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Metagenomic analysis facilitates ontogenetic investigations of microbiota composition and antimicrobial resistance in silver gull (Chroicocephalus novaehollandiae) chicks

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Yerbury, Maddison, Metagenomic analysis facilitates ontogenetic investigations of microbiota composition and antimicrobial resistance in silver gull (Chroicocephalus novaehollandiae) chicks, Bachelor of Science (Honours), School of Earth, Atmospheric and Life Sciences, University of Wollongong, 2021.
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Metagenomic analysis facilitates ontogenetic investigations of microbiota composition and antimicrobial resistance in silver gull (Chroicocephalus novaehollandiae) chicks

Abstract
The proliferation of antimicrobial resistance (AMR) in recent decades has been fuelled by the development, overconsumption and misuse of antibiotic therapeutics and non-medical antimicrobials. The evolution of AMR-bacteria is predominantly underpinned by the horizontal transfer of antimicrobial resistance genes (ARGs), which allows AMR to be transferred between bacterial species and accumulate within bacteria, even in the absence of antimicrobial selection pressures. It has been speculated that avian wildlife, in particular waterbird species such as gulls, act as reservoirs of AMR, facilitating the propagation of AMR-bacteria that can then be transmitted into humans. The current thesis, therefore, investigated whether silver gull (Chroiocephalus novaehollandiae) chicks may play a role in the evolution and proliferation of AMR-bacteria. Firstly, numerous DNA extraction protocols were trialled to optimise the extraction of DNA from multi-species microbiota samples. Metagenomic sequencing and analysis were then conducted for 60 temporal multi-species microbiota samples from 23 silver gull chicks, which had been subcultured for Enterobacteriaceae, a subpopulation of the microbiome enriched in AMR-associated bacteria. The data were examined in order to characterise bacterial and AMR composition and to analyse ontogenetic shifts in the microbiome to assess if the silver gull chick microbiome has the ability to accumulate and maintain AMR. Metagenomic analysis revealed a diverse Gammaproteobacterial community of 125 species and 30 genera, and the majority of species were of a genus containing pathogenic and AMR-associated species. Additionally, metagenomic analysis detected a large ARG pool, with 115 ARGs identified across all samples. These ARGs were dominated by Beta-lactams and Aminoglycoside resistance phenotypes, which are the most commonly used antibiotics in Australia. Ontogenetic analyses further showed that both bacterial and AMR profiles were in constant flux and varied between samples and within individual chicks. Additionally, both richness and composition measures for bacterial species and ARGs were significantly correlated, such that the ARG profile within a sample was correlated to the bacterial profile. The high diversity of AMR-associated bacteria and ARGs found in these samples indicates that the silver gull microbiome may act as an ‘ecological sponge’ of AMR-bacterial contamination in the environment, which transiently colonise the gut. Moreover, these findings indicate that ARGs are not accumulated and do not persist over time but are brought in with the transient bacteria they are associated with. The current thesis, therefore, concludes that silver gull chicks do not contribute to the proliferation and evolution of AMR-bacteria, and further, do not act as a 6 reservoir to support the propagation of AMR-bacteria. Moreover, this thesis presents the first longitudinal metagenomic study to examine the dynamic nature of the microbiome and AMR in a wildlife host. Future studies should investigate potential sources of the AMR contamination reflected in this silver gull population, and explore potential reservoirs of AMR, including wildlife populations and the non-human environment.

Degree Type
Thesis

Degree Name
Bachelor of Science (Honours)

Department
School of Earth, Atmospheric and Life Sciences

Advisor(s)
Bethany Hoye

Keywords
Microbiome, Wildlife, Resistome, Infection

This thesis is available at Research Online: https://ro.uow.edu.au/thsci/191

University of Wollongong

School of Earth, Atmospheric and Life Sciences

Metagenomic analysis facilitates ontogenetic investigations of microbiota composition and antimicrobial resistance in silver gull (*Chroicocephalus novaehollandiae*) chicks

Maddison Yerbury

This thesis is submitted in fulfilment of the requirements for the award of the degree

Bachelor of Science (Honours) (Dean's Scholar)

From

University of Wollongong

April 2021

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Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the degree of Bachelor of Science (Honours) (Dean's Scholar). It does not include any material published by another person without due reference within the text. The field and laboratory work presented in this thesis was performed by the author, except where acknowledged. This thesis has not been submitted for a degree at any other university.

