Horizontal Transfer of β-Lactam Resistance in the Mouse Gut Microbiota under Antibiotic Treatment

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ABSTRACT

The rise of β-lactam-resistant bacteria from agricultural settings, including food-producing animals and their related food products has become a significant public health concern. Consumption of food contaminated by such bacteria may cause infection as well as the transmission of resistance genes. Here we used a mouse model to assess the impact of different antibiotic treatments on the composition of the gut microbiota and any impact on the transfer of β-lactam resistance genes between donor and recipient bacteria. Mice were inoculated with β-lactam resistant *Escherichia coli* and an antibiotic-susceptible *Salmonella* Heidelberg strain. The mice were treated with either streptomycin, ampicillin or both antibiotics. Mouse feces were collected at regular intervals and processed using selective culture techniques to capture potential transfer of resistance genes. Gene transfer was confirmed by whole genome sequencing. DNA extracted from the feces was used for monitoring changes in microbial profiles by 16S rDNA sequencing. In the absence of antibiotic treatment, the inoculated bacteria were only transiently detected and no transconjugants were recovered from the mouse feces. In comparison, antibiotic treatment changed microbial profiles in the mouse gut, enhanced colonization of the bacterial isolates, and facilitated the transfer of the resistance genes into both *S.* Heidelberg and commensal *E. coli* recipient strains. The results of this study indicated that the use of multiple antibiotics may enhance infection of opportunistic β-lactam resistant bacterial pathogens relative to single antibiotics and pose a greater risk in terms of antibiotic resistance gene transfer. Such process might occur in clinical settings where patients are under prolonged antibiotic treatments. Information gained through this study together with future work will inform the development of new policies guiding the prudent use of antibiotics.

RÉSUMÉ

La montée des bactéries résistantes aux β-lactamines dans les milieux agricoles, y compris les animaux producteurs d'aliments et leurs produits alimentaires associés, est devenue un problème de santé publique important. La consommation d'aliments contaminés par de telles bactéries peut provoquer une infection ainsi que la transmission de gènes de résistance. Ici, nous avons utilisé un modèle de souris pour évaluer l'impact de différents traitements antibiotiques sur la composition du microbiote intestinal et l'impact sur le transfert de gènes de résistance aux β-lactames entre les bactéries donneuses et receveuses. Les souris ont été inoculées avec *Escherichia coli* résistant aux β-lactames et une souche de *Salmonella* Heidelberg sensible aux antibiotiques. Les souris ont été traitées avec de la streptomycine, de l'ampicilline ou les deux antibiotiques. Les excréments de souris ont été collectés à intervalles réguliers et traités en utilisant des techniques de culture sélectives pour capturer le transfert potentiel des gènes de résistance. Le transfert de gènes a été confirmé par séquençage du génome entier. L'ADN extrait des fèces a été utilisé pour surveiller les changements dans les profils microbiens par séquençage de l'ADNr 16S. En l'absence de traitement antibiotique, les bactéries inoculées n'ont été détectées que de manière transitoire et aucun transconjugant n'a été récupéré des fèces de souris. En comparaison, le traitement antibiotique a modifié les profils microbiens dans l'intestin de la souris, amélioré la colonisation des isolats bactériens et faciliter le transfert des gènes de résistance à la fois dans *S.* Heidelberg et dans les souches receveuses commensales d'*E. coli*. Les résultats de cette étude ont indiqué que l'utilisation de plusieurs antibiotiques peut augmenter l'infection d'agents pathogènes bactériens opportunistes résistants aux β-lactamines par rapport à des antibiotiques uniques et poser un risque plus élevé en termes de transfert de gènes de résistance aux antibiotiques. Cet processus peut se produire dans des contextes cliniques où les patients subissent des traitements antibiotiques prolongés. Les informations obtenues de cette

étude ainsi que les travaux futurs éclaireront l'élaboration de nouvelles régulations guidant l'utilisation prudente des antibiotiques.

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AAFC: Agri-Food and Agriculture Canada

AGP: Antibiotic growth promoter

Amp: Ampicillin

AmpC: Ampicillin class C

AMR: Antimicrobial resistance

CMY: Cephamycin

CTX-M: Cefotaxime-Munich

DNA: Deoxyribonucleic acid

DPI: Days post-infection

ESBL: Extended-spectrum β-lactamase

ESC: Extended-spectrum cephalosporins

GFP: Green-fluorescent protein

HGT: Horizontal gene transfer

HSD: Honest significant difference

Inc: Incompatibility

IRIDA: Integrated Rapid Infectious Disease Analysis

LAB: Lactic acid bacteria

LB: Luria-Bertani

LCM: Low complexity microbiota

MDR: Multidrug resistant/resistance

MGE: Mobile genetic elements

MRSA: Methicillin-resistant Staphylococcus aureus

NDM: New Delhi metallo-beta-lactamase

NML: National Microbiology Laboratory

OTU: Operational taxonomic unit

PBP: Penicillin binding protein

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PHAC: Public Health Agency of Canada

SCFA: Short chain fatty acid

SEM: Standard error of the mean

Str: Streptomycin

TEM: Temoneira

UV: Ultraviolet

WGS: Whole genome sequencing

XLT4: Xylose Lysine Tergitol 4

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Antimicrobial Resistance

The increasing abundance of antimicrobial resistant (AMR) bacteria has become a significant public health threat (McDonald et al., 2001; Vandal et al., 2015). The overuse of antibiotics globally has provided a selective pressure which promotes the propagation and dissemination of AMR bacteria (Scott et al. 2015; Brusselaers et al., 2011). In 2014, it was estimated that over 50% of antibiotics prescribed during primary care visits were unnecessary. The World Health Organization identified the "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* species) as the leading causes of hospital-acquired infections globally and species for which new antibiotics are urgently needed. Furthermore, the extensive use of antibiotics in animal agriculture has given rise to AMR foodborne pathogens such as *Salmonella* species and *Escherichia coli.* Without proper intervention, it is estimated that the annual mortality due to AMR infectious diseases could reach up to 10 million by 2050 (O'Neill, 2015).

Most of the antibiotics used today are derivatives of antimicrobial compounds naturally produced by environmental fungi or bacteria, namely *Streptomyces* species (Butler et al. 2006; Van der Meij et al. 2017). These bacteria evolved to produce antimicrobial metabolites to kill competitors and gain access to nutrients in the community; however, *Streptomyces* and other antimicrobial-producing microorganisms developed survival mechanisms to resist their own toxins. These mechanisms, in the form of AMR genes, have evolved over millions of years and have established themselves in some of the most critical human pathogens. D'Costa et al. (2011) surveyed 30,000-year-old permafrost and identified several genes with a high similarity to AMR genes from *Streptomyces* that confer resistance to β-lactam antibiotics, vancomycin, and

tetracycline. Another study identified genes with 99-100% identity to modern β-lactam, aminoglycoside, and phenicol resistance genes in *Staphylococci* isolated from ~3.5 million-year-old permafrost (Kashuba et al. 2017). Although the evolutionary history of AMR genes is not fully defined, it is important to note that these genes pre-date the use of antibiotics.

The discovery of the penicillin in 1928 greatly advanced modern medicine and reduced mortality associated with infectious diseases. However, even before the use of penicillin in 1942, there were signs that bacteria were capable of developing resistance to penicillin through the production of enzymes known as β-Lactamases (Abraham & Chain, 1940). These enzymes provide resistance by breaking open the core structure of the antibiotic (reviewed in section 1.3). As new antibiotics were introduced to compete with the development of resistance, the fear of reverting to a pre-antibiotic era declined. However, this relief was short-lived as bacteria developed resistance shortly after the introduction of each antibiotic. Further, as antibiotic use intensified, more bacterial species developed resistance. It became clear that there was an association with the consumption of antibiotics and the development of AMR.

A number of experiments during the twentieth century demonstrated that bacteria were able to develop resistance through the exchange of AMR genes. A study by Griffith et al. (1928) found that antibiotic-susceptible bacteria were able to acquire an antibiotic-resistant phenotype when cultured together with an antibiotic-resistant bacterial isolate. This process – later referred to as transformation – was determined to be a result of the movement of free DNA between the bacterial cells. Transformation, along with the processes of conjugation (1946) and transduction (1952) (reviewed in section 1.5) were found to be the main mechanism through which bacteria gain antibiotic resistance (Lederberg & Tatum, 1946; Zinder & Lederberg, 1952). These mechanisms are collectively grouped under the name horizontal gene transfer (HGT), which is

defined as the movement of genetic material between distantly-related organisms. Many *in vivo* studies have demonstrated the transfer of AMR genes between bacteria in the gut of rodents. Results have shown that the administration of antibiotics enhances colonization by AMR bacteria and that the gene transfer frequency is higher under antibiotic selective pressure (Fed et al. 2008). Other studies have shown that in certain circumstances antibiotic selection pressure is not required for gene transfer to occur and that the gene transfer frequency is higher in the absence of selection pressure (Schjorring et al. 2008; Stecher et al. 2012). However, *in vitro* studies have found that the administration of antibiotics to a growing bacterial culture can activate the transfer (*tra*) genes responsible for conjugation (Cantas et al. 2012; Møller et al. 2017). Furthermore, β-lactamase have been shown to induce the expression of silent β-lactamase genes (*bla* genes), resulting in the production of β-lactamases (Livermore and Yang, 1987; Sanders et al. 1997).

We are currently in an antibiotic crisis where infections caused by multi-drug resistant (MDR) pathogens require multi-drug therapy or are only treatable using last-resort antibiotics. The current fear is that the antibiotic pipeline will soon run dry and infectious diseases will again become major sources of mortality. Further research into the mechanisms through which bacteria develop resistance to antibiotics and transmit AMR genes is needed to identify factors associated with AMR and implement strategies to mitigate these risks.

1.2 β-Lactam antibiotics

1.2.1 Mechanism of action

β-Lactams are one of the most important antibiotics available today and have continued to save lives for approximately 100 years. They are currently the most widely used class of antibiotics, accounting for over 60% of all antibiotics used globally (World Health Organization,

2018). β-Lactams have a wide sprum of bioactivity and are a frontline drug for the ESKAPE pathogens and many foodborne pathogens. They act by disrupting the process of transpeptidation, which is the final stage in bacterial cell wall formation. During transpeptidation, penicillin-binding proteins (PBPs) cross-link the glycan chains of the peptidoglycan precursors, resulting in a mesh-like structure (van Heijenoort, 2001). β-Lactams are analogues of d-alanyl-d-alanine, which are found in the peptidoglycan precursor *N*-Acetylmuramic acid. The structural similarities between β-Lactams and d-alanyl-d-alanine allows β-Lactams to bind to the active site of the PBPs. As a result, β-Lactams prevent the binding of d-alanyl-d-alanine to the PBPs and disrupt cell wall synthesis (Figure 1.1) (Georgopapadakou et al. 1977).

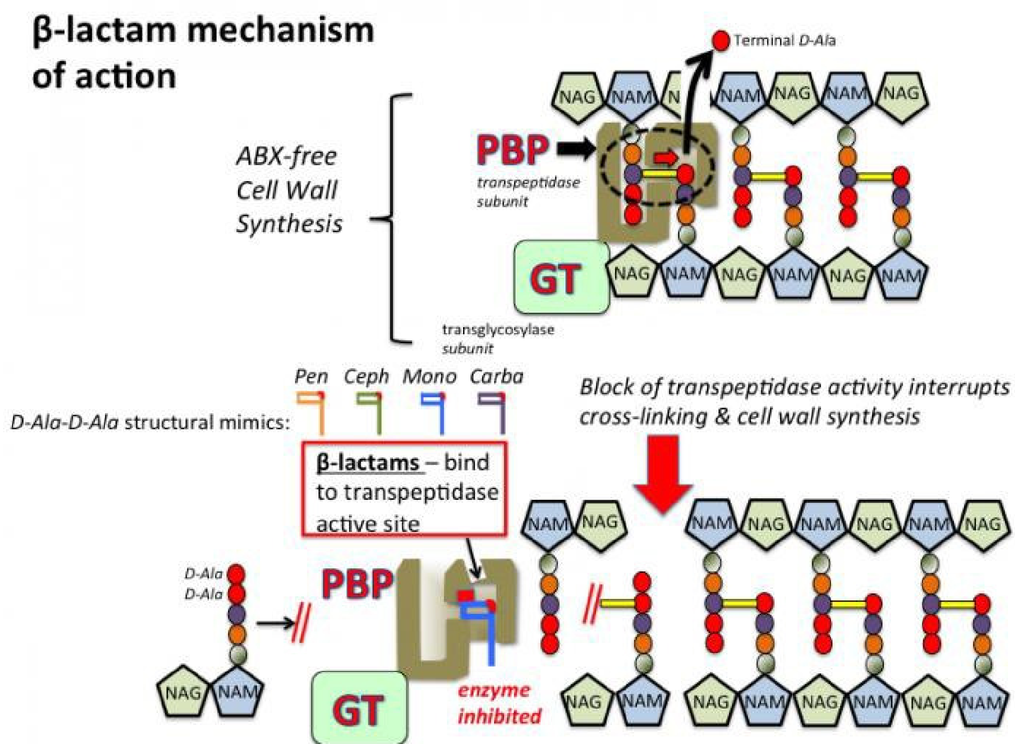

Figure 1.1. In the absence of antibiotics, d-alanyl-d-alanine binds to the active site of the PBP which catalyzes cross-linking of N-acetylmuramic acid, creating a mesh-like cell wall. β-Lactams bind to the PBP, thereby preventing the binding of d-ala-d-ala and interrupts cell wall synthesis. Figure obtained from Zango et al. (2019).

Each bacterial species has a set of PBPs to which unique β-Lactam compounds have different binding affinities (Georgopapadakou & Liu, 1980). The effect of each β-Lactam on the target bacteria is dependent on which PBP(s) the compound binds to. In *E. coli,* the inhibition of PBP1a and PBP1b by penicillin results in immediate cell lysis while the inhibition of PBP2 and PBP3 results in abnormal cell morphology and eventual cell death (Tomasz et al. 1979; Spratt et al. 1975). Conversely, most β-Lactams have a low binding affinity for PBP2a in methicillin-resistant *S. aureus* (MRSA), leaving it resistant to almost all anti-MRSA agents (Lim et al. 2002). The active binding site of PBPs can change as a result of point mutations or recombination with PBPs genes of other bacteria. This can reduce the binding efficiency of antibiotics with PBPs, thereby reducing the antibacterial activity of β-Lactams (Nagai et al. 2002). Reduced binding affinities to PBPs, in addition to other antibiotic resistance mechanisms such as the production of β-Lactamases, drives the search for new antibiotics and modifications to existing antibiotics in order to compete with the rapid development of AMR.

1.2.2 Penicillins

The first β-Lactams used in clinical practice were the penicillins (Figure 1.2), which had a broad-spectrum of activity that included both Gram-positive and Gram-negative bacteria. The basic structure of the first-generation penicillins consisted of a β-Lactam ring fused to a 5-membered thiazolidine ring (penam ring). The first clinically-used antibiotic (penicillin G) was discovered in 1928 by Alexander Fleming, who had identified a fungus growing on an agar plate that had killed the surrounding bacteria. Following isolation of the active compound and its

introduction into commercial practice in 1942, penicillin greatly advanced modern medicine and reduced mortality associated with *Staphylococcus aureus* infections (Lobanovska & Pilla, 2017). However, there were signs of bacterial resistance to penicillin beginning in 1942, when four *S. aureus* isolates from a hospitalized patient were not inhibited by penicillin (Rammelkamp et al. 1942). The semi-synthetic β-lactam methicillin was developed in response to the rapid emergence of penicillin-resistant *S. aureus* (Otto, 2012). However, the clinical success of methicillin was short-lived as MRSA began to appear just over one year later (Peacock et al. 2015). By the 1960s, over 80% of clinical *S. aureus* isolates were resistant to penicillin G (Lowy et al. 2003).

The aminopenicillins were developed out of a need for penicillins with a greater spectrum of activity. They are currently the most prescribed antibiotic class globally, with amoxicillin making up over 50% of all antibiotic prescriptions (Centers for Disease Control and Prevention, 2015). Their structure differs from the penicillins based on an amino group substitution on the phenyl acetamido side chain of the penam ring. This gives the aminopenicillins a greater ability to pass through the Gram-negative cell wall than penicillin G (Nestorovich et al. 2002). Furthermore, an increased binding affinity for PBP1 and PBP3 in most Gram-negative bacteria makes the aminopenicillins an effective antibiotic for the treatment of *E. coli, Shigella,* and *Salmonella* spp. infections (Zapun et al. 2008). Unfortunately, the dissemination of β-lactamases among Gram-negative bacteria has made the aminopenicillins ineffective against many clinically-relevant pathogens. The two remaining aminopenicillins on the market, ampicillin and its derivative amoxicillin, are still used in Gram-negative infections although they are often combined with a β-lactamase-inhibitor as a result of their instability towards β-lactamases (Betrosian et al. 2009). β-lactamase inhibitors protect the antibiotic from degradation by

irreversibly binding to the active site of the β-lactamase and inactivating the enzyme. The aminopenicillins are often administered along with the β-lactamase inhibitors clavulanic acid or sulbactam. These are effective at inhibiting class A β-lactamases and have been shown to reduce the minimum inhibitory concentration of the aminopenicillins towards *S. aureus*, *K. pneumoniae*, *Proteus mirabilis*, and *E. coli* (Cole et al. 1982; Brown et al. 1986).

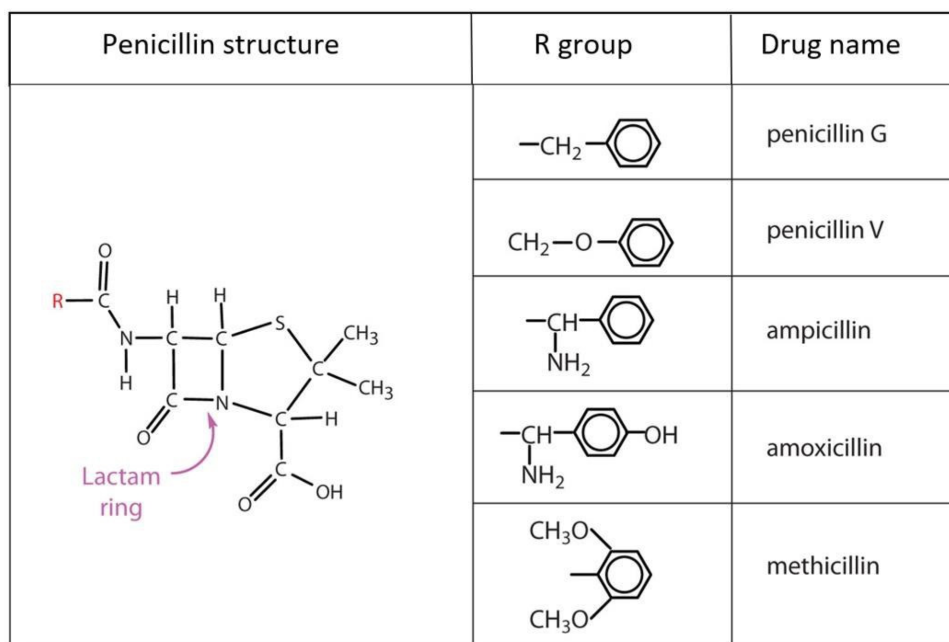

Figure 1.2. All penicillin antibiotics share the same 4-membered β-lactam ring fused with a 5-membered penam ring. The addition of an amino group at R gave rise to the aminopenicillins ampicillin and amoxicillin and enhanced their activity towards Gram-negative bacteria. Figure obtained from Wong et al. (2020).

