such as *Salmonella* and *Campylobacter* in poultry (Hinton et al., 1988). These SCFAs can reduce the colonization of pathogenic bacteria and the production of toxic metabolites through acidification of the diet (Dittoe et al., 2018). Regardless of the beneficial effects of SCFAs, the continuous use of SCFAs means that the long term effects need to be understood. A recent study showed that AIEC exposure to PA has led to PA adaption of the bacteria with phenotypic alterations including increased virulence (Ormsby et al., 2020). All the virulence traits that are used to characterise AIEC, such as biofilm formation, adhesion, invasion and intracellular replication within immune cells, were all increased due to repeated exposure to PA.

This chapter aimed to investigate the long-term effects of FA and PA exposure on AIEC and APEC. Previous work from the lab had established the importance of PA in driving phenotypic changes in AIEC leading to increased traits associated with virulence (Ormsby

et al., 2020). Given the widespread use of organic acids such as PA in the poultry industry, here we expanded this work to investgiate the effects of SCFAs on APEC strains also. APEC are opportunistic strains that can cause severe respiratory and systemic disease in chickens commonly termed colibacillosis. Another study has shown that UPEC and NMEC can cause a distinct disease syndrome in poultry, while APEC can cause human extraintestinal diseases (Martinez-Medina et al., 2011; Siek et al., 2005). Since recent studies indicated AIEC strains carry virulence-associated genes from ExPEC strains such as UPEC and NMEC, and the strain LF82 was found to be genetically similar to APEC, this provided further justification for inclusion of a group of APEC strains to investigate if similar responses to SCFAs would be seen in these pathotypes (Maturana, 2011).

The investigation of this study revealed that the alteration of the AIEC phenotype caused by PA-adaptation resulted in a significant increase in the net replication within macrophages. When ethanolamine, an extracellular carbon and nitrogen source that becomes accessible during intestinal inflammation was present, net replication also increased significantly within the macrophages and monocytes cells. This ability to exploit inflammation derived nutrient sources could be a factor that increases the prevalence of AIEC in IBDs. Indeed the *eut* operon was seen to be expressed in treatment-naïve paediatric CD patients in Glasgow, with those successfully treated and in remission showing reduced *eut* expression (Ormsby et al., 2019).

Contrary to PA-adaptation, FA-adaptation has a different effect on the *E. coli* strains, although this effect depends on the bacterial strain. FA-adaptation has an inhibitory effect on biofilm formation of the 601 APEC strain under anaerobic conditions. However, the other APEC strains were not affected by FA-adaptation. A different effect of FA-adaptation was seen in LF82. The biofilm formation of the adapted LF82 to FA was significantly increased both aerobically and anaerobically when FA was present as a carbon source. However, when other carbon sources such as PA and glucose were present, the biofilm formation of LF82 significantly decreased. This indicated the nutrients that are present in the microenvironment play role in how organic acids adaptation will affect *E. coli* strains.

Further investigation showed that FA-adaptation does not have a significant effect on AIEC virulence traits such as adhesion, invasion, motility, cell viability, acid tolerance, and net replication. The significant effect of FA-adaptation was on APEC strains, and it was an inhibitory effect. Invasion by APEC O1 and biofilm of the 601 strains were significantly decreased. this could support the theory that FA is a good antimicrobial although there was one worrying exception to its overall positive effects. FA-adaptation significantly increased

the adhesion of the 601 strain, this could be a potential risk of zoonotic disease because the cell line used was a human intestinal epithelial cell line. However, invasion by this strain was negligible indicating that colonisation rather than disease in the intestine was most likely, but this does not exclude migration of such strains to the urinary tract where it could potentially colonise.

Indeed, few limitations have been detected which could affect detecting the impact of FA-adaptation against the *E. coli* strains. First, the net replication of adapted AIEC and APEC strains to FA assay shows a decrease in the net replication of both AIEC and APEC over time. This could be interpreted as a killing assay. It is not clear what makes the net replication of both AIEC and APEC strains decrease over time, although both strains are recognised with their ability to replicate intracellularly within macrophages (Ormsby et al., 2020; Peng et al., 2018). If a time point 0 h was added and included with time points, it might help determine if the drop in net replication was reflecting bacterial killing or not. Second, strains such as CCN284 and 3770 had a high percentage of dead cells in the cell viability assay. In fact, cell death could impact bacterial recoveries by allowing gentamicin into infected cells, and the increased recovery of the *E. coli* strains could reflect variation in host cell death. Flow cytometry might be used to quantify cell viability to overcome this limitation. Third, the motility assays data did not show a significant difference between adapted strains to FA and non-adapted strains, and this could be attributed to several factors, such as the percentage of agar. The high percentage of agar (1.5%) used in the twitching motility assay could be a reason for the difficulty in detecting differences between *E. coli* strains. Other studies that measured the mobility of *E. coli* using different types of assays, such as swimming and swarming on soft agar, which had a lower percentage of agar (0.3%), showed a travel distance that could be measured easily (Fukuoka et al., 2003; Lippolis et al., 2014). Therefore, using a lower percentage of agar, such as soft agar, could assist in detecting differences between adapted strains to FA and non-adapted strains if it is present. Indeed, FA and PA-adapted *E. coli* strains were used in this study with the risk that *E. coli* may adapt by different mechanisms that could vary between the selected clones.

The key findings of this chapter were that long-term exposure to FA has a different impact on *E. co*li phenotypes than exposure to PA. In some cases, like in the case of FA-adaptation, the alterations caused by FA-adaptation for the most part led to attenuated strains, while post-PA-adaptation, more virulent strains may emerge. However, this chapter suggests that FA is a preferable antimicrobial to PA given traits associated with virulence were observed not to be increased, or in fact decreased, for the majority of strains under the majority of

assays. In addition, organic acids may act differently on bacteria in culture versus within the host, depending on their physiological state, also due to other factors that might impact the organic acids, such as the host immune responses and microbiome compositions, therefore further investigation needs to be done to determine the FA impact.

In addition, we have attempted to measure the virulence of APEC strains against chicken epithelial cells. However, this wasn't possible because; people refused to share them since they were being sold commercially, and when someone volunteered to let us use them in their lab to test APEC strains, the pandemic occurred, making this impossible.

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Chapter 4

The effect of organic acids on APEC and AIEC transcription

4.1 Outline

In this chapter, the effects of FA, which is commonly used as an antimicrobial in food and poultry, were examined on AIEC and APEC strains to determine its effect on bacterial transcription. Previous research conducted in our lab revealed that PA exposure, another extensively used antimicrobial, increases the expression of virulence genes in an AIEC strain. Here, using RNA-seq we determined the role of FA in driving phenotypic alterations in *E. coli* and how FA impacts vary between APEC strains.

4.2 Introduction

One of the major threats to human and animal health is the development of AMR. The widespread use of antibiotics in the livestock sector has been a major contributor to the rise of AMR in agriculture. For example, in the United States, more than 70% of antibiotics of human medical importance are utilised in animals, which raises a concern (Martin et al., 2015). Antibiotics have been widely used in animal husbandry to prevent disease, reduce infections, and promote animal growth. As a preventative measure, antibiotic use as a growth promoter is no longer allowed in the European Union but is still currently practised in other parts of the world (Wyrsch et al., 2016).

Moreover, antibiotic usage in poultry production raises the selective pressure for antibiotic-resistant bacteria (Diarra et al., 2014). *E. coli* are commensal bacteria that are prevalent in both humans and animals. Due to their prevalence, they are well recognised as indicator bacteria for antibiotic resistance in Gram-negative bacteria populations and serve as a model for studying the emergence of antibiotic resistance (Kaesbohrer et al., 2012). In addition, *E. coli* and other commensal bacteria may serve as a reservoir of antibiotic resistance genes that can be transmitted across bacterial species, including those that might cause disease in humans and animals. The effects of antibiotics used and the trends in the prevalence of antibiotic resistance in food-producing animals can be more accurately investigated in this indicator bacterium, than in food-borne pathogens (European Food Safety Authority, 2008).

A primary source of the transmission of antibiotic resistance in same spices bacteria is regarded to the exchange of genes, integrons, transposons, and plasmids (Benavides et al., 2021; Roth et al., 2019). Moreover, APEC strains that are mainly found in avian gut may

represent a zoonotic threat either by causing disease in human hosts or via horizontal gene transfer of plasmid-linked virulence-associated genes to human commensal strains (Bauchart et al., 2010; Tivendale et al., 2010). Importantly, many of the virulence-associated genes known to contribute to APEC virulence are linked to pathogenicity islands found on large transmissible plasmids (Johnson et al., 2006; Johnson, Siek, et al., 2006). Therefore, APEC strains were capable of infecting human cells and causing meningitis in rodent models (Kathayat et al. 2021). In addition, APEC strains have long been recognised as opportunistic infections in avian species. These strains can induce a variety of extraintestinal systemic infections in poultry, which are called colibacillosis (Collingwood et al., 2014; Mehat et al., 2021). Systemic infections such as Septicemia, omphalitis, cellulitis, yolk-sac infection, and enlarged head syndrome are among the disorders that APEC causes which can lead to significant morbidity, mortality, and carcass condemnation. All these factors contribute to large yearly economic losses in the poultry sector (Bélanger et al., 2011; Maturana, 2011).

Recent research has shown substantial variations in the distribution of virulence factors between APEC strains and commensal avian faecal *Escherichia coli* (AFEC) strains, indicating that APEC strains are well adapted to a pathogenic lifestyle (Kathayat et al., 2021; Mehat et al., 2021). APEC virulence mechanisms are likely to be comparable to those of other ExPEC strains, such as UPEC and NMEC both human diseases since they can cause disease in similar environments. A comparative analysis of the complete APEC genome sequence and genomes from diverse ExPEC sub pathotypes showed a strong similarity between these strains of *E. coli* which indicated that APEC could be a zoonotic risk (Kathayat et al., 2021; Mehat et al., 2021).

APEC categorization has generally been based on serotyping and virulence genotyping, with confirmation of virulence in day-old chicks or embryos serving as the gold standard for APEC designation. However, there is no consensus in the literature to define the APEC pathotype and the development of methodologies for its diagnosis because APEC pathogens are a highly heterogeneous group of microorganisms, and each isolate can harbour different associations of virulence factors, each capable of inducing avian colibacillosis (Mehat et al., 2021).

Organic acids are also capable of altering the gut microbiome composition like antibiotics and can similarly lead to inhibition of some microbes while promoting growth of others. For instance, organic acids that are used in poultry can inhibit the growth of pathogenic *Salmonella* yet promote growth of beneficial bacteria such as *Lactobacillus* (Dittoe et al., 2018). This process can occur by lower the pH of the environment and which make the

environment favourable for the acid acid-tolerant species such as *Lactobacillus spp*. This process is beneficial for the host because it helps to digest fibres that play role in the host's health (Venegas et al., 2019; Tugnoli et al., 2020). Research conducted in our lab showed that AIEC, which colonise the intestine of CD patients, have increased virulence gene expression after PA exposure. These results showed that various genes were upregulated that have a role in biofilm formation, stress responses, metabolism, membrane integrity, and transport of alternative carbon sources as a response to PA (Figure 4-1) (Ormsby et al., 2020). Overall, RNA-seq analysis showed that PA drives changes in genes associated with metabolism, biofilm formation, and stress which are fundamental to the AIEC pathotype (Figure 4-1). This was both a surprising and a worrying finding given the importance of PA in human food preservation and its use in agriculture as a preservative and food supplement.

In addition to PA, another food additive that is utilised extensively in the poultry industry is FA (Gharib et al., 2012; Polycarpo et al., 2017). Indeed, FA can potentially reduce enteric pathogenic bacteria in poultry, such as *E. coli*, *C. jejuni* and *Salmonella spp.* (Gharib et al., 2012; Polycarpo et al. 2017). A study demonstrated that the combination of only two acids (FA and PA) was sufficient to increase the weight gain of broilers while using them separately did not bring similar benefits (Roy et al., 2012). Moreover, in the previous chapter, it has been shown that organic acids could be associated with the alteration within *E. coli* phenotype (Ormsby et al., 2020). In addition, PA has been shown to affect the *E. coli* transcription. Therefore, we wanted to examine the effect of FA on *E. coli* transcription to understand the differences observed between PA and FA effects at the transcriptional level.

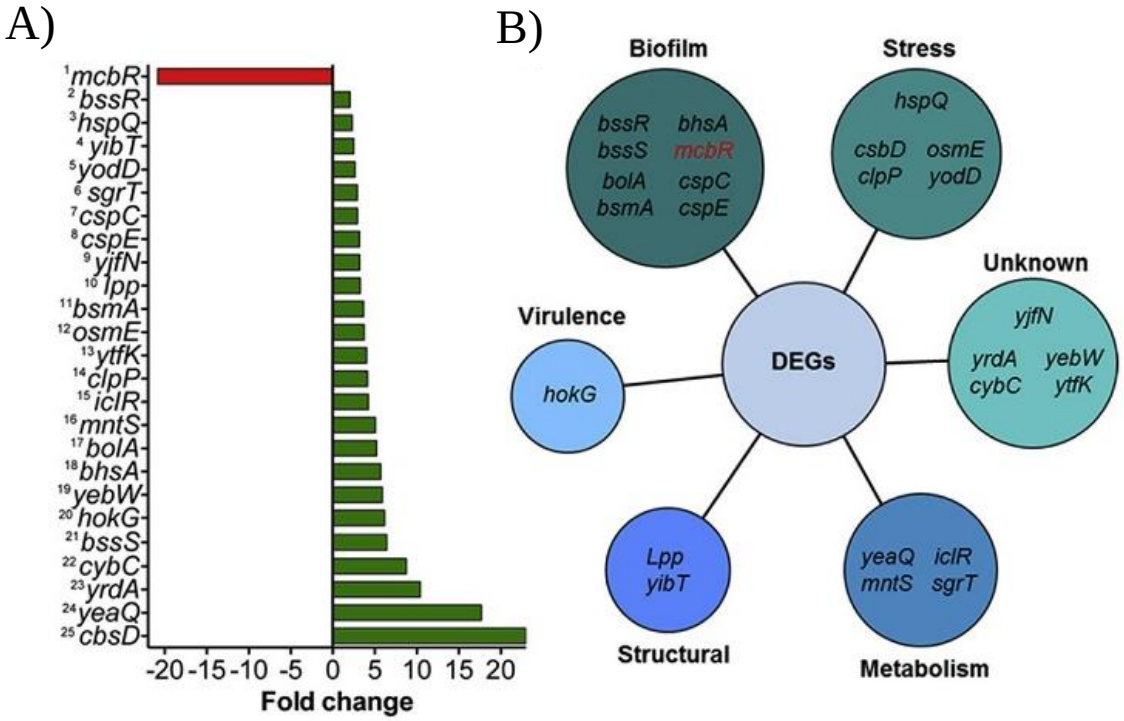


Figure 4-1 Adapted from Ormsby et al. 2020, it shows the significantly differentially expressed genes (DEGs) of LF82-PA are indicated in green (upregulated in LF82-PA) and red (downregulated in LF82-PA) (A). For clarity, genes related to ribosomal RNA coding regions were not labelled. DEGs are classified into functional categories (B).

4.3 Results

4.3.1 Formic acid effects on growth of *E. coli* strains

To determine how antimicrobials such as FA and PA affect the gene expression of APEC and AIEC, the effect of the antimicrobials on *E. coli* growth examined first because the growth rate has an essential role in gene expression and their analysis. Indeed, the growth rate of *E. coli* is regulated by sigma factors such as the primary σ factor (Wösten, 1998; Paget, 2015). Sigma factors are multi-domain subunits of bacterial RNA polymerase that play critical roles in transcription initiation, including the recognition and opening of promoters as well as the initial steps in RNA synthesis (Wösten, 1998; Paget, 2015). These factors are responsible for the bulk of transcription during growth in *E. coli* (Wösten, 1998; Paget, 2015). Therefore, growth curves for AIEC type strain LF82 and APEC strains 601 and TW731/12, were generated over a specific period in media supplemented with 20 mM FA. The results indicated that FA did not affect the growth of these strains in comparison to non-exposed strains. The purpose of this experiment was also to discover whether or not FA exposure affects the growth rate of bacteria to ensure equal bacterial cell numbers for RNA extraction stage, prior to RNA sequencing and gene expression analysis (Figure 4-2).

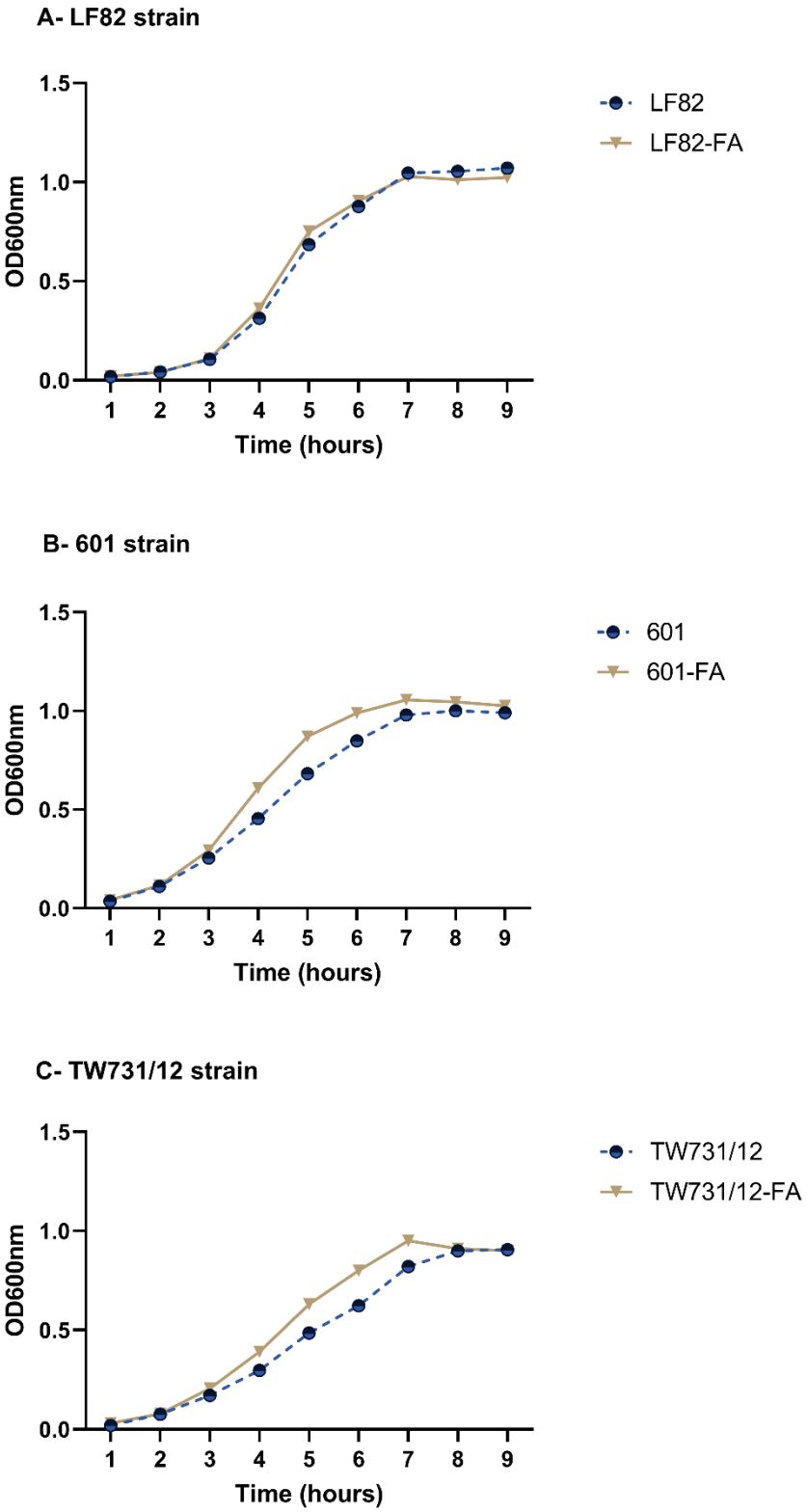


Figure 4-2 Growth curves of *E. coli* strains in present and absence of FA. Cultures of bacteria were grown overnight at 37°C. Cultures were diluted in media supplemented with 20 mM FA, then sample OD_{600} was measured every hour for 9 hours. Each data point is displayed as the mean of three independent biological replicates, and they were analysed using a two-way ANOVA.

4.3.2 RNA sequencing (RNA-Seq)

4.3.2.1 FA effect on gene expression in *E. coli* strains.

To determine the effects of FA on gene expression of *E. coli* strains, RNA-seq was performed on strains LF82, 601, and TW731/12. The AIEC type strain LF82 was examined alongside APEC strains to enable a comparison of LF82 response to FA to that of APEC strains. Indeed, it was demonstrated previously that LF82 genes associated with virulence increases significantly due to PA exposure so inclusion of LF82 here would also allow comparison with the effects of PA documented previously (Ormsby et al., 2020). The other APEC strains were selected based on their differing responses to FA exposure (Nash et al., 2010). FA exposure significantly increased adhesion of 601 to Caco-2 human intestinal cells, but no had effect on TW731/12, so inclusion of both strains would give a better understanding of the differing effects of FA on APEC strains.

The results of RNA-seq showed that FA had a major influence on *E. coli* strains, with over 300 genes changed in their gene expression (Figure 4-3). However, the number of shared expressed genes amongst *E. coli* strains is far less than the total number of significantly expressed genes (Figure 4-4 and Table 4-1).

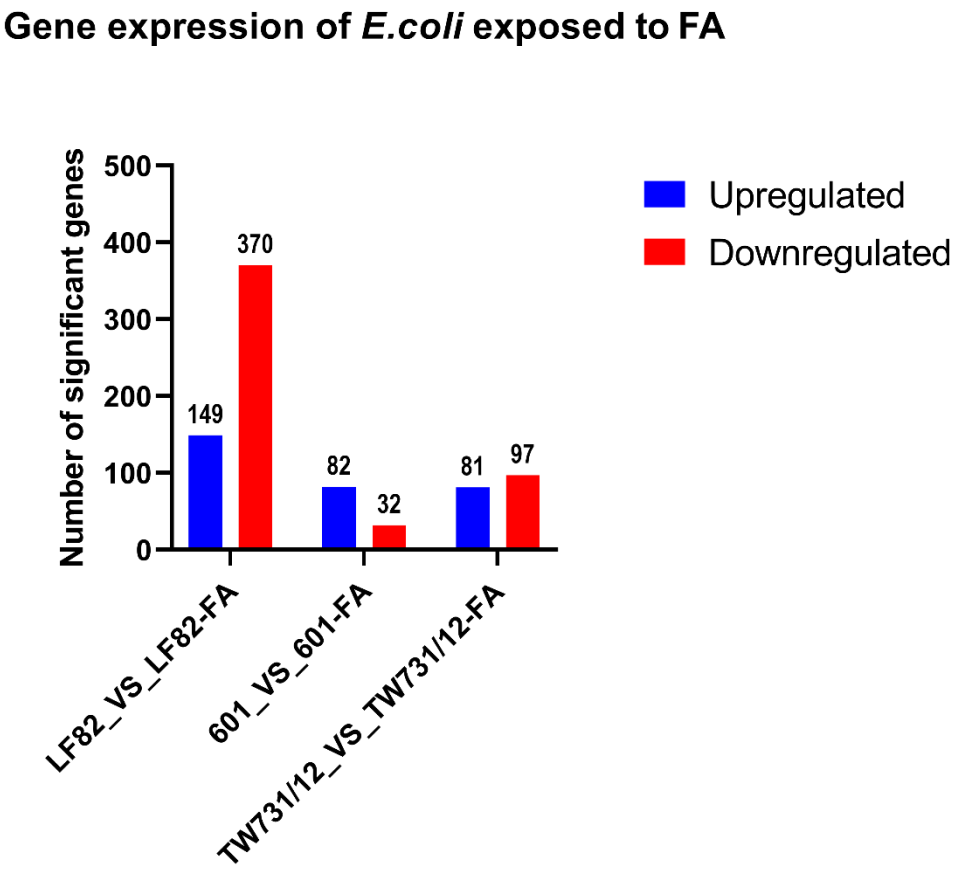
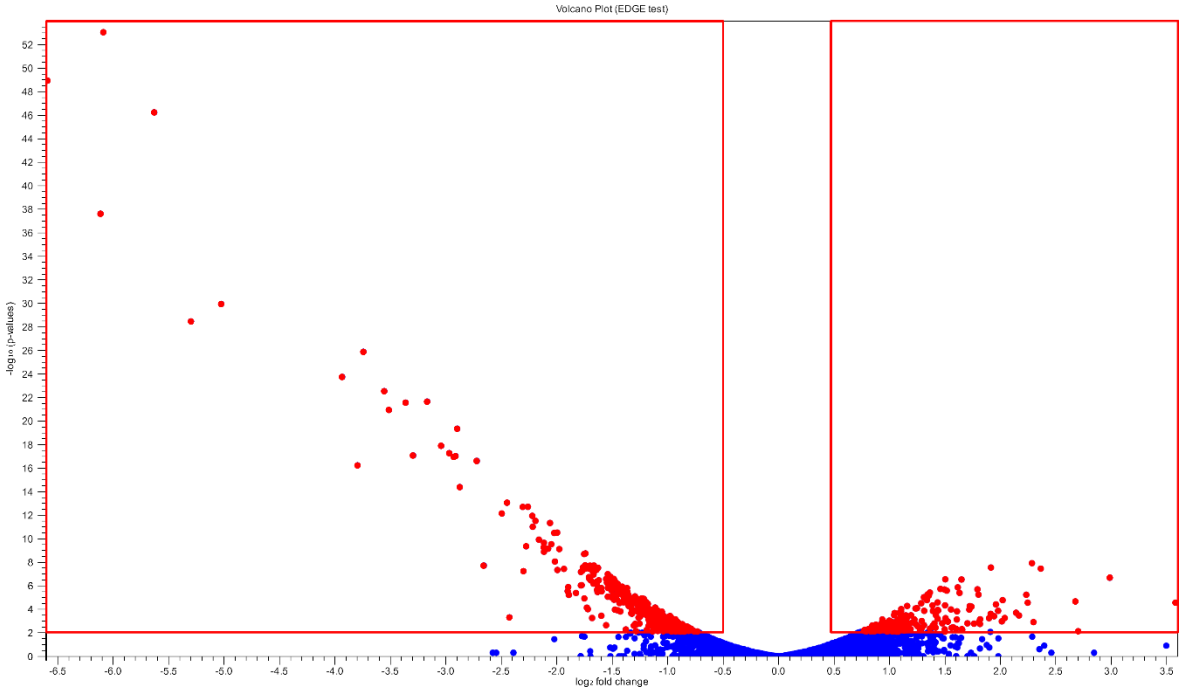
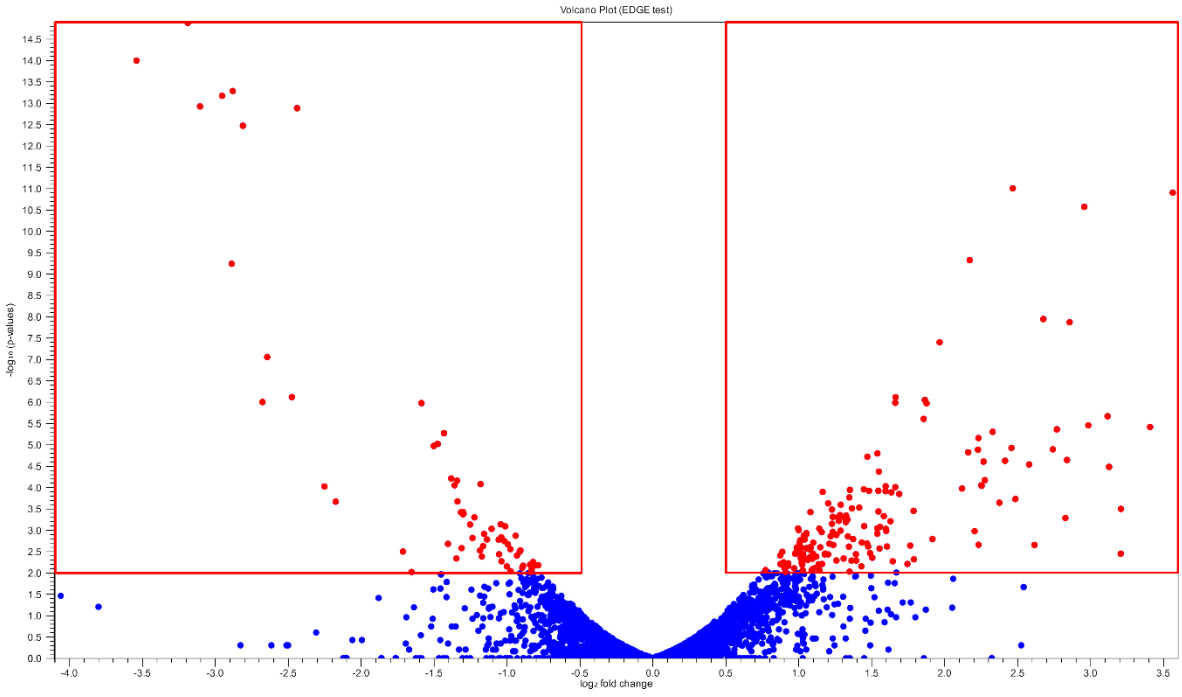