Maddison Yerbury

June 2021

Abstract

The proliferation of antimicrobial resistance (AMR) in recent decades has been fuelled by the development, overconsumption and misuse of antibiotic therapeutics and non-medical antimicrobials. The evolution of AMR-bacteria is predominantly underpinned by the horizontal transfer of antimicrobial resistance genes (ARGs), which allows AMR to be transferred between bacterial species and accumulate within bacteria, even in the absence of antimicrobial selection pressures. It has been speculated that avian wildlife, in particular waterbird species such as gulls, act as reservoirs of AMR, facilitating the propagation of AMR-bacteria that can then be transmitted into humans. The current thesis, therefore, investigated whether silver gull (*Chroiocephalus novaehollandiae*) chicks may play a role in the evolution and proliferation of AMR-bacteria. Firstly, numerous DNA extraction protocols were trialled to optimise the extraction of DNA from multi-species microbiota samples. Metagenomic sequencing and analysis were then conducted for 60 temporal multi-species microbiota samples from 23 silver gull chicks, which had been subcultured for Enterobacteriaceae, a subpopulation of the microbiome enriched in AMR-associated bacteria. The data were examined in order to characterise bacterial and AMR composition and to analyse ontogenetic shifts in the microbiome to assess if the silver gull chick microbiome has the ability to accumulate and maintain AMR. Metagenomic analysis revealed a diverse Gammaproteobacterial community of 125 species and 30 genera, and the majority of species were of a genus containing pathogenic and AMR-associated species. Additionally, metagenomic analysis detected a large ARG pool, with 115 ARGs identified across all samples. These ARGs were dominated by Beta-lactams and Aminoglycoside resistance phenotypes, which are the most commonly used antibiotics in Australia. Ontogenetic analyses further showed that both bacterial and AMR profiles were in constant flux and varied between samples and within individual chicks. Additionally, both richness and composition measures for bacterial species and ARGs were significantly correlated, such that the ARG profile within a sample was correlated to the bacterial profile. The high diversity of AMR-associated bacteria and ARGs found in these samples indicates that the silver gull microbiome may act as an ‘ecological sponge’ of AMR-bacterial contamination in the environment, which transiently colonise the gut. Moreover, these findings indicate that ARGs are not accumulated and do not persist over time but are brought in with the transient bacteria they are associated with. The current thesis, therefore, concludes that silver gull chicks do not contribute to the proliferation and evolution of AMR-bacteria, and further, do not act as a

reservoir to support the propagation of AMR-bacteria. Moreover, this thesis presents the first longitudinal metagenomic study to examine the dynamic nature of the microbiome and AMR in a wildlife host. Future studies should investigate potential sources of the AMR contamination reflected in this silver gull population, and explore potential reservoirs of AMR, including wildlife populations and the non-human environment.

Acknowledgments

I acknowledge the Dharawal people as the Traditional Owners of the land on which I have learnt, grown, and that this project was undertaken. I acknowledge that this land has never been ceded and I recognise my privilege to work on this Country, at the University of Wollongong and on the Five Islands. In studying the ecological questions examined in this thesis, I recognise that these problems exist as a result of social and environmental violence rooted in European colonisation. I pay my respects to Elders past, present, and emerging, and look to the knowledge and leadership of First Nations people now and always.

I would like to thank my supervisors, Bethany Hoye and Martina Sanderson-Smith, for their ongoing support throughout the process of my thesis, with its many unexpected hurdles. It was a challenge to bring together ecological and microbiological research for this unique study, and I thank you both for forming the link and developing this interesting project. I would also like to thank Kimberley Maute, Emma Proctor and Anuk Indraratna for showing me the ropes in the lab and field; you're help has been greatly appreciated.

Thank you to my friends who have provided necessary debriefs, advice and rest over coffee, dinners and outdoor escapes. Lasty I'd like to thank my incredible family, especially Mum, Dad and Alex. Thank you for the daily pep talks and for truly supporting me to do my best work while also making room for balance. You inspire me with your determination, energy and kindness, and I'm so grateful.