1.2.3 Cephalosporins

Cephalosporins are favoured over other β-lactam due to their innate stability towards β-lactamases (Figure 1.3) (Marshall et al. 1999). The first cephalosporin, known as cephalosporin C, was discovered in 1945 after being isolated from a fungus found in sewage water (Abraham, 1987). The core structure of the cephalosporins consists of a β-lactam ring fused with a

dihydrothiazine ring. The first and second generation cephalosporins have good bioactivity against methicillin-sensitive *Staphylococci* and *Streptococci* but only moderate bioactivity against Gram-negative bacteria. The third generation cephalosporins, also referred to as extended-spectrum cephalosporins (ESCs), are characterized by their "extended-spectrum" of antibacterial activity against Gram-negative pathogens although they have diminished activity against Gram-positive bacteria (Nath et al. 2020). Their stability against β-lactamases made ESCs useful for the treatment of drug-resistant infections caused by β-lactamase-producing bacteria. The ESCs ceftriaxone and cefotaxime had great clinical success; however, their misuse in hospitals and use of similar compounds in agriculture led to the dissemination of extended-spectrum β-lactamases (ESBLs) (Klein et al. 1995). Due to the strong correlation between the use of these antibiotics and the frequency of ESC resistant infections, the prudent use of ESCs has been encouraged for many years (Vibet et al. 2015; Gbaguidi-Haore et al. 2013). Risk assessments have found that an amoxicillin-clavulanic acid combination may be as effective as ceftriaxone and cefotaxime in the treatment of certain infections and that it has a lower risk for the selection and dissemination of ESBLs (Weiss et al. 2015; Batard et al. 2018). For this reason, the use of ESCs in agriculture has been restricted and their use in clinics is often reserved for non-ESBL producing bacterial infections.

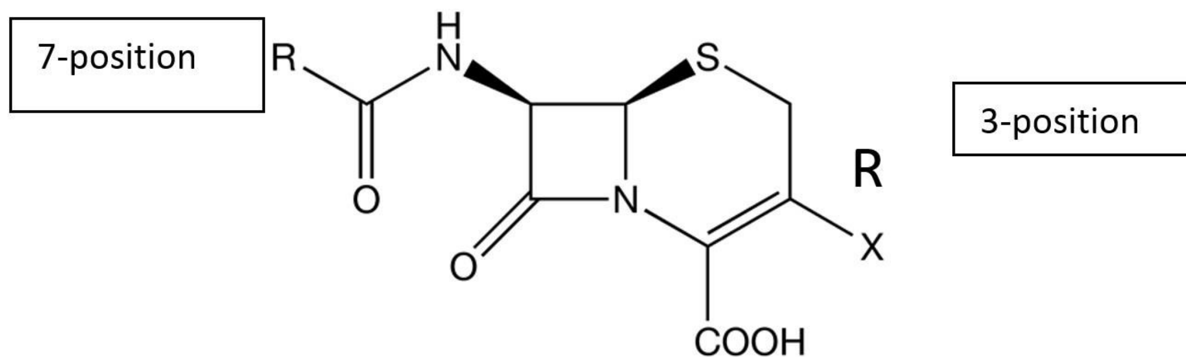

Figure 1.3. The chemical structure of cephalosporin C consists of a β-lactam ring fused with a thiazolidine ring. Modifications of cephalosporin C and other cephalosporins occur at C7 (R1) and C3 (R2). Substitutions at C7 lead to better stability against β-lactamases, while substitutions at C3 extend their serum half-life. Figure obtained from Wong et al. (2020).

1.3 Mechanisms of β-lactam Resistance

1.3.1 β-lactamases

The production of β-lactamases is the most common mechanism of β-lactam resistance in Gram-negative bacteria (Bush and Jacoby, 2010). β-lactamases are a group of enzymes that provide resistance to β-lactam antibiotics by hydrolyzing the β-lactam ring of the antibiotic (Figure 1.4). The first reported β-lactamase was discovered in 1940 when an enzyme isolated from penicillin-resistant *E. coli* inhibited the antibacterial activity of penicillin against *S. aureus* (Abraham and Chain, 1940). It was claimed that the introduction of penicillin gave rise to β-lactamase producing bacteria, although modern phylogenetic analyses indicate that β-lactamase-encoding genes, referred to as *bla* genes, have existed for over 2 billion years (Hamilton et al. 1979; Hall et al. 2004). While they all share the ability to deactivate β-lactams, β-lactamases are diverse. They are classified by the Ambler scheme (A, B, C, D), which is based on their amino acid sequences or their functional characteristics (1, 2, 3, 4), which includes their activity against different β-lactam classes and susceptibility to β-lactam inhibitors (Bush et al. 2013). Although there are over 4300 different β-lactamases, there are a select few that are more successful in

terms of their global spread and presence in the most important pathogens (Naas et al. 2017). Of utmost importance are ESBLs and AmpC (ampicillin class c) cephalosporinases, owing to their ability to deactivate ESCs and their prevalence in both foodborne and clinical pathogens (Madec et al. 2017; Tooke et al. 2019).

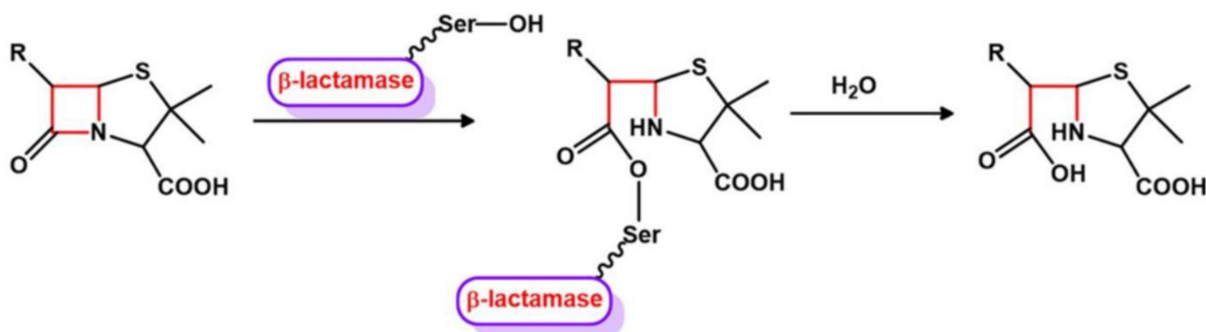

Figure 1.4. β-lactamase mechanism of action. The β-lactamase forms a noncovalent bond with the antibiotic and hydrolyzes the β-lactam ring with the hydroxyl group at the end of the serine residue. Figure obtained from Pan et al. (2017).

1.3.2 Extended-Spectrum β-lactamases

ESBLs are distinguished from other β-lactamases based on their ability to hydrolyze ESCs and their sensitivity to inhibition by β-lactamase inhibitors (Figure 1.4). The majority of ESBL variants are derivatives of the class A TEM-1 (Temoneira-1), and TEM-2 β-lactamases, each of which provide resistance to early generation cephalosporins and the penicillins (Bush & Jacoby, 2010). The use of ESCs, which are not hydrolyzed by TEM-1 and TEM-2, selected for point mutations in these β-lactamases that increased their hydrolytic activity towards ESCs. The first TEM-type ESBL was detected in 1982 in a ceftazidime-resistant *Klebsiella oxytoca* isolate. This enzyme, named TEM-12, was found to have developed after a patient was treated with ceftazidime for an AMR *K. oxytoca* infection. It was found that this enzyme differed from TEM-

1 by a substitution of arginine for serine at position 164 (Payne et al. 1990). While there are currently over 200 variants of the TEMβ-lactamases, TEM-1 has remained the most successful variant in terms of species distribution and prevalence (Pimenta et al. 2014; Bush & Bradford, 2016).

CTX-M (cefotaxime-Munich) β-lactamases are the most prevalent ESBLs detected globally (Cantón et al. 2012). Unlike TEM ESBLs, CTX β-lactamases did not arise as a result of point mutations but rather the mobilization of *bla* genes from *Kluyvera* spp. chromosome. The presence of antimicrobial compounds in the environment is believed to have mobilized ancient *bla* genes from the chromosome of *Kluyvera* spp. onto plasmids where they later spread to other *Enterobacteriaceae* species via HGT (Decousser et al. 2001; Humeniuk et al. 2002). *In vitro* studies have shown that the addition of cefotaxime or ceftazidime to the growth media of *Kluyvera* cultures can induce high-level expression of *bla*CTX genes and mobilize them onto plasmids (Lartigue et al. 2006; Nordmann et al. 2008). Two species in particular, *Kluyvera ascorbata* and *Kluyvera gerogiana,* have been found to carry homologs highly similar to CTX-M β-lactamases found in humans and animals (Olson et al. 2005; Margarita-Rodriguez et al. 2010). The success of CTX-M enzymes is in part due to their presence on plasmids that often carry AMR genes for other antibiotic classes. This allows CTX-M carrying bacteria to be selected for by several classes of antibiotics, a process called co-selection (Cantón & Ruiz-Garbajosa, 2011). Further, although they were originally detected in *Enterobacteriaceae*, their presence on broad host-range plasmids has enabled the spread of CTX-M enzymes into *P. aeruginosa* and *A. baumannii* (Celenza et al. 2006).

1.3.3 AmpC β-lactamases

AmpC β-lactamases, also known as cephalosporinases, constitute class C within the Ambler scheme of β-lactamase classification. They are occasionally classified as ESBLs due to their hydrolytic activity against ESCs; however, AmpC β-lactamases are are not inhibited by clavulanic acid (Jacoby et al. 2009). They are chromosomally encoded in several Gram-negative species, commonly referred to as SPACE organisms (*Serratia, Pseudomonas* or *Proteus, Acinetobacter, Citrobacter, Enterobacter*). The *ampC* gene is repressed in most AmpC producers by the regulatory protein AmpR although its expression can be induced through exposure to certain β-lactams (*e.g*. ampicillin, first-generation cephalosporins). When cells are exposed to β-lactam antibiotics, they induce the production of cell wall degradation products in the cell wall synthesis pathway. Normally, AmpD cleaves the residues from the degradation products, thereby preventing the binding to AmpR. However, with prolonged exposure to antibiotics, the degradation products continue to accumulate in the cytosol and, as a result, AmpD is unable to cleave off the residues (Jacobs et al. 1997; Guérin et al. 2015). The degradation products may then bind to AmpR, resulting in a derepression of *ampC* and a subsequent production of AmpC β-lactamases.

Plasmid-mediated AmpC β-lactamases have been found globally in both clinical and food-producing animal isolates. They have the same antimicrobial profile as chromosomal AmpC β-lactamases but commonly lack an AmpR regulatory protein. As a result, plasmid-mediated *ampC* genes are continuously expressed (Barnaud et al. 1998; Miriagou et al. 2004). The most common plasmid-mediated AmpC β-lactamases are the CMY (cephamycin) variants, which are highly similar to the chromosomal AmpC β-lactamases found in *C. freundii* (Pietsch et al. 2018). Like the CTX-M β-lactamases, plasmid-mediated AmpC enzymes are believed to have

been mobilized from the chromosome to plasmids through antibiotic pressure. The CMY-2 variant is the most widespread of AmpC β-lactamases globally and is a major cause of ESC resistance in foodborne non-typhoidal *Salmonella* (NTS) and *E. coli* (Miriagou et al. 2004). Treatment of infections caused by CMY-producing pathogens is difficult due to their resistance to clavulanic acid, the potential for *ampC* induction, and poor outcomes associated with the use of ESCs (Pai et al. 2004). Carbapenems or the fourth-generation cephalosporin cefepime have had clinical success to their resistance to degradation by CMY, low risk for *ampC* induction, and ability to rapidly penetrate the Gram-negative cell wall (Zanetti et al. 2003; Vimont et al. 2007).

1.4 Horizontal gene transfer

Horizontal gene transfer (HGT) is one of the most prominent mechanisms by which bacteria acquire AMR genes (Sanderson et al., 2016; Kelly et al., 2009). HGT involves the movement of genetic material between distantly-related organisms and plays an important in the evolution of bacteria. Consisting of conjugation, transformation, and transduction, HGT has facilitated the survival and persistence of bacteria by enabling the acquisition of AMR genes and virulence factors (Figure 1.4) (Burmeister, 2015). Gene transfer is most often mediated by mobile genetic elements (MGEs) such as bacteriophages and plasmids, although bacteria can uptake free DNA from the environment and integrate it into their genome through transformation (Arber et al. 2014; Aviv et al. 2016).

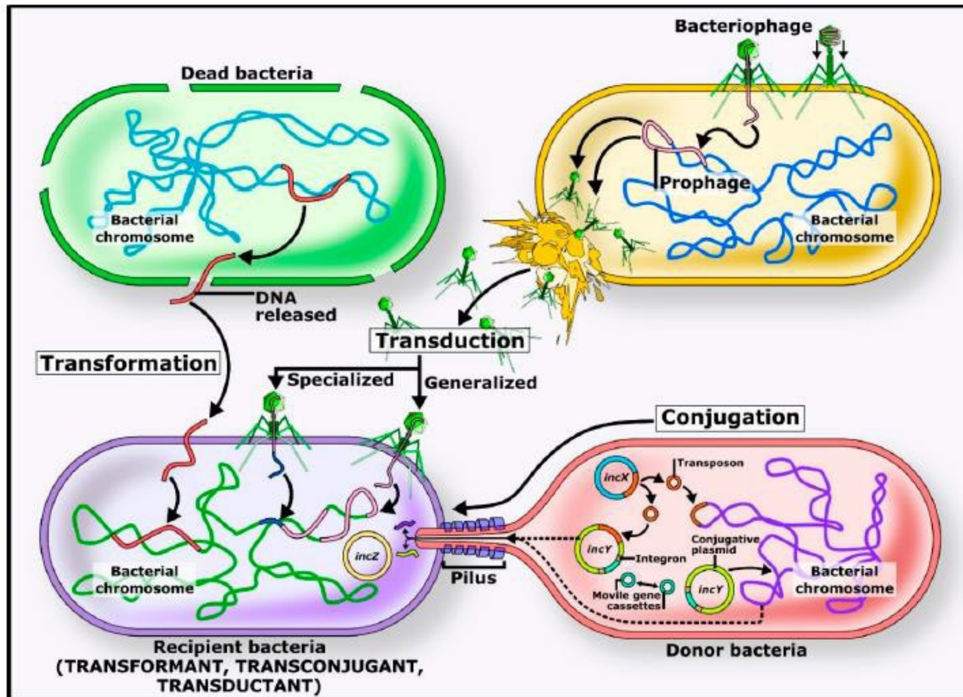

Figure 1.5. Mechanisms of horizontal gene transfer. Conjugation and transduction involve the transfer of AMR genes from donor to recipient bacteria via plasmids and bacteriophages, respectively. In transformation, AMR genes in the form of free DNA are transferred from lysed donor bacteria into recipient bacteria. Figure obtained from Bello-López et al. (2019).

1.4.1 Conjugation

Conjugation is the most common mechanism of HGT and has been linked to the development of resistance to most classes of antibiotics (Huddleston 2014). Conjugation is mediated by plasmids, which are extrachromosomal, self-replicating DNA molecules. In addition to the carriage of genetic material for replication and gene transfer, these extrachromosomal DNA molecules often carry genes essential for the growth of host bacteria under stressed conditions, such as those conferring resistance to antibiotics (AMR genes). Conjugation involves the transfer of plasmid DNA and genes from a donor bacterium to a recipient bacterium through direct cell-to-cell contact. Plasmids can be separated into conjugative, mobilizable, and non-

mobilizable subgroups. While conjugative plasmids contain all of the necessary components for conjugation, mobilizable plasmids need the assistance of conjugative plasmid machinery to achieve the transfer of plasmid DNA (San Millan & Maclean, 2017; Norman et al., 2009).

The process of conjugation begins when a tubular structure called a pilus extends from the donor cell and connects the donor and recipient cells (Figure 1.6). Following this, the relaxase – a component of the plasmid relaxosome - nicks the double-stranded plasmid DNA at the origin of transfer (*oriT)* (Garcillán-Barcia et al. 2009). After nicking the plasmid DNA*,* the relaxase becomes covalently bound to the plasmid DNA. This relaxase-DNA complex is then linked to the type IV secretion system (T4SS) of the donor cell by a coupling protein. The T4SS is a complex consisting of 11 proteins – VirB1 to VirB11 – which facilitate the formation of the pilus and transfer of plasmid DNA into the recipient cell (Zechner et al. 2017; Shintani et al. 2015). The specific function of each protein is not fully characterized although VirB1 has been shown to perforate the peptidoglycan layer and is crucial for pilus biogenesis (Llosa et al. 2002; Zupan et al. 2007). Along with VirB1, pilus biogenesis is mediated by VirB2 and VirB5, the latter of which is located at the end of the pilus and is responsible for adhesion of the pilus to the recipient cell. Finally, the ATPases VirB4 and VirB11 provide energy for the movement of the plasmid DNA through the pilus and into the recipient cell (Atmakuri et al. 2004; Ripoll-Rozada et al. 2013).

1.4.1.1 *Plasmids*

The relationship between plasmids and bacteria is complex. While plasmids may provide beneficial traits to the host, the cost of replicating and maintaining the plasmid places a metabolic burden on the host. Bacterial cells harboring plasmids replicate slower and have a growth disadvantage in plasmid-free communities. As a result, plasmids are prone to elimination

by the host in the absence of antibiotic selective pressure. Plasmid size is a major factor in terms of fitness cost on the host, with larger plasmids being associated with an increased metabolic burden. As a result, large plasmids often require antibiotic selective pressure in order to be stably maintained (Johnson et al. 2015). Prior to plasmid replication, bacterial cells require correct distribution of the plasmid copies in order to ensure inheritance. High copy number plasmids have a higher probability that both daughter cells will receive at least one copy of the plasmid; however, low copy number plasmids utilize partitioning systems to ensure that plasmids migrate to opposite cell poles before cell division (Ghosh et al. 2006). In order to maintain to minimize the metabolic burden and maintain a stable population of plasmid-bearing cells, the host cell is able to control the copy number of plasmids and replication rate, as well as utilize toxin-antitoxin (TA) systems which kill plasmid-free cells (Lobato-M´arquez et al. 2016; Page and Peti, 2016).

Plasmids are classified using an incompatibility-typing scheme. Incompatibility (Inc) is based on the inability of two plasmids with the same replication mechanism to stably co-exist in the same host. Traditional methods included introducing a plasmid into a bacterium carrying another plasmid and determining whether both plasmids remained in the daughter cells. However, PCR-based replicon typing and whole genome sequencing (WGS) have replaced culture-based Inc-typing and classify Inc types based on sequence homology.

IncF plasmids were the first conjugative plasmids to be discovered and among the first to be associated with AMR (de Toro et al. 2014; Partridge et al. 2018). While limited to a narrow host-range and the *Enterobacteriaceae* family, IncF plasmids are the most frequently encountered plasmid in *Enterobacteriaceae* globally (Villa et al. 2010). Additionally, IncF are one of the dominant carriers of ESBL/AmpC genes in food and food-producing animals (Casella et al. 2017; Yang et al. 2015).