Figure 4-3 Gene expression of *E. coli* exposed to FA. It shows the number of significantly expressed genes in LF82, 601, and TW731/12

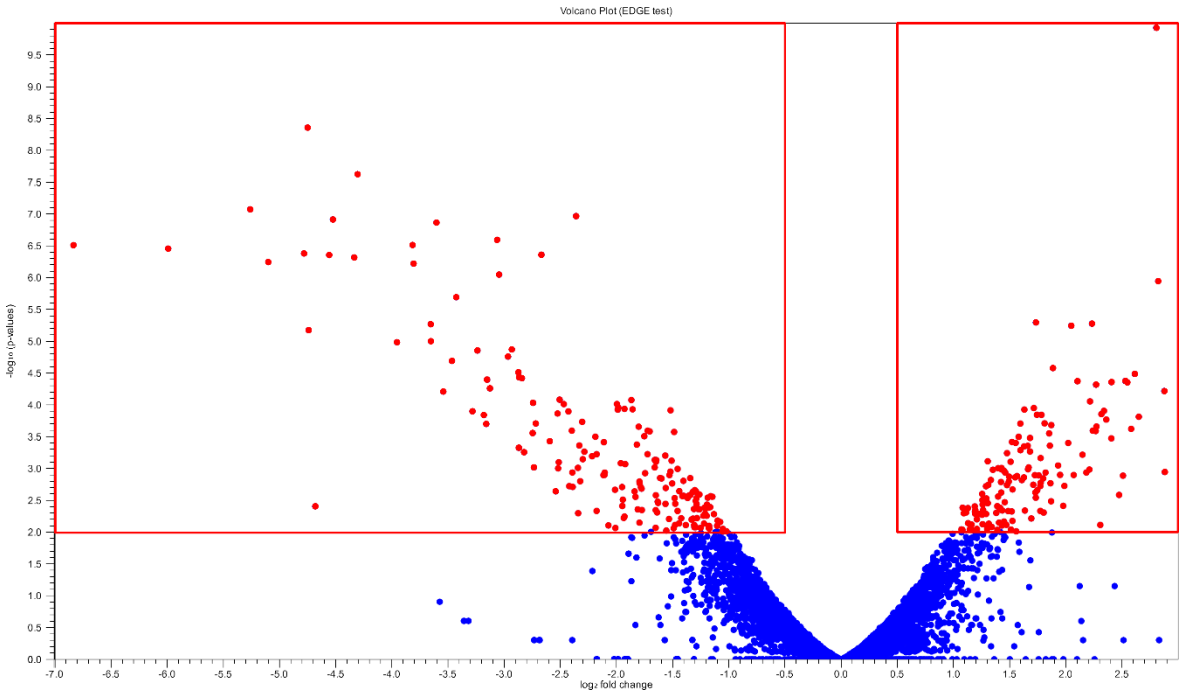
A-LF82


Volcano Plot (EDGE test)
-log10 (p-values)
log2 fold change

B-601


Volcano Plot (EDGE test)
-log10 (p-values)
log2 fold change

C-TW731/12



D- Venn diagram of *E. coli* exposed to FA

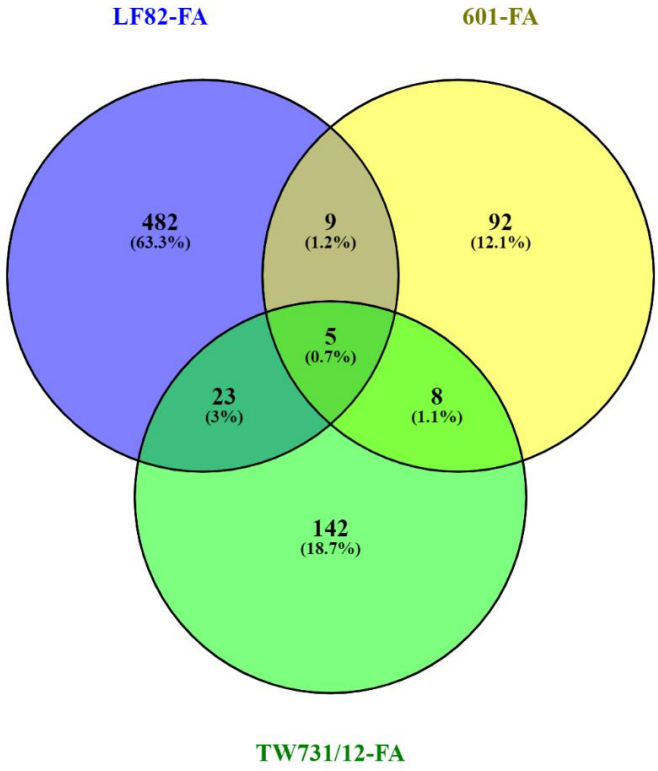


Figure 4-4 Volcano plots illustrate the differential gene expression between the exposed strain and non-exposed strain to FA, as determined by RNA-seq. Significance (Log10 p-value) and fold change cut-offs (log2) are indicated by the dashed and solid lines, respectively (A-C). Venn diagram shows the share of significantly differentially expressed genes (p-value ≤ 0.05) between the *E. coli* strains (D)

Genes	strain	RPKM (log_2)			Fold change	P-value	Function
		Bio-1 /FA	Bio-2 /FA	Bio-3 /FA			
bssR	LF82	7.45	7.58	7.83	2.54	0.000229	Induced in biofilms and motility regulation.
	601	8.28	7.58	8.56	2.34	0.04	
	TW731/12	8.07	10.05	9.91	5.31	0.00552	
dusC	LF82	5.15	4.31	5.28	1.98	0.02	tRNA-dihydrouridine (16) synthase
	601	5.40	5.26	4.77	-3	0.000239	
	TW731/12	5.73	7.06	7.29	4.7	0.0012	
purK	LF82	4.45	3.63	3.62	-8.24	4.4E-16	They are involved in Purine metabolism and Inosine monophosphate biosynthesis via de novo pathway
	601	4.88	2.64	5.89	4.85	0.00731	
	TW731/12	5.55	3.01	2.99	-4.32	0.02	
purM	LF82	4.75	2.88	3.53	-15.29	1.08E-21	
	601	5.54	2.64	6.58	6.69	0.00185	
	TW731/12	7.45	7.58	7.83	-5.53	0.00971	
purN	LF82	5.82	5.05	5.25	-7.83	1.74E-15	
	601	6.70	4.56	7.65	3.45	0.02	
	TW731/12	7.07	5.25	5.04	-3.89	0.03	
xanP	LF82	4.60	3.38	3.68	-13.39	9.06E-24	Transporter for xanthine
	601	4.95	3.06	5.63	4.7	0.00115	
	TW731/12	4.67	3.63	3.25	-3.99	0.00971	

Genes	strain	RPKM (log_2)			Fold change	P-value	Function
		Bio-1 /FA	Bio-2 /FA	Bio-3 /FA			
glmS	601	10.03	7.35	10.00	5.03	0.000876	Converting fructose-6P into glucosamine-6P using glutamine
	TW731/12	9.45	8.54	8.23	-4.94	0.01	
hycA	601	3.43	2.81	2.34	5.60	0.01	Formate hydrogenlyase regulatory protein
	TW731/12	1.11	4.45	4.96	5.70	0.04	
napG	601	3.68	-0.71	2.91	9.25	0.02	They are involved in electron transfer from ubiquinol to NapAB complex
	TW731/12	1.30	0.41	0.01	-7.31	0.02	
napH	601	3.51	-0.01	3.10	7.1	0.03	
	TW731/12	0.80	1.10	1.03	-5.73	0.04	
napA	601	3.88	0.35	3.47	4.62	0.05	
	TW731/12	1.34	1.62	1.33	- 4.33	0.04	
hycE	601	6.14	6.16	5.77	4.51	0.000000221	Formate oxidation
	TW731/12	3.50	6.28	6.54	3.87	0.04	

Table 4-1. Shared genes that are significantly differentially expressed between LF82, 601, and TW731/12 after exposed to FA. Names of genes that are significantly differentially expressed, the function of these genes and the strains where this differential expression was noted.

The differential gene expression analysis reveals that several genes were upregulated and downregulated related to bacterial membrane integrity, and stress responses. Genes such as *bssR*, *dusC*, *purN*, *purK*, *xanP*, and *purM* that were significantly differentially expressed, were found to be common in all the *E. coli* strains (Table 4-1). In fact, *bssR* was the only gene that was upregulated in all the *E. coli* strains. it was increased in the LF82, 601, and TW731/12 by fold changes of 2.54, 2.34, and 5.31, respectively (Table 4-1and Appendix II). This gene is associated with regulation of biofilm and motility in *E. coli* (Domka et al., 2006. In addition, it was shown that the increase of biofilm formation by *bssR* was related to differential expression of genes related to stress response (Domka et al., 2006). This suggested that FA was a stress factor for *E. coli* strains.

Furthermore, the other genes that were found to be common in all the *E. coli* strains are *purN*, *purK*, *xanP*, and *purM* and their gene expression was downregulated in LF82 and TW73/12, whereas it was upregulated in the 601 (Table 4-1 and Appendix II). The function of the genes is different and include metal ion binding, transport of xanthine, and purine biosynthesis. In addition, the cluster of *pur* genes was upregulated in the 601 and downregulated in TW73/12 (Table 4-4). In contrast, the gene expression of *dusC*, which was another gene found to be differentially expressed in all *E. coli* strains, was increased 1.98 and 4.7 fold in LF82 and TW73/12 respectively, but was downregulated 3 fold in strain 601. (Table 4-1 and Appendix II). This gene is involved in tRNA dihydrouridine synthase activity (Table 4-1).

Moreover, seven genes that are significantly expressed in APEC strains were found to be common. Genes associated with FA oxidation, including *hycE* and *hycA*, were increased significantly in both 601 and TW731/12. However, genes such as *napG*, *napH*, and *napA*, which are related to the electron transfer component of the periplasmic nitrate reductase complex NapAB, that facilitates electron flow between the membrane and periplasm, were increased significantly in 601 but downregulated in TW731/12. Indeed, the cluster of *nap* genes was upregulated in TW731/12 (Table 4-3 and Appendix II). Similarly, the gene expression of the *glmS* gene, which is involved in the glutamine and fructose 6-phosphate metabolic pathway, was increased in 601 but downregulated in the TW731/12 (Table 4-1 and Appendix II).

In response to FA exposure, the most significant upregulated and downregulated genes varied amongst *E. coli* strains. In the LF82 strain, the most impacted genes were *cstA* and

ytfE. The *cstA* gene, which is involved in peptide utilization during carbon shortage was increased by 11.96 fold change in the LF82 strain, which was the highest significant fold change. In contrast, *ytfE*, which is involved in the repair of iron-sulfur clusters damaged by stress responses, was the most downregulated in the LF82 strain, with a -96.47 fold drop (Table 4-2).

Furthermore, the most impacted genes in APEC strains differed from those in the LF82 strain. The most impacted genes in the APEC strains were *ilvC*, *gltJ*, *ygiW*, and *flgB* (Table 4-2). The *ilvC* gene that is involved in the biosynthesis of branched-chain amino acids such as isoleucine and valine, was the most upregulated in 601 with 11.84 fold change (Table 4-2). In contrast, the gene *gltJ*, which is involved in the transport of amino acids such as glutamate and aspartate across the bacterial membrane, was the most downregulated in the 601 strain by -9.12 fold reduction (Table 4-2).

Even though both 601 and TW731/12 are APEC strains, the most impacted genes vary across the strains. The gene *ygiW*, which is involved in stress response to acids, specifically hydrogen peroxide (H_2O_2), was the most upregulated gene in TW731/12 by 7.08 fold change, while the gene *flgB*, which is involved in forming the structural component of the flagellum which is the bacterial motility apparatus, was the most downregulated gene by 38.31 fold (Table 4-2). Moreover, the cluster of *flg* genes was upregulated in TW731/12 (Table 4-5). These genes again reflect a likely stress response while downregulation of flagella genes points towards a change in motility and perhaps biofilm formation in response to stress.

Overall, the significant changes in differential gene expression of AIEC and APEC indicate the FA has more impact on AIEC than APEC (Figure 4-4). Several genes in AIEC, both downregulated and upregulated, indicate that FA is a cause of significant stress to the bacterial strain. For instance, it has been shown that a mutation in one of the significantly changed genes, *ytfE*, renders *E. coil* strain more sensitive to nitric oxide stress (Justino et al., 2006). Unlike AIEC, the gene expression of APEC strains indicate that they are responding to FA stress by upregulating genes related to metabolism. For instance, *hycE* and *hycA* were upregulated in both APEC strains (Table 4-5 and Appendix II), and these genes are involved in regulating the formate metabolism via formate hydrogen lyase complex (Sinha et al., 2015). In fact, the cluster of *hyc* genes were upregulated in TW731/12 (Table 4-5). This could indicate that APEC strains tolerate FA stress more readily than AIEC.

Genes	Function	RPKM (log2)			Fold change	P-value	Strain
		Bio-1/FA	Bio-2/FA	Bio-3/FA			
cstA	Involved in peptide utilization during carbon starvation.	4.05	7.54	4.37	11.96	0.000852	LF82
yjbE	Uncharacterized protein	4.18	5.34	4.04	7.93	0.0000149	
metE	Methionine biosynthetic process	10.50	12.87	10.45	6.39	0.000701	
hmpA	Involved in nitric oxide detoxification in an aerobic process	4.91	5.08	4.96	-68	4.39E-50	
trpE	Biosynthesis of anthranilate that an intermediate in the biosynthesis of L-tryptophan	2.93	2.60	3.22	-69.23	2.94E-35	
ytfE	Involved in the repair of iron-sulfur clusters damaged by stress	4.68	5.58	4.88	-96.47	2.84E-46	
ilvC	Involved in the biosynthesis of branched-chain amino acids	8.01	5.88	8.66	11.84	7.08E-09	601
napF	Involved in the maturation of NapA, the catalytic subunit of the periplasmic nitrate reductase	5.02	0.46	4.78	10.63	0.000726	
napG		3.67	-0.71	2.91	9.25	0.02	
cspI	Activator, DNA-binding	7.99	7.15	7.65	-7.74	8.54E-11	
gltK	Amino acid transport	4.03	4.86	4.54	-8.6	1.11E-10	
gltJ		3.52	4.07	3.78	-9.12	6.65E-12	

Genes	Function	RPKM (log2)			Fold change	P-values	Strain
		Bio-1/FA	Bio-2/FA	Bio-3/FA			
ygiW	Cellular response to hydrogen peroxide	8.61	10.02	10.08	7.08	0.000306	TW731/12
gadC	Involved in glutaminase-dependent acid resistance	9.46	11.58	11.80	6.28	0.01	
hycF	Formate oxidation	2.50	3.92	4.27	5.85	0.00552	
hmpA	Involved in nitric oxide detoxification in an aerobic process	6.16	4.75	4.70	-27.49	0.000161	
ytfE	Involved in the repair of iron-sulfur clusters	4.46	4.13	4.38	-34.31	0.000181	
flgB	The structural component of the flagellum, the bacterial motility apparatus	-0.56	-0.12	1.34	-38.31	0.0000997	

Table 4-2. Most significantly upregulated and downregulated differentially expressed genes in *E. coli* strains and their function.

Genes	strain	RPKM (log2)			Fold change	P-value	Function
		Bio-1 /FA	Bio-2 /FA	Bio-3 /FA			
napA	601	3.88	0.34	3.47	4.62	0.05	They are involved in the periplasmic nitrate reductase NapAB complex that reduces nitrate to nitrite.
	TW731/12	1.34	1.62	1.33	- 4.33	0.04	
napB	601	-	-	-	-	Not significant	
	TW731/12	1.43	0.77	0.65	-6.58	0.01	
napF	601	5.02	0.46	4.78	10.63	0.0000148	
	TW731/12	-	-	-	-	Not significant	
napG	601	3.67	-0.71	2.91	9.25	0.02	
	TW731/12	1.30	0.41	0.01	-7.31	0.02	
napH	601	3.49	-0.01	3.09	7.1	0.03	
	TW731/12	0.80	1.10	1.03	-5.73	0.04	

Table 4-3. Cluster of genes that encode a periplasmic nitrate reductase are significantly differentially expressed within APEC strains after exposed to FA.

Genes	Strain	RPKM (log2)			Fold change	P-value	Function
		Bio-1 /FA	Bio-2 /FA	Bio-3 /FA			
purE	601	-	-	-	-	Not significant	They are involved in Purine metabolism and Inosine monophosphate biosynthesis via de novo pathway
	TW731/12	5.22	2.77	1.86	-5.03	0.02	
purK	601	4.88	2.64	5.89	4.85	0.00731	
	TW731/12	5.55	3.01	2.99	-4.32	0.02	
purL	601	5.45	2.64	6.62	7.92	0.000688	
	TW731/12	-	-	-	-	Not significant	
purM	601	5.54	2.64	6.58	6.69	0.00185	
	TW731/12	5.74	3.16	3.11	-5.53	0.00971	
purN	601	6.70	4.55	7.65	3.45	0.02	
	TW731/12	7.07	5.25	5.04	-3.89	0.03	
purD	601	4.31	2.49	5.35	4.35	0.00963	
	TW731/12	-	-	-	-	Not significant	
purH	601	4.40	1.95	5.51	5.98	0.00344	
	TW731/12	-	-	-	-	Not significant	

Table 4-4. Cluster of genes that involved in the purine biosynthesis pathway are significantly differentially expressed within APEC strains after exposed to FA.

Genes	Strain	RPKM (log2)			Fold change	P-value	Function
		Bio-1 /FA	Bio-2 /FA	Bio-3 /FA			
hycA	TW731/12	1.11	4.45	4.96	5.7	0.04	They are involved in formate oxidation, through transfer the formate to carbon dioxide.
hycD		2.06	3.36	3.22	4.2	0.04	
hycE		3.50	6.28	6.54	3.87	0.04	
hycF		2.50	3.92	4.27	5.85	0.00552	
flgB	TW731/12	-0.56	-0.12	1.34	-38.31	0.0000997	These genes play role in forming the structural component of the flagellum and the bacterial motility apparatus
flgC		0.08	0.41	0.97	-11.04	0.00335	
flgD		1.84	2.18	1.27	-20.17	0.000164	
flgH		1.99	1.68	1.33	-9.43	0.00247	
flgJ		1.37	1.29	1.39	-6.71	0.02	
flgK		3.38	6.26	5.74	-7.07	0.03	
flgL		4.94	7.30	6.87	-5.71	0.03	
flgM		5.36	6.97	6.37	-4.92	0.03	
flgN		5.97	7.38	7.17	-4.3	0.04	

Table 4-5. Cluster of genes that involved in the motility and formate oxidation are significantly differentially expressed within TW731/12 after exposed to FA.

In addition, when RNA-seq data for AIEC exposed to FA was compared with the previous data of PA exposure (Ormsby et al., 2020), results show that PA and FA have very different effects on AIEC. While FA exposure results in more than 300 genes being differentially regulated, PA exposure results in 25 genes differentially regulated (Figure 4-5). Most of the genes that were significantly changed due to FA exposure are related to stress response and membrane integrity, whereas PA induced changes in genes related to stress tolerance, biofilm formation and intracellular invasion genes such as *clpP*, *osmE*, and *bssS* (Table 4-6 and Appendix II).

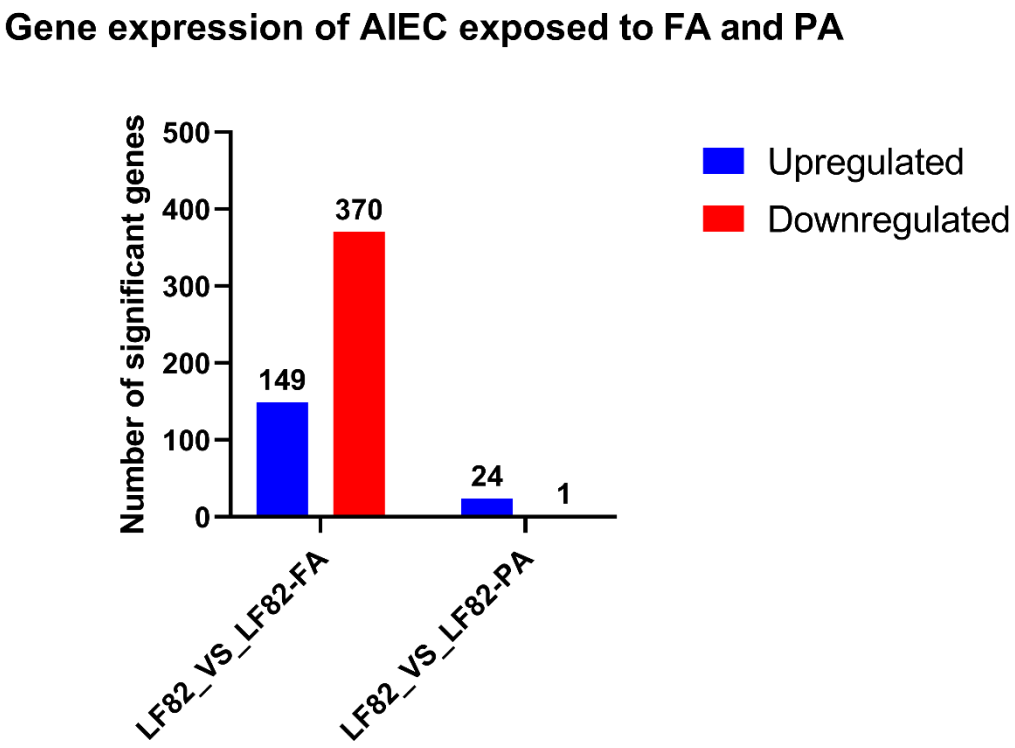


Figure 4-5. Gene expression of AIEC exposed to FA and PA. It shows the number of significantly expressed genes in LF82. The results of PA effect were adapted from Ormsby et al., 2020.

Genes	RPKM(log_2)			Function	Reference
	LF82-PA.1	LF82-PA.2	LF82-PA.3		
csbD	1.263	1.189	1	Stress response - overexpressed in virulent *E. coli.*	Amigo et al., 2016
yeaQ	1.0144	0.9486	0.1763	Function unknown.	-
yrdA	0.856	1.5558	-2	Carbonic anhydrase-like. Catalyse the rapid interconversion of CO_2 and H_2O to HCO_3	Merlin et al., 2003
cybC	0.9635	0.5753	0.5361	Electron-transport protein of unknown function.	-
bssS	0.5656	0.585	2.1342	Induced in biofilms. Motility regulation.	Domka et al., 2006
hokG	2.5261	-0.6666	2.1953	Toxin/Antitoxin system	
yebW	1.9745	1.3219	1.546	Function unknown.	-
bhsA (ycfR)	1.2869	2.4803	2.1177	Induced in biofilms. Stress response.	Zhang et al., 2007
bolA	4.3067	4.4209	4.6753	Switch from motility to biofilms.	Dressaire et al., 2015
mntS	4.0522	4.0959	3.1194	Optimize the Intracellular Concentration of Manganese	Martin et al., 2015
iclR	5.8071	6.1208	5.6424	Isocitrate lyase regulator. Repressor of *aceBAK* operon for glyoxylate shunt.	Molina-Henares et al., 2006
clpP	2.9373	2.6206	2.2357	Protease. Stress tolerance, biofilm formation and intracellular invasion.	Xie et al., 2013
ytfK	3.6041	4.591	4.0807	Regulated by *phoB*. Function unknown.	Baek and Lee, 2006
osmE	3.0942	2.7027	3.0461	Response to osmotic stress.	Gutierrez et al., 1995
bsmA (yjfO)	5.5163	5.3466	5.0492	Biofilm formation and stress response.	Weber et al., 2010

Genes	RPKM (log_2)			Function	Reference
	LF82-PA.1	LF82-PA.2	LF82-PA.3		
lpp	6.7733	5.9896	6.5633	Provides structural integrity to the outer membrane.	
yjfN	3.9964	4.0295	4.1506	Unknown. Downstream of *bsmA* (*yjfO*).	Weber et al., 2010
cspE	10.9825	10.4866	10.3823	Roles in membrane stress, motility, virulence and biofilm formation.	Michaux et al., 2017
cspC	3.5097	3.3937	2.7377	Roles in membrane stress, motility, virulence and biofilm formation.	Michaux et al., 2017
sgrT	6.8381	7.4463	6.4256	Increased in biofilms. Inhibits glucose use and encourages the utilisation	Raina and Storz, 2017
yodD	5.8432	5.8802	5.3847	Hydrogen peroxide, cadmium and acid stress	Lee et al., 2010
yibT	4.1683	4.9141	4.5033	Regulating membrane fatty acid composition	Si et al., 2016
hspQ	4.9667	5.0259	4.4899	Regulating membrane fatty acid composition	Puri et al., 2017
bssR	8.9034	9.1047	8.4232	Induced in biofilms. Motility regulation	Domka et al., 2006
mcbR	-16.6096	-16.6096	-16.6096	Regulator of biofilm formation.	Zhang et al., 2008

Table 4-6. Adapted from Ormsby et al., 2020, Reads Per Kilobase of transcript, per Million mapped reads (RPKM) values of differentially expressed genes following RNA-seq of LF82 exposed to PA.