Abbreviations

AGRF: Australian Genome Research Facility

AMR: Antimicrobial-resistance/resistant

ARG: Antimicrobial resistance gene

DBD: Diversity begets diversity

HTL: ‘hard to lyse’

LB: Lysogeny broth

PCoA: Principal Coordinate Analyses

PCR: Polymerase chain reaction

PERMANOVA: Permutational multivariate analysis of variance

RNase: Ribonuclease

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Appendix 6.9. Principle Coordination Analysis (PCoA) based on Jaccard distance of multi-species microbiota samples from silver gull chicks as dissimilarities in bacterial composition. Longitudinal samples were collected from silver gull chicks at the Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, before correction for low-resolution *Pseudomonas* species in samples 19-14-T1 and 18-19-T1. Samples are coloured and grouped by chick.

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1 INTRODUCTION

Antimicrobials, including antibiotic therapeutics, are agents that induce bacterial cell death or thwart cell growth by inhibiting the synthesis of bacterial cell walls, DNA, RNA, or protein (Kohanski et al. 2010). The development and commercial distribution of antibiotic medicines in the 1940s revolutionised the treatment of bacterial infections (San Millan 2018). Initially, these novel medicines decreased disease severity and mortality, and subsequently, bacterial infections were no longer the leading cause of disease deaths globally (Aminov 2017). However, shortly after introducing the first antibiotics, the apparent effectiveness of these treatments declined, and bacterial strains with reduced susceptibility to antibiotics became prevalent (Davies & Davies 2010). Antimicrobial resistance (AMR) has since proliferated as bacteria have evolved to overcome modifications made to existing therapeutics, as well as to the novel synthetic antimicrobials that have been developed (Davies & Davies 2010). Antimicrobial resistant (AMR) bacteria have also evolved resistance to other antimicrobials such as heavy metals and biocides (Jackson et al. 2011). AMR-bacteria are now found across the globe, and resistance to all classes of antibiotics has been detected (Spellberg et al. 2013).

The widespread overuse of antimicrobials in clinical and veterinary medicine, in addition to their misuse as non-medical agents, has propelled the spread of AMR among bacteria (San Millan 2018). Pathogenic bacteria that have been controlled for decades are now acquiring resistance to multiple antibiotics, thus reducing treatment options for even common infections (WHO 2018). For instance, the US alone records 2.8 million antibiotic-resistant infections every year (CDC 2019). These resistant bacterial infections place additional strains on healthcare systems due to the high cost of care for patients who require expensive medications and experience prolonged illnesses (WHO 2018). In 2014, it was estimated that each year at least 700,000 deaths are a result of AMR (O'Neill 2016). By 2050, increased AMR could see related deaths increase to 10 million per year, with the global cost reaching 100 trillion USD (O'Neill 2016).

In addition to AMR prevalence in human populations, AMR is also hosted by numerous wild animal species, likely a result of antimicrobial pollutants and AMR-bacteria from human and agricultural waste (Vittecoq et al. 2016). The spread of AMR is therefore not confined to humans and is likely inextricably linked to the non-human environment (Djordjevic &

Morgan 2019). It is therefore necessary to take a holistic, One Health approach, which encompasses the interactions of and between humans, animals and the environment, to combat the AMR health crisis (Walsh 2018). Until recently, bacterial AMR research has focused primarily on pathogenic bacteria in clinical settings (Djordjevic & Morgan 2019), however, a greater focus on the role of the environment and wildlife in the evolution of AMR-bacteria is required. Moreover, it is apparent that an increased understanding of the mechanisms underlying the accumulation, persistence and transmission of AMR is vital to understand these interactions between human and natural environments and to subsequently prevent the proliferation of AMR-bacteria (CDC 2019).

1.1 Evolution of antimicrobial resistance

Antimicrobials and AMR are ancient phenomena originating in environmental bacteria within dynamic microbial communities (Arnold et al. 2016; Dcosta et al. 2011). Resistance to antimicrobials occurs via various molecular mechanisms, which are each coded for by specific genes (Kohanski et al. 2010). Beta-lactamase genes, for example, encode enzymes that degrade beta-lactam proteins, therefore inactivating these antimicrobials (Kapoor et al. 2017). These resistance genes originally evolved as protective mechanisms from antimicrobial-producing bacteria (Frost et al. 2005) either as a result of chromosomal mutations (Furuya & Lowy 2006) or point mutations on plasmids (Frost et al. 2005). Greater use of antimicrobials generates persistent selection for resistance carrying genes and mutations, and therefore the proliferation of resistant strains (San Millan 2018).