IncI-complex plasmids have been associated with the spread of several ESBLs among *Salmonella* and *E. coli*. While this complex includes replicons I, K, B, and Z, IncI1 in particular is the dominant vehicle for the carriage of the CTX-M-1 ESBL variant. CTX-M-1-producing *E. coli* is commonly isolated from both humans and food-producing animals globally, suggesting that IncI1 plasmids may play a role in the dissemination of β-lactam resistance from farm-to-fork (Girlich et al. 2007; Marcade et al. 2009). The *bla*CMY-2 gene has been found on IncI2 plasmids from avian commensal *E. coli* and *Salmonella* isolates. In combination with a higher prevalence of *bla*CMY-2 in animal isolates, it is believed that the spread of CMY and other β-lactamases across species may occur in the gut of food-producing animals (Johnson et al. 2007).

IncN plasmids are also considered to be epidemic resistance plasmids, being found in human, environmental and animal bacterial isolates globally. They tend to be highly stable, conjugate at a high frequency and have a broad host-range that includes many species within the *Enterobacteriaceae* family (Carattoli et al. 2011; Dolejska et al. 2013). The IncN and IncI plasmid lineages are often associated with the spread of *bla*CTX-M-1, the reservoir of which appears to be avian *E. coli* (Johnson et al. 2007; Moodley et al. 2009). Although IncN plasmids are frequently detected in nosocomial pathogens such as *Enterobacter cloacae* and *Klebsiella* species, the carriage of *bla*CTX-M-1 on IncN plasmids is almost exclusively limited to *E. coli.*

IncA/C plasmids have a broad host-range that includes many members of the *Enterobacteriaceae* family. Originally referred to as plasmid RA1 and classified as IncA, this plasmid was found to be compatible with several Inc types. IncA and IncC plasmids were later combined into the IncA/C complex after further work revealed they were closely related (Datta and Hedges, 1974). Currently, IncA/C complex plasmids are highly prevalent in *Salmonella enterica* and often encode multi-drug resistance (MDR). With the application of whole genome

sequencing (WGS), it has been determined that CMY-2 β-lactamases are most commonly found on IncA/C replicons where they typically co-reside with genes encoding resistance to chloramphenicol/florfenicol, tetracycline, and streptomycin (Lindsey et al. 2009).

1.4.2 Transduction

Transduction is a mechanism of horizontal gene transfer in which genetic information is transferred between bacteria through bacteriophages. Bacteriophages are viruses that infect and replicate within bacteria (Torres-Barceló et al. 2018). Through transduction, bacteriophages can transfer any bacterial DNA, such as antimicrobial resistance genes, chromosomal fragments, or mobile genetic elements from a donor to recipient bacteria (Mazaheri Nezhad Fard et al. 2011; Varga et al. 2012). Though they require a host to replicate, bacteriophages are capable of independent existence outside of a bacterial cell (Jensen et al. 1998). As a result, the donor cell and any recipient cells do not have to be in close proximity or present at the same time for transduction to occur (Muniesa et al. 2013). Together these factors make phages a suitable carrier for AMR genes within different biomes (Muniesa et al. 2011).

It is unclear what role phages play in the spread of AMR genes. Much research has been dedicated to plasmid-mediated transfer of AMR genes and less to the role of phages. Wastewater and run-off from agricultural lands have been demonstrated to be reservoirs of phage-packaged β-lactam resistance genes. Colomer-Lluch et al. (2011, 2014) found a high abundance of phage-packaged *bla*TEM and *bla*CTX-M genes in Tunisian and Spanish animal manure and wastewater. Due to the structure of phages, they can survive in the environment longer than their bacterial hosts and free DNA. As a result, phages may persist for long periods of time in the environment (E.g. soil) and give rise to new AMR bacterial strains. This presents a risk to public health as phage-packaged AMR genes may transduce into foodborne pathogens upon the

application of manure onto crops which are later ingested by humans via contaminated food (Blahova et al. 1993, Brown-Jaque et al. 2018).

1.4.3 Transformation

Natural transformation is a process of HGT that involves the uptake, integration and expression of free DNA in the host cell. Unlike conjugation and transduction, the uptake of DNA is not dependent on MGEs but upon the uptake of DNA released by donor cells (Thomas and Nielsen, 2005). Like transduction, transformation does not require cell-to-cell contact, so the donor and recipient cells do not have to be present at the same time. The role of transformation in the spread of AMR genes is not well characterized. There are currently no *in vivo* studies demonstrating the transformation of AMR genes between bacteria. Attempts have been made to demonstrate the transformation of exogenous DNA into the microbiota of healthy rats; however, no transformants have been detected (Nordgård et al. 2012). This may be a result of low transformation frequencies, the low level of naturally transformable bacteria, or a lack of competent bacteria in the experimental conditions. While transformation may play less of a role than conjugation and transduction in the development of AMR bacteria, further information on the process of transformation may help understand how bacteria acquire AMR genes from species outside the host range of plasmids or bacteriophages.

1.5 The gut microbiota

The human gut microbiome is a complex community of microorganisms composed of up to 10^{13} bacterial cells belonging to over 1,000 different species. It is dominated by four phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Qin et al. 2010; Sender et al. 2016). The highly diverse and densely populated gut microbiota plays a major role in gut

homeostasis and host health. The production of short-chain fatty acids (SCFAs) and metabolites by the gut microbiota provides energy to the host and other gut microbes, as well as aids the host in the metabolism of ingested food (Macfarlane et al. 2003; Marin et al. 2015). Further, the production of SCFAs and bacterial toxins known as bacteriocins protect the host from colonization by pathogens (Schamberger et al. 2002; Hammami et al. 2013). The Firmicutes:Bacteroidetes ratio has often been an indicator of gut health and disease states (D'Argenio et al. 2015). The composition of the gut microbiota can be influenced by factors including but not limited to age, diet, antibiotic use, and geographical area (Yatsunenko et al. 2012; Gajer et al. 2012; Pérez-Cobas et al. 2013; David et al. 2014). While the optimal ratio and composition differs between groups of people, a disruption in the balance of the gut microbiota can lead to health consequences.

1.5.1 Bacteroidetes

Bacteroidetes are Gram-negative obligate anaerobes that reside with the mammalian gut (Gibiino et al. 2018). They are normally considered commensals within the gut although can cause serious infections if displaced into the blood or other tissues. Bacteroidetes species have been shown increase nutrient availability for other commensal bacteria through the upregulation of polysaccharide utilization loci, which are lacking in certain Firmicutes. A study by Mahowald et al. (2009) found that with greater access to nutrients, the Firmicutes species *Eubacterium rectale* upregulated the enzyme phosphate acetyltransferase which increased production of butyrate by *Bacteroides thetaiotaomicron.* The production of butyrate is beneficial to the host as it provides both energy to colon epithelial cells and resistance to colonization of the gut by pathogens (Rios Covian et al. 2017).

Bacteroidetes also play an antagonistic role against other bacteria in order to maintain their own niche and protect the host from pathogens. Jacobson et al. (2018) found that *Bacteroides* species were able to inhibit the growth of *Salmonella enterica* serovar Typhimurium *in vivo* through the production of the SCFA propionate which caused a decrease in the *S.* Typhimurium intracellular pH. A study by You et al. (2019) demonstrated that mice lacking Bacteroidetes were susceptible to infection by *Vibrio cholerae* although resistance to infection was restored in germ-free mice colonized with *Bacteroides vulgatis.* This was found to be a result of increased butyrate and propionate production by *B. vulgatis.*

1.5.2 Firmicutes

Firmicutes are Gram-positive, spore-forming, facultative and obligate anaerobes. Most microorganisms in the human gastrointestinal tract are within Clostridia class clusters XIVa and IV, which play a major role maintaining human health (Kim et al. 2017; Lopetuso et al. 2013). Like Bacteroidetes, many members of Clostridia contribute to gut homeostasis through the production of SCFAs. Takahashi et al. (2016) found that patients with Crohn's disease had lower levels of the butyrate-producing Clostridia *Eubacterium, Faecalibacterium, and Ruminococcus* in comparison to healthy controls. *Clostridium butyricum* has been shown to inhibit the growth *Salmonella, E. coli,* and *Helicobacter pylori* (Shimbo et al. 2005; Zhao et al. 2017). Woo et al. (2011) demonstrated a significant growth inhibition of *E. coli* O157:H7 and Shiga-toxin production in mice prophylactically treated with *C. butyricum* MIYAIRI 588 (CBM588). Further, Takahashi et al. (2004) found that *C. butyricum* prevented the adhesion of *E. coli* O157:H7 to Caco-2 cells.

Another dominant member of Firmicutes are Lactic Acid Bacteria (LAB), which are well known for their use as probiotics and in food production systems. LAB are distinguished

from other Firmicutes based on their production of lactic acid as an end product of carbohydrate fermentation. Common species include *Enterococcus, Lactobacillus* and *Bifidobacterium* although *Lactobacillus* is the largest genus, with over 56 species (Ganji-Arjenaki et al. 2018; Naabar and Mikelsaar, 1998). Several studies have demonstrated the ability of *Lactobacillus acidophilus* and *Bifidobacteria* to inhibit the adherence to and invasion of human cells by the pathogens *E. coli, Yersinia paratuberculosis* and *S.* Typhimurium (Bernet et al. 1993; Bernet et al. 1994). LAB and other Firmicutes also utilize bacteriocins to persist in the gut and protect the host. Bacteriocins are a heterogenous family of peptides with antimicrobial activity (Cotter et al. 2005). While they are produced by most bacteria, the majority of characterized bacteriocins are produced by Firmicutes and are generally associated with positive disease outcomes (Hammami et al. 2013). Bacteriocins function as a barrier to colonization by pathogens, often without having a significant impact on the gut microbiota (Kommineni et al. 2015). Rea et al. (2010) found that *Bacillus thuringiensis* produced the bacteriocin Thuricin CD which was able to inhibit the growth of the opportunistic pathogen *C. difficile in vivo* but had no inhibitory effect on several commensal LAB strains. Another study by Ramasamy et al. (2015) demonstrated the production of a broad-spectrum bacteriocin by *L. acidophilus* with inhibitory activity against *E. coli, Streptococcus* spp., *Bacillus subtilis,* and *P. aeruginosa.*

1.5.3 Dysbiosis of the gut microbiome

The gut microbiota provides a barrier to colonization by pathogens; however, perturbation of the gut microbiota can result in dysbiosis – a disruption in the microbial balance – where the gut microbiota are no longer able to protect against colonization (Schubert et al., 2015; Pérez-Cobas et al. 2013). This is often due to an alteration in the Firmicutes:Bacteroidetes

ratio, where a reduction in one population favours the growth of the other (Panda et al. 2014). This can lead to an increased susceptibility to infection or the overgrowth of indigenous pathogens normally suppressed by the commensal bacteria (Staley et al. 2017). Susceptible patients can then become infected with exogenous bacteria where they can develop a life-threatening disease or become a reservoir for patient-to-patient transmission.

Antibiotic treatment can have a significant impact on the composition of the gut microbiota. Research into the impact of antibiotic use on colonization resistance began in the 1950s when Bohnhoff et al. (1954, 1962) found that pre-treating mice with streptomycin reduced the infectious dose of *Salmonella* Enteriditis by 10,000-fold. Since then, antibiotic pre-treatment has become a common practice in many animal-infection models (Hentges et al. 1985; Barthel et al. 2003; Nilsson et al. 2019). Many studies have explored the impact of antibiotics on the gut microbiota using metagenomic sequencing. Burdet et al. (2019) found that cefotaxime and ceftriaxone reduced species richness and microbial diversity in healthy humans. Both antibiotics increased the Bacteroidetes population and reduced the Firmicutes and the Gram-negative bacterial populations. Gibson et al. (2016) found similar results where cefotaxime reduced the Gram-negative bacterial population (*E. coli* and *Klebsiella* species); however, treatment with ampicillin and ticarcillin resulted in a significant increase in the abundance of *E. coli* and *K. pneumoniae*.

Consistent with earlier studies on streptomycin-treated mice, modifications in the gut microbiota often precede colonization of the gut by pathogens. Isaac et al. (2017) found that vancomycin treatment suppressed the Firmicutes population in the mouse gut and increased susceptibility to colonization by *Enterococcus.* Lewis et al. (2015) found that vancomycin increased susceptibility to colonization by several Gram-negative pathogens. In the same study,

it was found that metronidazole did not have a significant impact on the gut microbiota, nor did it significantly increase the susceptibility to exogenous colonization.

1.5.4 The gut microbiome as a source of AMR genes

1.5.4.1 The human gut resistome

In addition to the increased risk for colonization of the gut microbiota, the use of antibiotics may select for and enrich indigenous AMR bacteria and their corresponding AMR genes. The use of metagenomic sequencing has shown us that the gut microbiota is a rich reservoir of AMR genes (Shterzer and Mizrahi, 2015; Jeong et al. 2019). The mere presence of AMR genes in the gut is not a concern as the gut microbiota suppresses pathogenic bacteria; however, a dysbiosis induced by antibiotics may allow the overgrowth of these pathogens which may act as either a donor or recipient of AMR genes. Raymond et al. (2016) found that patients treated with the second-generation cephalosporin cefprozil experienced blooms of cefprozil-resistant *E. cloacae* carrying *bla*ACT (AmpC-type) genes. Owing to their ability to produce AmpC β-lactamases, blooms of *E. cloacae* are often seen in patients treated with penicillins and cephalosporins. Gibson et al. (2016) found that individuals treated with cefotaxime had increased numbers of *Enterobacter cloacae* harboring *bla*CMY genes.

As conjugation is dependent on physical contact between cells, the enrichment of AMR bacteria as a result of antibiotic treatment increases the risk for the transfer of AMR genes within the gut. Martino et al. (2019) reported a case where five different AMR *Enterobacteriaceae* species (*Klebsiella quasipenumoniae, E. coli, Citrobacter freundii, Serratia marcescens,* and *E. cloacae*) harboring an identical *bla*NDM-1 (New Delhi metallo-beta-lactamase)-harboring plasmid were isolated from a single patient undergoing ESC therapy. The exact route of

transmission could not be confirmed although it was speculated that the *bla*NDM-1 carrying plasmid originated from a *K. quasipenumoniae* infection and later transferred into the indigenous *Enterobacteriaceae*. Luzzaro et al. (2004) identified a case where an ESC-resistant *E. cloacae* infection developed resistance to carbapenems after carbapenem treatment. The carbapenem-resistant *E. cloacae* isolate was found to carry *bla*VIM-4, which was also identified in an indigenous *K. pneumoniae* isolate. The carbapenem therapy likely selected for and enriched the indigenous *bla*VIM-4 carrying *K. pneumoniae* which was spread to the ESC-resistant *E. cloacae.*

1.5.4.2 The gut resistome of food-producing animals

Animal agriculture is one of the main contributors to the spread of AMR bacteria (Baron et al. 2014). Antibiotics are utilized heavily in animal agriculture, typically as either antibiotic growth promoters (AGPs) or for the prevention of infectious disease (prophylactic) in food-producing animals. AGPs and prophylactic antimicrobials are injected or supplemented into the feed of food-producing animals at a sub-therapeutic dose. It has been found that the gut microbiome of animals treated with subtherapeutic doses of antibiotics have higher levels of AMR genes than unmedicated animals (Looft et al. 2011).

Food-producing animals are a large reservoir of ESBL and AmpC-producing bacteria. Poultry in particular have been found to be carriers of CTX-M and CMY-2 producing *E. coli* and NTS (Börjesson et al. 2013; Madec et al. 2015). A sampling of retail chicken meat samples in the Netherlands found that 94% of samples carried ESBL-producing *E. coli.* An assessment of Dutch human *E. coli* isolates found that 35% of these carried the same ESBL (E.g. *bla*CTX-M-1) genes which were carried on genetically identical plasmids to the retail meat (Leverstein van Hall et al. 2011). Surveillance studies in Canada have also found genetically similar plasmids carrying

*bla*CMY-2 in NTS samples of human, abbatoir poultry, and retail poultry (Martin et al. 2012; Edirmanasinghe et al. 2017).

The prevalence of ESBL and AmpC producing foodborne pathogens is associated with the use of antibiotics in animal agriculture. A strong correlation between the spread of *bla*CMY-2 and use of the second-generation cephalosporin cefoxitin in agriculture was observed in Canada. As a result of a similar antibacterial mechanism, the use of cefoxitin caused cross-resistance to the ESC ceftriaxone although the prevalence of CMY-2 producing NTS decreased following the cessation of cefoxitin in 2014 (Canadian Integrated Program for Antimicrobial Resistance Surveillance, 2016 annual report). A comprehensive meta-analysis by Tang et al. (2017) found that the reduction of antibiotic use in animal agriculture decreased the prevalence of AMR bacteria in animals by 15%, decreased the prevalence of MDR bacteria in animals by 24-32%, and decreased the prevalence of AMR bacteria in humans by 24%. This is similar to other meta-analyses of conventional agriculture versus organic agriculture where antibiotic-free farms typically had lower levels of AMR bacteria (Wilhelm et al. 2009; Young et al. 2009; Smith-Spangler et al. 2012).

1.6 In vivo horizontal gene transfer

Animal studies have been done in an attempt to elucidate the factors influencing the transfer of AMR genes and development of AMR bacteria. Stecher et al. (2012) demonstrated the transfer of a colicin-encoding plasmid from *Salmonella* Typhimurium to commensal *E. coli* in the gut of low complexity microbiota (LCM) and streptomycin pre-treated mice. Feld et al. (2008) compared the transfer of the erythromycin resistance gene *ermB* in gnotobiotic (germ-free) and streptomycin pre-treated mice. In both studies, the transfer frequency was lower in the streptomycin pre-treated mice which was believed to be a result of re-growth of the gut

microbiota and elimination of the inoculated bacteria. Other studies have found that the transfer of AMR genes is enhanced by antibiotic selective pressure. Bahl et al. (2003) found that the transfer of *ermB* between *E. faecalis* in gnotobiotic rats was dependent on antibiotic concentration. *E. faecalis* transconjugants appeared first in mice treated mice with a high dose (50 μg/ml) of erythromycin although the levels were statistically similar to the low 5 (μg/ml) and medium doses (10 μg/ml) throughout the experiment. Similarly, Johnson et al. (2015) found that only a high dose of chlortetracycline resulted in the transfer of an IncA/C plasmid between *E. coli* strains in pig gut.

More recent studies have used molecular approaches to determine how the composition of the gut microbiome influences AMR gene transfer. The veterinary antibiotics enrofloxacin and florfenicol have been shown to reduce the Firmicutes population in the chicken gut and enhance the transfer of the multidrug efflux pump gene *OqxAB* from *Salmonella* to commensal *E. coli* (Chen et al. 2016). Marosevic et al. (2014) found that the transfer of an *ermB*-carrying plasmid from *E. faecalis* to the chicken gut microbiota (*Enterococcus, Streptococcus*) was enhanced by the administration of tylosin, lincosamide, or streptogramin B. It was found that these antibiotics reduced the relative abundance of *Lactobacillus* species with concurrent increases in *Lachnospiraceae, Enterococcus* species, and *Streptococcus* species. Ye et al. (2019) found that the transfer of *bla*NDM-1 from *K. pneumoniae* to *E. coli* was influenced by the composition of the gut microbiome and varied widely between antibiotics.