Moreover, the results showed that LF82 exposed to PA and FA share three genes that are significantly expressed. Genes such as *bssR*, *yibT*, and *yodD* were upregulated in both LF82 that are exposed to PA and FA (Figure 4-6 and Table 4-7). These genes are involved in biofilm formation, motility, regulating membrane fatty acid composition, and acid stress. The results indicate that both FA and PA induced a stress response in AIEC, but the stress in PA AIEC adapted to this stress and became more virulent by increasing expression of several virulence factors.

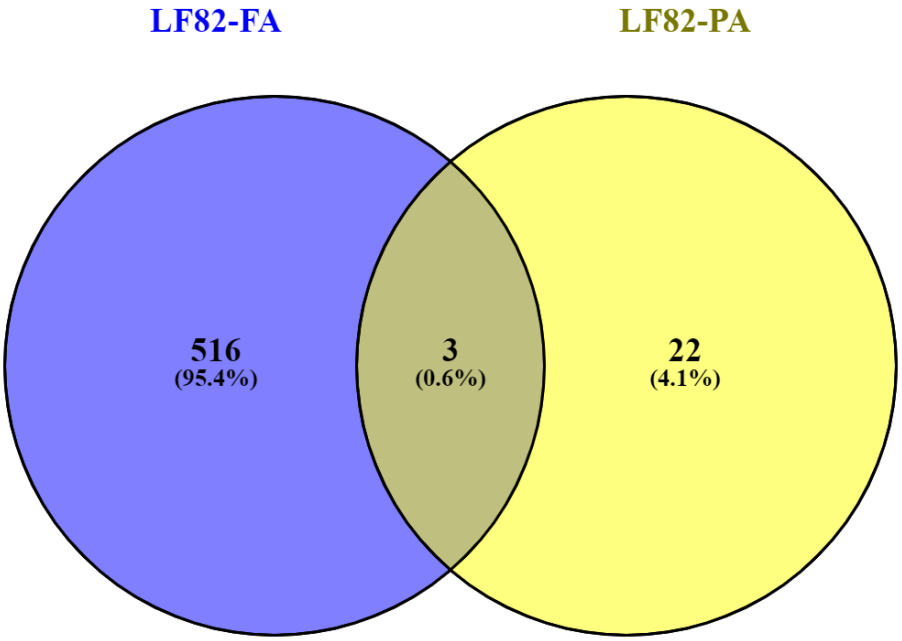


Figure 4-6. Venn diagram indicating the number of shared genes that were significantly differentially expressed (p-value ≤ 0.05) between the AIEC exposed to FA and exposed to PA.

Genes	Function	Fold Change in LF82-PA	Fold Change in LF82-FA
bssR	Induced in biofilms and motility regulation.	8.42	2.54
yibT	Regulating membrane fatty acid composition	4.50	3.50
yodD	Hydrogen peroxide, cadmium, and acid stress	5.84	6.39

Table 4-7. Shared genes that are significantly expressed between FA exposed and PA exposed in AIEC and their function.

4.4 Discussion

Organic acids such as FA and PA have been widely used as antimicrobials in many fields. These organic acids are effective antimicrobials because they can inhibit the growth of pathogenic bacteria such as *Salmonella* and *Campylobacter* (Dittoe et al., 2018). Even though organic acids are effective against pathogenic bacteria, several studies indicated that organic acids such as PA can alter the AIEC strain phenotype leading to increased adhesion, invasion, and biofilm formation (Ormsby et al., 2020; Pace et al., 2021; Viladomiu et al., 2021). Intriguingly, AIEC strains have been shown to be genetically similar to APEC strains that cause diseases such as colibacillosis and, as shown in several studies, PA also alters the traits associated with the virulence of AIEC strains (Kathayat et al., 2021; Ormsby et al., 2020). Here I focused on probably the most commonly used organic acid antimicrobial in poultry feed, FA, determining if exposure to this organic acid could drive changes in gene expression related to genes associated with virulence in *E. coli* similar to PA.

The number of genes significantly differentially expressed in the *E. coli* strains in our results indicated that FA significantly affected the *E. coli* strains. Several genes were related to bacterial membrane integrity and the stress response indicating that FA has altered or potentially damaged the membrane of the bacteria (Appendix II), which is expected given how FA functions as an antimicrobial (Kovanda et al., 2020).

However, in the growth experiments, the results indicate that FA exposure did not have impact on the *E. coli* strains growth even though several studies stated that FA inhibits the bacterial growth such as *E. coli* (Gadde et al., 2017; Hernández et al., 2006; Luise et al., 2020; Thompson et al.,1997) . In fact, there are differences in the growth trials between our study and those indicating FA inhibits *E. coli* (Gadde et al., 2017; Hernández et al., 2006; Luise et al., 2020; Thompson et al.,1997) . In this study, growth experiment conducted *in vitro*, whereas previous studies did growth experiments mostly *in vivo*. In addition, the experimental duration in previous studies differs from the experimental period in this study. The duration of the trial was up to 35 or 42 days, and the effect of FA on *E. coli* examined in pigs and chickens (Gadde et al., 2017; Hernández et al., 2006; Luise et al., 2020; Thompson et al.,1997). In contrast, the experiment in this study lasted 9 hours and the effect of FA assessed on *E. coli* growth.

In addition, FA was added to water or animal feed in different concentrations such as 10 grams into 1 kilograms of feed or 0.5% in drinking water in these studies (Gadde et al., 2017; Hernández et al., 2006; Luise et al., 2020; Thompson et al.,1997). While in this study, the

used concentration of FA was 20 mM. However, in this study, it is unclear why FA did not have an impact on the *E. coli* strains' growth in this study, but this could indicate that FA might act differently on bacteria in culture versus the gut depending on their physiological state.

Furthermore, the *bssR* gene was upregulated in all the *E. coli* strains due to FA exposure. Previous studies indicated that *bssR* functions as a biofilm repressor, but it is thought to function as a biofilm regulator during stress responses (Domka et al., 2006; Ren et al., 2004). Biofilm formation is a well-known survival strategy adopted by bacteria during stress, so *bssR* upregulation here is unsurprising (Watnick et al., 2000). *bssR* regulates biofilm formation and multiple genes involved in catabolite suppression and the stress response, as well as controlling signalling pathways, uptake and export, quorum sensing and the stationary-phase signal (Domka et al., 2006). Furthermore, carbon fluxes that regulate bacterial cell growth by coordinating carbon, play an essential role in biofilm formation by influencing *bssR* gene expression. Indeed, the type and quantity of carbon influence *bssR* gene expression (Domka et al., 2006). For instance, *bssR* functioned as an inhibitor for biofilm formation in the minimal microbial growth medium (M9) and LB containing glucose. However, this function as an inhibitor was not observed in the same media when the glucose was absent (Domka et al., 2006).

Moreover, the findings of this study on carbon correspond with the results of biofilm formation assays in Chapter 3. The results showed that biofilm formation of AIEC adapted to FA was significantly increased. Still, when a carbon source such as PA or glucose was present, the biofilm of AIEC was significantly decreased, which indicates that carbon availability could influence the role of the *bssR* gene when it is upregulated. Although transcription of the *bssR* gene was upregulated in all the strains, the biofilm formation of 601 and TW731/12 strains were affected differently than LF82.

Transcription of this gene was also upregulated in AIEC exposed to PA and biofilm formation of AIEC was increased as well (Ormsby et al., 2020). Biofilm formation is not just a strategy of bacteria to survive extreme environments but also a strategy used by pathogens during infection (Watnick et al., 2000). For instance, it has been shown that AIEC can form biofilm on IECs to increase colonisation (Palmela et al., 2018). Indeed, data indicate that AIEC exposed to PA has several upregulated genes associated with virulence in addition to the *bssR* gene. In contrast, AIEC exposed to FA has several upregulated genes associated with bacterial membrane integrity and stress responses, but not genes associated

with virulence. This may suggest that the upregulation *bssR* gene promotes survival in AIEC exposed to FA and colonisation in AIEC exposed to PA.

Unlike *bssR*, *hycA* and *hycE* were only upregulated in the APEC strains 601 and TW731/12 strains in response to FA exposure. These genes are involved in several biological processes such as anaerobic respiration, glucose catabolic process, and FA oxidation through the formate hydrogenlyase (FHL) complex (McDowall et al., 2014). Furthermore, during anaerobic fermentative growth, when all exogenous electron acceptors are absent, *E. coli* performs a mixed-acid fermentation, utilising glucose as the only carbon and energy source and producing FA, succinate, acetate, lactate, and ethanol as products. When extracellular FA levels reach a crucial level, with a concomitant drop in environmental pH, the FA is transported back into the cell cytoplasm via a specific transporter where the membrane-bound FHL complex produces CO_2 and H_2 (McDowall et al., 2014). The upregulation of these genes indicates that APEC strains can regulate the effects caused by FA, which could enable the APEC strains to inhabit an environment with higher FA levels. However, these genes were not identified among the significantly expressed genes in AIEC exposed to FA. It is unclear why AIEC did not similarly respond to FA as APEC strains did. It is apparent in the environment that AIEC is exposed to FA significantly less than APEC strains because AIEC colonises the human gut, and FA levels are thought to be lower compared to the animal gut.

Furthermore, not all *E. coli* strains have a similar response to FA. For instance, the gene *flgB*, which is involved in bacterial motility by forming the flagellar basal body protein, was significantly upregulated in 601 but downregulated in TW731/12. Moreover, flagellar motility and chemotaxis, which is the directed migration of cells in response to concentration gradients of extracellular signals, are essential for the effective colonisation and infection of hosts (Chaban et al., 2015; Erhardt, 2016; Matilla et al., 2018). However, FA adaptation did not affect the motility of bacterial strains. The high percentage of agar thought to be reason for not detecting no differences in motility, but strain like TW731/12 showed a downregulated gene expression of motility genes. However, it is unclear why, in response to FA, one APEC strain increased flagellar gene expression while the other downregulated this gene.

Successful colonisation of the GIT by enteric bacteria mainly depends on their ability to penetrate or disrupt the viscous mucus layer to reach a favourable niche. For example, MUC2-deficient mice are prone to spontaneous inflammation and less resistant to bacterial infection demonstrating the significance of the mucus barrier in maintaining gut homeostasis

(van der Sluis et al., 2006; Bergstrom et al., 2010; Zarepour et al., 2013). Therefore, the upregulation of the *flgB* gene in 601 could indicate that FA acts as a positive signal that increases bacterial motility in the face of stress or nutrient deprivation, but FA may have a side effect of increasing the potential of these strains to become more adherent and invasive through this increased motility.

Genes such as *napA, napG*, and *napH* were only upregulated in the 601 strain, but not in TW731/12 strains. In fact, the whole cluster of genes was downregulated in TW731/12. These genes are involved in electrons transfer in the periplasmic nitrate reductase NapABC complex. This complex plays an essential role in bacterial growth under anaerobic conditions using nitrate as an electron acceptor via periplasmic nitrate reductase activities. Bacteria can utilise inorganic nitrogen compounds (e.g., NO^{-}_{3}, NH^{+}_{4}) and ammonia can be directly used to synthesise proteins and nucleic acids, while NO^{-}_{3} can be stored and converted to ammonia via nitrate and nitrite reduction (Brondijk et al., 2002; Richardson et al., 2001; van Alst et al., 2009). In addition, it has been shown that the NapABC complex supports the growth of *E. coli* in the lumen of the inflamed gut (Spees et al., 2013; Winter et al., 2013). Furthermore, a study indicated that macrophages utilise nitric oxide to kill invading organisms as part as the innate immune system (Sparacino-Watkins et al., 2014). A correlation between the presence of nitrate and nitrite reductase enzymes and an organism's insensitivity to nitric oxide implies that nitrate reductase may have a role in microbial survival (Sparacino-Watkins et al., 2014).

In addition, the gene expression of *iss* gene supports the hypothesis that organic acids could alter *E. coli* phenotype and the zoonotic transmission of these altered *E. coli* strains. Indeed, *iss* gene expression was upregulated significantly in 601, but not in TW731/12, and this gene is associated with increased serum survival and surface exclusion, a process that occurs during conjugation. Indeed, a genetic element is transmitted from a bacterial donor to a recipient cell through a connecting channel during conjugation. This is the primary cause of the spread of antibiotic resistance. Conjugative elements may have exclusion systems that inhibit the element's transfer to a cell that already contains it (Chuba et al., 1989; Gago-Córdoba et al., 2019; Johnson et al., 2008). Indeed, *iss* was identified as being significantly more associated with APEC strains than with faecal isolates from healthy birds (Pfaff-McDonough et al., 2000; Rodriguez-Siek et al., 2005).

In the past the *iss* gene has been identified as a distinguishing trait of avian ExPEC but not human ExPEC (Johnson et al., 2006). However, recently *iss* has been identified in UPEC and NMEC strains. Moreover, this gene is encoded for several virulence factors and carried

by the virulence plasmid ColV. Several studies have demonstrated a link between APEC virulence and the possession of ColV plasmids (Ginns et al., 2000; Johnson et al., 2002; Ewers et al., 2004; Tivendale et al., 2004; Vandekerchove et al., 2005). Moreover, the ColV plasmid is suggested to be associated with zoonotic transmission from poultry to humans due to the fact that APEC strains that have the ColV plasmid can induce diseases such as meningitis and urinary tract infection in mice models (Liu et al., 2018; Jørgensen et al., 2019). Also, because of the presence of poultry specific ColV plasmids in human ExPEC isolates (Zhuge et al., 2019).

Another gene that was found significantly upregulated in 601, but not TW731/12 was *ireA*. This gene functions as an iron-regulator in ExPEC. During natural infection, the initiation, progression, and transmission of most bacterial infections depend on the ability of the invading pathogen to acquire iron from the complicated environment (Li et al., 2016). Indeed, extra-intestinal sites have low iron; therefore, various bacterial receptors chelate iron ions. However, the function of *ireA* in APEC strains remains unclear, but a recent study showed that an *ireA* deletion mutant showed a significant decrease in adhesion to epithelial cells and resistance to environmental stress compared to the wild-type strain which indicates that it has a role in the adhesion and stress resistance (Russo et al., 2001). Therefore, upregulating the gene in the 601 strain due to FA exposure suggests FA exposure could be a potential risk because it increases factors associated with virulence. Moreover, the results in Chapter 3 showed that 601 adhesions to IECs increased significantly after FA adaption, although IECs are a human cell line and 601 is an APEC strain.

Perhaps most interestingly in the context of these SCFAs being employed as antimicrobials, the gene expression of AIEC exposed to PA differed significantly from that of AIEC exposed to FA. While PA increased the expression of virulence genes, FA regulates the genes related to bacterial membrane integrity and stress responses (Ormsby et al., 2020). Even though FA and PA induce different gene expression in AIEC, osmotic stress was induced by both organic acids. The gene *osmE* was significantly upregulated in AIEC exposed to PA, while the gene *osmY* was significantly upregulated in AIEC exposed to FA. Both genes regulate the osmotic stress response, but the difference is that *osmY* is more related to hyperosmotic stress, whereas *osmE* is an osmotically inducible gene (Gutierrez et al., 1995; Yim et al., 1992). These findings indicate that FA has a significant effect on the AIEC membrane.

In addition, *ytfE* was significantly downregulated in AIEC exposed to FA, and this gene is a Di-iron-containing protein involved in the repair of iron-sulfur clusters that are damaged by

oxidative or nitrosative stress conditions. A study showed that mutation of *ytfE* gene made the *E. coil* strains more sensitive to nitrosative stress (Justino et al., 2007). Unlike the transcription data that show some genes related to the stress responses were upregulated, the data from the growth experiments do not indicate that bacterial strains were stressed.

To conclude, organic acids have different effects on *E. coli* transcription. While FA has more impact on the genes involved in the stress responses, metabolism, and membrane integrity, PA has more impact on genes associated with virulence factors. The AIEC and APEC strains had significantly different transcriptional responses to FA exposure with FA inducing a significant downregulation of genes in the AIEC strain LF82 in general and a shift towards a stress response. In addition, APEC strains, while more similar in their response, also had subtle differences in their transcriptional responses to FA exposure. Several genes were significantly upregulated in 601, but not in TW731/12 and these genes have been linked to persistence and survival of APEC, which indicate that 601 could survive the antimicrobial effect of FA.

Chapter 5

The impact of organic acids on *E. coli* within *in vitro* fermentation gut model

5.1 Outline

The influence of both FA and PA, which are routinely used as antimicrobials in food and poultry, on *E. coli* was investigated in this chapter using an *in vitro* fermentation gut model. In the previous two chapters, it has been shown that FA has a different effect on the *E. coli* phenotypes, affecting traits associated with the virulence of some strains while others were not affected. For instance, strains such as 601 and LF82, their traits that are associated with virulence, such as biofilm formation and adhesion, were altered by FA. In contrast to FA, PA has increased more traits of AIEC strains that are associated with virulence such as intracellular replication. To gain a better understanding of these organic acids, and how they impact the *E. coli* strains and the chicken gut microbiome, an *in vitro* fermentation gut model that is commonly used to investigate the impact of dietary additives on the human gut microbiome and fibre fermentation capacity was used. This model could detect SCFA and microbiome alterations, which are essential factors affecting *E. coli* and other pathogens. Since SCFAs can be used as a source of energy, and microbiome alterations could shift the balance between the beneficial bacteria and the pathogens, these factors will be investigated.

5.2 Introduction

Providing a safe supply of food is a major worldwide challenge, and chickens are considered part of this challenge because they are the most prevalent livestock in the world, and there is a high demand for their products (Pandit et al., 2018). More than 60 billion chickens are produced each year, with output expected to rise considerably over the next 20 years (Pandit et al., 2018). In addition, poultry is susceptible to a potentially large number of pathogens, due to their eating habits and husbandry practices (Pandit et al., 2018). *Campylobacter* and *Salmonella* are highly prevalent in poultry, particularly commercial chicken meat, which is often implicated as the primary dietary carrier of infection for humans via consumption of raw or undercooked infected poultry meat and products. Transmission of these organisms from poultry to humans via contaminated food or water is a serious public health threat because these foodborne pathogens can cause serious illness (Sridapan et al., 2021). The global number of *Campylobacter* and non-typhoidal *Salmonella* cases were estimated to be more than 95 and 78 million, respectively in 2010 (Sridapan et al., 2021). Another microorganism that causes problems in the poultry industry is *E. coli*. The commercial poultry industry relies on raising large quantities of birds at high stocking densities, particularly in the broiler production system, which allows bacterial infections, such as *E. coli* infections, to occur (Swelum et al., 2021). In poultry, *E. coli* causes a variety of disease

syndromes such as yolk sac infection (omphalitis), respiratory tract infection, perihepatitis, and septicaemia. These disease syndromes can result in high mortality, poor weight gain, and poor flock uniformity. This will eventually lead to economic losses for producers (Lutful, 2010; Swelum et al., 2021).

One of the solutions that are used to overcome this challenge is antimicrobial agents (AMAs) and antibiotics are one of the AMAs that are used to reduce pathogens and enhance livestock production and feed efficiency (Brown et al., 2017). The antibiotic consumption patterns in agriculture vary across regions and countries, and even antibiotics that have been banned in other countries, including developed countries, are still being used in most developing countries (Brown et al., 2017). Antibiotics that are used as growth promoters are usually administered in relatively low concentrations, ranging from 2.5 mg/kg to 125 mg/ kg (ppm), depending on the drug type and animal species (Brown et al., 2017). For instance, tylosin is an antibiotic that is used extensively in chicken farms, it is recommended to add 50 mg/kg to chicken feed to promote growth (Brown et al., 2017; Kim et al., 2012). Tylosin is also commonly used to treat diarrhoea and inflammation in the digestive tracts of other mammals, such as dogs, cats, and cows. Tylosin has a broad spectrum of activity against Gram-positive organisms and a narrow spectrum of activity against Gram-negative organisms. Other antibiotics such as tetracycline, streptomycin, gentamicin, penicillin, and erythromycin also, are used as growth promoters (Page et al., 2012). Some of these antibiotics are medically important for human use and this raises a concern about antibiotic resistance development in zoonotic pathogens, as livestock are reservoirs of important bacterial pathogens of humans. Because of this concern, many countries have regulated the use of antibiotics as growth promoters, and EU countries banned the practice. (Brown et al., 2017).

For a long time, using prophylactic and in-feed growth-promoting antibiotics was the preventative strategy for dealing with ongoing problems (Swelum et al., 2021). However, the poultry industry and food chain industry have shifted toward using antibiotic alternatives such as organic acids, prebiotics, probiotics, bacteriophages, enzymes, and phytogenics due to concerns about the spread of antibiotic-resistant bacteria (Gadde et al., 2017; Zhu et al., 2021). Because of their antibacterial properties, organic acids have been considered a potential alternative to antibiotic growth promoters (AGPs) (Gadde et al., 2017). Chemically, organic acids utilised in food animal production can be classified as either simple monocarboxylic acids (e.g., FA, acetic, PA, and butyric acids) or carboxylic acids with hydroxyl groups (e.g., lactic, malic, tartaric, and citric acids) (Gadde et al., 2017; Tugnoli et al., 2020). Organic acids can be utilised separately as organic acids or their salts

(sodium, potassium, or calcium) or as blends of multiple acids or their salts in feed or drinking water (Gadde et al., 2017; Zhu et al., 2021). Over the years, organic acid usage has been shown to have considerable advantages in swine and poultry production. Fumaric acid addition in broiler chicken diets has been found to boost weight growth and feed efficiency (Gadde et al., 2017). When butyric acid was added to the broiler diet, the results of improved growth performance were similar (Gadde et al., 2017). The antibacterial effect of organic acids is specific to species; therefore, the addition of organic acids causes a decrease in *E. coli*, *Salmonella*, *Campylobacter*, and other potentially pathogenic bacteria, which results in a beneficial effect on the gut health of the host (Zhu et al., 2021).

The microbial community in the GIT of chickens (i.e., broilers and hens) plays a crucial role in determining health and productivity (Oakley et al., 2014; Stanley et al., 2014). The section of the chicken GIT with the highest population density of microbial community is the ceca, a pair of blind-ended sacs that connect to the large intestine (Clench et al., 1995; Rychlik, 2020). Due to its more widespread and diversified microbial population and longer transit time of digest (12–24 h), the caecum is not only the primary region for bacterial fermentation but also pathogen colonisation (Sergeant et al., 2014; Varki et al., 2017). Consequently, most studies on the chicken gut microbiome have mostly focused on the ceca microbial communities (Glendinning et al., 2020). Therefore, understanding the GIT and its role is critical, particularly for agricultural and industrial processes, because the GIT affects poultry health, and hence meat quality and nutritional content (Mota de Carvalho et al., 2021). The gut and GIT conditions (e.g., pH and enzymes) will also influence feed digestion and nutrient absorption, as they directly influence the number of gut bacteria, their viability, and metabolism. Furthermore, the animal breed influences the chicken's growth rate, feed intake, weight gain, and conversion ratio (Mota de Carvalho et al., 2021).

Animal testing is one of the most renowned methodologies for predicting the effectiveness and influence of dietary additives on the gut microbiota, but ethical considerations and costs can limit these applications (Macfarlane et al., 2007). Intestinal *in vitro* models can circumvent some of these constraints by allowing reproducible experimentation under standardised conditions and, more importantly, by providing the opportunity to investigate the complexity of gut microbiomes and the functional relatedness of specific bacterial species in the absence of a host (Payne et al., 2012). One of these types of models is an *in vitro* fermentation model, which provides a useful tool for assessing treatment-related changes in microbiota metabolism and composition that is independent of the host (Tanner et al., 2014). In addition, the model has been used to investigate how different types of

dietary additives, artificial sweeteners, and domestic hygiene products affect the composition of the gut microbiome and the capacity for fibre fermentation in healthy individuals. It was shown that maltodextrin and the aspartame-based sweetener affected gut microbiome composition and production of SCFAs but did not induce dysbiosis. Instead, it had a beneficial effect by inhibiting the growth of *E. coli*, thus also promoting *Bifidobacterium* and correspondingly increasing the production of acetic acid and PA (Gerasimidis et al., 2020). In addition, there is a study successfully managed to provide a close reproduction of the composition and activity of the chicken caecal microbiota *in vivo* by using *in vitro* fermentation model called PolyFermS (Asare et al., 2021). This indicates that *in vitro* fermentation models could be beneficial in studying the microbial community in the GIT of chickens.

5.3 Results

5.3.1 Microbiome abundance within *in vitro* fermentation chicken gut model

The chicken GIT is rich in diverse bacterial communities, with each bacterium adapted to its ecological niche and coexisting with other bacterial species in the same community (Shang et al., 2018). The composition and function of these communities have been shown to vary depending on the age of the bird, location in the GI tract, and dietary components (Shang et al., 2018). Microbiota composition and complexity increase significantly in distal parts of the intestinal tract (caecum and colon), even though colonic microbiota is variable due to chicken intestinal physiology and may resemble either ileal or caecal microbiota (Rychlik, 2020). To determine how organic acids such as FA and PA, which are used as a dietary component in the chicken diet, impact the chicken microbiome, *in vitro* fermentations experiments were performed. The fermentations were conducted with chicken faecal contents and with or without chicken feed, as a nutrient source for the fermentation. Seven fermentation conditions were applied in this model to mimic the chicken gut which includes (chicken faecal samples supplemented with chicken feed, and/or PA or FA). Other samples were spiked with the APEC strain, which was 601. APEC was used as a source of spiked in *E. coli* to determine *E. coli* numbers in case of numbers in the gut were deficient (Table 5-1).