Bacteria can acquire genetic information through two different mechanisms, either by inheritance through cell division or through horizontal gene transfer (Furuya & Lowy 2006). Horizontal gene transfer describes the genetic exchange between different bacterial cells of the same or different species (Thomas & Nielsen 2005). There are three main types of horizontal gene transfer: transformation, transduction and conjugation (Frost et al. 2005). Transformation involves the uptake of exogenous DNA released from a lysed cell, allowing genetic material to be integrated into the recipient cell's chromosome or its associated plasmids (Frost et al. 2005; Griffith 1928) (Fig 1.1). Transduction is mediated by bacteriophages that package chromosomal DNA as they replicate, which is then inserted into

a new host cell to be integrated chromosomally (Frost et al. 2005; Zinder & Lederberg 1952) (Fig 1.1). Conjugation requires direct contact between cells formed by a conjugational bridge or sex pilus, which then enables the transfer of mobile genetic elements such as plasmids or transposons (Frost et al. 2005; Lederberg & Tatum 1946) (Fig 1.1). These methods facilitate the transfer of a wide range of genes, including those encoding AMR (Furuya & Lowy 2006).

The acquisition of antimicrobial resistance genes (ARGs) in an individual bacterium through mutation, cell division or horizontal transfer leads to the evolution of microbial populations. Importantly, this evolution is distinct from that in multicellular organisms, as the acquisition of ARGs can occur horizontally between individuals and species. This process of bacterial evolution is not limited to vertical inheritance, and the population of organisms exposed to such selection can therefore be multi-species and diverse. The evolution of a multi-species bacterial population occurs as a result of the proliferation of acquired AMR through a combination of clonal expansion and gene exchange. The acquisition of resistance in an individual bacterium and the evolution of a bacterial population are, therefore, distinct yet interlinked processes facilitating the proliferation of AMR.

Transfer of genetic information between cells was first described by Griffith (1928), who discovered that virulence factors could be transformed between pneumococcal strains *in vivo*. Since those initial studies, evidence of the horizontal transfer of ARGs has been found in human, animal, and environmental studies, collectively demonstrating the adaptive potential of ARG associated genetic elements to promote the evolution of pathogenic bacteria (Davies & Davies 2010). Early investigations of ARG transfer were conducted in animal models and environmental bacteria. For example, one study in human volunteers demonstrated gene transfer between resistant mutants of each person's own *Escherichia coli* and fecal flora by assessing phenotypic resistance profiles to determine resistant marker patterns (Anderson et al. 1973). In environmental bacteria, Graham and Istock (1979) demonstrated genetic exchange between strains of *Bacillus subtilis* containing linked resistance genes by identifying triple transformants (Graham & Istock 1979). Since the development of commercial DNA sequencing, the role of horizontal gene transfer in the proliferation of AMR-bacteria has been demonstrated by genomic analysis. For example, one clinical study found identical ARG sequences in different pneumococcal lineages from clinical outpatients in Nairobi (Kell et al. 1993). Sequencing technology has also been utilised to understand ARG transmission *in vivo*, including one study that tracked the transfer of the *vanA* resistance

gene from *Enterococcus faecium* of chicken origin to those of human origin, after both were ingested by human volunteers (Lester et al. 2006). Sequence analyses in animals have also supported these findings; for example, closely related ARG sequences have been identified in different bacterial species within the same swine host, demonstrating the potential for the cross-species transmission of these ARGs (Frye et al. 2011). Collectively, these studies reveal the potential for ARGs to transfer between bacterial strains and species, highlighting the importance of horizontal gene transfer in the spread of resistance to clinically important antibiotics.