1.7 Project rationale

Much *in vivo* research has shown that streptomycin or β-lactam administration can facilitate the transfer of AMR genes and result in the development of AMR bacteria (covered in 1.6). Streptomycin plus ampicillin has been shown to be effective at treating AMR human infections in comparison to either antibiotic alone (Dodge et al. 1997; Oill et al. 1981; Fuurstead et al. 1987). Further, these antibiotics and other combinations of β-lactams (E.g. amoxicillin, penicillin) with aminoglycosides (E.g. gentamicin) are still prescribed for clinical use and for use in food-producing animals (Chowdhury et al. 2015; Manyi-Loh et al. 2018).

Animal models have shown that the choice of antibiotic can greatly impact the risk of AMR gene transfer and that the transfer is influenced by the gut microbiota composition. There are currently no animal models comparing the impact of single versus multiple antibiotics on the gut microbiota composition and any subsequent influence on the transfer of AMR genes. This study will use 16S rDNA sequencing in tandem with culture-based microbiological techniques to determine how the composition of the mouse gut microbiota influences the development of AMR bacteria under different antibiotic treatment regimens.

1.8 Hypothesis

Dual-course antibiotic treatments will induce a more significant change in the gut microbiome composition and enhance colonization of the introduced AMR bacteria. This will result in a greater transfer frequency of AMR genes. The AMR gene transfer frequency will be positively correlated with the degree of microbial disruption.

1.9 Objectives

I. Determine the impact of different antibiotic treatments on AMR gene transfer by comparing gut colonization and transfer frequency with changes in the composition of the gut microbiome.

II. Determine the impact of antibiotic treatment on AMR gene transfer between the inoculated bacteria and the gut microbiota using whole genome sequencing.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strains

The bacterial strains used are listed in Table 2.1. The *E. coli* O80:H26 donor and *S.* Heidelberg donor isolates were provided by Dr. Richard Reid-Smith at the Public Health Agency of Canada (PHAC), Guelph, Canada. The 10 *S*. Heidelberg recipient isolates were provided by Dr. Michael Mulvey at PHAC, Winnipeg, Canada. Dr. Patrick Boerlin at the University of Guelph, Guelph, Canada provided the *S.* Bredeney donor isolate. Liquid bacterial cultures were grown at 37°C in Difco™ Luria-Bertani (LB) broth containing the appropriate antibiotics (Table 1) (Becton Dickinson, USA). Solid bacterial cultures were grown at 37°C on Difco™ LB agar.

ID	Strain	Source	Type	Antibiotic
C546	*Salmonella* Bredeney	Turkey	Donor	Cefotaxime (4 mg/l)
C551	*Escherichia coli* O80:H26		Donor	Cefotaxime (4 mg/l)
C553	*Salmonella* Heidelberg		Donor	Cefotaxime (4 mg/l)
C439	*Escherichia coli* CV601gfp		Recipient	Rifampicin (50 mg/l) & Kanamycin (50 mg/l)
C565	*Salmonella* Heidelberg	Human blood	Recipient	None
C566	*Salmonella* Heidelberg	Human stool	Recipient	None
C567	*Salmonella* Heidelberg	Human blood	Recipient	None
C568	*Salmonella* Heidelberg	Human blood	Recipient	None
C569	*Salmonella* Heidelberg	Human stool	Recipient	None
C570	*Salmonella* Heidelberg	Human blood	Recipient	None
C571	*Salmonella* Heidelberg	Human stool	Recipient	None
C572	*Salmonella* Heidelberg	Human stool	Recipient	None
C573	*Salmonella* Heidelberg	Human blood	Recipient	None
C574	*Salmonella* Heidelberg	Human stool	Recipient	None

Table 2.1. Bacterial strains and their respective growth conditions.

2.2 Preservation and storage of bacteria

Selected donors, recipients and transconjugants recovered from the mouse feces were preserved at -80°C using the Microbank™ (Pro-lab Diagnostics, Canada) system. This system uses cryovials containing 25 porous beads immersed in a cryopreservative which allows bacteria to adhere to the surface of the beads. To preserve a bacterial isolate, 2-3 colonies of an 18-24 hour pure bacterial culture were scraped from the surface of the agar using an inoculating loop and the colonies were immersed in the cryopreservative. The colonies were dislodged from the inoculating loop by scraping the loop against the inner wall of the cryovial. The cryovials were then inverted 4-5 times and the cryopreservative was aspirated into a separate sterile 2.0 ml Nalgene cryovial. The cryovials were placed directly onto dry-ice and transferred to a -80°C freezer. When a solid culture was needed, a single bead was removed from the cryovial using a sterile 10 µl disposable inoculating loop and placed onto the surface of LB agar. The bead was streaked on the first quadrant using the inoculating loop. The bead was then removed using sterile forceps and the rest of the plate was streaked for colony isolation using a new inoculating loop. When a liquid culture was needed, the bead was placed directly into LB broth using the inoculating loop. The solid and/or liquid cultures were then incubated at 37°C for 18-24 hours.

2.3 *In vitro* conjugation assays

Prior to the *in vi*vo mouse inoculations, the bacterial suspensions were adjusted to a McFarland standard, which provides an estimate of bacterial cell density by measuring the turbidity of the suspension. The McFarland standard used for the mouse inoculations was 1.0 which corresponds to a cell density 3.0×10^8 CFU/ml, respectively.

2.3.1 Verification and calibration of the DensiCHEK™ instrument

The turbidity of the McFarland standards was measured using a DensiCHEK™ instrument. Prior to measuring the McFarland standards, the DensiCHEK™ instrument was verified according to Manufacturer-supplied standard tubes. The verification kit uses 4 standards (0, 0.5, 1.0, 2.0 McFarland). The tubes were inverted 5-6 times prior to inserting into the DensiCHEK instrument to ensure even distribution of the suspension. After reading the turbidity of the calibration tubes and ensuring they fall within the accepted range, the DensiCHEK™ instrument was zeroed using phosphate buffered saline (PBS). To do this, a single sterile culture tube (12 x 75 mm, Simport™ Scientific) was filled with 2 ml PBS and inserted into the instrument. After pressing ZERO, the tube was rotated until the reading was complete.

2.3.2 Conjugation assay procedure

In vitro conjugation assays were performed to assess transferability of the plasmids between donor and recipient bacteria (Table 1.1) prior to the *in vivo* mouse experiments. The conjugation assays using *E. coli* CV601gfp were performed by technicians at AAFC in London, Ontario. The donor cultures were grown overnight in 10 ml LB broth containing 4 mg/L cefotaxime on an incubator shaker (200 rpm at 30°C). After 18-24 hours, the culture was centrifuged for 10 minutes at 3,800 g. The supernatant was discarded and the pellets were washed once by resuspending the pellet in 10 ml of antibiotic-free LB broth and centrifuging again for 10 minutes. The supernatant was again discarded and the pellets were resuspended in 1 ml of 1/10X LB broth. The *E. coli* CV601gfp culture was grown in 150 ml LB broth containing 50 mg/L rifampicin and 50 mg/L kanamycin on an incubator shaker (200 rpm at 30°C). After 18-24 hours, the culture was centrifuged for 10 minutes at 3,800 g, washed once in 40 ml of

antibiotic-free LB broth, and centrifuged again for 10 minutes at 3,800 g. The supernatant was discarded and the pellet was resuspended in 40 ml of 1/10X LB broth. The donor and recipient cultures were adjusted to a final OD600=1 in 1/10X LB broth and allowed to sit for 1h at room temperature. The cultures were then set up in a 1:10 donor to recipient mating mixture as follows: 500 µl of the recipient culture was inoculated into 4.5 ml of 1/10X LB broth, and vortexed briefly. The donor cultures (50 µl) were then added to the recipient culture in 1/10X LB, and vortexed briefly. Donor-only and recipient-only mixtures (50 µl and 500 µl each in 4.5 ml 1/10X LB broth) were included as controls. All mating mixtures were then incubated statically overnight at 30°C.

Donors, recipients, and transconjugants were enumerated by spread-plating the overnight mating mixes on LB agar plates with the appropriate antibiotics. *E. coli* CV601gfp transconjugants were enumerated by spread-plating 100 µl of the 10-fold dilution series onto Chromocult agar (Sigma-Aldrich, USA) with 4 mg/l cefotaxime, 50 mg/l rifampicin and 50 mg/l kanamycin. *E. coli* CV601gfp recipients were enumerated by spot-plating 5 µl of the dilution series onto Chromocult agar with 50 mg/l rifampicin and 50 mg/l kanamycin. All donors were enumerated by spot-plating 5 µl of the dilution series onto Chromocult agar with 4 mg/l cefotaxime. Donor only and recipient only mixtures were spot-plated onto the transconjugant selective media to show that their growth is inhibited under transconjugant selective conditions. After 24 hours of incubation at 30°C, the colonies were counted and transconjugation frequency was expressed as transconjugants/donors.

2.4 Preparation for *in vivo mouse experiments*

2.4.1 Mice used in the in vivo experiments

Four-week old female C57BL/6 mice were chosen based on their susceptibility to *Salmonella* infection. This is due to a lack of a functional mouse *Nramp1* gene that influences the availability of the nutrient iron for *Salmonella.* Female mice were chosen due to their less aggressive nature and ease of handling. All mice were purchased from Charles River Laboratories (Massachusetts, USA).

2.4.2 Preparation of bacterial inoculum for mouse experiments

Single colonies were first obtained by streaking a Microbank™ (Pro-lab Diagnostics, Canada) bead onto freshly prepared LB agar containing the appropriate antibiotics (Table 1.1). After 18-24 hours of growth at 37°C, a single colony was inoculated into 10 ml of LB broth. The cultures were grown overnight on a platform shaker by shaking at 200 rpm (37°C). One milliliter of the overnight cultures was then inoculated into fresh LB broth and shaken for 3 hours at 250 rpm to achieve log-phase of growth.

After removing the flasks or culture tubes from the shaking incubator, the cells were pooled into 50 ml centrifuge tubes (35 ml per tube) and centrifuged for 10 minutes at 10,000 g. The supernatant was poured into a waste container and the cells were resuspended in 35 ml of PBS by pipetting up-and-down. The suspension was centrifuged again for 10 minutes at 10,000 g. The supernatant was removed and the pellet was resuspended in 2 ml of PBS. A 200 µl aliquot of the suspension was diluted in 1800 µl of PBS (1:10) and vortexed for 5 seconds. The tube was then measured using the DensiCHEK™ instrument. If the reading was higher than the desired McFarland standard, the bacterial suspension was diluted by adding a small volume of PBS.

After mixing by pipetting up-and-down several times, a 200 µl aliquot was diluted 1:10 in 1800 µl of PBS and measured again using the DensiCHEK™ instrument. This procedure was followed until a 1.0 McFarland standard was reached. If the initial reading was below the desired turbidity, the bacterial suspension was centrifuged for 10 minutes at 10,000 g to concentrate the cells. After removing the supernatant, the pellet was resuspended in a smaller volume of PBS, taking caution not to over dilute the suspension. The suspension was then adjusted to a 1.0 McFarland standard by following the above steps.

2.4.3 Preparation of antibiotics

All antibiotics used for the mouse experiments were prepared in water. Ampicillin was administered via drinking water at a concentration of 0.16 mg/ml. Every cage of mice received a bottle containing 50 ml of a 0.16 mg/ml ampicillin solution, which was refilled daily. Prior to the beginning of the experiment, 8 mg of ampicillin (Sigma-Aldrich, USA) was weighed out and placed into separate 50 ml centrifuge tubes for each cage and day. When refilling the ampicillin solutions, each centrifuge tube was filled with 50 ml of water and vortexed to make the 0.16 mg/ml solution. The bottles were refilled with the fresh solution, being cautious not to mix bottles between cages. For the streptomycin pre-treatment, each mouse was administered 20 mg streptomycin. The solutions were prepared by dissolving 400 mg of streptomycin (Sigma-Aldrich) in 2 ml of water. Each mouse was administered via oral gavage with 0.1 ml of the 200 mg/ml solution for a total of 20 mg of streptomycin per mouse.

2.4.4 Mouse inoculation

Administration of streptomycin as well as the bacterial suspensions was given by oral gavage. The syringes (1 ml) (Becton Dickinson, USA) were filled with 100 µl of the inoculum and the mice were immobilized by grasping the loose skin on the back of the neck. The mice

were held in an upright position and the gavage needle (5.1 cm) (Fisher Scientific, USA) was inserted into the mouth and down the esophagus toward the stomach. The plunger of the syringe was depressed slowly to administer the inoculum. The mice were monitored after inoculation for any signs of distress.

2.5 *In vivo* selection of a recipient strain

A preliminary experiment was conducted to find a plasmid-free recipient strain suitable for use in the *in vivo* horizontal gene transfer experiments. Sixteen groups of mice in total were used to assess how well each strain colonized the mouse gut. Seven groups (4 per group) were pre-treated with streptomycin (20 mg per mouse), followed by inoculation with 0.1 ml of a different *S.* Heidelberg strain (10^8 CFU/ml) per group. Seven additional groups were each inoculated with the respective *S.* Heidelberg strain but without streptomycin pre-treatment. Two groups served as controls: one group was pre-treated with streptomycin and the other did not receive any treatments. The fecal samples were collected over a period of 10 days, homogenized, and cultured on XLT4 agar for *S.* Heidelberg counts.

2.6 *In vivo* horizontal gene transfer experiment

2.6.1 Experimental design

An *in vivo* mouse experiment was conducted to assess the impact of antibiotic treatment on the gut microbiota and horizontal gene transfer of AMR genes. Four groups of mice were used in total (six mice per group). The donor *E. coli* O80:H26 strain was selected from the conjugation assays based on its high transfer frequency and easy differentiation from *S.* Heidelberg on Chromocult agar. The recipient *S.* Heidelberg C570 strain was selected from the

preliminary mouse experiment (section 2.5) based on its ability to colonize the mouse gut with and without streptomycin pre-treatment. The experimental design can be seen in figure 2.1.

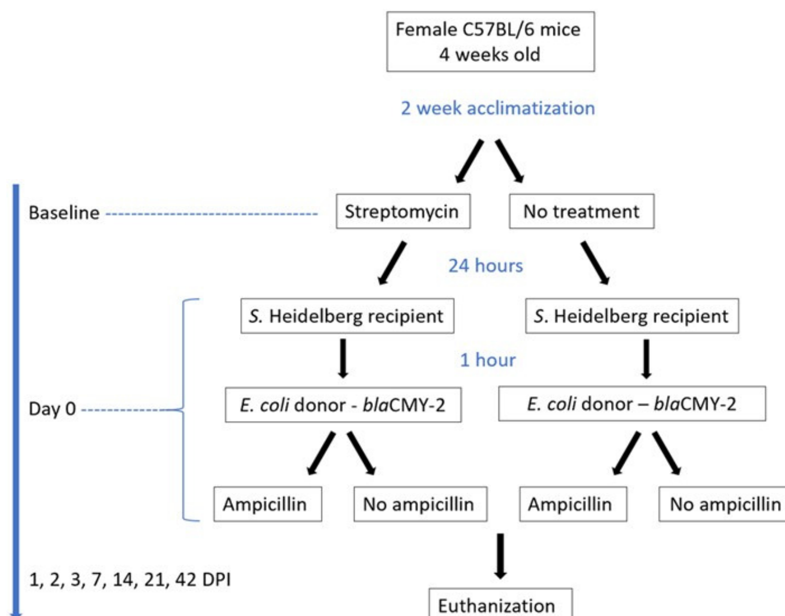

Figure 2.1. Experimental design for the *in vivo* horizontal gene transfer experiment. Groups Str and Str-Amp were pre-treated with streptomycin 24 hours prior to inoculation of the bacterial strains. Recipients were inoculated one hour prior to inoculation of the donor strain. Ampicillin treatment began the same day as inoculation. Samples were collected on 1 day post-inoculation (DPI), 2 DPI, 3 DPI, 7 DPI, 14 DPI, 21 DPI, and 42 DPI. Str =streptomycin Amp=ampicillin

2.6.2 Sample collection

Fecal samples were collected by first placing the individual mice into clean, empty cages. After 30-60 minutes, fecal pellets were collected from the cages using sterile forceps and placed into sterile 1.5 ml Nalgene cryovials (Nalgene, USA). Samples were stored at -80ºC until culturing. Fecal samples were also collected directly from the mouse for molecular work. This was done by holding the restrained mice over a sterile 1.5 ml Nalgene cryovial until at least 2

fecal pellets were collected. The cryovials were immediately placed in dry-ice and stored at -80°C until DNA extraction.

2.6.3 Culturing of mouse feces

Fecal samples were thawed on ice prior to processing. The samples were weighed in 2.0 ml Axygen™ microcentrifuge tubes (Corning, Inc., USA) and suspended in 1.0 ml PBS. A single sterile 5 mm stainless steel bead (Qiagen, Germany) was placed into the tube and the samples were homogenized at 10 Hz for 30 seconds using a TissueLyser II (Qiagen, Germany). The homogenized samples were then serially diluted tenfold from 10^{-1} to 10^{-8} in PBS and plated onto selective and differential media. *S.* Heidelberg C570 recipients were enumerated by spot-plating 5µl of the dilution series onto XLT4 agar with 50 mg/L rifampicin. The donor *E. coli* was enumerated by spot-plating 5 µl of the dilution series onto Chromocult agar with 4 mg/L cefotaxime, and the transconjugants were enumerated by spread-plating 100 µl of the dilution series onto XLT4 agar with 4 mg/L cefotaxime and 50 mg/L rifampicin. After 24 hours of growth at 37°C, the colonies were counted. The recipient counts were calculated by subtracting the number of colonies on the transconjugant plates from the number of the colonies on the recipient plates. The transconjugation frequency was expressed as transconjugants/donors.

2.7 Whole genome sequencing of transconjugants

Horizontal gene transfer was confirmed using whole genome sequencing (WGS) of the recovered transconjugants. WGS was performed at National Microbiology Laboratory (NML) at Guelph (Guelph, Ontario) using both Illumina and Oxford Nanopore technologies. Genomic DNA extraction of the putative transconjugants was performed on the Qiagen Advanced XL

instrument (Qiagen, Germany) using the Qiagen EZ1 DNA tissue kit (Qiagen, Germany), as per the manufacturer's protocol. Illumina sequencing was done on a Miseq instrument (Illumina, USA) using the MiSeq Reagent Kit v3 and Nextera XT DNA Library Preparation Kit (Illumina, USA). Oxford Nanopore sequencing was performed Oxford Nanopore MinION™ sequencer using a SQK-RBK004 Rapid Barcoding Kit and FLO-MIN106 flow cell (Oxford Nanopore Technologies, U.K.). Draft assemblies from Illumina raw reads were constructed on the Integrated Rapid Infectious Disease Analysis (IRIDA) platform using the St. Petersburg genome assembler (SPAdes) pipeline. Demultiplexing, base-calling and quality filtering of Nanopore raw reads was done using Oxford Nanopore Technologies' Albacore v2.1.3. Hybrid *de novo* assemblies of short and long reads were produced using the Unicycler v0.4.3 assembly pipeline. Characterization of plasmids from draft assemblies was done using MOB-suite which provides *in silico* predictions of the replicon type, relaxase type, mating-pair formation type and transferability of the plasmid.