Moreover, in order to study the effect of FA and PA accurately, we used three groups as controls which included chicken faeces (CF) alone, chicken faeces spiked with APEC strain 601 (CF_601), and chicken faeces spiked with strain 601 and with chicken fibre feed added (CF_601_Feed). This group of controls will establish the microbiome composition and

SCFA levels in the absence of SCFA supplementation. In case the target organism, *E. coli*, number was low, the second control was spiked with *E. coli* (Table 5-1). This would enable the determination of any specific effects on *E. coli*. In addition, fibre feed is important factor in the diet because it can modulate SCFA production through increasing microbiome metabolism (Gerasimidis et al., 2020). Therefore, we included fibre feed in the experiment, along with a fibre feed control, to examine its effects along with FA and PA (Table 5-1).

Fermentation conditions	**Abbreviation**
Chicken faecal sample	CF
Faecal sample, and APEC strain	CF_601
Faecal sample, APEC strain, and FA	CF_601_FA
Faecal sample, APEC strain, and PA	CF_601_PA
Faecal sample, APEC strain, and chicken feed	CF_601_Feed
Faecal sample, APEC strain, FA, and chicken feed	CF_601_FA_Feed
Faecal sample, APEC strain, PA, and chicken feed	CF_601_PA_Feed

Table 5-1. The seven fermentation conditions that were used in *in vitro* fermentation model.

Following *in vitro* fermentation, at different time points (0, 6, and 24 hours) microbial DNA was extracted from the original faecal or from fermentation samples for 16S rRNA gene sequencing. Sequencing the 16s rRNA gene which encodes a small subunit of the ribosome with a highly conserved region in all the bacteria allowed us to distinguish between bacteria, to the genus and species level, and determine the relative abundance of each sequence.

Analysis of the composition of the microbial community showed that *Firmicutes* are the most abundant phylum under all the fermentation conditions followed by; *Fusobacteriota, Proteobacteria, Cyanobacteria, and Bacteroidota* (Figure 5-1; Appendix III). Furthermore,

a heatmap was generated to shows the abundance of the top 35 genera from all the samples (Figure 5-1B).

The findings show that fermentation duration may play a role in shaping the composition of the microbial community. In all fermentation conditions for instance, the abundance of the *Fusobacteriota* phylum is relatively lower at the 24 h time point compared to the 0 h and 6 h time points, while the abundance of the *Firmicutes* phylum is relatively higher at the 24 h time point when it is compared to the other time points (Figure 5-1A). Another factor has an effect on the microbial composition which is the diet. The presence of chicken feed in the fermentation increased the abundance of the *Cyanobacteria* phylum in certain fermentation conditions when compared to fermentation conditions without chicken feed (Figure 5-1A).

Overall, the fermentation conditions containing FA and PA did not show significant differences in their microbial composition; however, under FA fermentation conditions at 6 h, *Fusobacteriota* is relatively more abundant than PA fermentation conditions at the same time point (Figure 5-1A). These results suggest that fermentation duration and diet have a greater influence on the genus abundance of the microbial composition than FA and PA, which might improve the understanding of the spectrum of SCFAs impact.

Beta diversity analysis was used to assess the differences between microbial communities across the conditions. To compare microbial communities between each pair of community samples, a square matrix of distance or dissimilarity was calculated to reflect the dissimilarity between certain samples, such as Unweighted Unifrac (UPGMA) and Weighted Unifrac distance (WPGMA). The data in this distance matrix represented graphically using PCoA and PCA. The data again showed that there was a difference in the microbial community composition between the fermentation conditions based on the fermentation duration and the diet (Figure 5-2). The main findings from this overview were that the major drivers of change in the fermentation were the duration of fermentation and the presence or absence of chicken feed (Figure 5-1 and 5-2).

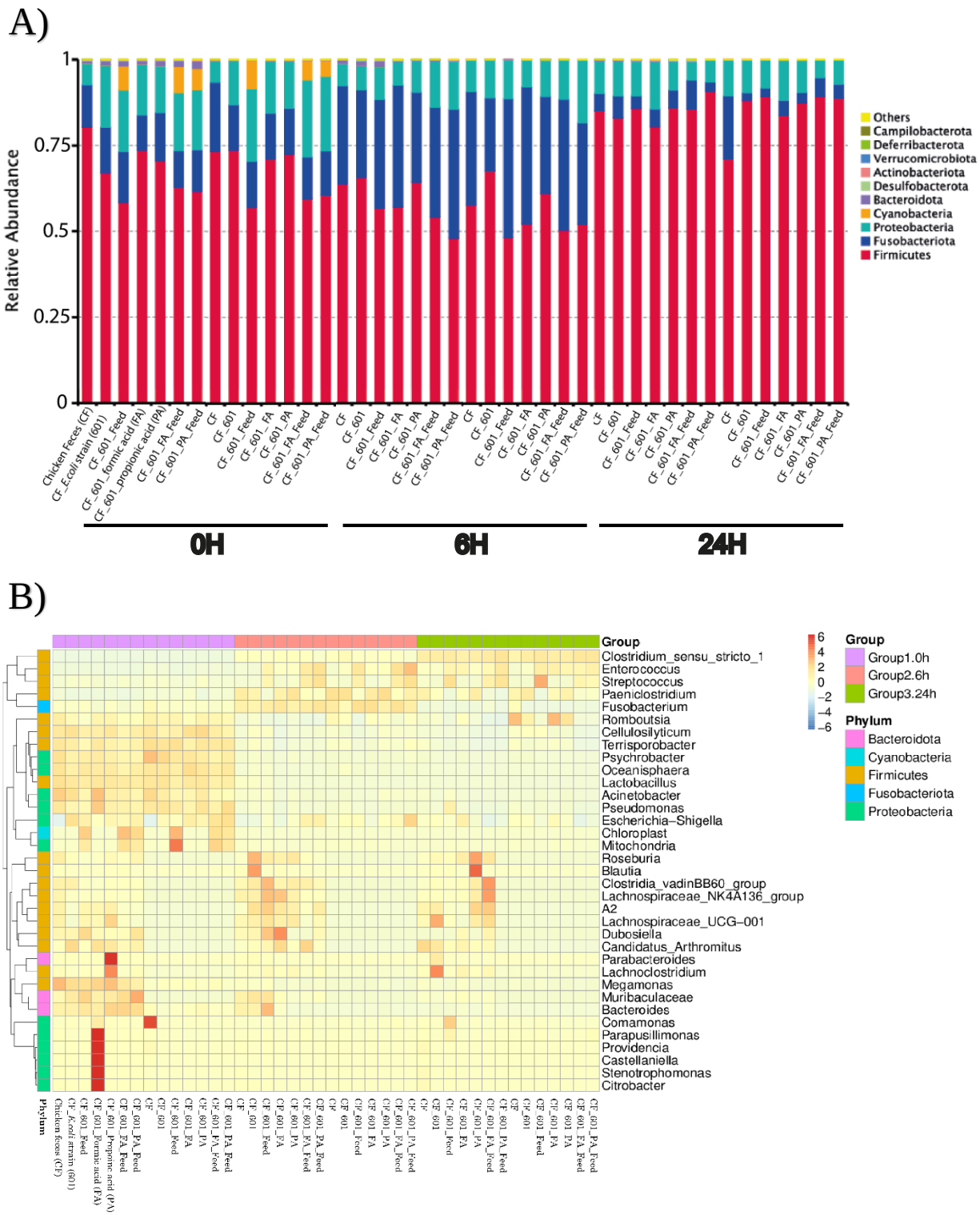
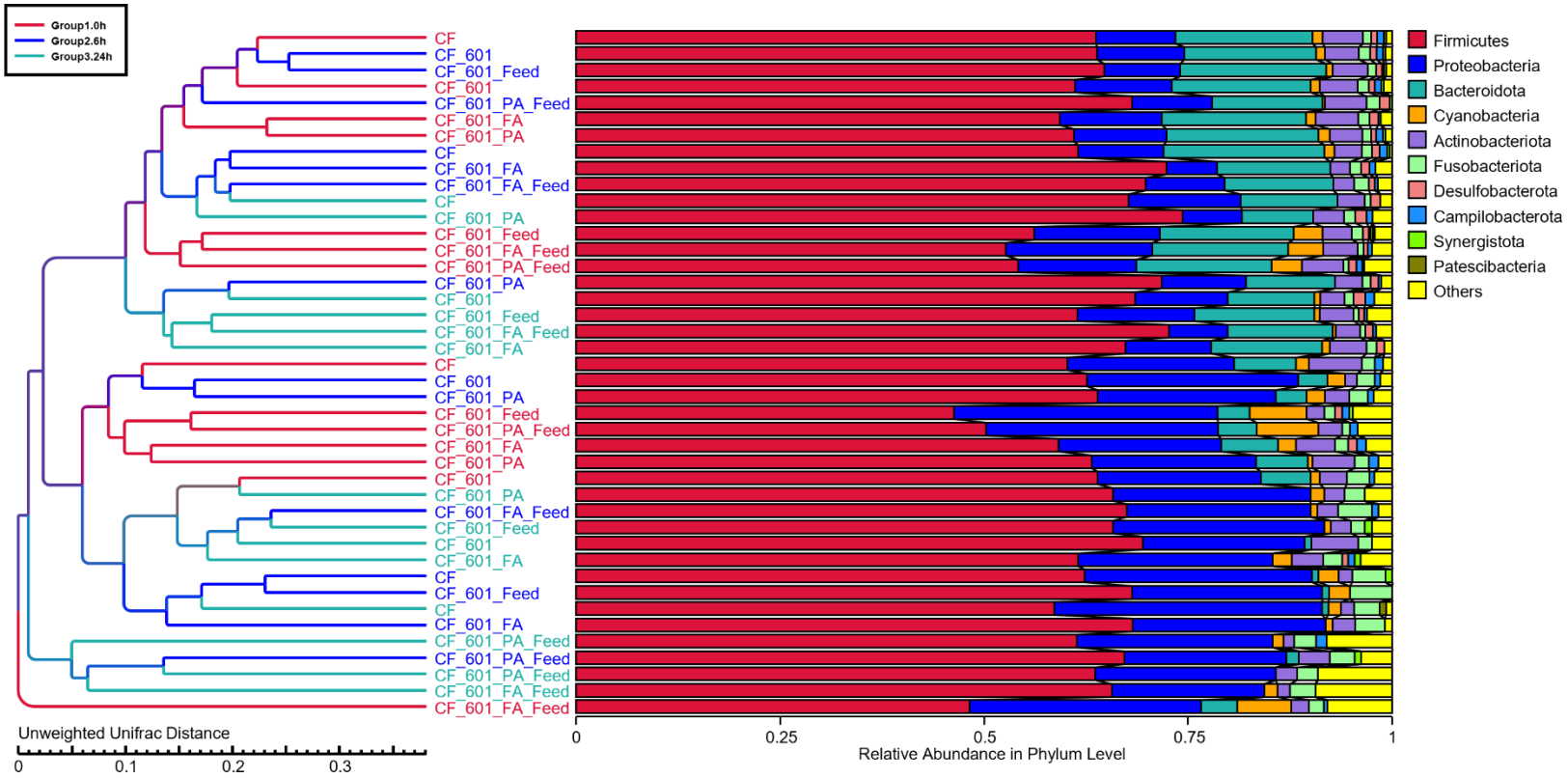


Figure 5-1. Composition of microbial community analysis. (**A**) The top 10 taxa in relative abundance from each phylum across the fermentation conditions. (**B**) The top 35 genera abundance clustered in a heatmap showing each fermentation condition and the duration of fermentation.

A)


Group1.0h
Group2.6h
Group3.24h
CF
CF_601
CF_601_Feed
CF_601
CF_601_PA_Feed
CF_601_FA
CF_601_PA
CF
CF_601_FA
CF_601_FA_Feed
CF
CF_601_PA
CF_601_Feed
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CF
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CF_601
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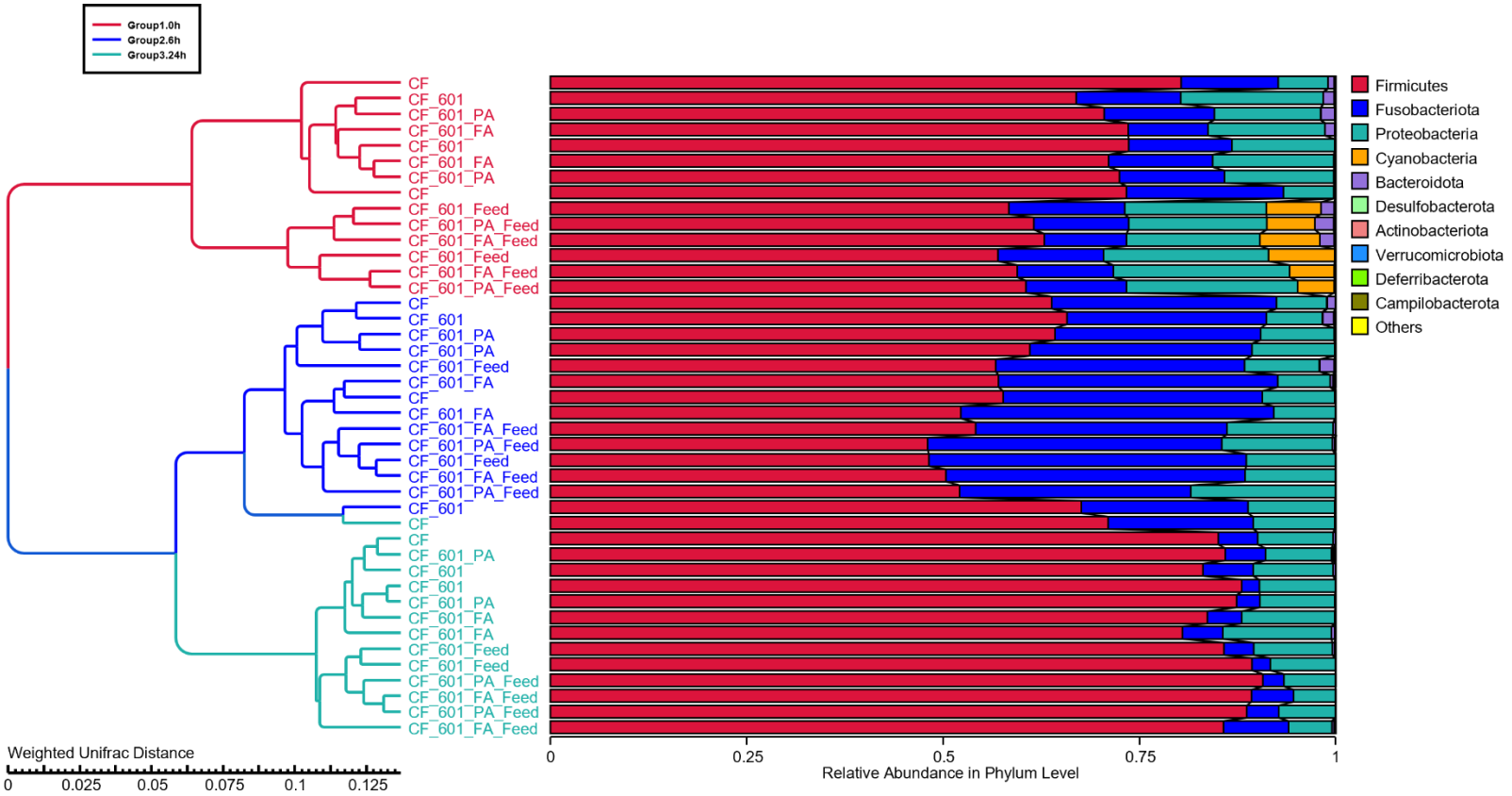
CF
CF_601_Feed
CF
CF_601_FA
CF_601_PA_Feed
CF_601_PA_Feed
CF_601_PA_Feed
CF_601_FA_Feed
CF_601_FA_Feed
Unweighted Unifrac Distance
0
0.1
0.2
0.3
0
0.25
0.5
0.75
1
Relative Abundance in Phylum Level
Firmicutes
Proteobacteria
Bacteroidota
Cyanobacteria
Actinobacteriota
Fusobacteriota
Desulfobacterota
Campilobacterota
Synergistota
Patescibacteria
Others

B)


Group1.0h
Group2.6h
Group3.24h
CF
CF_601
CF_601_PA
CF_601_FA
CF_601
CF_601_FA
CF_601_PA
CF
CF_601_Feed
CF_601_PA_Feed
CF_601_FA_Feed
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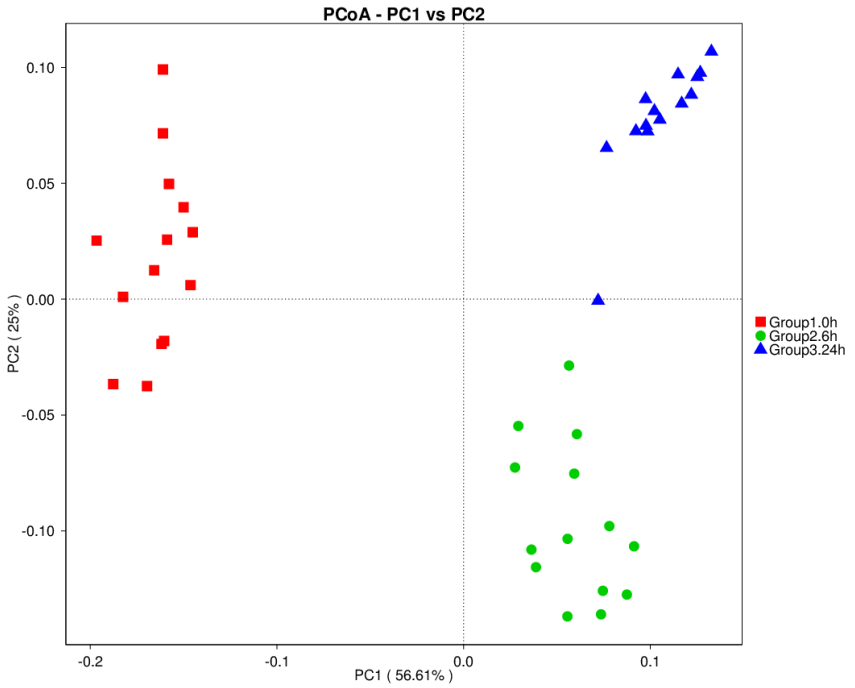
CF_601
CF_601
CF_601_PA
CF_601_FA
CF_601_FA
CF_601_Feed
CF_601_Feed
CF_601_PA_Feed
CF_601_FA_Feed
CF_601_PA_Feed
CF_601_FA_Feed
Weighted Unifrac Distance
0
0.025
0.05
0.075
0.1
0.125
0
0.25
0.5
0.75
1
Relative Abundance in Phylum Level
Firmicutes
Fusobacteriota
Proteobacteria
Cyanobacteria
Bacteroidota
Desulfobacterota
Actinobacteriota
Verrucomicrobiota
Deferribacterota
Campilobacterota
Others

C)



D)

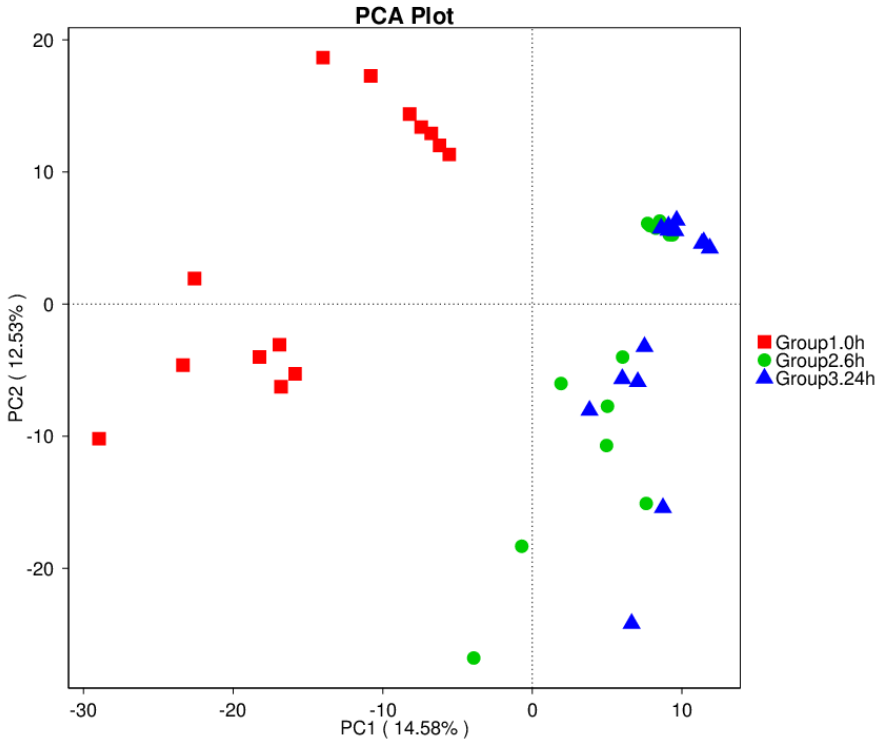


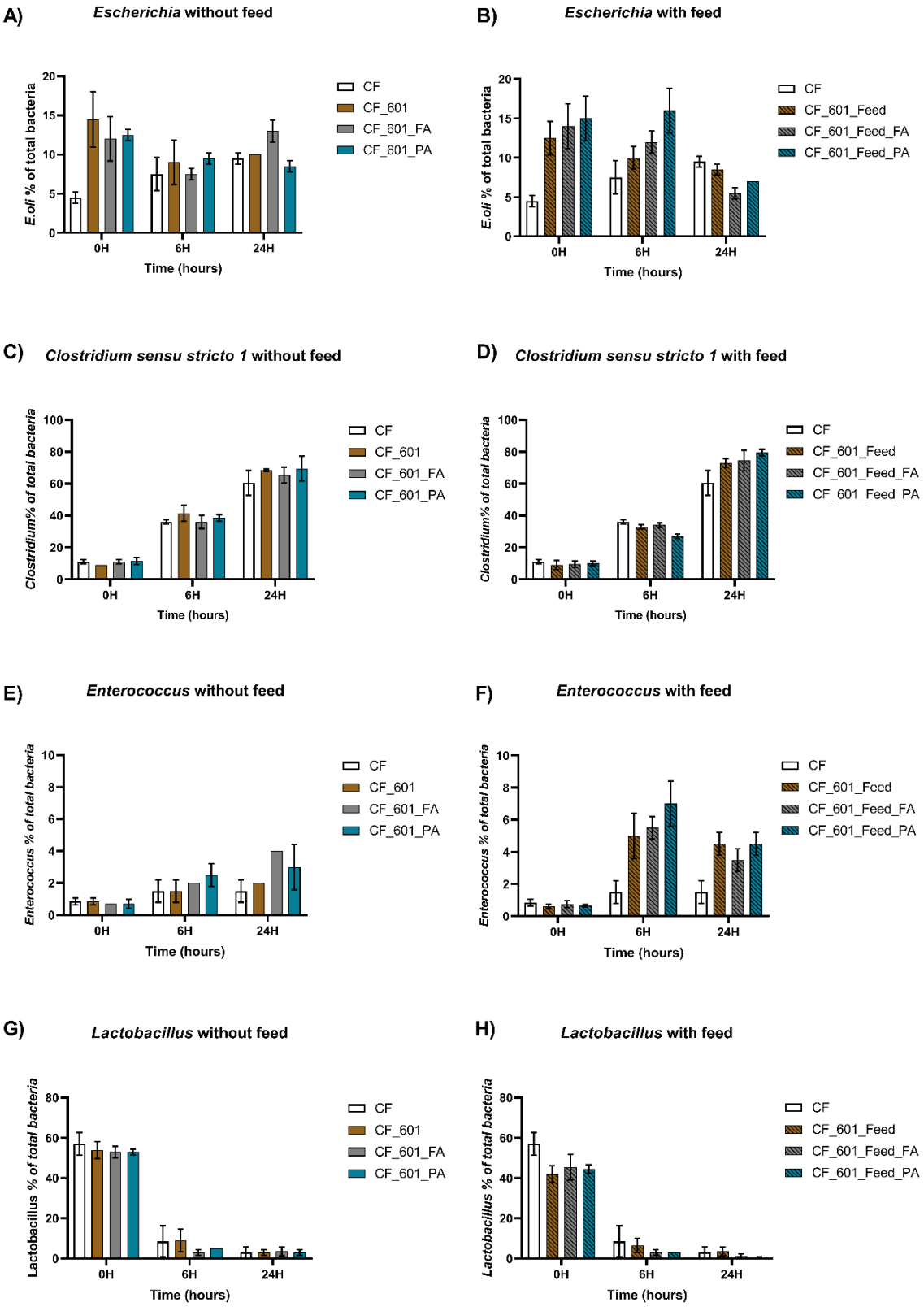
Figure 5-2. Analyses of Beta diversity under the different fermentation conditions. (**A**) UPGMA, **(B)** WPGMA, (**C**) PCoA, and (D) PCA show the differences in the microbial community composition between the fermentation conditions based on the dissimilarity factors such as fermentation duration and diet.

To understand further the effects that SCFAs have on the microbiome of the GIT, the abundance of bacterial genera in fermentation conditions was investigated. Bacterial genera including *Escherichia*, *Clostridium*, and *Enterococcus* which contain known opportunistic pathogens of the chicken gut (e.g. *E. coli*, and *Clostridium perfringens*), as well as bacterial genera like *Lactobacillus* and *Fusobacterium* that are beneficial to the poultry gut were investigated (Gadde et al., 2017; Schreier et al., 2021).

The main finding relating to *Escherichia* was that PA increased *Escherichia* abundance in the presence of chicken feed at 6 h when compared to controls (Figure 5-3B). However, a similar increase in their abundance is not observed when the chicken feed is absent (Figure 5-3A). FA unlike PA did not show any significant increase in *Escherichia* abundance at either the 6 h or 24 h fermentation time points when chicken feed was present or absent (Figure 5-3A-B). Furthermore, there was no significant difference between FA and PA presence when comparing *Escherichia* abundances under all fermentation conditions.

Clostridium abundance increased over time in groups with FA or PA whether feed was present or not (Figure 5-3C-D). FA and PA increased *Clostridium* abundance in comparison to controls, but there was no significant difference between FA and PA in regards of *Clostridium* abundance (Figure 5-3C-D). Moreover, both FA and PA significantly affected *Enterococcus* abundance under several fermentation conditions. In the fermentation without feed, FA increased *Enterococcus* abundance significantly at the 24 h time point (Figure 5-3E), but in the fermentation containing feed it was the addition of PA that increased *Enterococcus* abundance significantly (Figure 5-3F).

The abundance of bacterial species thought to be beneficial to the poultry gut was affected by both SCFAs used. *Lactobacillus* abundance decreased gradually over time in all groups (Figure 5-3G-H). Unlike *Lactobacillus*, the major change in *Fusobacterium* abundance is in the group with no feed (Figure 5-3I). FA increased the abundance of *Fusobacterium* significantly at 6 h (Figure 5-3I).


A)
Escherichia without feed
E.oli % of total bacteria
Time (hours)
0H
6H
24H
CF
CF_601
CF_601_FA
CF_601_PA
B)
Escherichia with feed
E.oli % of total bacteria
Time (hours)
CF
CF_601_Feed
CF_601_Feed_FA
CF_601_Feed_PA
C)
Clostridium sensu stricto 1 without feed
Clostridium% of total bacteria
Time (hours)
D)
Clostridium sensu stricto 1 with feed
Clostridium% of total bacteria
Time (hours)
E)
Enterococcus without feed
Enterococcus % of total bacteria
Time (hours)
F)
Enterococcus with feed
Enterococcus % of total bacteria
Time (hours)
G)
Lactobacillus without feed
Lactobacillus % of total bacteria
Time (hours)
H)
Lactobacillus with feed
Lactobacillus % of total bacteria
Time (hours)

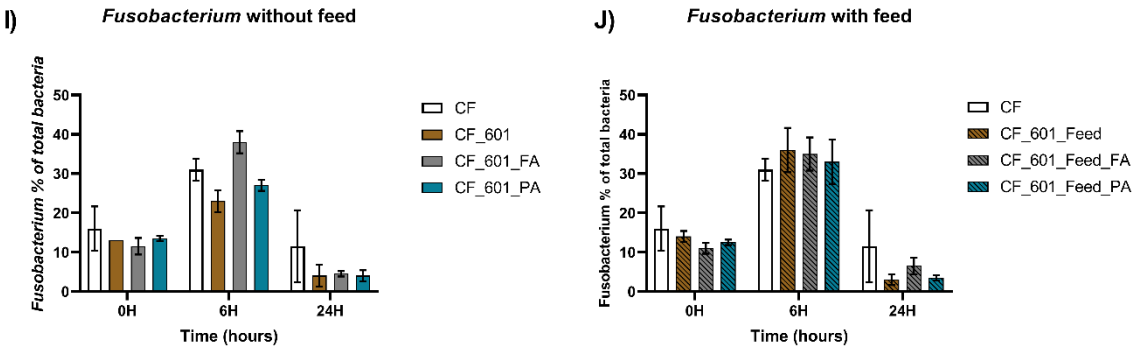


Figure 5-3. The relative abundance of genera under each fermentation condition. Data are expressed as mean ± SD; data were analysed using a two-way ANOVA.

To gain a better understanding of how PA and FA impact bacterial survival during fermentation, bacterial recovery was performed using HiChrome media, a selective medium designed to identify *E. coli* and coliforms in various environments. The main results show that FA has a different impact on bacterial recovery than PA and this impact varies depending on feed presence in the fermentation.

Furthermore, FA and PA have less bacteria recovered than the CF_601 which is considered as a second control in the no feed group (Figure 5-4B). Indeed, the elevated levels of *E. coli* in the bacterial recovery conditions such as CF_601, CF_601_FA, and CF_601_PA relative to CF could be associated with the injection of 601 strain within the fermenters. In addition, when comparing PA and FA groups, PA had higher bacterial recovery than CF and was also higher than CF_601_ FA when feed wasn't present (Figure 5-4B-C). Additionally, PA had higher bacterial recovery than CF_601 in the 24 h time point, but not the 6 h time point (Figure 5-4B-C).

In the feed group, both FA and PA have a higher *E. coli* recovery than the controls (CF and CF_601) in the fermentation condition at the 6 h time point when chicken feed is present (Figure 5-4E), however when comparing PA with FA, there is no significant difference in *E. coli* recovery (Figure 5-4E). In contrast, bacteria were recovered at lower levels in PA treated samples compared to the control (CF_601_Feed) when the feed is present (Figure 5-4F). In the FA condition, it was unclear if the FA treated samples bacteria were not recovered due to the effects of FA supplementation or user error (Figure 5-4F). However, all samples were processed simultaneously, incubated together and then counted. While this result disagrees with counts performed via 16s rRNA gene amplification it may be that cells treated with FA are in a stressed state and are more difficult to quantify by this method.

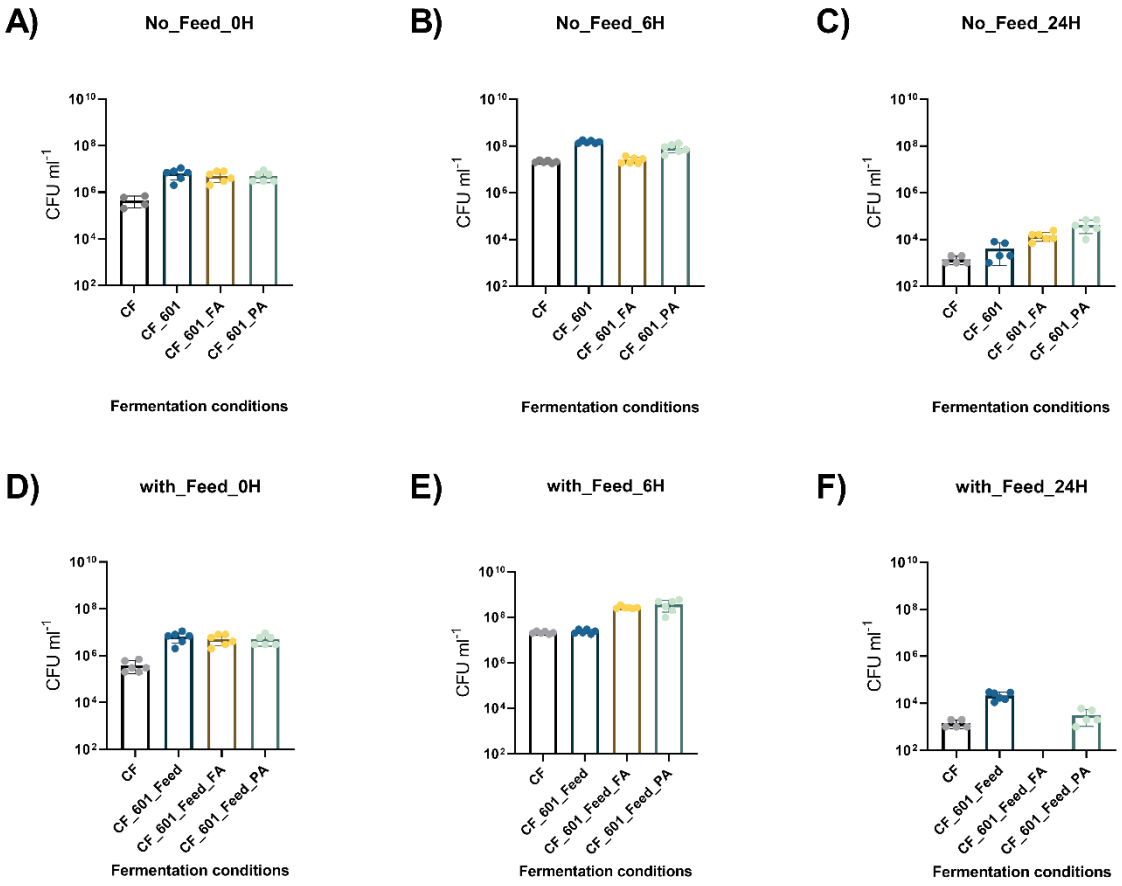


Figure 5-4. Bacterial recovery from various fermentation conditions within *in vitro* fermentation gut model. A selective HiChrome media to recover *E. coli* and other coliforms was used at 0 h, 6 h, and 24 h (A-F). Data are expressed as mean ± SD; data were analysed using a one-way ANOVA.

5.3.2 Quantitative changes in *E. coli* and the gut microbiota within in vitro fermentation chicken gut model

To better understand how FA and PA influence the gut microbiota within the *in vitro* fermentation gut model further work was carried out to quantify *E. coli* and total bacteria. The quantity of *E. coli* and total bacteria was measured using quantitative real-time PCR with *E. coli* specific primers to detect *E. coli* genomic DNA alongside bacteria specific primers to detect total bacteria. The overall results show the quantity of *E. coli* and total bacteria in all fermentation conditions are gradually increasing over time until 6 h and then decrease (Figure 5-5).

Moreover, the fermentation conditions that contain chicken feed have a higher quantity of *E. coli* than fermentation conditions that don't have chicken feed at 6 h (Figure 5-5B). Another significant difference between fermentation conditions can be seen in the total bacteria at 6 h (Figure 5-5). In the presence of the chicken feed, the FA fermentation condition have significantly fewer bacteria than the control (Figure 5-5E). However, there is no significant difference between FA and PA in the presence or absence of chicken feed at 6 h (Figure 5-5E). Similar results can be observed at 24 hours in both conditions with and without chicken feed (Figure 5-5G).

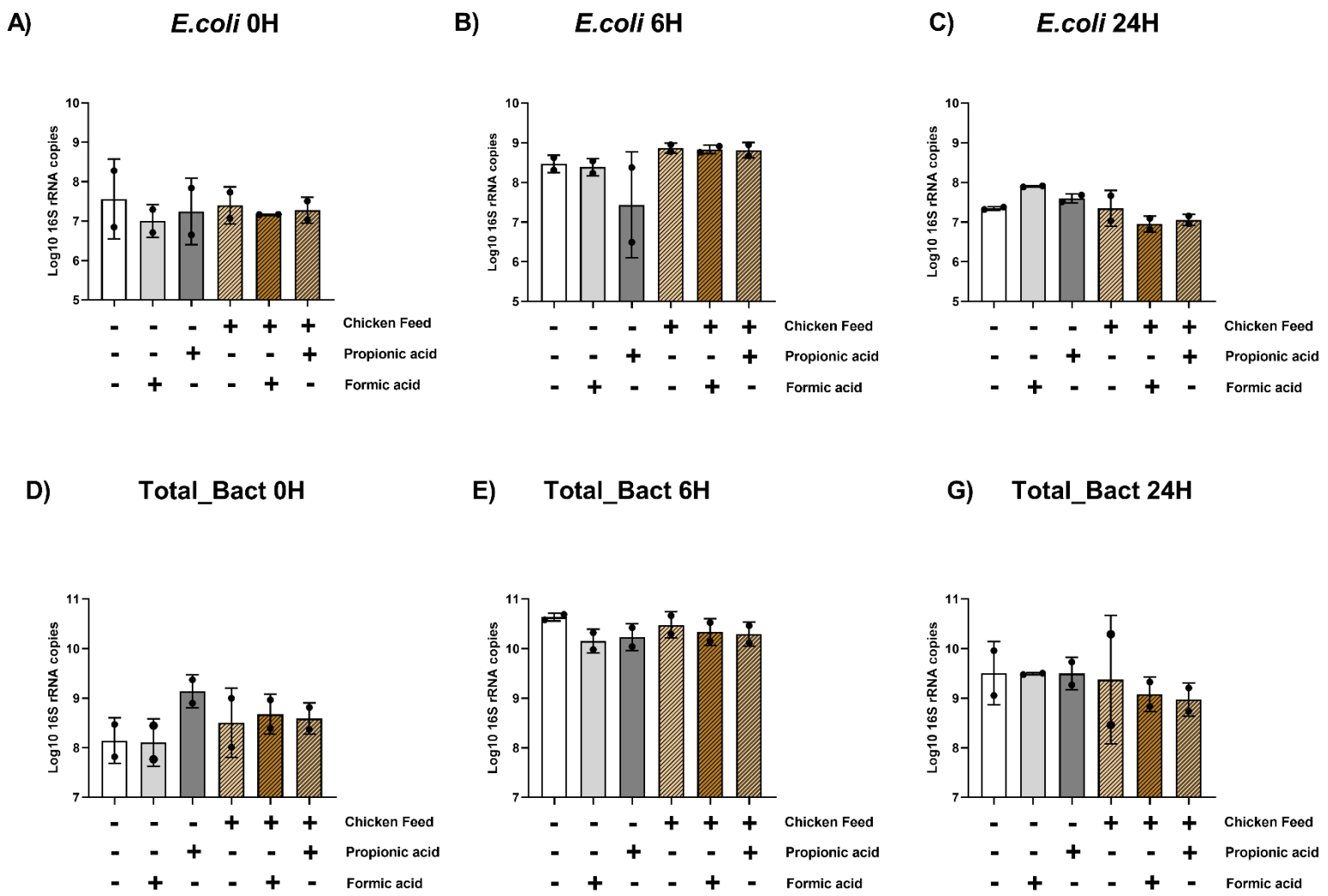


Figure 5-5. Abundance of *E. coli* and total bacteria under the different fermentation conditions. The bacterial quantity is represented by Log10 16S ribosomal RNA gene copy number/ millilitre. Data are expressed as mean ± SD; data were analysed using a one-way ANOVA.

5.3.3 SCFA abundance within *in vitro* fermentation chicken gut model

SCFA abundance within the *in vitro* fermentation model was measured by gas chromatography to understand how FA and PA impact SCFA production. The results show that PA has more effect on SCFA abundance under several fermentation conditions. Total SCFA abundance was measured, and this metric includes abundance of; acetic acid, FA, PA, butyric acid, citric acid, caprylic acid, benzoic acid, valeric acid, isobutyric acid, isovaleric acid, and isosteric acid, and it shows that PA increased the abundance of total SCFA (Figure 5-6A-B). In addition, PA supplementation into the fermentation has increased the abundance of total SCFA when it's compared to FA (Figure 5-6 A-B).

The SCFAs acetic acid, PA, and butyric acid are found at high concentrations in the chicken gut as the most abundant SCFAs and these are generated through colonic fermentation of dietary fibres (Liu et al., 2021). Given their importance to host physiology their abundance in response to FA and PA supplementation was examined separately. Analysis showed that acetic acid was the most abundant SCFA under all fermentation conditions (Figure 5-6C-D). Acetic acid abundance at 6 h was reduced by FA and PA when compared to the control (Figure 5-6C). However, the opposite can be seen at 24 h, with acetic acid abundance increased by FA and PA supplementation in comparison to the control (Figure 5-6D). In the chicken feed group, the acetic acid abundance was not affected by FA or PA supplementation and again the presence of feed in the fermentation appeared to negate, or at lease reduce, the efficacy of SCFA supplementation (Figure 5-6C-D).

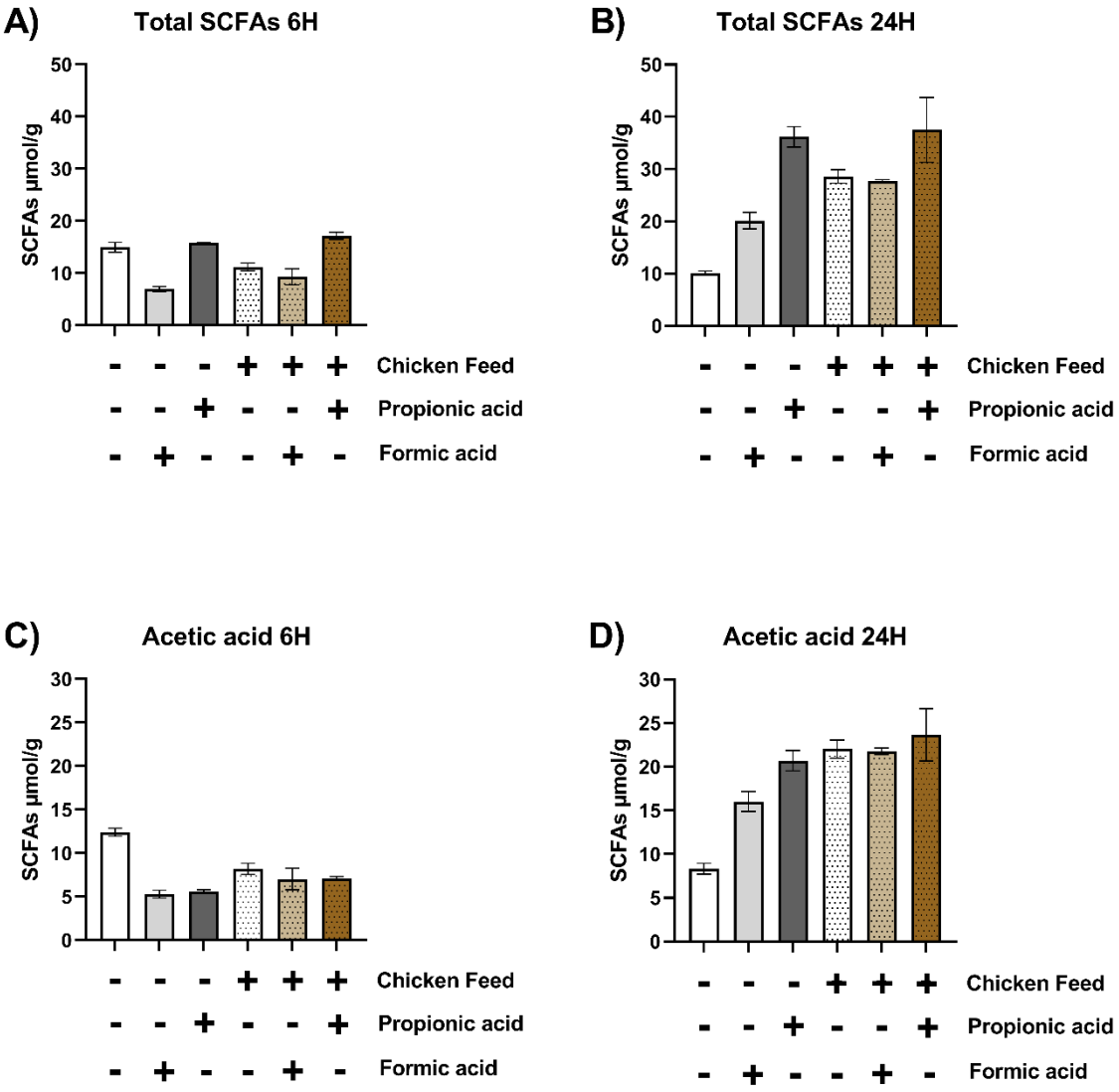
The second SCFA that was examined separately is PA and data showed PA was higher under the fermentation conditions which it was supplemented as expected. Although it is unclear if this increase is all through supplementation of PA or whether addition of PA has encouraged outgrowth of PA producing members of the microbiota, such as *Clostridia*. FA supplementation had no effect on PA levels (Figure 5-6E-F).

Butyric acid was the third of the three most abundant SCFAs examined separately, and its abundance was affected by supplementation of both FA and PA (Figure 5-6G-H). Butyric acid abundance reduced significantly in 6 h due to PA addition under all conditions, including both with and without chicken feed (Figure 5-6G). Under conditions where chicken feed was added, PA also reduced butyric acid abundance when compared to FA

(Figure 5-6G). Given the importance of butyric acid to intestinal cells, its reduction through PA supplementation would likely be detrimental to chicken gut health.

Of all SCFAs butyric acid was the most significantly affected by FA and PA, likely reflecting the susceptibility of butyric acid producing bacteria to these SCFA antimicrobials. It was, along with acetic acid, the most increased SCFA in response to chicken feed addition but also showed the most varied response when fermentations with and without chicken feed were compared. In the presence of chicken feed, FA promoted an increase in butyric acid production, but this likely positive effect was negated when feed was added with a slight reduction in butyric acid production. However, unlike FA, PA supplementation reduced butyric levels consistently across all time points, whether feed was present or not (Figure 5-6).

Lastly, branched chain fatty acids (BCFA) were examined. BCFAs are generated from the fermentation of branched amino acids, and undigested protein reaching the colon (Heimann et al., 2016). The results show that PA has increased the abundance of isobutyric acid in the no feed group (Figure 5-6J). Moreover, the abundance of isobutyric acid and isovaleric acid is lower when chicken feed is present at both 6 h and 24 h (Figure 5-6I-L). However, the levels of these BCFAs were extremely low in comparison to other SCFAs and this is likely due to the feed being used being high in fibre and having a low protein content.


A)
Total SCFAs 6H
SCFAs µmol/g
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10
20
30
40
50
- - - + + + Chicken Feed
- - + - - + Propionic acid
- + - - + - Formic acid
B)
Total SCFAs 24H
SCFAs µmol/g
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10
20
30
40
50
- - - + + + Chicken Feed
- - + - - + Propionic acid
- + - - + - Formic acid
C)
Acetic acid 6H
SCFAs µmol/g
0
5
10
15
20
25
30
- - - + + + Chicken Feed
- - + - - + Propionic acid
- + - - + - Formic acid
D)
Acetic acid 24H
SCFAs µmol/g
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- - - + + + Chicken Feed
- - + - - + Propionic acid
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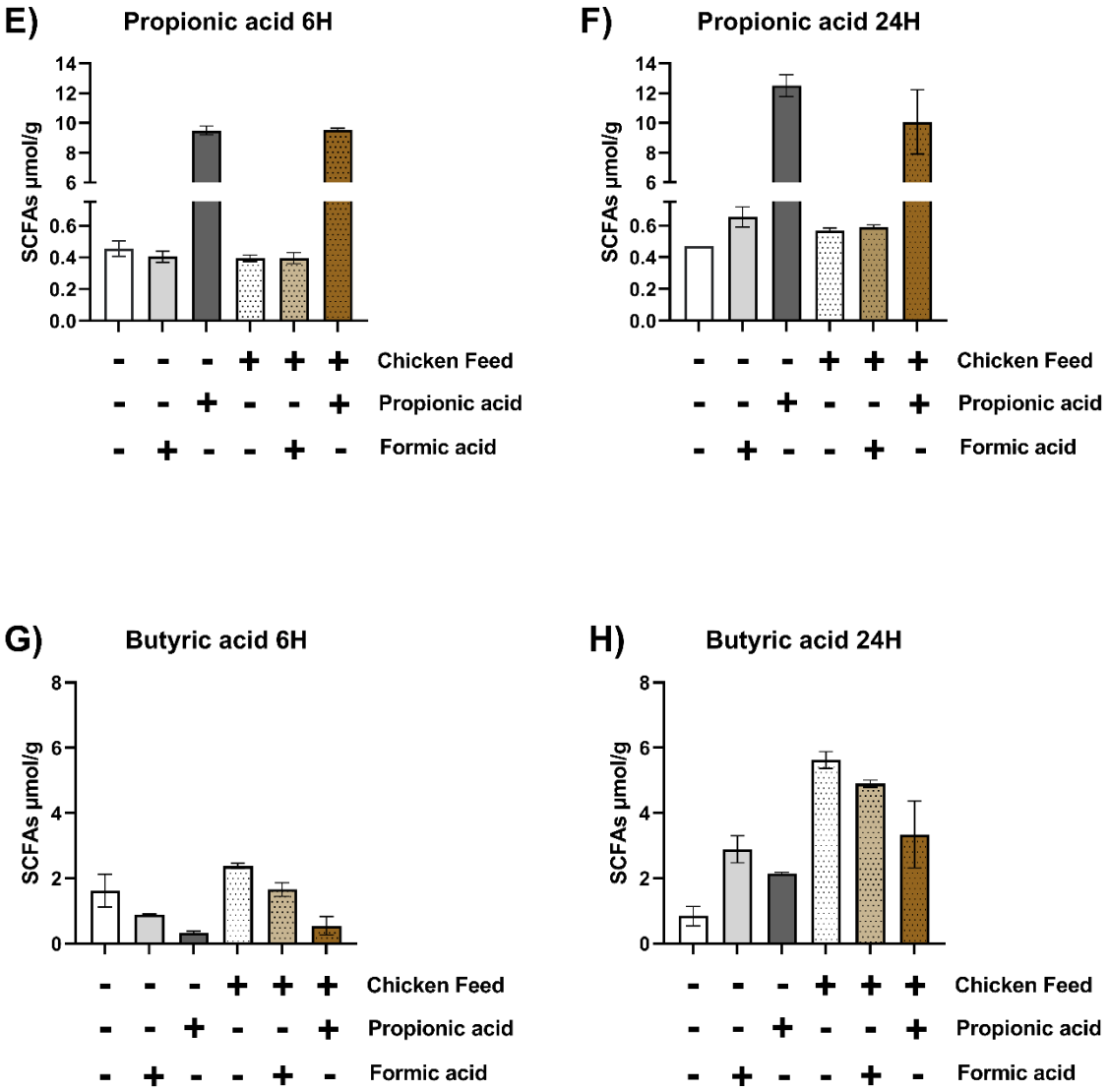
E)
Propionic acid 6H
SCFAs µmol/g
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12
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8
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0.6
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0.0
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- - + - - + Propionic acid
- + - - + - Formic acid
F)
Propionic acid 24H
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SCFAs µmol/g
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- - + - - + Propionic acid
- + - - + - Formic acid
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Butyric acid 24H
SCFAs µmol/g
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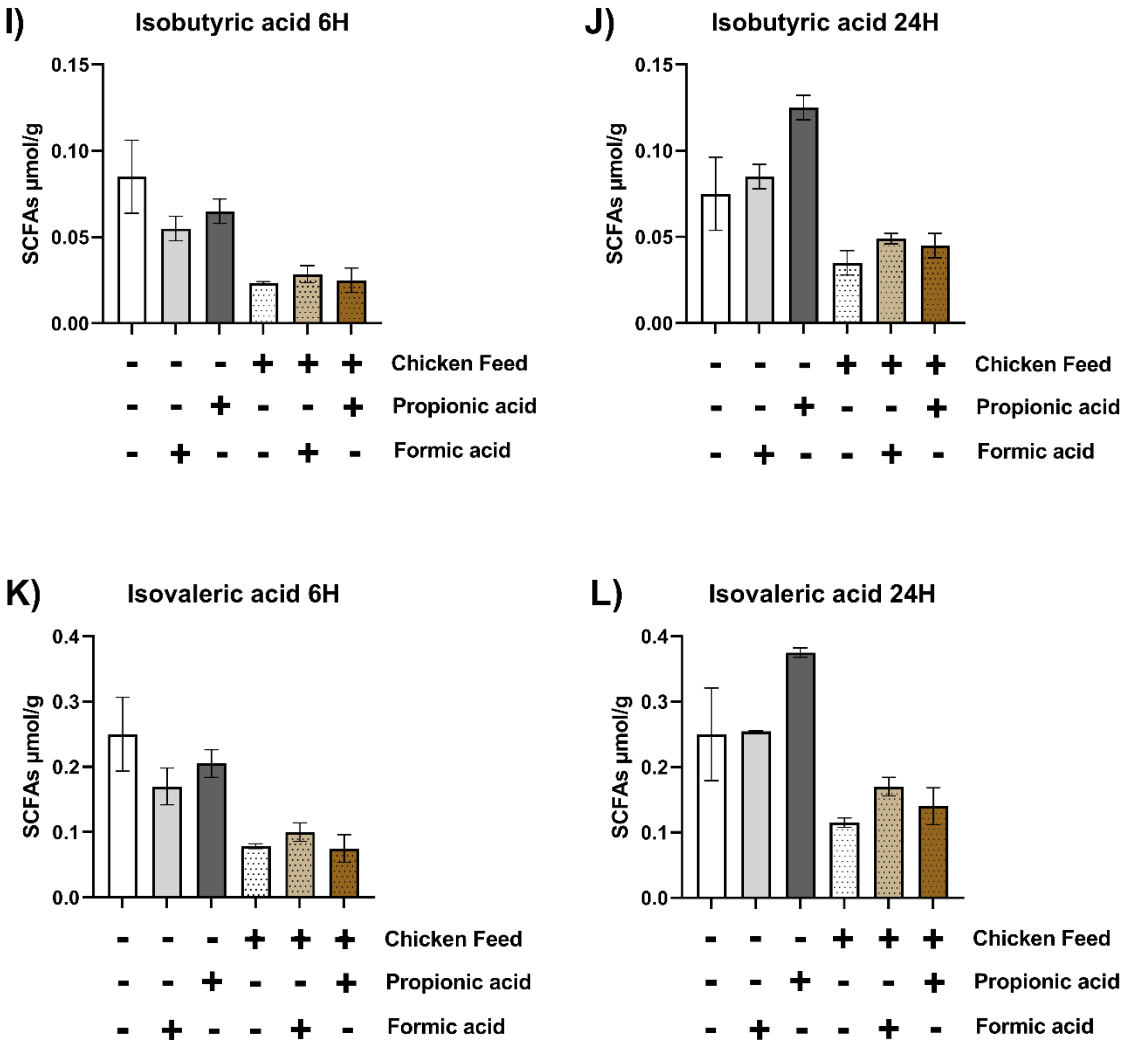

Figure 5-6: Individual SCFA and BCFA abundance within fermentation conditions at different time points. Data are expressed as mean ± SD; data were analysed using a one-way ANOVA

5.4 Discussion

In this chapter, we aimed to investigate the impact of FA and PA on the gut microbiome, including *E. coli*, by using an *in vitro* fermentation gut model. This model was previously used to study the effect of food additives and fibre fermentation capacity in the human gut (Gerasimidis et al., 2020). There are several *in vitro* fermentation gut models including batch fermentation models or dynamic fermentation models and each model serves a specific function (Verhoeckx et al., 2015). A batch fermentation model was chosen in this study because it gives the ability to assess and examine the impact of supplemented substances on the physiology and biodiversity of gut microbes (Verhoeckx et al., 2015). Additionally, the effect of SCFAs on the gut microbiota in this case, could be examined using quantitative and qualitative molecular techniques, and the impact on metabolic activity could be measured by analysing the synthesis of SCFAs or other metabolites (Verhoeckx et al., 2015).