2.8 Gut microbiota profiling

2.8.1 Extraction of genomic DNA for 16S rDNA sequencing

Genomic DNA for 16S rDNA metagenomic sequencing was extracted using the NucleoSpin® Soil kit (Macherey-Nagel, Germany). The DNA was extracted as per the manufacturer's protocol using a single fecal pellet. The fecal samples were transferred to the NucleoSpin® Type A lysis tubes using sterile forceps and placed into an ice bucket. The protocol was performed on ice until the mechanical lysis step. After placing the fecal samples in the Type A lysis tubes, 700 µl of lysis buffer SL2 and 150 µl of the Enhancer SX was added. The tubes were then removed from ice and homogenized using the FastPrep-24™ 5G instrument (5

m/s for 30 seconds). The tubes were centrifuged for 2 minutes at 11,000 g to pellet contaminants. The clear supernatant underneath the top foam layer was then transferred to a new 2.0 ml Axygen™ microcentrifuge tube (Corning, Inc., USA). In the tubes containing the supernatant, 150 µl of buffer SL3 was added and the tubes were vortexed for 5 seconds. The tubes were incubated at 4°C for 5 minutes and centrifuged for 1 minute at 11,000 g to precipitate contaminants. The supernatant (~700 µl) was transferred to a NucleoSpin® Inhibitor Removal Column and centrifuged for 1 minute at 11,000 g to filter the lysate. The inhibitor removal column was discarded and 250 µl of buffer SB1 was added to the lysate. The SB1-lysate mixture was vortexed for 5 seconds and loaded onto the NucleoSpin® Soil Column to bind the DNA. Due to the large volume of SB1-lysate mixture (>1.0 ml), the mixture was loaded in two stages. After adding 550 µl of the mixture, the column was centrifuged for 1 minute at 11,000 g. The flowthrough was poured from the collection tube and the remaining mixture (~500 µl) was loaded and centrifuged. After the second half of flowthrough was poured off, the column was placed back into the collection tube. The silica membrane in the NucleoSpin® Soil Column was then washed four times with 30 second centrifuge steps (11,000 g) between each wash. Briefly, 500 µl of buffer SB1 was added to the column and centrifuged. The flowthrough was poured off and the column was placed back into the collection tube. This was repeated for the remaining 3 wash steps as follows: 550 µl of SW1 was added to the column and centrifuged, 650 µl of SW2 was added, vortexed for 2 seconds and centrifuged; finally, 650 µl of SW2 was added, vortexed for 2 seconds and centrifuged. The flowthrough was poured off and the column was centrifuged for 2 minutes at 11,000 g to eliminate any remaining SW2 buffer. The column was then transferred into a new 1.5 ml microcentrifuge tube (Fisher Scientific, USA). The DNA was eluted into the new microcentrifuge tube by adding 100 µl of buffer SE, incubating for 1 minute

at room temperature (with the lid open) and centrifuged for 30 seconds at 11,000 g. The column was discarded and the purified DNA was stored at -20°C until further processing.

2.8.2 16S rDNA sequencing

DNA was extracted from the fecal samples using the NucleoSpin® Soil DNA extraction kit (Macherey-Nagel, Germany), as per manufacturer's protocol, using 700 µl of Buffer SL2 and 150 µl of Enhancer SX. The samples were homogenized on a FastPrep®-24 instrument (MP Biomedicals, USA) for 30 seconds at 5 m/s. The DNA was eluted in 100 µl of Buffer SE. Prior to library preparation, the extracted DNA was quantified and normalized using the Quant-iT™ dsDNA Assay Kit (Invitrogen™, USA). The 16S rDNA sequencing was performed at Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield using Illumina technologies. The sequencing was done on a Miseq instrument (Illumina, USA) using the MiSeq Reagent Kit v3, Nextera XT DNA Library Preparation Kit (Illumina, USA) and Nextera XT Index Kit (Illumina, USA). Library preparation was done according to Illumina's 16S Metagenomic Sequencing Library Preparation protocol. Validation and quality control were assessed by sequencing the 20 Strain Even Mix Whole Cell Material (ATCC® MSA-2002™) mock microbial community (American Type Culture Collection, USA). Data analysis was done using the QIIME 2™ microbiome platform with a bioinformatics pipeline developed at Canadian Food Inspection Agency, Ottawa Laboratory Carling.

2.8.3 Parameters used for gut microbiota profiling

The impact of different treatment conditions on the gut microbiota profile was assessed by comparing species richness, microbial diversity, and the relative abundance of bacterial phyla, families, and genera. Species richness is a measure of the number of species present in a sample

and was determined using observed OTUs (operational taxonomic units). The Shannon Index is a measure of diversity that generates a number based on both species richness and species evenness (the relative abundance of each species in an environment).

CHAPTER 3: RESULTS

3.1 Selection of a donor strain from conjugation assays

Prior to the *in vivo* HGT experiment, conjugation assays from the three donor isolates to *E. coli* CV601gfp (Table 2.1) were performed to determine which donor would be the most suitable. *E. coli* CV601gfp was used as its GFP marker allowed it to be distinguished from other bacteria by viewing the agar plates under ultraviolet (UV) light. The donor strain was chosen from these assays based on its transfer frequency to *E. coli* CV601gfp. *E. coli* O80:H26 had the highest transfer frequency followed by *S.* Heidelberg C553 and *S.* Bredeney C546 (Table 3.1). As a result, it was selected for future use as a donor strain in the *in vivo* horizontal gene transfer experiments.

ID	Strain	Type	Plasmid	Motility	AMR gene	Transfer frequency
C546	*Salmonella* Bredeney	Donor	IncN	Conjugative	*bla*CTX-M-1	5.21E-05
C551	*Escherichia coli* O80:H26	Donor	IncI2	Conjugative	*bla*CMY-2	7.69E-04
			IncY	Mobilizable	*bla*TEM-1B	
C553	*Salmonella* Heidelberg	Donor	IncA/C2	Conjugative	*bla*CMY-2	2.98E-04
					*bla*TEM-1B	
C439	*Escherichia coli* CV601	Recipient				

Figure 3.1 Results from the conjugation assays. *E. coli* O80:H26 was selected as a donor strain based on its high transfer frequency and easy differentiation from the *S.* Heidelberg recipient on culture media.

3.2 Selection of *S.* Heidelberg C570 as a recipient strain

A significant difference in the recovery of *S.* Heidelberg was observed between strains (Figure 3.2) and between the streptomycin pre-treated and non-treated groups. *S.* Heidelberg recovery was higher in the streptomycin pre-treated mice (Panel A) for all seven strains in comparison to the non pre-treated mice (Panel B). The *S.* Heidelberg levels decreased after 1 DPI for all strains. By 7 DPI, *S.* Heidelberg C569, C572, and C574 had decreased to below the

detection limit in the pre-treated mice. By 10 DPI, only *S.* Heidelberg C565, C566, and C570 were recovered from the pre-treated mice although strain C566 was significantly lower. Strains C565 and C570 were considered for use as recipient strains as both were recovered from the feces of pre-treated mice up until 10 DPI (>10^6 CFU/g). Due to a greater number of mice colonized by *S.* Heidelberg C570 in comparison to strain C565 on 10 DPI (3 replicates for C570, 2 replicates for C565), this strain was chosen for future *in vivo* HGT experiments.

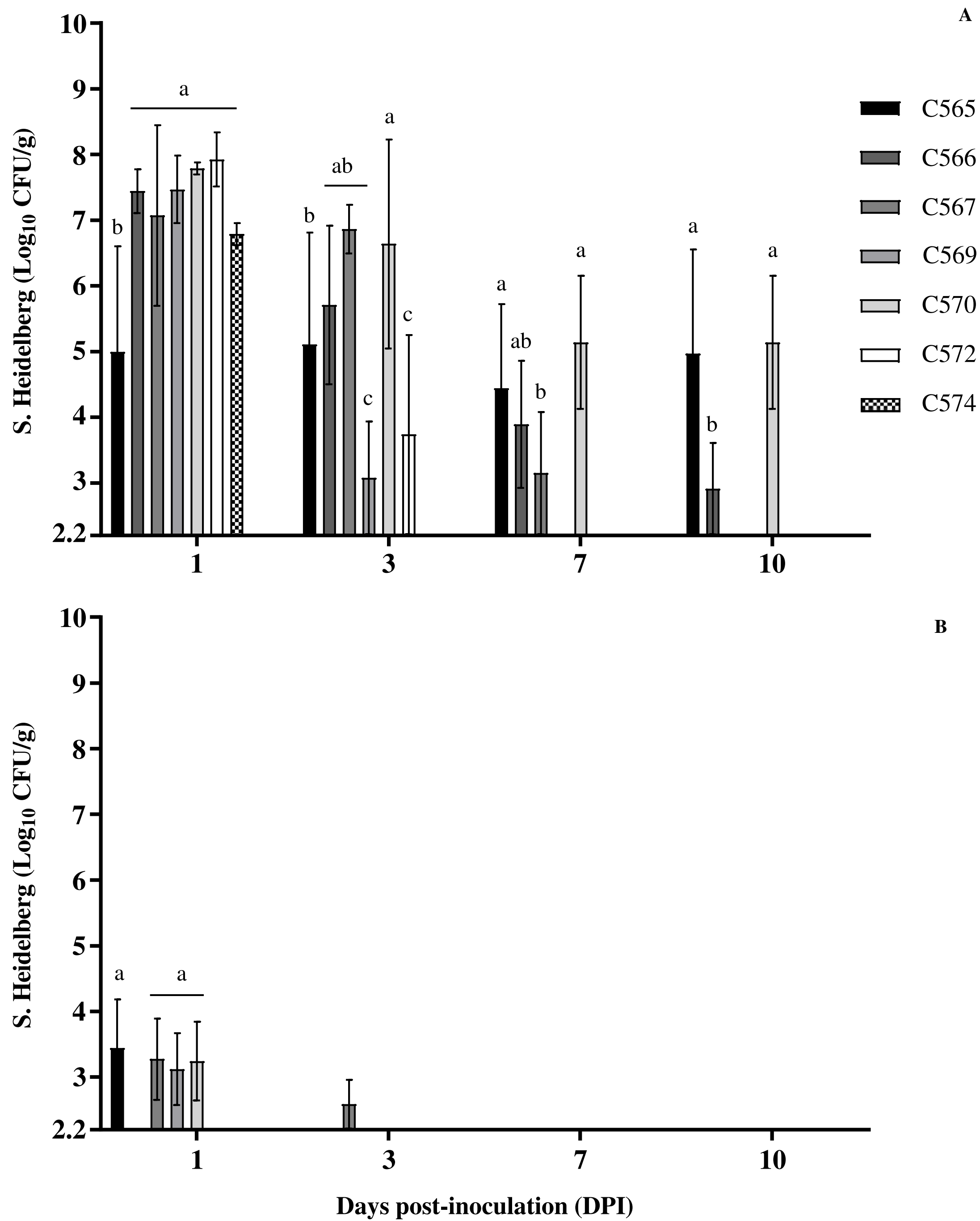

Figure 3.2 Bacterial counts of *S.* Heidelberg strains from streptomycin pre-treated (Panel A) and non-treated mice (Panel B) over 10 days post-inoculation. Letters were used to indicate significant ($p \leq 0.05$) differences between strains as determined by one-way ANOVA with Tukey's HSD. The error bars represent standard error of the mean (SEM).

3.3 Culturing of mouse feces from *in vivo* horizontal gene transfer experiments

3.3.1 Recovery of bacteria from E. coli O80:H26 and *S. Heidelberg C570 infected mice.*

The donor *E. coli* O80:H26 and recipient *S.* Heidelberg C570 strains were recovered from all groups on 1 DPI (Figure 3.3, Panel A). Antibiotic treatment had a significant impact on *E. coli* colonization as the bacterial counts were significantly greater in the ampicillin, streptomycin, and the streptomycin-ampicillin treated groups in comparison to the control group (no antibiotic). It was found that the recovery of *E. coli* was highest in streptomycin-ampicillin treated mice, followed by streptomycin and ampicillin alone. Conversely, the streptomycin-ampicillin treatment resulted in a lower recovery of *S.* Heidelberg in comparison to streptomycin and ampicillin alone. Transconjugants were only recovered from the antibiotic treated groups. The transconjugant counts were similar in the streptomycin-ampicillin and streptomycin treated mice; however, they were significantly lower in the ampicillin treated mice in comparison to streptomycin alone.

By 2 DPI, the *E. coli* and *S.* Heidelberg counts in the control group had decreased to below the detection limit (Figure 3.3, Panel B). Like 1 DPI, the *E. coli* counts were greatest in the streptomycin-ampicillin treated mice followed by the streptomycin and ampicillin treated mice. This was also observed with the recovery of *S.* Heidelberg on 2 DPI. The transconjugant levels in both the streptomycin and streptomycin-ampicillin treated groups increased by 2 DPI and remained statistically similar to each other. This trend was also observed on 3 DPI (Figure 3.3, Panel C) with increases in the transconjugant levels and slight decreases in the *E. coli* and *S.* Heidelberg counts. By 7 DPI, the *E. coli* counts were significantly lower in the streptomycin treated mice in comparison to the ampicillin alone and streptomycin-ampicillin groups. The

transconjugant levels in the ampicillin treated mice increased significantly from 3 DPI; meanwhile, the transconjugant counts in the streptomycin treated groups decreased significantly from 3 DPI. Between groups on 7 DPI, transconjugants were significantly lower in the streptomycin and ampicillin treated mice in comparison to the streptomycin-ampicillin treated mice. No significant changes from 3 DPI were observed in the *S.* Heidelberg counts in any group.

By 14 DPI, the *E. coli* counts in the streptomycin treated mice had decreased to below the detection limit (Figure 3.3, Panel E). The *S.* Heidelberg counts in these mice were now significantly lower than the streptomycin-ampicillin treated mice although the transconjugant counts remained statistically similar to the streptomycin-ampicillin treated mice. *E. coli* and *S.* Heidelberg were only recovered from one ampicillin treated mouse on 14 DPI and the transconjugants had decreased to below the detection limit (Figure 3.3, Panel F). By 21 DPI, *E. coli, S.* Heidelberg and transconjugants were only recovered from the streptomycin-ampicillin treated mice (Figure 3.3, Panel G). The counts for *E. coli, S.* Heidelberg, and transconjugants did not decrease significantly by 42 DPI although transconjugants were only recovered in 2 replicates (versus 4 on 21 DPI) (Figure 3.3, Panel H).

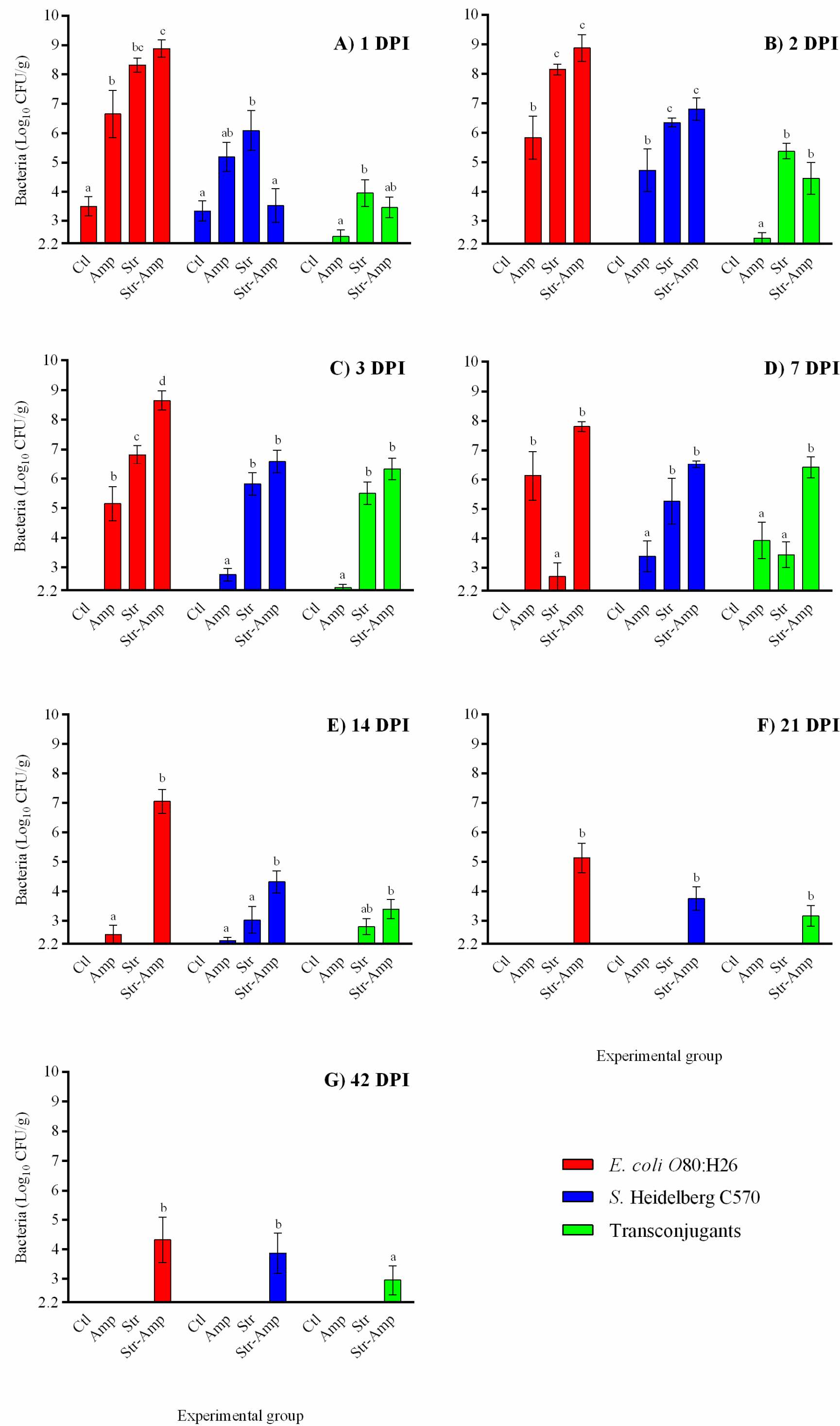

Figure 3.3 Mean recovery of *E. coli* O80:H26, *S.* Heidelberg C570, and transconjugants from 1 DPI-42 DPI. Estimates of the recipient recovery were obtained by subtracting the total number of colonies recovered on transconjugant media from the total number of colonies on recipient media. Letters used to indicate significant ($p \leq 0.05$) differences between groups as determined by one-way ANOVA with Tukey's HSD. Error bars represent standard error of the mean (SEM).

3.3.2 Transfer frequency

Transfer frequency is a measurement of number of transconjugants that developed per donor bacterium and is expressed as transconjugants/donors. Transconjugants were not recovered at all from the control, antibiotic-free mice and as a result the transfer frequency is reported as below the transfer frequency detection limit (-9 Log). On 1 DPI, the transfer frequency was significantly greater in the streptomycin and streptomycin-ampicillin treated mice in comparison to the control and ampicillin treated mice (Figure 3.4). On 7 DPI, the transfer frequency for the streptomycin treated mice was significantly lower than the ampicillin and streptomycin-ampicillin treated mice and was similar to the control mice.