On the other hand, the limitations of this type of model can be seen over longer periods, where the conditions in the batch culture are altered due to substrate depletion and the accumulation of the end products of microbial metabolism. However, this model has been used successfully for short term effects of dietary changes on the human gut microbiome while the effects of organic acid microbials are known to be rapid, inducing significant changes within the gut within days (Feye et al., 2020; Gerasimidis et al., 2020; Ormsby et al., 2020). Therefore, this *in vitro* batch fermentation model was expected to offer valuable insights into the SCFAs effect on the chicken gut (Gadde et al., 2017). Although the *in vitro* fermentation models could provide outcomes are closely similar to *in vivo* models, there are differences between the *in vitro* and *in vivo* models due to several factors including the host immune system. For instance, the host immune system plays an important role in shaping the gut microbiome composition which in turn could affect other vital processes like the host metabolism (Bäckhed et al., 2015; Dollé et al., 2016). A study indicated that Immunoglobulin A (IgA) plays a role in shaping the intestinal microbiota, and they observed that through a comparison between the impact of breastfeeding on infant gut microbiome and the impact of stopping breastfeeding on infant gut microbiome, and results indicate that stopping breastfeeding led to the maturation of the infant gut microbiome, indicating that the critical amount of IgA secreted in the mother's milk seems to play a central role in the regulation of microbiota composition (Bäckhed et al., 2015).

Recent evidence points towards a potential link between APEC that can colonise the gastrointestinal and respiratory tracts of chickens and AIEC, a pathogenic strain that promotes inflammation in the human intestine (Palmela et al., 2018; Kathayat et al., 2021). It has been shown that the AIEC type strain LF82 is genetically similar to APEC (Lestrange et al., 2017; Ormsby et al., 2020; Swelum et al., 2021). APEC strains are opportunistic pathogens that cause significant losses in the poultry industry, but it is not clear what makes APEC strains pathogenic and if there is a potential link between APEC and AIEC. Therefore, we wanted to investigate the effect of SCFAs on APEC strains, such as the 601 strain and to determine, whether APEC persistence shows a similar increase to AIEC strains in response to the presence of PA.

Initial analysis of the fermentation data demonstrated that the phyla present represented known microbes within the ceca of mature laying chickens, such as *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Actinobacteriota*, *Deferribacterota*, *Fusobacteriota*, and *Verrucomicrota* (Khan et al., 2020). Relative abundance and heat map clusters showed that *Firmicutes*, *Fusobacteriota*, *Protrobacteria*, *Cyanobacteria*, and *Bacteroidota* were the most abundant phyla under all fermentation conditions and the main differences found across all fermentation conditions were due to two factors, feed presence and fermentation duration. Moreover, significant changes in microbial composition of the fermentation between the FA treatment and PA treatment were not observed in phyla level, even though FA and PA treatment have been associated with the alteration of the poultry gut microbiome. It is unclear whether the PA and FA concentration is considered as being too low to have an impact on the microbiome composition within *in vitro* fermentation gut model. Therefore, using different concentration of FA would be helpful to determine the FA effects.

Because of the increase in the abundance of *Escherichia* over the first 6 h under fermentation conditions with PA supplemented along with chicken feed, an additional species-level investigation was done by recovering bacterial species. The main findings indicate that more *E. coli* was recovered from the PA conditions than the FA conditions when the feed was present versus when it was not. This could be associated with the inhibitory effect of FA that has been described in several studies (Gadde et al., 2017; östling et al., 1993). Indeed, the findings from previous work in the thesis (Chapters 4) indicate that FA causes more stress against *E. coli* than PA. In addition, these findings indicates that in some conditions the impact of PA could be affected by other factors such as the diet. Even though is reported that organic acids and fibre diet are effective in reducing the *E. coli* number in broilers gut (Dittoe

et al., 2018; Sabour et al., 2019). Given previous findings from the lab that continuous PA exposure altered the phenotype of AIEC by enhancing virulence traits such as biofilm formation, adhesion, invasion, and acid tolerance (Ormsby et al., 2020), these findings showing PA can also increase *E. coli* levels in a mock chicken intestine, raise concerns about using PA as an antimicrobial in poultry.

SCFAs have been linked to a variety of health benefits in chickens, including energy production, regulation of intestinal blood flow, mucin production, enterocyte growth and proliferation and intestinal immune responses (Ma et al., 2021; Mota de Carvalho et al., 2021). It is thought that supplemented SCFAs stimulate SCFAs synthesis in the host, which has beneficial effects on the host, and this could occur through altering the microbial composition (Dittoe et al., 2018). Therefore, SCFAs have been quantified and measured within *in vitro* fermentation model. In SCFAs analysis, two-time points, 6h and 24h were selected to measure and quantify SCFAs. Several factors influenced this decision, such as the limited access to gas chromatography and the past experiences of using *in vitro* fermentation model. Indeed, previous experiences of using this specific gas chromatography with a flame ionisation detector, indicate that detecting SCFAs at 0 h could be challenging due to the low intensity of SCFAs because of that it was recommend selecting 6h and 24h as time points. Additionally, a study of intestinal digestion of chicken related to starch indicates that the length of time that food is retained in the digestive tract of poultry averages between 5 and 6 h (Svihus et al., 2019). Because of these factors, the time points (6h and 24h) were selected in detecting SCFAs.

Detection of FA has proven to be one of the challenges in quantifying SCFAs. Although gas chromatography with a hydrogen flame ionisation detector is one of the most robust techniques for detecting various fatty acids and, it difficult to detect FA signal using this specific type of gas chromatography because FA contains only one carbon atom, which gives a low sensitivity (Hamano et al., 2020; Hong et al., 1999; Xu et al., 2010; Zervas et al., 2001). Even though SCFAs extractions procedures were intended to increase SCFAs sensitivity to gas chromatography, the signal for FA remained weak.

The SCFAs results show that PA has increased the total production of SCFAs when the feed is provided compared to when it is not. However, this increase in the total production of SCFAs could be associated with PA addition. Indeed, the increase in SCFA production is reported to enhance the poultry's nutritional digestibility and growth performance which indicated the importance of increasing the SCFAs production (Ma et al., 2021; Mota de

Carvalho et al., 2021). Even though PA has showed a beneficial effect which is increasing SCFAs abundance within *in vitro* fermentation gut model, previously it has shown that PA can alter *E. coli* phenotype and increase traits that are associated with virulence factors (Ormsby et al., 2020).

Acetic acid was detected as the most abundant of all the SCFAs. Acetic acid increased production is considered an important factor in the ability of *Bifidobacteria* to inhibit enteropathogens which indicates importance impact of acetic acid (Mota de Carvalho et al., 2021). Acetic acid levels increased over time, particularly in the presence of chicken feed at 24 h, but both FA and PA also increased acetic acid abundance in the absence of chicken feed at 24 h within *in vitro* fermentation gut model. Indeed, acetic acid is associated with improved glucose and lipid metabolism by promoting anti-lipolytic action in addition to promoting the increased proliferation of crypt cells (He et al., 2020). The results indicate that both PA and FA could be correlated with effects of stimulating acetic acid production. While butyric acid is often regarded as more important in the context of human health, acetic acid also plays role a crucial role in butyrate production through bacterial cross-feeding and is regarded as important for intestinal health (Mota de Carvalho et al., 2021).

PA significantly decreased the abundance of butyric acid under conditions where the feed is present and absent. Butyric acid is considered the primary energy source for colonic epithelial cells, stimulates intestinal epithelial proliferation and turnover, regulates immune responses, and increases mucin production (Ma et al., 2021; Mota de Carvalho et al., 2021). Since butyric acid plays such an essential role in the immune response of the host, reducing butyric acid production would be considered a negative impact chicken health. FA in contrast increased butyric acid abundance when chicken feed was present within *in vitro* fermentation gut model. With one of the main goals of using SCFAs in poultry food to improve the health and the growth of the chicken (Makowski et al., 2022), these findings suggest that FA is preferable to PA as it is likely better for chicken to increase the butyric acid abundance.

Lastly, PA was noted to have an effect on the abundance of isobutyric acid, which is a BCFA. Elevated concentration of isobutyric acid indicates that microbes have shifted their metabolism from carbohydrate sources such as fibres, which predominantly result in butyric acid production, to a protein-based metabolism resulting in isobutyric acid production. PA itself is a by-product of protein metabolism and its addition to the fermentation reaction here may have signalled other microbes to adopt a protein-centric metabolism. This alteration in

metabolism highlights the impact of organic acid supplementation on the gut microbiota and how addition of a single SCFA could be used to direct metabolism to prefer energy sources such as protein, if required. However, as stated earlier these potential benefits are offset by a number of negatives, including decreased butyric acid production and the potential selection for enteropathogens by the addition of these acids to the diet.

The increase of SCFAs production in poultry is thought to be associated with shifting the microbial composition (Dittoe et al., 2018; Sabour et al., 2019). Since PA treatment increased total SCFAs, it could indicate that a shifting in microbial composition occurred. Therefore, the quantitative changes in *E. coli* and total bacteria were detected, and the quantitative changes in *E. coli* and total bacteria 16S rRNA gene counts indicate that microbial compositions are shifting. The data show that there is no significant difference between FA and PA in total *E. coli*, but total bacterial quantity is reducing in FA treatment. These findings indicate that FA could alter the microbiota of the gut. Since FA increases butyric acid abundance, this effect could be a beneficial effect. addition, PA has increased total SCFAs abundance, but had no effect on the quantity of *E. coli*, and total bacteria, which might indicate a potential change in the bacterial species or other factors that influenced the SCFAs abundance. Overall, PA and FA the effects vary, and their effects could be depended on other factors such as diet and fermentation period.

Chapter 6

Conclusion remarks and future work

6.1 Objective of the thesis

The overall aim of this project was to investigate the phenotypic alterations in AIEC after organic acid treatment, their association with CD and any similar role for organic acids in promoting virulence in related APEC strains. Indeed, AIEC is distinguished from other *E. coli* strains by virulence traits that include potent adhesion, invasion, biofilm formation, and intracellular replication within immune cells such as macrophages (Palmela et al. 2018). Moreover, AIEC was isolated from CD at a higher rate relative to healthy individuals (Darfeuille-Michaud et al., 2004). Therefore, AIEC has been linked with CD, but it remains unclear what drives AIEC pathogenesis.

A recent study from our lab indicated that exposure to PA could alter the phenotype of AIEC and increase its virulence (Ormsby et al., 2020). As well as being in high concentrations in the human gut, PA is widely used as an antimicrobial in different sectors such as agriculture and food animal production. We hypothesised that environmental factors such as organic acids, including PA, could have a role to play in CD by altering the phenotype of AIEC in the environment prior to colonizing the human gut. Also, of interest was the fact that AIEC are both evolutionarily and phylogenetically related to APEC (Nash et al., 2010). Similar to AIEC, it is still unclear what drives APEC virulence, but they remain a significant burden in the poultry industry (Guabiraba et al., 2015). Given APEC strains are often exposed to antimicrobials such as organic acids through their widespread use in poultry, APEC was included in our investigation to understand how organic acids impact the phenotype of both AIEC and APEC and to determine if widespread organic acid could be a potential driver for phenotype alterations of theses *E. coli* strains.

6.2 Summary of main findings

In this study, we characterised the phenotype of AIEC and APEC in the presence of FA and PA and detected alterations in the traits of both pathotypes as per the data shown in Chapter 3. Both strains are known have virulence traits such as adhesion, invasion, and biofilm formation (Palmela et al., 2018; Kathayat et al., 2021). Our data showed that AIEC adapted to PA has increased net replication within macrophages. These results correspond with a study aimed to identify the PA effects on AIEC strain (Ormsby et al., 2020). In addition, these AIEC strains when adapted to PA, significantly increased their ability to replicate intracellularly within macrophages in the presence of ethanolamine. This carbon source becomes available during intestinal inflammation and is a known carbon and nitrogen source

for *Salmonella typhimurium* during infection (Thiennimitr et al., 2011). This could indicate that AIEC may utilise this nutrient to promote their persistence in the inflammatory phase and evidence was seen for this with ethanolamine operon expression from *E. coli* increased in paediatric CD patients, and a similar phenomenon has been seen with *Salmonella*. (Ormsby et al., 2019; Rogers et al., 2021). To validate these findings, the gene responsible for ethanolamine utilisation in AIEC was deleted, and net replication of AIEC exposed to PA no longer increased significantly in the presence of ethanolamine. Additionally, along with net replication within macrophages, PA adaptation increased adhesion and invasion of AIEC, the main characteristic virulence traits of the AIEC pathotype.

Unlike PA, FA had a very different impact on AIEC and APEC. FA adaptation increased biofilm formation of AIEC, but not net replication, adhesion, or invasion. However, some challenges have been detected in some experiments, which could affect the detection of FA-adaptation impact. The net replication of FA adapted *E. coli* strains shows a decrease over time which could indicate that the net replication was not measured accurately. Indeed, it's unclear if the net replication drop is due to bacteria killing or FA-adaptation impact. Another challenge was measuring the cell viability of macrophages infected with *E. coli* adapted to FA. Some strains have a high percentage of cell death, and this may affect bacterial recoveries by allowing gentamicin into infected cells, and the increased recovery of the *E. coli* strains could reflect variation in host cell death. In addition, measuring motility was another challenge because it needs to be more clarified whether no differences were detected due to a high percentage of agar or other factors. In fact, the transcription data shows a cluster of motility genes were downregulated in TW731/12 exposed to FA. Therefore, further investigation needs to be done to determine how FA-adaptation impacts motility. In general, the data indicate that FA adaptation has a different impact on virulence traits of AIEC than PA adaptation. In addition, APEC strains were unaffected by FA-adaptation, except for strain 601. The adherence of 601 to human IECs increased significantly after FA adaptation, indicating a potential zoonotic disease risk.

Moreover, RNA-seq data from Chapter 4 showed that FA exposure had a distinct stress response rather than an increase gene associated with virulence. Even though RNA-seq data indicate FA exposure caused stress to the *E. coli* strains, the growth assays didn't show a similar effect. Similar results have been seen in different assays like acid tolerance, motility and net replication where genes are upregulated but no impact on the assays. Therefore, validation of gene expression would be helpful in determining the effect of FA exposure.

Overall, the findings indicate that continuous exposure to organic acids can alter the phenotype of AIEC and increase factors associated with virulence. This expanded our understanding of AIEC traits virulence and its role in CD. Moreover, the effects of organic acids on APEC strains vary depending on the strains, and this suggests that organic acids could be effective against a particular strain, while promoting traits associated with virulence of another. This work improves our comprehension of the impact of the organic acids that are used as antimicrobials but raises fundamental questions about their use and their potential for increasing risk of zoonotic transfer of pathogens from organic acid treated poultry. While SCFAs are effective against a number of poultry pathogens, the results presented here show that we still are unclear on their exact mechanism of action and any potential inadvertent effects on the non-target pathogens.

To acquire a better understanding of how FA and PA affect the factors associated with virulence of AIEC and APEC, the gene expression of a number of strains was analysed in their presence using RNA-seq in Chapter 4. Based on the number of genes that were significantly differentially expressed in AIEC and APEC, FA had a significant effect on all *E. coli* strains. Several upregulated DEGs were associated with bacterial membrane integrity and stress responses, indicating that FA caused a significant stress on AIEC and APEC. However, not all strains of *E. coli* responded similarly to FA. In APEC strain TW731/12, FA downregulated genes essential for bacterial motility such as *flgB*. Since motility is essential for virulence, it indicates FA is again exhibiting antimicrobial effects. Indeed, a study stated that motility and antibiotic resistance could be related by showing mutations in genes previously implicated in antibiotic resistance resulting in loss of swarming motility of *Salmonella* Typhimurium (Kim et al., 2003).

PA had a different impact on AIEC when compared to FA. The data previously generated in our lab aligned with the results in this study. It showed that PA exposure significantly affects genes associated with virulence such as toxicity and biofilm formation in AIEC (Ormsby et al., 2020). Moreover, these distinct changes in transcription could indicate that FA is likely a more effective antimicrobial in the context in which it is used than PA. In addition, the difference between PA and FA in their effects on APEC and AIEC could be attributed to the acidity of these organic acids (Zhang et al., 2009). The antimicrobial efficacy of organic acids depends on factors such as their ability to pass through bacterial membranes and their acidity (Zhang et al., 2009). FA might be more efficient and transferrable to the bacterial membrane than other organic acids since it is more acidic than PA and is considered one of the simplest carboxylic acids (Tugnoli et al., 2020).

Further, the impact of FA and PA on *E. coli* was investigated using an *in vitro* fermentation gut model as described in Chapter 5. We have developed this fermentation model which is an *in vitro* fermentation batch model that mimics the chicken gut to understand how PA and FA affect *E. coli* and microbiome. This model is simplistic but gave interesting data, such as the significant increase in *Escherichia* when both PA and chicken feed were present in the fermentation. Moreover, *E. coli* was readily recovered under these fermentation conditions while FA had significant less *E. coli* recovered, again indicating FA could inhibit *E. coli*. Unlike FA, more *E. coli* were recovered from fermentations with PA which could indicated that PA was less inhibitory effect than FA. In addition, FA and PA both had effects on SCFA production, a potentially important finding in the context of chicken gut health. PA increased total SCFAs but decreased the abundance of butyric acid that has an essential role in immune responses of the host and colonic health (Makowski et al., 2022; Załęski et al., 2013). In contrast, FA increased the abundance of butyric acid in the presence of feed.

6.3 Conclusions

In conclusion, our findings highlighted the importance of phenotypic alterations in AIEC and their association with adaptation to the intestinal conditions observed in CD. Our data provides insights into the role of FA and PA in phenotypic alteration of AIEC and add further weight to the hypothesis that environmental factors can be associated with the aetiology of CD. Moreover, the effect of organic acids on APEC strains differs widely depending on the strains, with some strains of APEC, like strain 601, exhibiting a similar response to AIEC when adapted to FA. In future we hope this might contribute to our understanding of pathogenicity of this diverse pathotype, with APEC strains exhibiting a surprisingly diverse response to different signals and antimicrobials. Moreover, the common practice of continuously using organic acids as antimicrobials in livestock could result in an alteration of APEC strains, which might be a potential for zoonotic disease. Overall, PA and FA can induce phenotypic alterations in AIEC and APEC, resulting in increasing factors associated with virulence within bacterial strains. In contrast, FA and PA are effective as antimicrobials in the context of other bacterial pathogens of poultry such as *Salmonella* and *Campylobacter*, but their potential for adverse long-term effects on APEC strains will continue to be a concern.

6.4 Future work

We have shown that AIEC can adapt to conditions similar to those in CD. Therefore, the alterations we see would likely provide AIEC with a significant advantage in the CD intestine. In that context, we investigated the FA and PA as environmental factors that can cause alteration in the phenotype of AIEC. In addition, the phenotypic alteration of APEC was investigated since APEC is evolutionarily and phylogenetically related to AIEC. Moreover, APEC was investigated due the fact that it causes a potential risk of zoonotic disease. Indeed, this study has generated several unaddressed questions that require further investigation. To thoroughly understand how FA alter the phenotypic of APEC strains, they need to be examined on an avian cell line. Our findings show FA adaptation has no effect on net replication, adhesion, or invasion of APEC strains except for 601, where it has significantly increased adhesion. Indeed, these factors were examined on human cell lines; therefore, examining the effect of FA on avian cell lines might provide a better understanding of how long-term exposure to FA alters the APEC strains which their pathogenicity remains unclear.

Furthermore, a limitation was detected in the cell viability assay. It is unclear the reason for the high percentage of dead cells in a few strains, and this could affect the interpretation of data. Therefore, using Flow cytometry to measure cell viability might help overcome this limitation. In fact, flow cytometry can be used to count the cells and checked viability though the dyes that bind to DNA such as propidium iodide. This dye is not able to cross the cell membrane, however in the case of dead or dying cells, on which the plasma membrane is compromised, and this dye can readily permeate and through intercalation bind to the cell DNA and generate a specific fluorescent signal. Indeed, this would be useful to determine the impact of adapted strains to FA on cell viability. Another limitation was detected in the motility assays. The high percentage of agar that is used in the twitching motility assay could be the reason for not detecting the differences between the adapted strains to non-adapted. Therefore, using different low agar motility assays, such as the swarming and swimming assays, can help investigate whether the agar percentage is a factor for not detecting motility differences between *E. coli* strains. In addition, several cluster of genes can be validated such as *hyc*, *flg*, *nap,* and *pur* to determine how the FA exposure impact the bacterial strains.

Furthermore, we investigated the effects of FA and PA on *E. coli* independently using the *in vitro* fermentation gut model. Using a combination of organic acids as an antimicrobial agent is a novel strategy in agriculture. Thus, the effects of FA and PA mixtures on *E. coli*

strains can be investigated. Moreover, measuring SCFAs was part of our investigation to determine the effect of organic acids. However, one of the SCFAs cannot be measured by gas chromatography. This type of gas chromatography has limitations in detecting compounds such as FA that have only one carbon atom. Therefore, using a different type of gas chromatography, such as gas chromatography-mass spectrometry or another instrument, such as Nuclear magnetic resonance spectroscopy, will aid in measuring FA. This work emphasises the need to investigate the long-term effect of SCFAs that are used as antimicrobials and provides methods that can be used for future work.

Chapter 7

Appendices

I. Appendix

- Publication related to this work.

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Inflammation associated ethanolamine facilitates infection by Crohn's disease-linked adherent-invasive *Escherichia coli*

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ABSTRACT

Background: The predominance of specific bacteria such as adherent-invasive *Escherichia coli* (AIEC) within the Crohn's disease (CD) intestine remains poorly understood with little evidence uncovered to support a selective pressure underlying their presence. Intestinal ethanolamine is however readily accessible during periods of intestinal inflammation, and enables pathogens to outcompete the host microbiota under such circumstances.
Methods: Quantitative RT-PCR (qRT-PCR) to determine expression of genes central to ethanolamine metabolism; transmission electron microscopy to detect presence of bacterial microcompartments (MCPs); *in vitro* infections of both murine and human macrophage cell lines examining intracellular replication of the AIEC-type strain LF82 and clinical *E. coli* isolates in the presence of ethanolamine; determination of *E. coli* ethanolamine utilization (*eut*) operon transcription in faecal samples from healthy patients, patients with active CD and the same patients in remission following treatment.
Results: Growth on the intestinal short chain fatty acid propionic acid (PA) stimulates significantly increased transcription of the *eut* operon (fold change relative to glucose: >16.9; *p*-value <.01). Additionally ethanolamine was accessible to intra-macrophage AIEC and stimulated significant increases in growth intracellularly when it was added extracellularly at concentrations comparable to those in the human intestine. Finally, qRT-PCR indicated that expression of the *E. coli eut* operon was increased in children with active CD compared to healthy controls (fold change increase: >4.72; $P < .02$). After clinical remission post-exclusive enteral nutrition treatment, the same CD patients exhibited significantly reduced *eut* expression (Pre *vs* Post fold change decrease: >15.64; $P < .01$).
Interpretation: Our data indicates a role for ethanolamine metabolism in selecting for AIEC that are consistently overrepresented in the CD intestine. The increased *E. coli* metabolism of ethanolamine seen in the intestine during active CD, and its decrease during remission, indicates ethanolamine use may be a key factor in shaping the intestinal microbiome in CD patients, particularly during times of inflammation.
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1. Introduction

Adherent-invasive *E. coli* (AIEC) are over-represented in the ileal microbiota of Crohn's disease (CD) patients, being present in 51.9% of mucosal samples from CD patients compared with 16.7% in healthy controls [1–5]. Alterations in the gut microbiota composition of patients

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Research in context

Evidence before this study

Adherent-invasive *Escherichia coli* (AIEC) have been implicated in the aetiology of Crohn's disease (CD), being isolated in consistently greater numbers from CD patients compared to healthy controls. The reasons underlying this association however are poorly understood. Additionally the ability of AIEC to replicate to high numbers within macrophages indicated that a readily available carbon source must be present in the intestine.

Added value of this study

In this study we have determined that the intestinal short chain fatty acid propionic acid acts as a signal for AIEC to alter their metabolism and increase their use of ethanolamine, an intestinal metabolite known to be used by pathogens during times of inflammation. To date the rapid replication of AIEC in macrophages has been unexplained, but we have shown here that this rapid intracellular growth can be facilitated by the presence of extracellular levels of ethanolamine comparable to those in the human intestine. Lastly, we have shown the clinical relevance of our findings by detailing the increased metabolism of ethanolamine by *E. coli* in pediatric patients with active CD, and a significant reduction upon remission in the same patients.

Implications of all the available evidence

Our study has revealed an important role for PA as a signaling molecule for AIEC, allowing it to adapt to life in the inflamed CD intestine through the use of ethanolamine. The ability to utilize ethanolamine, which is released during times of intestinal inflammation, renders AIEC able to out-compete commensal microbes under the conditions seen in CD. The increased *E. coli* metabolism of ethanolamine seen in pediatric CD patients with active disease, when compared to healthy controls and those in remission, strongly suggests that ethanolamine is a key metabolite in the shaping CD microbiome.

suffering from CD are well reported with the majority of studies reporting an increase in the abundance of Proteobacteria, of which AIEC are members, and a decrease in Firmicutes [6,7]. While AIEC strains harbor genetic similarity to extra-intestinal pathogenic *E. coli* (ExPEC) in terms of phylogenetic origin and virulence genotype, the factors underlying their virulence have proved more difficult to identify [8]. In addition, the discovery of AIEC strains across all five major diverse phylogroups of *E. coli* means that an overarching explanation for the origin and virulence of AIEC has remained out of reach.

Intestinal pathogens utilize various mechanisms to outcompete the host intestinal microbiota, thus increasing their ability to persist and cause disease. These mechanisms include induction of inflammation, direct or indirect killing of commensals, or exploitation of alternative carbon sources [9–11]. Intestinal pathogens use a variety of carbon sources during infection: *Escherichia coli* and *Clostridium perfringens* using sialic acid [12,13]; Enterohaemorraghic *E. coli* (EHEC) consume galactose, hexuronates and ribose [14]; while *Yersinia enterocolita* and *Salmonella enterica* serovar Typhimurium use the adenosyl-cobalamin, 1,2-Propanediol degradation (1,2-PD; *pdu*) and tetrathionate operons in concert to catabolize 1,2-PD under the anaerobic conditions found in the gut [15]. Recent evidence also suggested a role for 1,2-PD metabolism during adherent-invasive *E. coli* (AIEC) colonization with the *pdu* operon shown to be overrepresented within this CD-associated pathotype and possibly playing a role in driving systemic inflammation, although other work has questioned this link [16–18].

Along with 1,2-PD, phosphatidylethanolamine, a ubiquitous component of host cell membranes, is abundant in the inflamed intestine and is readily hydrolysed into ethanolamine and glycerol [19,20]. Ethanolamine can be used as a carbon and nitrogen source by a variety of intestinal pathogens such as *S.* Typhimurium, enterohaemorrhagic *E. coli* (EHEC), *Enterococcus faecalis*, *Listeria monocytogenes* and *Clostridium difficile* [21–25]. Inflammation associated with infection renders 1,2-PD and ethanolamine available for metabolism as reduced tetrathionate is released allowing its use as a terminal electron acceptor for growth on these carbon sources. The inflammatory environment of the CD intestine similarly offers access to these alternative carbon sources as tetrathionate is again released and available to facilitate 1,2-PD and ethanolamine metabolism [21,23,26,27]. Although critical to outgrowth of intestinal pathogens during inflammation, many bacteria cannot readily use these carbon sources [17,22,28].

Recently, we have shown that exposure of AIEC to propionic acid (PA), an abundant intestinal short chain fatty acid (SCFA), results in modulation of the key phenotypic traits of the AIEC pathotype, rendering PA-exposed bacteria more adherent, invasive and persistent [29]. This is in stark contrast to the antimicrobial and anti-virulence effects PA exerts on other intestinal pathogens such as *S.* Typhimurium and *Campylobacter spp.* [30–38]. Here we show that the intestinal SCFA PA causes AIEC to significantly increase its ethanolamine metabolism. To overcome the toxic by-products associated with ethanolamine use, AIEC synthesize and then excrete bacterial microcompartments (MCPs). Additionally, ethanolamine added extracellularly to macrophages, at concentrations comparable to those of the human intestine, stimulated rapid intracellular proliferation of AIEC. Finally, we determined the clinical relevance of these findings, establishing that despite *E. coli* numbers remaining unchanged in patients with active CD, ethanolamine use was significantly increased. However, ethanolamine metabolism was significantly reduced in these same patients upon treatment leading to clinical remission.

2. Methods

2.1. Bacterial strains and growth conditions

Strains used in this study are listed in Supplementary Table S1 and were routinely grown at 37 °C at 180 rpm in Lysogeny broth (LB) or M9 minimal medium ([20% M9 salts (32 g $Na_2H_2PO_42H_2O$, 12.5 g NaCl, 2.5 g NH_4Cl, 7.5 g KH_2PO_4 and 400 ml H_2O], 0.1% trace metal solution, 0.2 mM $MgSO_4$, 0.02 mM $CaCl_2$, 1 mM thiamine, 0.01% 5 g/l $FeCl_3$, 0.01% 6.5 g/l ethylenediaminetetraacetic acid (EDTA), 0.1% taurocholic acid and dH_2O) supplemented with D-glucose (10 mM), sodium propionate (PA; 20 mM), 1,2-propanediol (1,2-PD; 20 mM) or ethanolamine (ethanolamine; 20 mM). Strains for infection were grown overnight in 10 ml cultures of RPMI-1640 supplemented with 3% fetal calf serum (FCS; heat-inactivated) and 2 mM L-glutamine before being back-diluted the following morning into 10 ml of the same media. These were then grown at 37 °C at 180 rpm to an optical density at 600 nm (OD_{600}) of 0.6 before further dilution to give a final multiplicity of infection (MOI) of 10. For transmission electron microscopy (TEM), isolates were grown in No-Carbon-E (NCE) media supplemented with 20 mM glucose or PA at 37 °C, to an OD_{600} of 0.6. For real-time PCR (qRT-PCR), bacteria were grown aerobically in NCE media [39]. Twenty millimolar PA, 1,2-PD, ethanolamine or D-glucose were added with 200 nM cyano-cobalamin to act as an electron acceptor [40]. Cultures were grown overnight in LB, washed three times in NCE media with no carbon source added, and inoculated 1:100 into 10 ml NCE media containing each respective carbon source. Cultures were grown until mid-log phase (OD_{600} of 0.6) and used for RNA-extraction. *eutR* deletion strains were generated by Lambda red-mediated mutagenesis as previously

described [41] using the primers *eutR* KO For and eutR KO Rev (Supplementary Table S2). *eutR* deficient strains were confirmed using the primers Δ*eutR* Check For and Δ*eutR* Check Rev All chemical suppliers are listed in Supplementary Table S4.

Clinical isolates (B94, B115, B122 and B125) were from the "Bacteria in Inflammatory bowel disease in Scottish Children Undergoing Investigation before Treatment" (BISCUIT) study [42]. All isolates were recovered from patients with Crohn's disease. The median (range) age was 13.7 (11.2 to 15.2), height z-score was −0.4 (−2.0 to 0.2), weight z-score was −0.7 (−3.4 to −0.1), and BMI z-score was −1.3 (−4.0 to 0.4). Symptom duration prior to diagnosis was median 7.5 months [5 to 12]. 50% had granulomas present on initial histology. Phenotypes by Paris criteria [43] at diagnosis were: B94- colonic, non-stricturing/non-penetrating (L2, B1); B115- colonic, non-stricturing/non-penetrating (L2, B1); B122- ileocolonic, stricturing (L3, B2); B125- ileocolonic, non-stricturing/non-penetrating (L3, B1). This study is publically registered on the United Kingdom Clinical Research Network Portfolio (9633).

2.2. Transmission electron microscopy

Five microlitre suspension droplets were placed onto the glow discharged surface of 300 mesh Formvar/carbon coated nickel grids and left to settle for 2 min. Grids were then placed sample side down onto a 30 μl droplet of 2% ammonium molybdate for 30 s prior to air-drying. Samples were viewed on a FEI Tecnai T20 TEM running at 200 kV and images captured using a GATAN Multiscan 794 camera and GATAN Digital Imaging software (DM4 converted to TIFF).

2.3. Total RNA extraction

Bacterial cultures were grown as above and mixed with two volumes of RNAprotect reagent, before incubating for 5 min at room temperature. Total RNA was extracted and genomic DNA removed as described previously [44].

2.4. Patient faecal samples

The whole bowel movement was collected, stored in a cool bag under anaerobic conditions (Oxoid™ AnaeroGen™) and transferred to the laboratory within three hours of defecation [45]. The whole sample was homogenized with mechanical kneading and aliquots were stored in RNAlater at −70 °C.

Samples were collected from 10 newly diagnosed, treatment naïve children (11.4 (Q1:8.5, Q3:15.3 years; 5 female) with active CD undergoing an 8-week induction treatment with exclusive enteral nutrition (EEN) with a polymeric casein-based liquid feed (Modulen) as described previously [46]. No other food was allowed. A first sample was collected before EEN initiation and another at the end of the eight-week treatment. A single faecal sample was collected from healthy children with no family history of inflammatory bowel disease to serve as a control group. Children with CD and healthy controls were matched for age and gender. Participants that had received antibiotics three months before or during the study were excluded.

Disease activity was monitored during the course of the EEN treatment using the weighted pediatric CD Activity Index (wPCDAI) [47]. Faecal calprotectin (FC), an established marker of colonic inflammation was measured with the Calpro ELISA kit as described previously (Supplementary Table S3) [48].

2.4.1. Ethics statement

Patients were recruited from the pediatric gastroenterology clinics at the Royal Hospital for Children, Glasgow and healthy controls from the same background community using leaflet advertisement. Children with CD were diagnosed according to the revised Porto criteria [49]. All participants and their carers signed informed consent. The study was approved by the NHS West of Scotland Research Ethics Committee (14/WS/1004) and was registered at www.clinicaltrials.gov (NCT02341248).

2.5. Total RNA extraction from faecal samples

Faecal samples were stored at −80 °C in RNAlater. To extract RNA, samples were thawed on ice before brief centrifugation to remove RNAlater. Approximately 250 μg of faecal material was then subjected to RNA isolation using the RNeasy PowerMicrobiome Kit. RNA quantity and quality was estimated using a NanoDrop (ThermoFisher Scientific) spectrophotometer and DNA depletion confirmed through PCR using 16S primers (Supplementary Table S2).

2.6. Quantitative real-time PCR (qRT-PCR)

cDNA was generated from total RNA using an Affinity Script cDNA multi-temp Synthesis Kit following the manufacturer's instructions. Levels of transcription were analysed by qRT-PCR using PerfeCTa SYBR Green FastMix. Individual reactions were performed in triplicate within each of three biological replicates. The 16S rRNA gene was used to normalize the results. RT-PCR reactions were carried out using the CFX Connect Real-Time PCR Detection System (BIO-RAD Laboratories, Inc.) according to the manufacturer's specifications and the data were analysed according to the $2^{-\Delta\Delta CT}$ method [50]. All primers used are listed in Supplementary Table S2.

2.7. Cell culture and maintenance

The RAW264.7 murine macrophage cell line obtained from the American Type Culture Collection (ATCC) was maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin. THP-1 (ECACC 88081201) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) as growing cultures. Cells were maintained in 10% FCS, 2 mM L-glutamine and penicillin/streptomycin. All cells were maintained at 37 °C and 5% CO_2 with regular media changes.

2.8. Cell culture infection

Both RAW264.7 macrophages and THP-1 cells were seeded at 1×10^5 cells per well of a 24-well plate 48 h prior to infection. THP-1 cells were differentiated for 24 h in the presence of 200 nM phorbol 12-myristate 13-acetate (PMA) to activate macrophages. After activation, the medium was removed, and the cells washed prior to infection to remove dead or non-adherent cells and left for a further 24 h. RAW264.7 macrophages were treated with 100 ng/ml lipopolysaccharide to induce an activated state 24 h after seeding. Infections for both cell types were carried out in RPMI media supplemented with 3% FCS and L-glutamate. Infections were carried out at an MOI of 10. After 1 h the bacteria that had not been internalized were killed by adding 50 μg/ml gentamycin sulfate (Sigma-Aldrich) and the infection allowed to proceed.

2.9. Statistical analysis

All statistical tests were performed with GraphPad Prism software. All replicates in this study were biological; that is, repeat experiments were performed with freshly grown bacterial cultures or mammalian cell lines, as appropriate. Technical replicates of individual biological replicates were also conducted, and averaged. Values are represented as means ± standard deviation. Significance was determined using *t*-tests (multiple and individual as indicated in the figure legends) and ANOVA (one-way or two-way) corrected for multiple comparisons with a Tukey's *post hoc* test (as indicated in the figure legends). qRT-PCR data was log-transformed before statistical analysis. For patient

samples statistical analyses were preformed using GraphPad Prism, with data analysed by one-way ANOVA followed by Dunns multiple comparisons post-test. Values were considered statistically significant when P values were $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

3. Results

3.1. The intestinal short chain fatty acid PA stimulates AIEC degradation of ethanolamine

We previously observed that pre-exposure of the wild type AIEC strain LF82 to PA (termed LF82-PA), resulted in a more virulent phenotype [29]. Comparing growth of LF82-PA on ethanolamine as a sole carbon source relative to wild type LF82, indicated that LF82-PA could utilize ethanolamine more rapidly resulting in higher biomass (doubling time [OD_{600nm} 0.1 to 0.2] LF82-PA: 2.5 h; LF82: 6.7 h) (Fig. 1). No difference between the LF82 and LF82-PA strains was noted previously in rich nutrient media, indicating this was not a universal increase in growth rate post-PA exposure [29]. Subsequent deletion of *eutR*, the regulator of the *eut* operon, removed the ability of LF82 and LF82-PA to grow on ethanolamine (Fig. 1). Despite long-term exposure, LF82 was unable to efficiently metabolise the other predominant intestinal SCFAs, acetate and butyrate, as sole carbon sources (Supplementary Fig. S1).

3.2. Ethanolamine degradation occurs in bacterial microcompartments (MCPs)

In the presence of the intestinal SCFA PA, transmission electron microscopy (TEM) revealed the release of outer membrane vesicles containing pentagonal shapes that we speculated to be MCP containing vesicles (Fig. 2a). MCPs are utilized by bacteria for growth on 1,2-PD and ethanolamine as their metabolism releases the toxic by-products propanol and ethanol that can then be sequestered into the MCP to protect the bacteria. We first examined the ability of LF82 to express MCPs from either the *eut* or *pdu* operons, which are known to encode MCPs. Outer membrane vesicles were not detected during growth on glucose (Fig. 2a).

To further understand the role of MCPs in ethanolamine metabolism and their link to intestinal SCFA levels, qRT-PCR examination of the genes central to PA metabolism (*prpB*) and MCP production (*eutS* and *pduC*) was undertaken in the presence of the relevant carbon sources. This revealed that, as expected, transcription of *prpB* was highest during growth on PA (fold change relative to glucose: 24.8; p-value <.0001); *pduC* was highest during growth on 1,2-PD (fold change relative to glucose: 28.2; p-value <.05); and *eutS* was highest during growth on ethanolamine (fold change relative to glucose: 76.7; p-value <.001). However, growth on the intestinal SCFA PA stimulated significantly

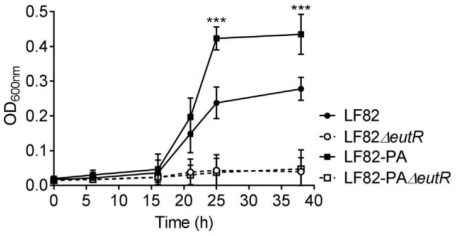

Fig. 1. PA stimulates AIEC degradation of ethanolamine. Anaerobic growth (OD_{600nm}) of LF82, LF82-PA and their corresponding *eutR* knock out mutants in minimal media (NCE) supplemented with ethanolamine (20 mM), $MgSO_4$ (1 mM), trace metals and sodium thiosulphate (40 mM). Data were analysed using a two-way ANOVA with Tukey; $p <$ .001 ***. Only significant differences between LF82 and LF82-PA are shown. There were no significant differences between mutant strains at any time point.

increased transcription of the *eut* operon (fold change relative to glucose: >16.9; p-value <.01) while the *pdu* operon was not significantly affected by PA presence (fold change relative to glucose: >8.4; p-value 0.79). Therefore exposure of LF82 to PA induces significant metabolic changes that adapt bacteria to utilize a carbon and nitrogen source readily available in the inflamed intestine.

3.3. Extracellular ethanolamine stimulates increased AIEC intramacrophage replication

Rapid intracellular replication in macrophages is a key phenotypic trait in AIEC [1,3,51]. However the mechanism behind this increased replication has remained elusive. In order to examine the effect of human intestinal levels of extracellular ethanolamine on the intramacrophage replication of LF82, we infected RAW264.7 macrophages with LF82 and LF82 that had been exposed to PA (LF82-PA) [22,31]. Supplementation of ethanolamine did not affect replication of wild type LF82 after 24 h at any concentration (Fig. 3). However, replication of LF82-PA within macrophages significantly increased in response to ethanolamine in a dose dependent manner (Fig. 3). Ethanolamine addition did not affect replication of the LF82Δ*eutR* strain or LF82-PAΔ*eutR*, which are unable to metabolise ethanolamine due to the removal of the regulator of the ethanolamine utilization operon *eutR* (Fig. 3). While RAW264.7 cells are commonly used to study AIEC virulence, to ensure the observed effects were not specific to murine macrophages this was repeated within the human monocyte THP-1 cell line where again ethanolamine significantly increased intracellular replication in a dose-dependent and *eut* dependent fashion (supplementary Fig. S2). Collectively, these data indicate that the intestinal short chain fatty acid PA induces increased survival and replication of LF82 within macrophages in the presence of concentrations of ethanolamine found in the human intestine.

3.4. The PA-driven enhanced intracellular replication phenotype is mirrored in other clinical AIEC isolates

E. coli isolated from intestinal biopsies of pediatric patients with active CD were examined for their ability to replicate intracellularly in macrophages in the presence of extracellular ethanolamine. The characteristics of these isolates has been determined previously with all isolates exhibiting an AIEC phenotype after PA-exposure with an ability to; adhere to and invade intestinal epithelial cells, replicate within macrophages and form biofilms both aerobically and anaerobically [29]. PA pre-exposure resulted in significantly increased intracellular replication in comparison to the unexposed wild type in two of four strains, whilst in the two others the increase was not significant (Supplementary Fig. S3). The increasing concentrations of ethanolamine significantly increased intracellular replication of isolates B115 and B125 in a dose dependent manner. Due to an inability to metabolise PA, we were unable to generate an adapted strain of the commensal *E. coli* F-18 strain. In any case, F-18 showed a distinct lack of intracellular replication. Collectively, these data indicate that the intestinal SCFA PA induces increased survival and replication of LF82 and clinically relevant *E. coli* within macrophages in the presence of physiologically relevant concentrations of ethanolamine.

3.5. Expression of the eut operon correlates with inflammatory status in pediatric CD patients

Given the significance of ethanolamine utilization in facilitating *in vitro* infection by LF82 we examined faecal samples from pediatric CD patients, before and after treatment, compared to healthy controls to determine any relevance of the *eut* operon to disease status. All CD patients had active disease at treatment initiation (wPCDAI >12.5). By the end of treatment with exclusive enteral nutrition (EEN), (the first-line therapy for pediatric CD), 8/10 (80%) of patients had clinically

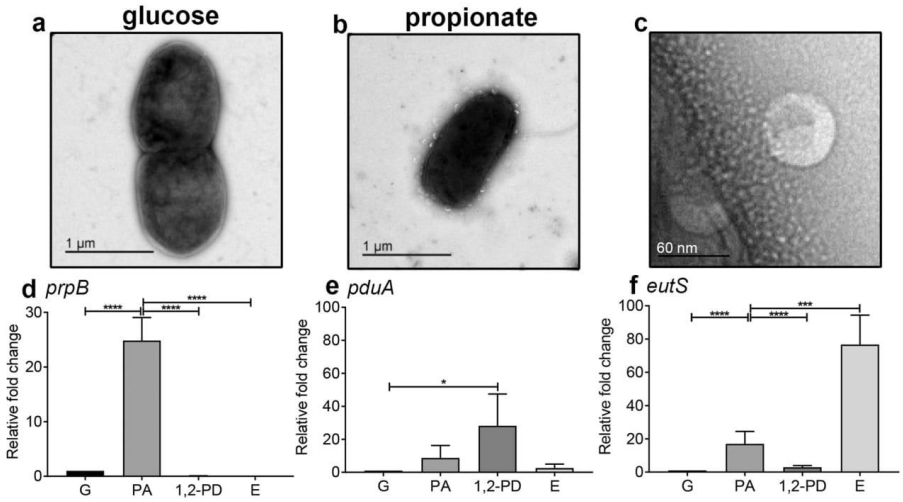

Fig. 2. Growth on PA stimulates the production of bacterial MCPs for the utilization of ethanolamine as a carbon source. For TEM, cultures of LF82 were grown in NCE media supplemented with cobalamin (200 nM) and either (a) glucose or (b) PA, at a final concentration of 20 mM. (c) A close up of a MCP-containing outer membrane vesicle from a PA supplemented culture is shown. qRT-PCR was conducted on LF82 grown in NCE media supplemented with cobalamin (200 nM) and either glucose (G), propionic acid (PA) 1,2-propanediol (1,2-PD) or ethanolamine (E) at a final concentration of 20 mM. Relative fold change of (d) *prpB*, (e) *pduC* and (f) *eutS* were measured relative to their expression in the presence of glucose, using 16S rRNA as an internal control. Four independent biological replicates were performed. Data are expressed as relative fold change ± SD and were analysed using a one-way ANOVA with Tukey; $p < .05$ *; $p < .001$ ***.

improved (wPCDAI decrease >17.5), with 6/10 (60%) of patients having entered clinical remission (mean wPCDAI treatment start: 42.25 [23.4]; wPCDAI treatment end: 11 [12.4]). There was also a significant decrease in faecal calprotectin levels during the course of treatment from initial mean values of 1561 mg/Kg (SD:596) at the start of treatment, decreasing to 1037 mg/Kg (592, 1614) by the end of EEN (Supplementary Table S3).

Quantitative RT-PCR (qRT-PCR) indicated an increase in *E. coli eutS* expression in children with active CD compared to healthy controls (Fig. 4; Fold change increase: >4.72; $P < .02$). A significant decrease in *eutS* expression was observed post-EEN treatment across all CD patients (Pre *vs* Post Fig. 4; Fold change decrease: >15.64; $P < .01$). This drop in *eutS* copy number was most significant in patients with reduction in their faecal calprotectin levels as an indicator of colonic inflammation (Supplementary Table S3). Subsequent analysis of total *E. coli* numbers using 16S gene copy number revealed no significant differences between healthy controls, CD patients pre-treatment and the same patients post-treatment (Supplementary Fig. S4). This indicates that the observed differences in *eutS* copy number are not due to fluctuating *E. coli* levels but due to transcriptional differences.

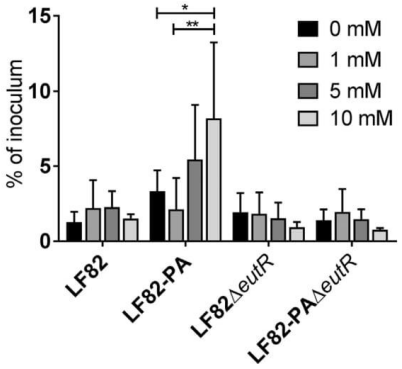

Fig. 3. Extracellular ethanolamine increases intracellular replication of LF82-PA. Intramacrophage (RAW264.7) survival and replication of wild type, PA-adapted, and LF82ΔeutR at 24 h post-infection with or without ethanolamine supplementation. For all values, the mean ± SD of three independent biological replicates are shown. Statistical analyses were preformed using GraphPad Prism, with data analysed by two-way ANOVA ($p < .05$ *; $p < .01$ **; $p < .001$ ***).

4. Discussion

The role of AIEC in the pathology of CD has remained an enigma since the recovery of the first strain from a CD patient nearly 20 years

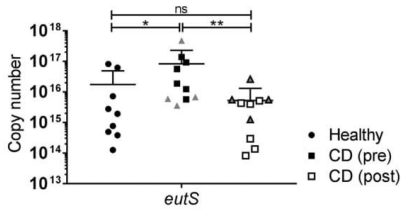

Fig. 4. qRT-PCR *of eutS* in healthy patients, active Crohn's disease patients and Crohn's disease patients following EEN. Ethanolamine utilization was determined by abundance of *eutS* transcripts. *eutS* was amplified using primers designed against LF82. Transcript levels were normalized to 16S rRNA transcripts. Healthy samples ($n = 9$); Crohn's disease samples pre-treatment (CD pre; $n = 10$); Crohn's disease samples post-treatment (CD post; $n = 10$). Crohn's disease samples pre- and post-treatment were paired. Patients were marked as responders (squares) and non-responders (triangles) based on their drop in calprotectin levels (Supplementary Table S1). Statistical analyses were preformed using GraphPad Prism, with data analysed by one-way ANOVA followed by Dunns multiple comparisons post-test ($p < .05$ *; $p < .01$ **).

ago [52]. Subsequent work indicating their predominance in both CD and colorectal cancer patients has led to numerous studies looking for a common denominator linking all distantly related AIEC strains that are now found widely spread across all *E. coli* subtypes [53,54]. Here we present evidence indicating a common intestinal carbon and nitrogen source, ethanolamine, can be readily used by the AIEC type strain LF82 and other AIEC strains isolated from CD patients. The significance of ethanolamine in AIEC virulence however is three-fold; its utilization is stimulated by the intestinal short chain fatty acid PA; it is available to AIEC enabling increased growth within macrophages when added extracellularly at intestinal concentrations, and its utilization within the intestine directly correlates with inflammatory status of pediatric CD patients.