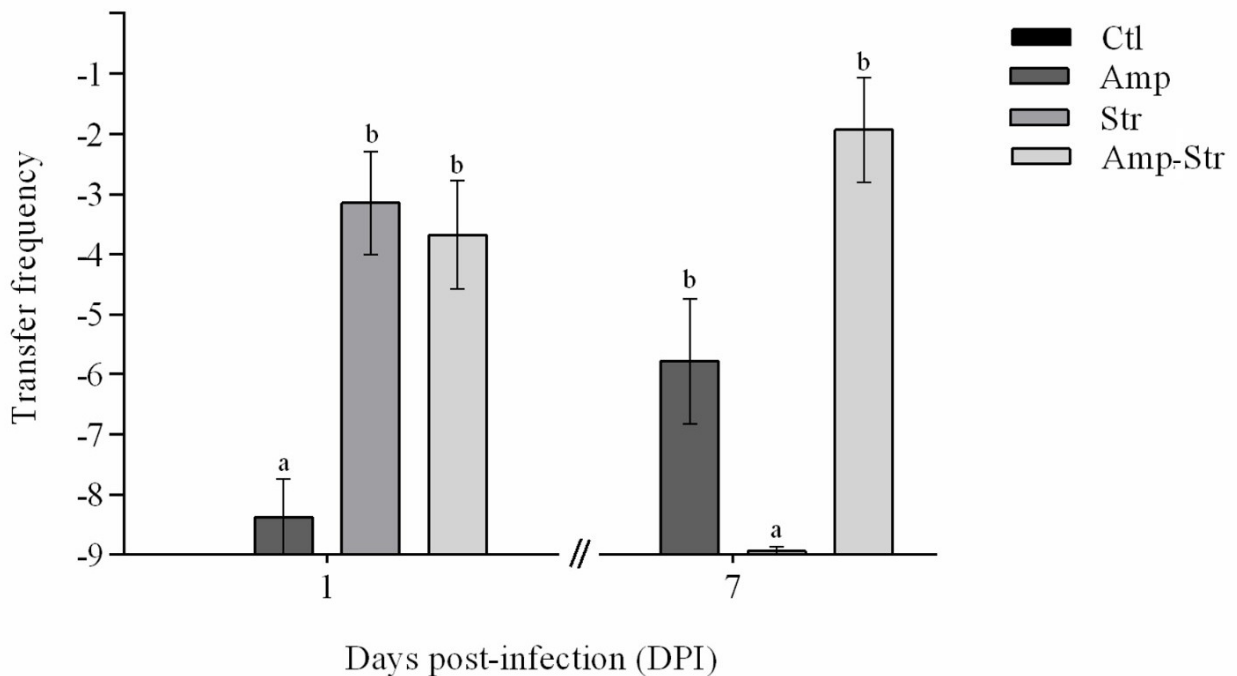

Figure 3.4 Mean transfer frequency on 1 DPI and 7 DPI. The transfer frequency is expressed as transconjugants/donors. Letters used to indicate significant ($p \leq 0.05$) differences between groups as determined by one-way ANOVA with Tukey's HSD. Error bars represent standard error of the mean (SEM).

3.4 Whole genome sequencing

A total of 45 isolates from 1 DP1, 3 DPI, 7 DPI, 14 DPI, 21 DPI, and 42 DPI were sent for WGS to confirm transconjugants (Table 3.1). WGS of the isolates confirmed that all of the *S.* Heidelberg isolates were indeed transconjugants based on the presence of IncI2 plasmids carrying *bla*CMY-2. Based on completed Illumina and Nanopore hybrid assemblies, 18 of the *S.* Heidelberg isolates also carried IncI1 plasmids, none of which carried any known AMR genes. The IncI1 plasmids were identified in *S.* Heidelberg isolates from both the streptomycin and streptomycin-ampicillin treated mice. Interestingly, they were only identified in isolates from 3 DPI onwards. The six *E. coli* putative transconjugants sent for sequencing (4 from 14 DPI, 2 from 42 DPI) were also confirmed to be transconjugants, based on the presence of IncI2 plasmids carrying *bla*CMY-2. The *E. coli* transconjugants had the serovar O2:H6, suggesting that they are of commensal origin. Out of the six *E. coli* O2:H6 transconjugants, one was confirmed by Illumina and Nanopore hybrid assemblies to carry an IncI1 plasmid. IncI1 appeared to be present in the other five isolates based on the Illumina assemblies, but was not present in the isolates based on hybrid assemblies. Hybrid assemblies also revealed the presence of *bla*CMY-2 on both the IncI2 plasmid and chromosome of the six *E. coli* O2:H6 isolates.

Group	DPI	Genus	Species	Serovar	IncI1	IncI2	*bla*CMY-2	
							Chromosome	Plasmid
Str	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	1	*Salmonella*	*enterica*	Heidelberg		x		x
Str	2	*Salmonella*	*enterica*	Heidelberg		x		x
Str	2	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	2	*Salmonella*	*enterica*	Heidelberg		x		x
Str	3	*Salmonella*	*enterica*	Heidelberg		x		x
Str	3	*Salmonella*	*enterica*	Heidelberg		x		x
Str	3	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str	3	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	3	*Salmonella*	*enterica*	Heidelberg		x		x
Str-Amp	3	*Salmonella*	*enterica*	Heidelberg		x		x
Amp	7	*Salmonella*	*enterica*	Heidelberg		x		x
Str	7	*Salmonella*	*enterica*	Heidelberg		x		x
Str	7	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	7	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	7	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str	14	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str	14	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	14	*Escherichia*	*coli*	O2:H6	x	x	x	x
Str-Amp	14	*Escherichia*	*coli*	O2:H6	x	x	x	x
Str-Amp	14	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	14	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	14	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	14	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	14	*Escherichia*	*coli*	O2:H6	x	x	x	x
Str-Amp	14	*Escherichia*	*coli*	O2:H6	x	x	x	x
Str-Amp	21	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	21	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	21	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	21	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	21	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	42	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	42	*Escherichia*	*coli*	O2:H6	x	x	x	x
Str-Amp	42	*Escherichia*	*coli*	O2:H6		x	x	x
Str-Amp	42	*Salmonella*	*enterica*	Heidelberg	x	x		x
Str-Amp	42	*Salmonella*	*enterica*	Heidelberg	x	x		x

Table 3.1. WGS of the recovered transconjugants. All isolates sent for WGS were confirmed to have acquired the *bla*CMY-2 gene. The *E. coli* isolates were found to carry *bla*CMY-2 on the IncI2 plasmid as well as in the chromosome. WGS revealed the presence of an IncI1 plasmid in the *S.* Heidelberg and *E. coli* O2:H6 transconjugants from 3 DPI onwards.

3.5 16S rDNA sequencing

3.5.1 Observed OTUs

The number of observed OTUs was used as a measure of total species richness, with a higher number of OTUs indicating a greater number of species. The number of observed OTUs in the control mice did not change significantly over the course of the experiment. The observed OTUs of the streptomycin and streptomycin-ampicillin treated mice dropped significantly following the initial streptomycin treatment (Figure 3.5, Panel B). By 1 DPI, the number of observed OTUs decreased in the ampicillin treated mice although they still remained similar to the control mice (Figure 3.5, Panel C). The number of observed OTUs on 1 DPI remained similar between the streptomycin and streptomycin-ampicillin treated mice and both remained significantly lower than the control and ampicillin treated mice. By 2 DPI, the number of observed OTUs in the ampicillin treated mice decreased significantly from the control mice and was similar to the streptomycin and streptomycin-ampicillin treated mice (Figure 3.5, Panel D). By 7 DPI, the number of observed OTUs in the streptomycin treated mice increased to control levels and remained so for the rest of the experiment (Figure 3.5, Panel E). The number of observed OTUs in the ampicillin and streptomycin-ampicillin treated mice further decreased from 2 DPI and remained similar to each other. By 14 DPI, the number of observed OTUs in the ampicillin treated mice increased to control levels and remained so for the rest of the experiment (Figure 3.5, Panel F). During this time and for the rest of the experiment, the number of observed OTUs in the streptomycin-ampicillin treated mice was similar to the other antibiotic groups but remained significantly lower than the control mice (Figure 3.5, Panel F-H).

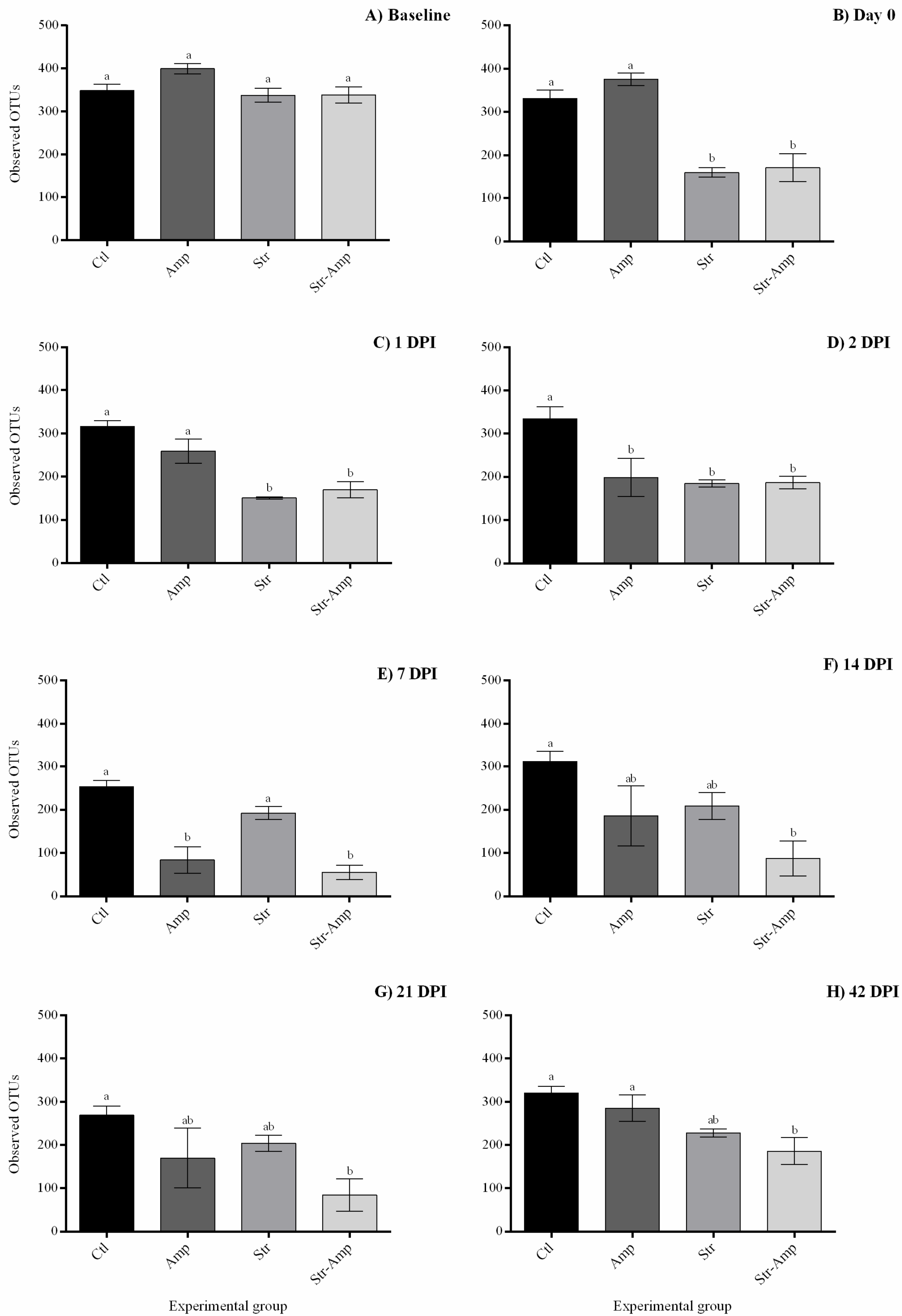

Figure 3.5. Results from 16S rDNA sequencing showing shifts in the number of observed OTUs under different treatment conditions. Letters used to indicate significant ($p \leq 0.05$) differences between groups over time as determined by one-way ANOVA with Tukey's HSD.

3.5.2 Shannon's diversity index

The Shannon index (H) was also used to calculate alpha diversity (Figure 3.6). Unlike the observed OTUs, the Shannon index takes into consideration both species abundance and species evenness. A higher H value indicates a more diverse community. The Shannon index of the control mice did not change significantly over the course of the experiment. The Shannon index of the streptomycin and streptomycin-ampicillin treated mice dropped significantly following treatment (Figure 3.6, Panel B). By 1 DPI, following the commencement of ampicillin, the Shannon index for the ampicillin treated mice dropped significantly below the control mice but remained similar to the streptomycin and streptomycin-ampicillin treated mice (Figure 3.8, Panel C). By 2 DPI, the Shannon index for the streptomycin treated increased to the level of the control mice and remained so for the rest of the experiment (Figure 3.6, Panel D-H). The Shannon index for the ampicillin and streptomycin-ampicillin treated mice remained significantly lower than the control mice and were similar to each other (Figure 3.6, Panel D). By 7 DPI, the Shannon index for the streptomycin-ampicillin treated mice decreased further and was no longer statistically similar to the ampicillin treated mice (Figure 3.8, Panel E). After the withdrawal of ampicillin treatment on 7 DPI, the Shannon index began to increase for both the ampicillin and streptomycin-ampicillin treated mice. By 14 DPI, the Shannon index increased to control levels and remained so for the rest for the experiment (Figure 3.6, Panel F). The Shannon index for the streptomycin-ampicillin treated mice increased from 7 DPI to 14 DPI but was still significantly lower than the control level). By 21 DPI, the Shannon index for the streptomycin-ampicillin treated mice increased to control levels and remained so for the rest of the experiment (Figure 3.6, Panel G-H).

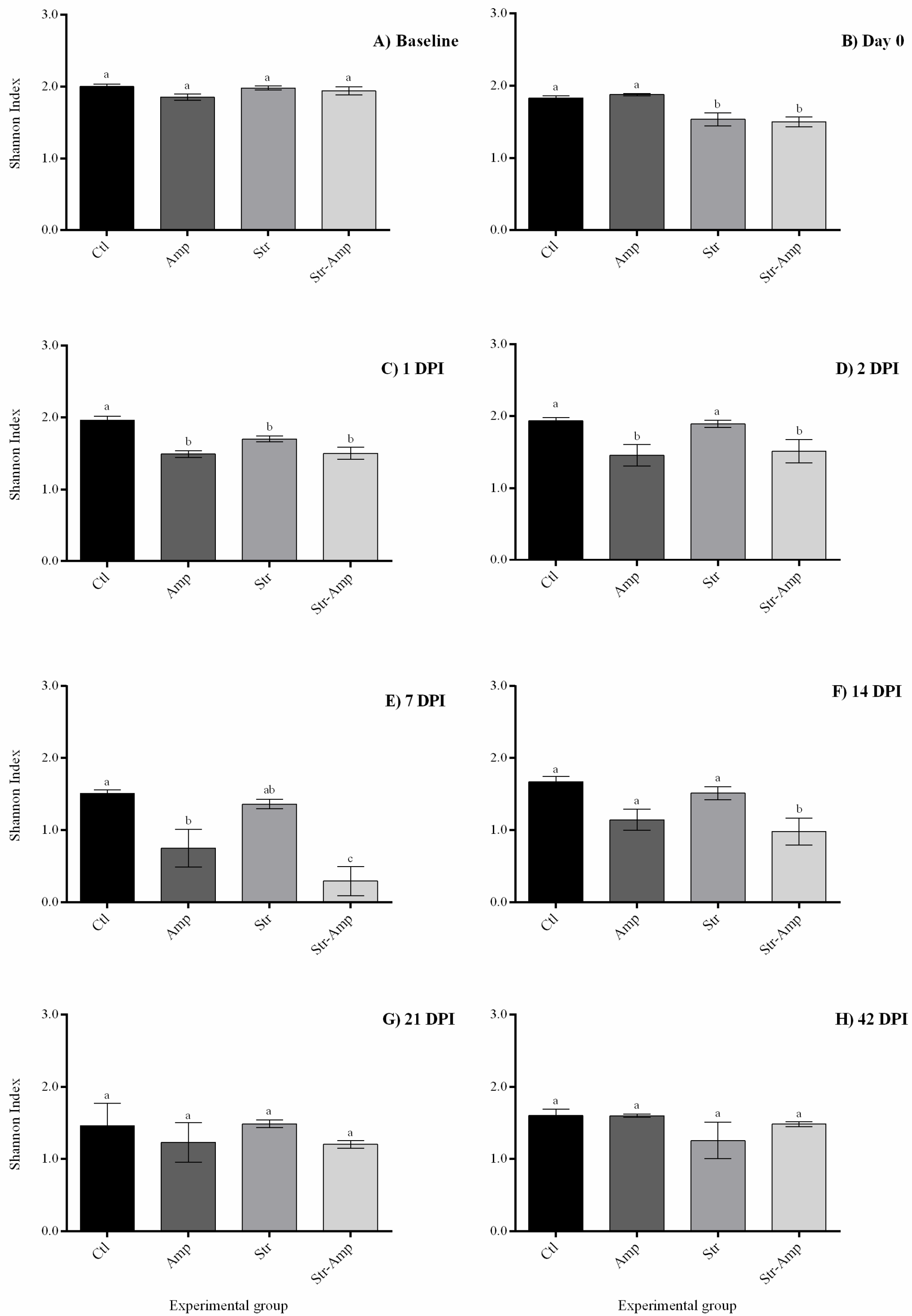

Figure 3.6. Results from 16S rDNA sequencing showing shifts in the Shannon index under different treatment conditions. Letters used to indicate significant ($p \leq 0.05$) differences between groups over time as determined by one-way ANOVA with Tukey's HSD.