Our previous work indicated that prior-exposure of AIEC to PA results in a pathogen with enhanced abilities to adhere to and invade intestinal epithelial cells, tolerate acidic conditions, form better biofilms and persist in an animal model of infection [29]. Here we show that PA pre-exposure also acts as a metabolic signal, stimulating degradation of ethanolamine. The action of PA as a positive signal for colonization by AIEC of the inflamed intestine is in stark contrast to that of pathogens such as *Campylobacter* spp. and *S.* Typhimurium, where the high PA concentration of the caecum and colon have been shown to represses virulence and colonization [33–38]. This ability to withstand the toxic effects of PA and use it as a positive inducer of virulence sets AIEC and a small number of other pathogens such as *Mycobacterium tuberculosis/avium* apart [55–57].

Notably, after exposure to PA the LF82-PA strain was more readily able to utilize ethanolamine as a carbon source, resulting in an increased rate of growth and increased biomass (Fig. 1). This observation was not surprising, given that growth on PA resulted in expression of *eut* (Fig. 2f) but it also enabled LF82-PA to significantly increase intra-macrophage replication (Fig. 3). Rapid intra-macrophage growth is a recognized feature of the AIEC pathotype [1,3,51] and may play a critical role in facilitating persistence in the intestine given numbers of macrophages and dendritic cells are increased in the mucosa of CD patients [58]. As ethanolamine is also more readily available for consumption under these conditions and our work demonstrates that increased extracellular ethanolamine is available to AIEC, ethanolamine may be a crucial carbon source in facilitating AIEC persistence in immune cells in the intestine [21]. Similarly, increased intra-macrophage proliferation due to the presence of extracellular ethanolamine has been demonstrated previously in *S.* Typhimurium indicating additional persistence strategies such as inhibition of programmed cell death may be needed to facilitate long-term intracellular ethanolamine use [25,59]. The driver for predominance of AIEC in CD, as opposed to other intestinal enterobacterial pathogens such as *S.* Typhimurium and *Y. enterocolitica*, remains unknown. However the ability of AIEC to positively respond to the normally antimicrobial SCFA PA may underlie this unique ability. While other intestinal pathogens are directly inhibited by this potent antimicrobial, AIEC can utilize PA, incorporate it, and respond to its presence [29,33–38]. Therefore, in the presence of PA, upregulation of the *eut* operon may give AIEC a distinct competitive advantage in the inflamed, ethanolamine replete CD intestine. Whether this capability is conserved across a wide range of *E. coli* strains or is distinct to AIEC is not yet known, but if widely conserved it may explain why *E. coli* as opposed to other bacteria dominate the CD microbiome. Indeed, utilization of ethanolamine may benefit AIEC through induction of a positive feedback loop whereby ethanolamine use leads to AIEC proliferation resulting in further inflammation and ethanolamine release.

We observed the secretion of MCPs inside OMVs during growth on PA (Fig. 2b and c). The suggested association between AIEC and the 1,2-PD utilization operon (*pdu*) and the close metabolic relationship between 1,2-PD degradation and PA production lead us to hypothesize that upregulation of the *pdu* operon would be responsible for their production [16,17,60]. Surprisingly, our analysis indicated these PA-induced MCPs were encoded by the *eut* operon which allows for degradation of ethanolamine (Fig. 2f). Ethanolamine confers on a number of pathogens an important metabolic advantage in out-competing the host microbiota during episodes of inflammation [21–24]. The *eut* derived MCP is known to enhance *E. coli* and *S. enterica* proliferation in diverse environments, including on food products, in *Caenorhabditis elegans* models of infection, during growth on bovine intestinal content, and in a murine model of infection [21,22,24,27]. It is therefore plausible that PA exposure provides AIEC with an enhanced ability to survive within regions of the intestinal tract that other pathogens cannot, signaling for upregulation of the genes necessary for ethanolamine metabolism.

As our *in vitro* findings indicated that *eut* metabolism conferred a significant growth advantage on AIEC in the context of infection, we next sought to investigate the significance of this in the context of AIEC predominance in CD patients. Using faecal samples taken over an eight week period we determined the levels of *eut* expression in patients both before and after induction treatment and compared these to healthy controls. All CD patients had undertaken an exclusive enteral nutrition (EEN) diet for the eight-week period after the initial sample was taken and faecal calprotectin levels were used to assess changes in colonic inflammation and to assign patients to either responder or non-responder groups (Supplementary Table S3). *eutS* levels were increased by >4.7 fold in CD patients compared to healthy controls indicating a significant increase in ethanolamine use in these patients (Fig. 4). However this effect was reversed by EEN treatment with both inflammation and *eutS* levels significantly reduced (>15.6 fold) and *eutS* levels not being significantly different post-treatment to healthy controls. While the availability of ethanolamine during inflammation is not surprising given previous reports, the direct correlation between CD activity and *E. coli eutS* transcript levels was unexpected. Samples from those patients who responded best to the treatment were shown to have the most significant reduction in *eutS* levels, further indicating a correlation between ethanolamine use and *E. coli* in the CD intestine. This is possibly explained by the observed drop in intestinal propionate levels seen after EEN treatment [45]. This reduction in the signaling molecule for AIEC metabolism of ethanolamine would lead to the type of *eut* transcription drop observed here. Further validation of this in another dataset of patients that have undergone a different mode of treatment induction would be a useful extension of this work.

This work highlights a new AIEC phenotypic trait that exhibits a direct correlation with severity of CD. Monitoring of *eut* expression within the CD intestine shows potential as a useful biomarker for monitoring severity of CD and overgrowth of *E. coli* in CD, perhaps predicting useful interventions using direct targeting of *E. coli* to alleviate disease [45]. This work also raises the possibility that other pathogens associated with CD may utilize similar metabolic strategies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.071.

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Declarations of interest

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Author contributions

MJO developed the initial concept, designed and performed the experiments, analysed the data and prepared the manuscript; ML recruited participants, collected and curated samples; SAJ helped develop the initial concept, assisted in experimental design and critically appraised the manuscript; AM provided technical assistance throughout the study; GF conducted intra-macrophage replication experiments; GLH and RH supplied clinical isolates and patient specific details from the BISCUIT study; RP and EP extracted DNA from faecal samples and conducted qPCR; RKR, ML and RH recruited participants and followed them up; KG & RKR designed the clinical study, applied for funding and ethical approvals; RKR, UZI, KG supervised and co-ordinated the clinical study; DMW developed the initial concept, designed the experiments, analysed the data and prepared the manuscript. All authors contributed to editing the manuscript for publication.

General

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II. Appendix

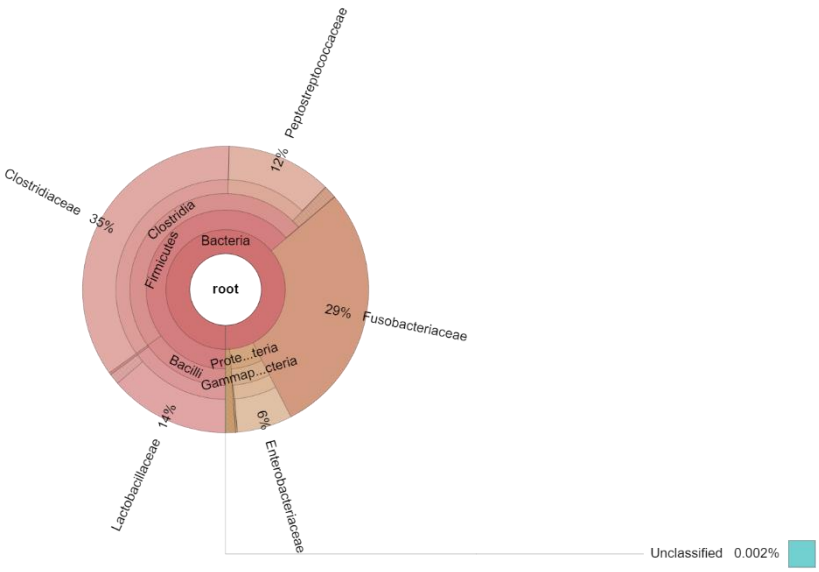
Appendix II, contain a file of RNA-seq data of *E. coli* exposed to FA.

Go to: Gene expression of *E. coli* strains exposed to FA

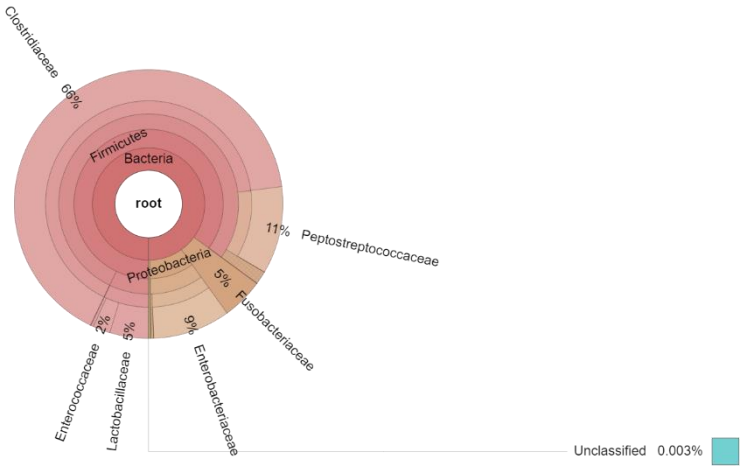
III. Appendix

The Krona graph displays the relative abundance of annotated taxa in *in vitro* fermentation samples from different fermentation conditions.

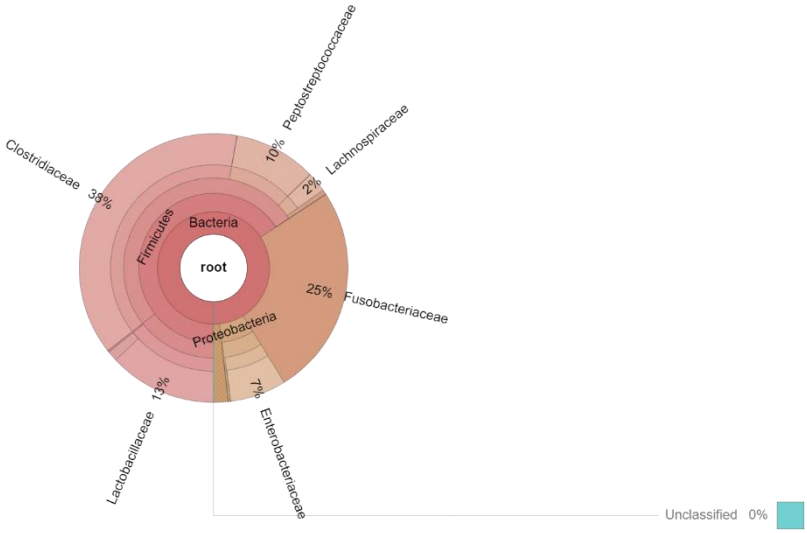
A. CF at 6h



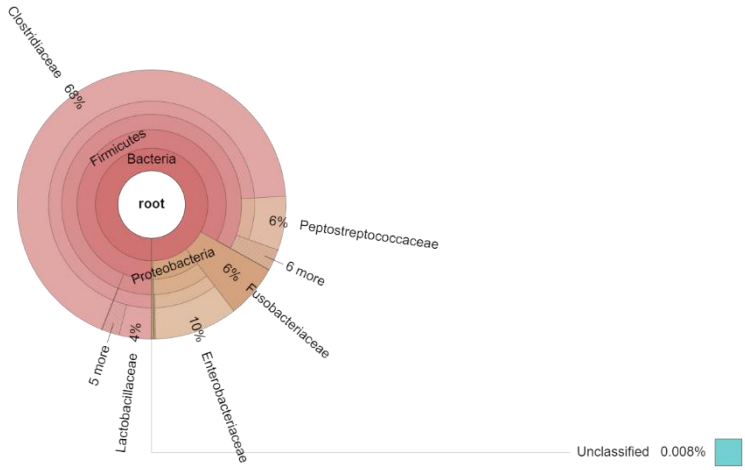
B. CF at 24h



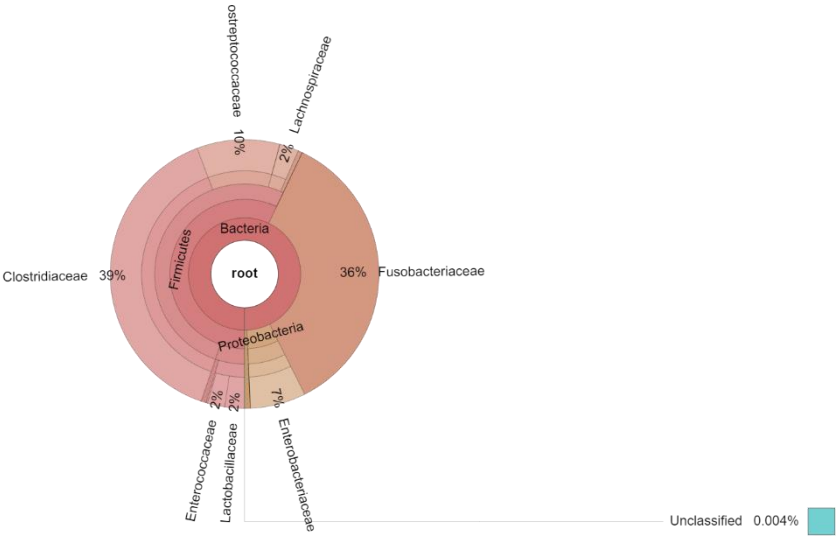
C. CF_601 at 6h



D. CF_601 at 24h



E. CF_601_ FA at 6h



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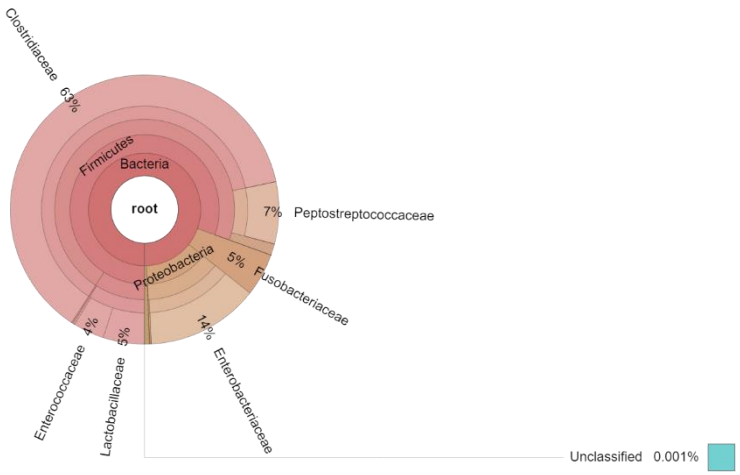

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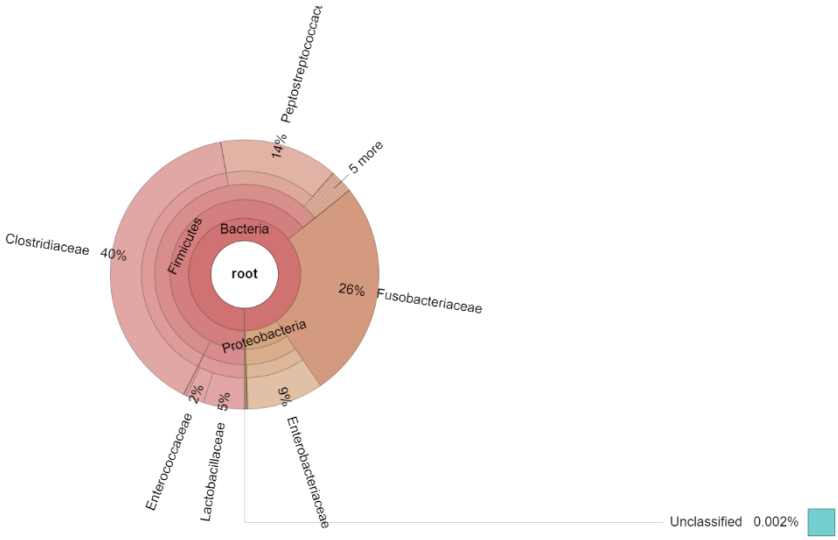

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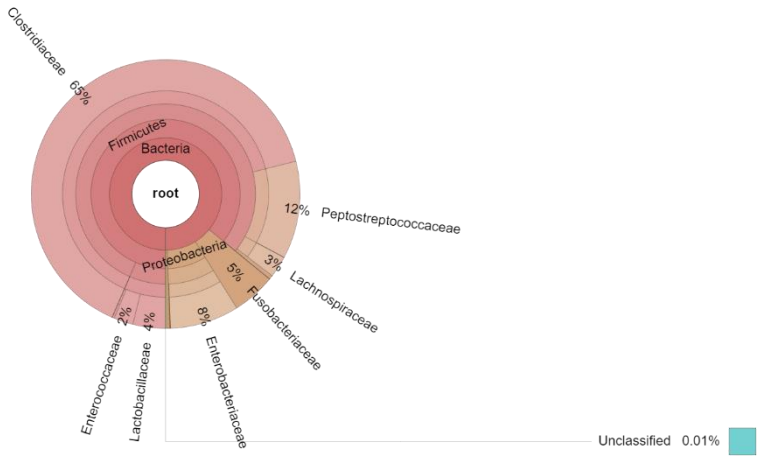

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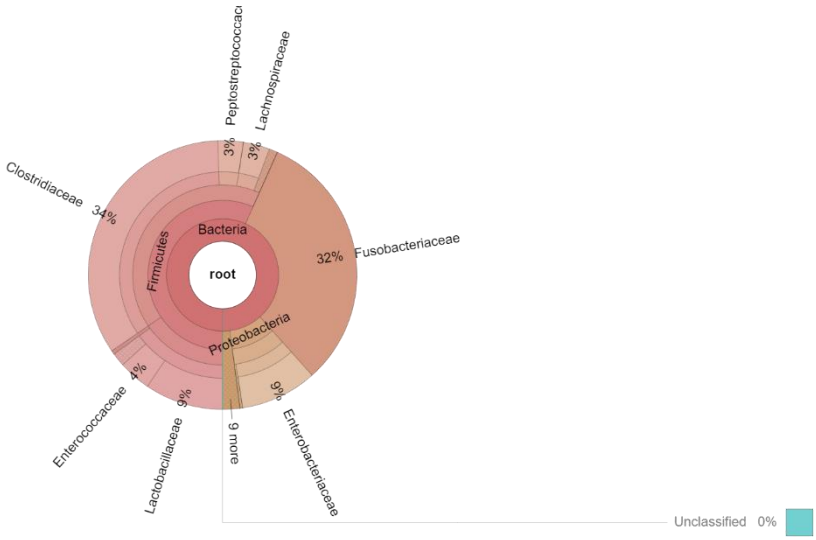

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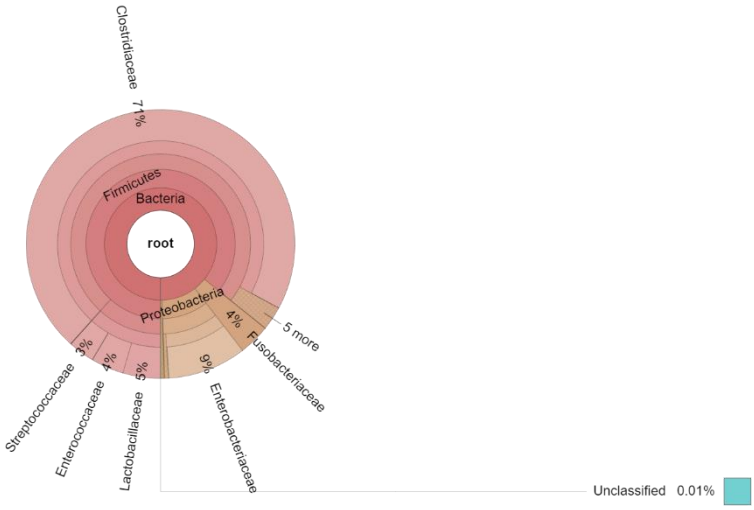

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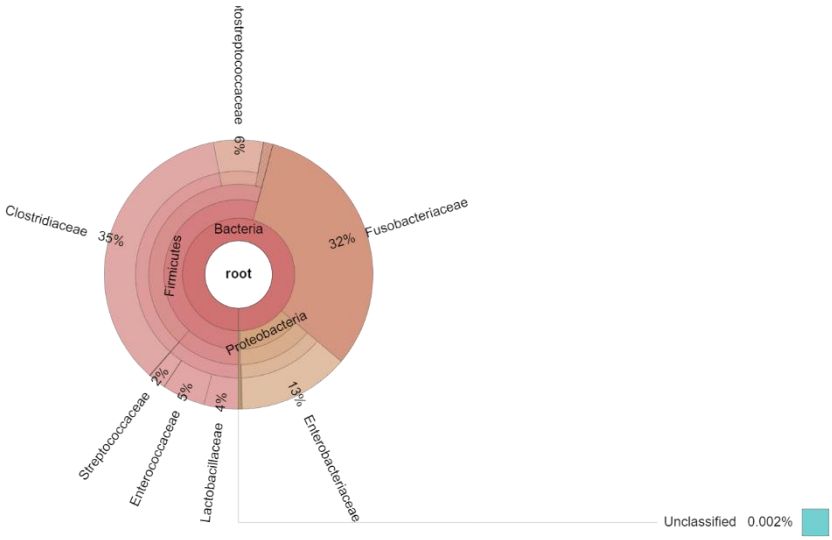

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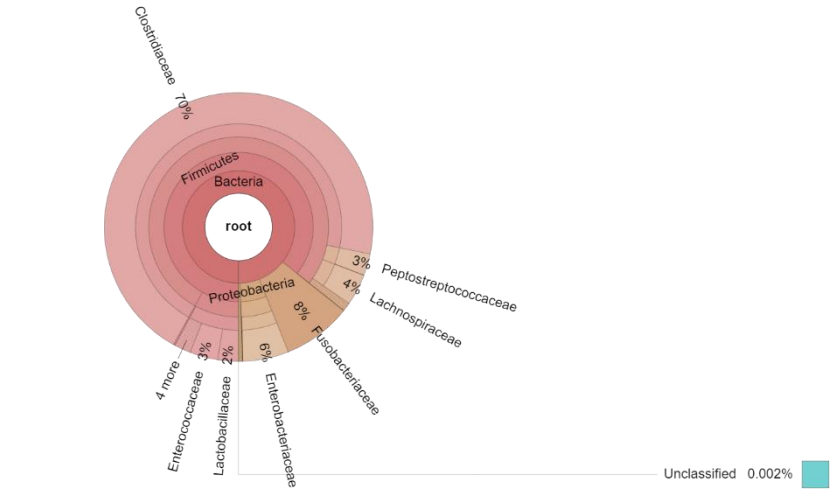

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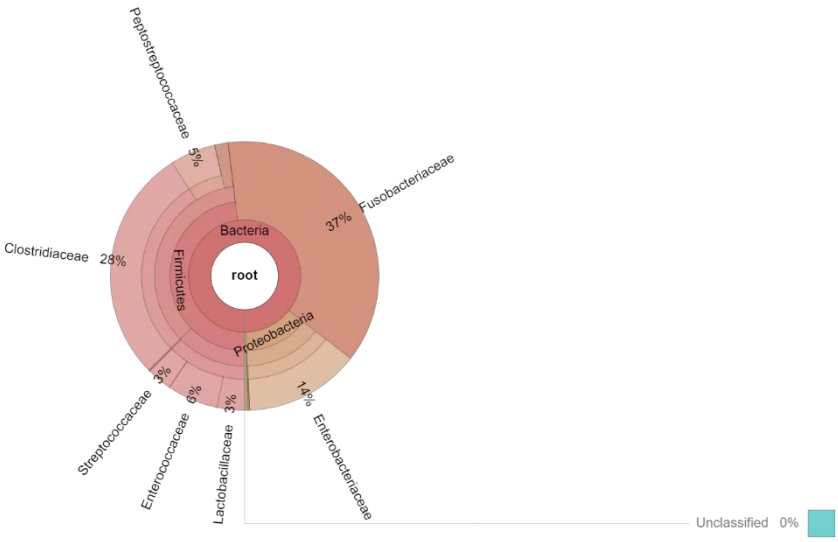

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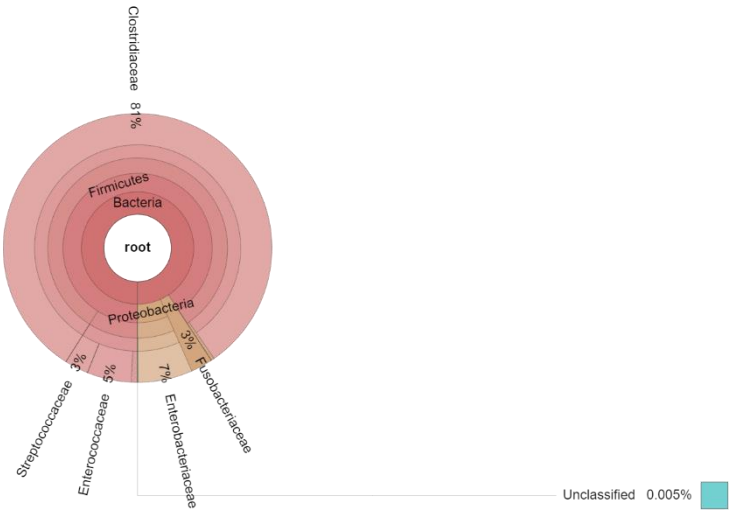

Figure 7-1. The relative abundance of families under each fermentation condition. (A-G) fermentation condition without feed, and (H-N) fermentation condition supplemented with feed.

Chapter 8

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