3.5.3 Impact of antibiotics on phylum diversity

Alterations in the Bacteroidetes, Firmicutes, and Proteobacteria were analyzed as they dominate the gut microbiome and play an important role in gut homeostasis (section 1.5). There were no significant alterations in the gut phyla composition of the control mice during the observation period. The mean relative abundance of Bacteroidetes, Firmicutes and Proteobacteria for the control mice over time was 41%, 54%, and <1%, respectively. On day 0, streptomycin was found to have a profound impact on the gut phyla composition as there were significant alterations in all three phyla of interest in the gut microbiota of the streptomycin and streptomycin-ampicillin treated mice (Figure 3.7, Panel B). In comparison to the control group, the relative abundance of Firmicutes in these mice was significantly lower and corresponded with significant increases in the Bacteroidetes and Proteobacteria populations. As ampicillin did not begin until later on day 0, there were no changes in the gut phyla composition of the ampicillin treated mice on day 0. By 1 DPI, the relative abundance of Firmicutes continued to decrease in the streptomycin and streptomycin-ampicillin treated mice and the Bacteroidetes and Proteobacteria populations increased (Figure 3.7, Panel C). Ampicillin treatment also resulted in decreases in the relative abundance of Firmicutes and increases in Bacteroidetes (relative to control mice), although not as great as the impact of streptomycin-ampicillin. This trend continued on 2 DPI with streptomycin-ampicillin having the greatest impact on all three phyla (Figure 3.7, Panel D). By 7 DPI, the relative abundance of Bacteroidetes and Firmicutes in the streptomycin treated mice increased and were similar to those of the control mice (Figure 3.7, Panel E). With continued ampicillin treatment, the Proteobacteria population continued to increase and the Firmicutes population decreased. The mean relative abundance of Firmicutes in the ampicillin and streptomycin-ampicillin treated mice decreased from 34% and 37% on 2 DPI, respectively, to 5% and 4% on 7 DPI. Further, the mean relative abundance of Proteobacteria in

the ampicillin and streptomycin-ampicillin treated mice increased from 1% and <1% on 2 DPI, respectively, to 49% and 82% by 7 DPI. After withdrawal of ampicillin on 7 DPI, the relative abundance of all three phyla began to return to baseline levels in the ampicillin and streptomycin-ampicillin treated mice. By 14 DPI, the relative abundance of Firmicutes in all groups were statistically similar (Figure 3.7, Panel F). The relative abundance of Proteobacteria in the ampicillin and streptomycin-ampicillin treated mice decreased from 7 DPI to 27% and 26%, respectively, although both were still significantly greater than the control and streptomycin treated mice. As the relative abundance of Firmicutes and Proteobacteria began to return to control levels in the ampicillin and streptomycin-ampicillin treated mice, the Bacteroidetes relative abundance decreased significantly relative to the control and streptomycin treated mice. By 21 DPI, the relative abundance of Proteobacteria was still significantly greater in the ampicillin and streptomycin-ampicillin mice in comparison to the control and streptomycin treated mice (Figure 3.7, Panel G). The relative abundance of Bacteroidetes in the streptomycin-ampicillin treated mice was similar to the control and streptomycin treated mice, although the relative abundance was still significantly lower in the ampicillin treated mice. By 42 DPI, the relative abundance of all three Phyla were statistically similar to each other (Figure 3.7, Panel H).

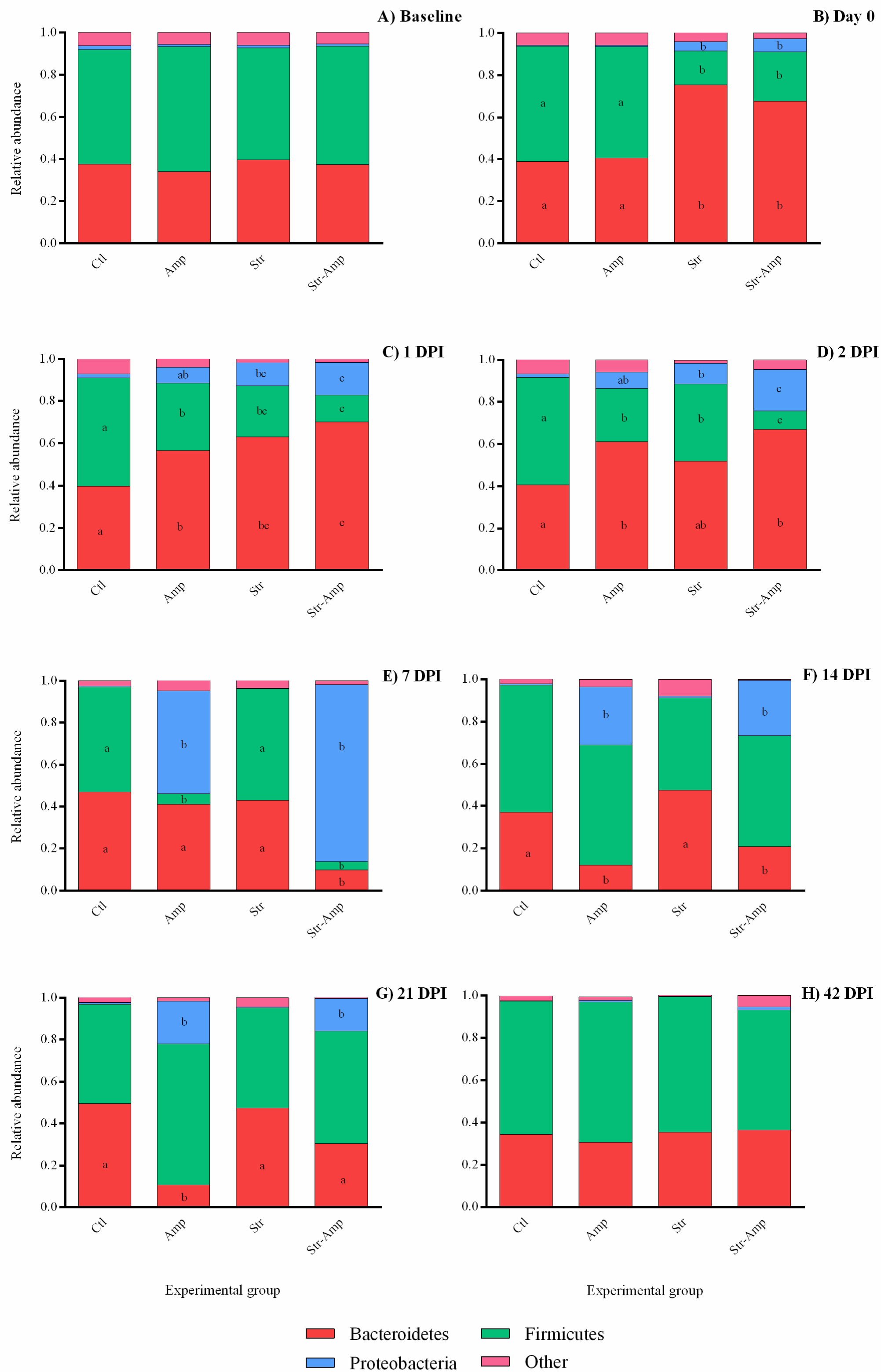

Figure 3.7 Results from 16S rDNA sequencing showing the shifts in the gut microbiota phylum composition under different treatment conditions. Letters used to indicate significant ($p \leq 0.05$) differences over time within families as determined by one-way ANOVA with Tukey's HSD. Data points with no letters are classified as "a" and statistically similar to all other experimental groups.

3.5.4 Impact of antibiotics on family diversity

The families *Bacteroidaceae, Lactobacillaceae, Lachnospiraceae, Ruminococcaceae* and *Enterobacteriaceae* were analyzed this study. *Lactobacillaceae, Lachnospiraceae,* and *Ruminococcaceae* are members of the Firmicutes phylum and play an important role in colonization resistance against pathogens through the production of SCFAs and bacteriocins. The *Enterobacteriaceae* family contains the *Escherichia* and *Salmonella* genera and other opportunistic pathogens. There were no significant alterations in the gut family composition of the control mice during the observation period. Over the entire observation period, the mean relative abundance of *Bacteroidaceae, Lactobacillaceae, Lachnospiraceae, Ruminococcaceae* and *Enterobacteriaceae* in the control mice did not change significantly. *Bacteroidaceae* was the most abundant family in the control mouse gut microbiota, with an average relative abundance of 36%. *Lachnospiraceae* and *Ruminococcaceae* were the most abundant members of the Firmicutes phylum in the control mouse gut microbiota, with an average relative abundance of 25% and 16% over the observation period, respectively. *Lactobacillaceae* and *Enterobacteriaceae* made up 7% and <0.1% of the gut microbiota, respectively.

Like the alterations of the gut at the phyla level, streptomycin treatment had an immediate impact on several gut families. On day 0, there were significant decreases in the relative abundance of *Lachnospiraceae* and *Ruminococcaceae* in the streptomycin and streptomycin-ampicillin treated mice in comparison to control mice (Figure 3.8, Panel B). The mean relative abundance of *Lachnospiraceae* in the streptomycin and streptomycin-ampicillin treated mice decreased from 20% and 21% on baseline (Figure 3.8, Panel A), respectively, to 1% and 3% on day 0. Similarly, the mean relative abundance of *Ruminococcaceae* in the streptomycin and streptomycin-ampicillin treated mice decreased from 16% and 18% on

baseline, respectively, to 1% and 2% on day 0. Streptomycin also had immediate impacts on the *Bacteroidaceae* and *Enterobacteriaceae* populations. On day 0, the relative abundance of both *Bacteroidaceae* and *Enterobacteriaceae* populations in the streptomycin treated mice increased significantly from baseline and were significantly greater than the control and ampicillin treated mice.

The trends in the gut microbiota composition on 1 DPI and 2 DPI were nearly identical with the exception of changes in the relative abundance of *Bacteroidaceae.* On 1 DPI, the relative abundance of the *Lachnospiraceae* and *Ruminococcaceae* in the streptomycin treated mice remained significantly lower than the control mice while the relative abundance of *Bacteroidaceae* and *Enterobacteriaceae* remained significantly greater (Figure 3.8, Panel C). Unlike on day 0, the relative abundance of *Lactobacillaceae* on 1 DPI was significantly greater in the streptomycin treated mice in comparison to the control mice. There were no significant changes in the gut family composition on 1 DPI in the ampicillin and streptomycin-ampicillin treated mice in comparison to the streptomycin only treated mice with the exception of *Lactobacillaceae*. Ampicillin treatment had an additional impact on *Lactobacillaceae* in comparison to the streptomycin treated and control mice as the relative abundance of *Lactobacillaceae* was significantly lower in the ampicillin and streptomycin-ampicillin (only 2 DPI) treated mice (Figure 3.8, Panel D).

Similar to the phylum level, the family composition of the streptomycin treated mice was almost back to control levels on 7 DPI with the exception of *Ruminococcaceae,* which was still significantly lower than the control mice (Figure 3.8, Panel E). The relative abundance of *Lachnospiraceae, Ruminococcaceae,* and *Lactobacillaceae* were still significantly lower in the ampicillin and streptomycin-ampicillin treated mice in comparison to control and streptomycin

treated mice. The relative abundance of *Enterobacteriaceae* in the ampicillin and streptomycin-ampicillin treated mice increased from 2 DPI to 7 DPI, going from 6% and 16% on 2 DPI, respectively, to 49% and 84% on 7 DPI.

By 14 DPI, the relative abundance of *Ruminococcaceae* in all antibiotic groups was still significantly lower than control mice (Figure 3.8, Panel F). The *Lactobacillaceae* population was similar across all groups on 14 DPI and the relative abundance of *Lachnospiraceae* was similar in all groups except the streptomycin-ampicillin treated mice (significantly lower than all other groups). The relative abundance of *Enterobacteriaceae* in the ampicillin and streptomycin-ampicillin treated mice had decreased slightly between 7 DPI and 14 DPI although was still significantly greater than the control and streptomycin treated mice. As observed at the phyla level with Bacteroidetes, the relative abundance of *Bacteroidaceae* in the ampicillin and streptomycin-ampicillin treated mice decreased significantly on 14 DPI (in comparison to control and streptomycin treated mice) as members of the Firmicutes phylum (e.g. *Lachnospiraceae, Ruminococcaceae,* and *Lactobacillaceae*) began to increase.

By 21 DPI, the relative abundance of *Lachnospiraceae* was similar across all groups and the relative abundance of *Lactobacillaceae* was similar between all groups except the ampicillin treated mice (Figure 3.8, Panel G). The *Ruminococcaceae* population returned to control levels in all but the streptomycin-ampicillin treated mice. The *Enterobacteriaceae* population decreased slightly from 14 DPI but was still significantly greater than the control and streptomycin treated mice. Finally, the relative abundance of the *Bacteroidaceae* increased to control levels in the streptomycin-ampicillin mice but was still significantly lower in the ampicillin treated mice. By 42 DPI, the relative abundance of *Bacteroidaceae*, *Lachnospiraceae*, and *Enterobacteriaceae* was similar across all groups (Figure 3.8, Panel H). However, the relative abundance of

Ruminococcaceae was still significantly lower in the streptomycin and streptomycin-ampicillin treated mice. Further, the relative abundance of *Lactobacillaceae* was significantly greater in the streptomycin treated mice in comparison to the control mice.

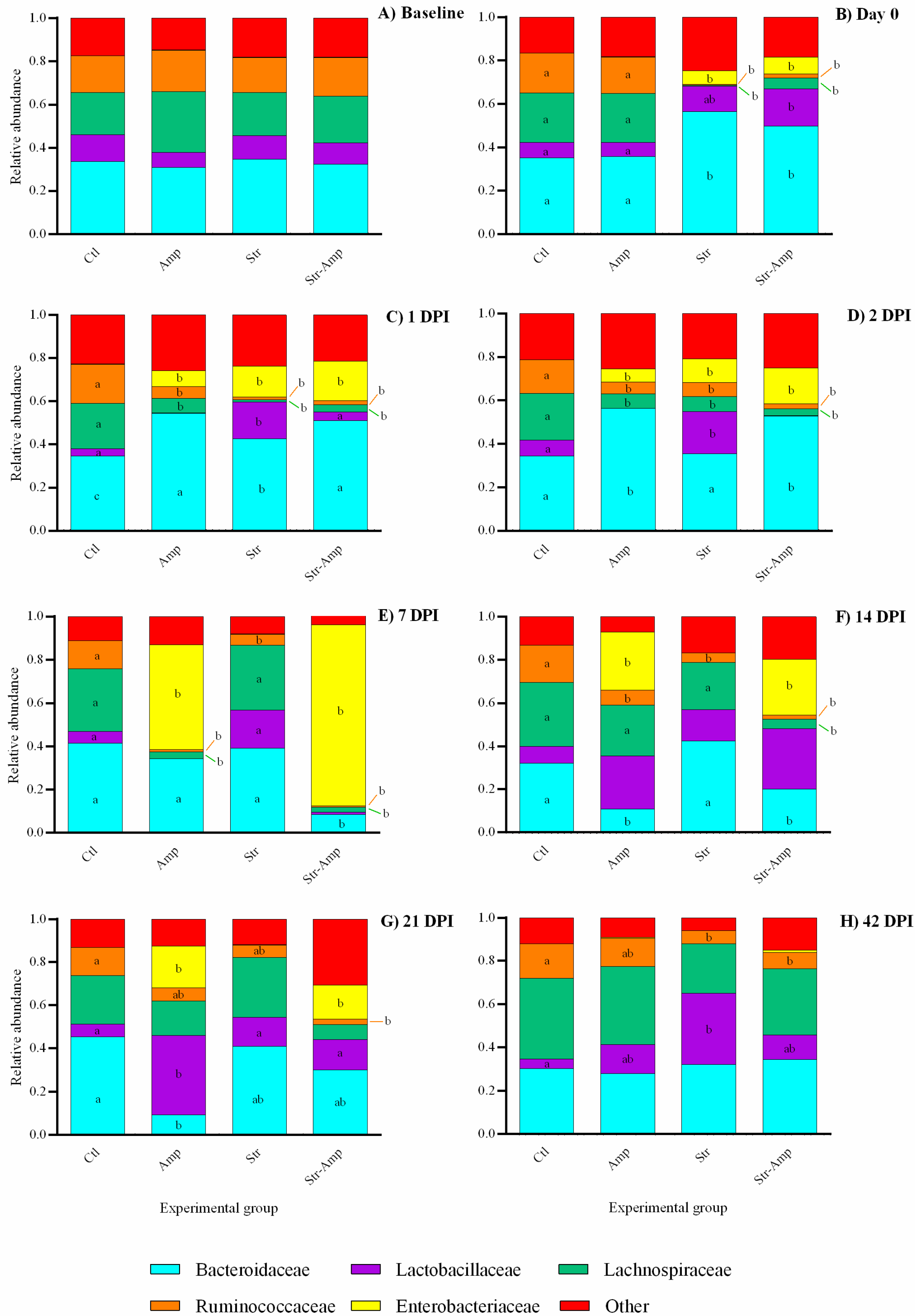

Figure 3.8 Results from 16S rDNA sequencing showing the shifts in the gut microbiota family composition under different treatment conditions. Letters used to indicate significant ($p \leq 0.05$) differences over time within groups as determined by one-way ANOVA with Tukey's HSD. Data points with no letters are classified as "a" and statistically similar to all other experimental groups.

3.5.5 Impact of antibiotics on genus diversity

Within the *Enterobacteriaceae* family, it was found that *Salmonella, Proteus, Klebsiella, Escherichia-Shigella,* and *Enterobacter* were the dominant genera. Over the course of the study, only *Escherichia-Shigella* and *Proteus* were detected in the control group, neither of which changed significantly. Together, the relative abundance of these genera did not exceed 0.2% of the gut microbiota on any collection day. Streptomycin had an immediate impact on the gut microbiota. On day 0, the relative abundance of *Escherichia-Shigella* in the streptomycin and streptomycin-ampicillin treated mice was significantly greater in comparison to the control mice (Figure 3.9, Panel B). On 1 DPI, the *Enterobacter* genus was detected in the ampicillin, streptomycin, and streptomycin-ampicillin treated mice while the *Klebsiella* genus was detected in the ampicillin treated mice (Figure 3.9, Panel C). *Salmonella* was detected in all three antibiotic groups although the relative abundance was only significant in the streptomycin treated mice. The *Proteus* genus was not significantly impacted by ampicillin nor streptomycin as the relative abundance did not change significantly in comparison to the control group. There were no significant changes in the trends of any genera by 2 DPI (in comparison to 1 DPI group significance) with the exception of the detection of *Klebsiella* in the streptomycin-ampicillin treated mice (Figure 3.9, Panel D). By 7 DPI, *Klebsiella* was only detected in the streptomycin treated mice while *Enterobacter* and *Salmonella* were only detected in the streptomycin-ampicillin treated mice (Figure 3.9, Panel E). *Escherichia-Shigella* was detected in all groups on 7 DPI although the relative abundance was only significantly greater in the streptomycin-ampicillin treated mice relative to the control group. *Enterobacter* was detected in both ampicillin groups although neither was significantly greater than the control mice and both were statistically similar to each other. The relative abundance of *Salmonella* was inconsistent with the

culture results in that *Salmonella* was not detected by 16S rDNA sequencing although it was detected by culture. This may be attributed to a number of reads that were not unassigned to any specific genus and as a result considered unknown. By 14 DPI, the relative abundance of *Escherichia-Shigella* increased from 7 DPI in the ampicillin and streptomycin-ampicillin groups, both of which were also significantly greater than the control and streptomycin treated mice. *Salmonella* was detected in the ampicillin and streptomycin-ampicillin treated mice on 14 DPI although the relative abundance was 1ess than 1% (Figure 3.9, Panel F). *Klebsiella* and *Enterobacter* were detected in the streptomycin-ampicillin treated mice, each of which up less than 1% of the gut microbiota. By 21 DPI, *Klebsiella, Salmonella,* and *Enterobacter* were detected again in both the ampicillin and streptomycin-ampicillin treated mice. During this time, all of the detected genera were statistically similar to the control mice (Figure 3.9, Panel G). By 42 DPI, *Enterobacter, Klebsiella*¸ and *Salmonella* were detected only in the streptomycin-ampicillin treated mice and each made up less than 1% of the gut microbiota (Figure 3.9, Panel H).

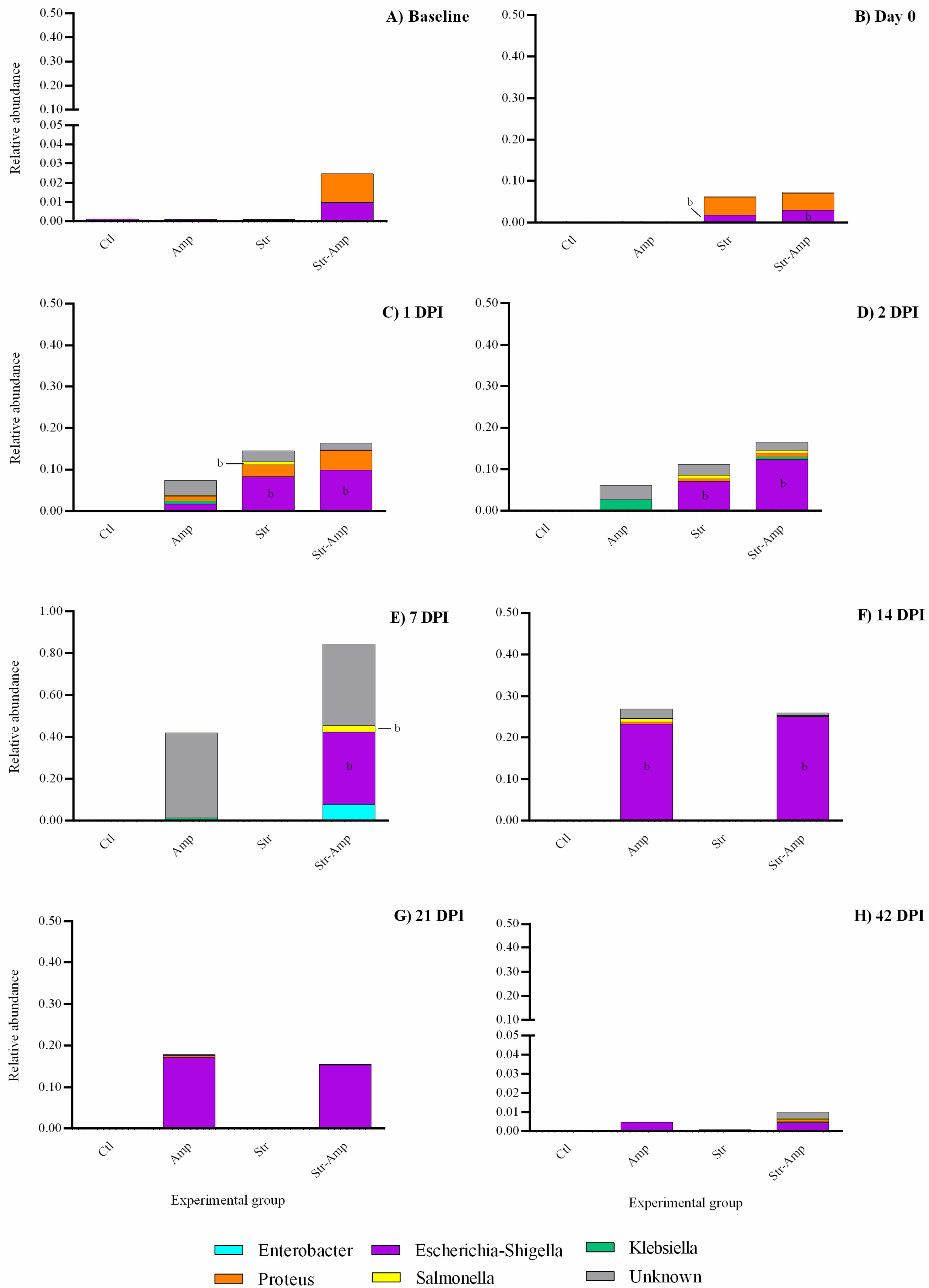

Figure 3.9 Results from 16S rDNA sequencing showing the shifts in the gut microbiota genus composition under different treatment conditions. Letters used to indicate significant ($p \leq 0.05$) differences over time within groups as determined by one-way ANOVA with Tukey's HSD. Data points with no letters are classified as "a" and statistically similar to all other experimental groups.

CHAPTER 4: DISCUSSION

4.1 General Discussion

Bacteria have exchanged AMR genes via horizontal gene transfer (HGT) for billions of years as an evolutionary mechanism to survive the production of antimicrobial compounds. *In vivo* models are useful tools in assessing the risk factors for AMR gene transmission outside of an environment optimized for conjugation. Unlike transformation and transduction, bacterial conjugation is cell contact-dependent and requires the donor and recipients to be present in the same place at the same time. Many of the pathogens at-risk for the development of AMR (E.g. *Enterobacteriaceae*) are kept at low levels due to the protective action of the gut microbiota. However, it is well known that the use of broad-spectrum antibiotics may result in the overgrowth of these pathogens. This leaves the host susceptible to infection by exogenous bacteria but may also increase the risk of AMR gene transfer as a result of increased levels of indigenous plasmid-free recipients. The use of β-lactam antibiotics and immune suppression are considered the biggest risk factors for the acquisition and/or development of AMR bacteria (Ramen et al. 2018). This study utilized a 16S rDNA sequencing-culture based approach to determine how the modification of the gut microbiota composition via different antibiotics (streptomycin, ampicillin, streptomycin-ampicillin) effects the transmission of AMR genes in the mouse gut.

4.1.1 Bacterial colonization is influenced by the composition of the gut microbiota

In this study we found that the recovery of the *E. coli* donor strain, *S.* Heidelberg recipients, transconjugants, and the transfer frequency were greatest in the presence of antibiotic treatment. The absence of antibiotic treatment resulted in low recovery or levels below the

culture detection limit. Although donor and recipients were recovered from the control, antibiotic-free group on 1 DPI, there was no observed AMR gene transfer, and both fell below the detection limit by 2 DPI. An analysis of the gut microbiota by 16S rDNA metagenomic sequencing showed that antibiotic treatment had a significant impact on its composition which varied between antibiotic treatments. Ampicillin treatment had a progressive impact on the observed OTUs and Shannon Index with the values dropping significantly by 2 DPI and continuing until 7 DPI. Following the withdrawal of ampicillin on 7 DPI, the number of observed OTUs and the Shannon Index increased to control levels. This is consistent with several human studies demonstrating a decline in species richness and the Shannon Index in patients undergoing ampicillin therapy (Fouhy et al. 2012; Anukam et al. 2017). The donor *E. coli* levels did not change significantly during ampicillin treatment; however, there was a significant reduction of the donor levels as the observed OTUs and Shannon Index began to increase. Streptomycin treatment alone had a great impact on the number of observed OTUs and Shannon Index which recovered earlier than the ampicillin treated mice. Although the microbiota recovered earlier in the streptomycin treated mice relative to the ampicillin treated mice, transconjugants were recovered up until 14 DPI (versus 7 DPI for the ampicillin treated mice). Streptomycin pre-treatment and ampicillin had a synergistic effect on species richness as the observed OTUs index did not recover to baseline levels by the end of the experiment. Further, in this group the donor and transconjugants were recovered up until 42 DPI. It appears as if the long-term impact of ampicillin-streptomycin on the gut microbiota and selection of donors and transconjugants allowed a longer colonization than streptomycin or ampicillin alone. This is similar to a study by Croswell et al. (2009) who found that a streptomycin-bacitracin combination reduced the copy number of the 16S rDNA gene and increased colonization of the

mouse gut with *Salmonella* Typhimurium in comparison to streptomycin treatment alone. While the number of OTUs in the streptomycin-ampicillin treated mice did not return baseline levels, the Shannon Index increased significantly after withdrawal of ampicillin and reached levels statistically similar to baseline levels by 42 DPI. This is not reported in the literature although considering that the Shannon Index also takes into consideration species evenness (the abundance of each species), it is likely that those species who were not eliminated were able to recover after ampicillin withdrawal. In the case of the species richness, antibiotic cocktails have been shown to create a germ-free status in mice, with the elimination of certain species and incomplete recovery altogether (Reikvam et al. 2011).

We next wanted to determine whether the colonization of the mouse gut with the introduced bacteria and the AMR gene transfer frequency was correlated with the modification of certain taxa. We found that antibiotic treatment had a major impact on certain taxa and correlated with the recovery of the introduced bacteria. A reduction in short-chain fatty acid (SCFA) – producing bacteria has been associated with susceptibility to enteric disease. Several metagenomic studies have found that patients with irritable bowl disease (IBD) have a lower level of SCFA-producing Firmicutes belonging to the *Lachnospiraceae* and *Ruminococcaceae* families and high levels of Proteobacteria in comparison to healthy controls (Frank et al. 2007; Machiels et al. 2014). *Lachnospiraceae* and *Ruminococceae* are the most abundant butyrate-producing bacteria in the human and mouse gut microbiota (Louis and Flint, 2009). Reeves et al. (2012) found that pre-conditioning germfree mice with *Lachnospiraceae* reduced *Clostridium difficile*-induced mortality by 80%. In our study, colonization of the donor *E. coli*, transconjugants, and the AMR gene transfer frequency had a negative correlation with the relative abundance of *Lachnospiraceae* and *Ruminococcaceae*. Streptomycin pre-treatment had

an immediate impact on the *Lachnospiraceae* and *Ruminococcaceae* populations, reducing their combined relative abundance from 38% to 5% of the gut microbiota. The relative abundance of both populations returned to baseline levels by 7 DPI which also resulted in a reduction in the *Enterobacteriaceae* population and in the recovery of donors and transconjugants. The gut microbiota of groups receiving ampicillin alone or streptomycin-ampicillin was affected for a longer period of time. In these groups, the *Enterobacteriaceae* populations did not return to control levels until 21 DPI and the *Ruminococcaceae* population did not recover to control levels for the rest of the experiment in the streptomycin-ampicillin group. These results are consistent with the culture data where the *E. coli* donors and *S.* Heidelberg transconjugants of the streptomycin-only mice were either significantly lower or depleted by 7 DPI. However, the donor and transconjugants were recovered from those receiving ampicillin-only or streptomycin-ampicillin were recovered up until 14 DPI. As expected, the continued ampicillin treatment (versus one-time streptomycin pre-treatment) had a greater impact on the gut microbiota and which enabled these bacteria to colonize for a longer period of time.

The relative abundance of the *Lactobacillaceae* population was not significantly correlated with the colonization of the introduced bacteria nor the transfer frequency. Streptomycin treatment had no impact on the *Lactobacillaceae* population although the ampicillin alone and ampicillin-streptomycin treatments significantly reduced the relative abundance of *Lactobacillaceae* during the ampicillin treatment. After the withdrawal of ampicillin, the *Lactobacillaceae* increased significantly on 14 DPI and decreased back to baseline levels by 21 DPI. This corresponded with a decrease in the *Enterobacteriaceae* population and a lower recovery of donors and transconjugants. There are no studies reporting the interactions of the *Lactobacillaceae-Enterobacteriaceae* populations following antibiotic

treatment; however, *Lactobacillaceae* produce a large amount of *Enterobacteriaceae*-inhibiting bacteriocins (Ilayajara et al. 2011; Abdulwahab et al. 2013).

There was no significant correlation between the colonization of the introduced bacteria and the abundance of the total Bacteroidetes and *Bacteroidaceae* populations. During the ampicillin and streptomycin-ampicillin treatment, these populations were significantly higher in comparison to control levels and resulted in a decrease in the Firmicutes:Bacteroidetes ratio. Further, as the Firmicutes:Bacteroidetes ratio decreased, the *Enterobacteriaceae* population began to expand. This is consistent with several mouse studies reporting an expansion of Bacteroidetes and Proteobacteria following ampicillin treatment (Schubert et al. 2015; Rodrigues et al. 2017). Interestingly, the Bacteroidetes and *Bacteroidaceae* populations decreased and Proteobacteria made up the majority of the mouse gut microbiota on 7 DPI for the ampicillin and streptomycin-ampicillin treated mice. This was more prominent in the streptomycin-ampicillin treated mice. However, it doesn't appear that the Bacteroidetes were negatively impacted by ampicillin as the levels were slightly elevated during treatment and Bacteroidetes are typically resistant to the action of β-lactams (Edwards et al. 1997; Steifel et al. 2015).

The 16S rDNA samples were also analyzed at the genus level to determine why many of the bacteria were at low levels on 7 DPI despite the significant increase in Proteobacteria and *Enterobacteriaceae*. It was found that the *Enterobacteriaceae* population was a mixture of *Escherichia-Shigella, Salmonella, Klebsiella, Enterobacter, Proteus,* and *Cronobacter*. The sensitivity of 16S rDNA sequencing makes it difficult to determine *Enterobacteriaceae* trends and interactions at the genus level as a result of the large number of reads assigned to "unknown." Only six *Enterobacteriaceae* genera were detected when analyzing the 16S rDNA data at the genus level although there are at least 27 genera in this family (Adeolu et al. 2016).

From day 0 to 2 DPI there were no significant differences between the relative abundance of *Escherichia-Shigella* of the streptomycin and streptomycin-ampicillin treated mice. Unlike the diversity metrices, phylum and genus analyses, ampicillin did not have any significant effects on *Escherichia-Shigella* until after antibiotic treatment at which point it was statistically similar to that of the streptomycin-ampicillin treated mice. This is like a study by Ye et al. (2019) who observed a blooming of *Escherichia-Shigella* following ampicillin treated mice. We also detected *Klebsiella* and *Enterobacter* in the ampicillin and streptomycin-ampicillin treated mice which is consistent with Gibson et al. (2016).

4.1.2 AMR gene transfer is enhanced by antibiotic treatment

Through an analysis of the gut microbiota under different treatment conditions over time, we found that the AMR gene transfer was influenced by an antibiotic-induced dysbiosis. Gene transfer only occurred in mice treated with streptomycin, those receiving ampicillin treatment, or those treated with both. This was found to correlate with the extent of dysbiosis, with greater AMR gene transfer frequencies correlating with a greater impact on species richness and diversity. Further, a greater degree of dysbiosis was also correlated with a longer persistence of the transconjugants over time.

Putative transconjugants from the antibiotic treated mice were sent for WGS to confirm transfer. All *Salmonella* isolates were confirmed to have acquired the *bla*CMY-2 carrying IncI2 plasmid from the donor *E. coli* O80:H26. Although the *S.* Heidelberg transconjugants were detected on 42 DPI in the streptomycin-ampicillin group, they were only found in two mice and were near the detection limit. It is likely that the transconjugant population would be eliminated as the gut microbiota continued to recover. Several commensal *E. coli* O2:H6 isolates were also

found to have acquired *bla*CMY-2, which was found on both the IncI2 plasmid and the *E. coli* O2:H6 chromosome. The transfer of AMR genes into commensal bacteria has been reported in *in vivo* studies although none have found the integration of these genes into the chromosome. Card et al. (2017) demonstrated the transfer of a *bla*CTX-M-1 carrying IncI1 plasmid from *S.* Typhimurium to seven commensal *E. coli* clones in an *in vitro* chicken gut model. Stecher et al. (2012) found that inflammation enhanced the transfer of a colicin-encoding plasmid from *S.* Typhimurium to commensal *E. coli* in the mouse gut. Chromosomal integration may be a common phenomenon in *in vivo* models; however, gene transfer is often confirmed with methods other than WGS such as PCR (Johnson et al. 2015; Hadziabdic et al. 2018). Chromosomal integration of AMR genes may not be a concern in a commensal isolate. However, the transfer of AMR genes into resident opportunistic pathogens such as *C. difficile* is a risk as the host could develop an AMR infection if they become immunocompromised. Further, the presence of the *bla*CMY-2 carrying conjugative plasmid in a host-adapted *E. coli* strain is a risk as it may continue to persist without antibiotics and act as a donor to other *Enterobacteriaceae* in the gut or an ingested bacterium. Aviv et al. (2016) demonstrated the transfer of an MDR plasmid from a *S.* Typhimurium isolate into commensal *E. coli* and *Lactobacillus* species. A second *in vivo* experiment found that the re-inoculated commensal *E. coli* isolate was able to transfer the MDR plasmid into a plasmid-free *S.* Typhimurium isolate. Another notable observation was that the *E. coli* O2:H6 transconjugants were only recovered from the streptomycin-ampicillin mice. Further, the *E. coli* O2:H6 transconjugants were only found in samples from 14 DPI onwards. By 14 DPI the donor *E. coli* in the streptomycin treated mice was below the detection limit and was significantly lower in the ampicillin treated mice relative to the streptomycin-ampicillin treated

mice. It is possible that as the streptomycin-ampicillin mice experienced a post-antibiotic bloom of *Escherichia-Shigella,* the high donor levels facilitated transfer of CMY-2 into *E. coli* O2:H6.

WGS also revealed the presence of IncI1 plasmids in the *S.* Heidelberg and *E. coli* O2:H6 transconjugants from both the streptomycin and streptomycin-ampicillin groups. The transfer of plasmids into commensal bacteria is widely reported but there are no studies demonstrating the transfer of plasmids from the commensal bacteria into an inoculated recipient. The IncI1 plasmids did not contain any known AMR genes which led to the question of where they came from and why they transferred to *S.* Heidelberg and *E. coli.* As discussed previously, IncI1 plasmids are found mainly in *E. coli* and *Salmonella* although they have been shown to disseminate among many members of the *Enterobacteriaceae* family (Carattoli et al. 2018). However, plasmids place a significant metabolic burden on the host and are prone to elimination when no benefit is given to the host. We did not do any investigation to determine the source of the plasmids, although it is likely that the host was a member of *Enterobacteriaceae* family. Without fully characterizing the plasmids, it can only be speculated that the plasmids carried genes encoding a beneficial trait to the host such as bacteriocin or biofilm-encoding genes, which have been found to be carried on IncI1 plasmids (Kaldone et al. 2019). These would give the transconjugants an advantage by gaining the ability to inhibit competitors via bacteriocin production or resist the action of antibiotics by growing in biofilms.

4.2 Conclusion

In this study we found that antibiotic treatment had a significant impact on the composition on the gut microbiome which in turn enhanced bacterial colonization and the subsequent transfer of AMR genes. The experimental setup allowed us to assess the transfer of *bla*CMY-carrying IncI2 plasmid from *E. coli* O80:H26 to *S.* Heidelberg with and without the selective pressure of ampicillin and/or streptomycin. We found that AMR gene transfer was enhanced by streptomycin, ampicillin, and streptomycin-ampicillin treatments although their effect on the gut and the transfer frequencies differed between treatments. As hypothesized, the degree of microbial disruption correlated with the colonization of the donor and recipient strains and the persistence of transconjugants over time. Streptomycin-ampicillin treatment had a long-lasting impact on the number of species present in the gut as well as the relative abundance of important genera. Further, streptomycin-ampicillin treatment enabled the persistence of transconjugants longer than the other antibiotic treatment groups and facilitated the transfer of the IncI2 plasmid into indigenous *E. coli.*

Our results suggest that while multiple-antibiotic treatment may broaden the antibacterial therapy in comparison to single antibiotics, this may have a greater impact on the gut microbiota and increase the risk of AMR gene transfer. Relative to single antibiotics, multiple antibiotic treatment can create a long-lasting reservoir of AMR genes in the gut which may spread to other indigenous bacteria or ingested bacteria. Hopefully, this information can influence further research into the identification of antibiotics and/or combinations that pose the greatest risk in terms of developing AMR and contribute to the knowledge that will guide the prudent use of antibiotics in the future.

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