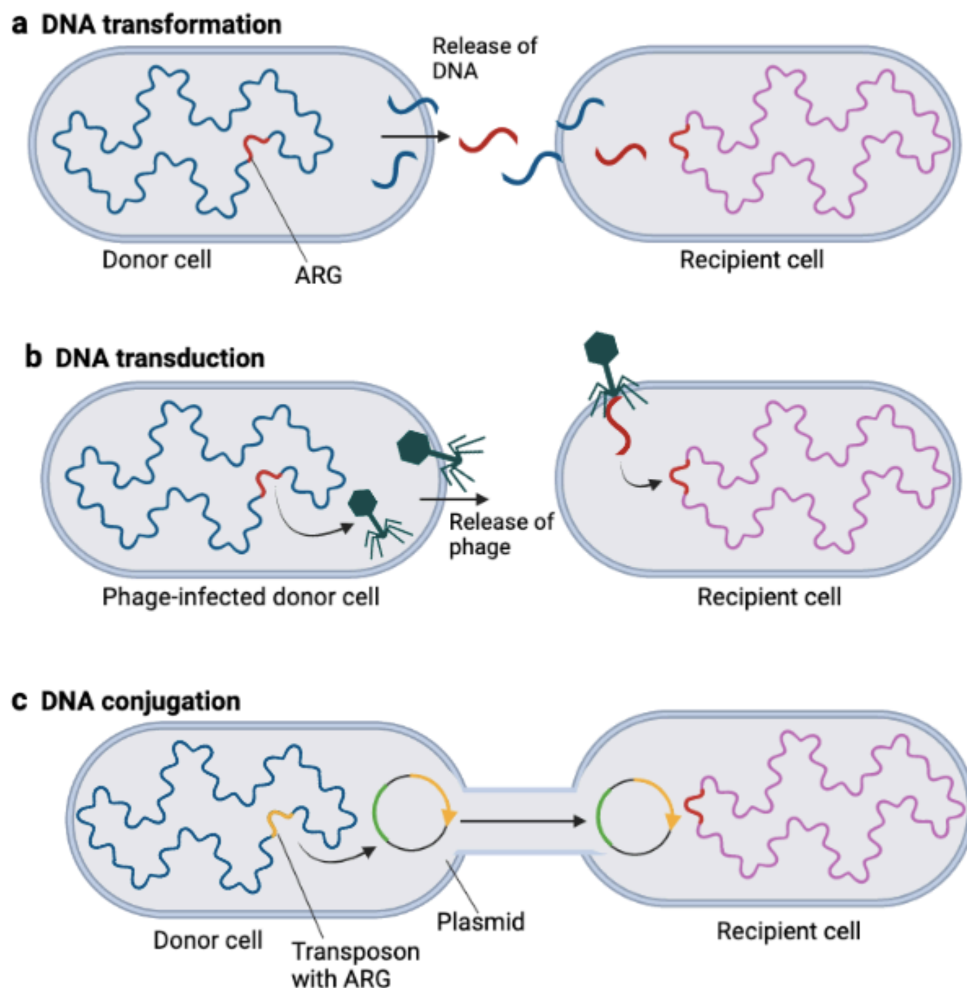


Figure 1.1. Horizontal gene transfer between bacteria (adapted from Furuya & Lowy 2006; created in BioRender.com). **(a)** Transformation occurs when a cell uptakes exogenous DNA released from a nearby cell; **(b)** Transduction describes the transfer of bacterial DNA during the life cycle of bacteriophages; **(c)** Conjugation involves the formation of a bridge between two cells, which allows the direct transfer of bacterial DNA.

Horizontal transfer of ARGs also significantly contributes to the development of multi-drug resistance (Forsberg et al. 2012; Sun 2018). Bacteria expressing multi-drug resistance exhibit reduced susceptibility to multiple antimicrobials, and the more agents a bacterium is resistant to, the more difficult an infection is to treat (O'Neill 2016). Resistance to each antimicrobial is coded for by one or more specific genes, which can accumulate within a single bacterial cell (Nikaido 2009). Multiple ARGs encoded on an individual genetic element can then be spread by horizontal transfer, disseminating multi-drug resistance to susceptible bacteria in a single transfer event (Nikaido 2009). Horizontal transfer of multi-drug resistance is concerning as it likely promotes the rapid and widespread proliferation of AMR between bacterial species, even in the absence of selection pressure from antimicrobials (Nikaido 2009). The accumulation and propagation of multiple resistance genes is, therefore, a serious threat, particularly in cases of bacteria that have developed resistance to all available treatments (Nikaido 2009). For example, methicillin-resistant *Staphylococcus aureus* encodes resistance to all first-line antibiotics, and has continued to acquire resistance as new antibiotics, such as vancomycin, have been introduced as treatments (Nikaido 2009). Moreover, horizontal transfer of multi-drug resistance can contribute to a shared ARG pool between environmental and clinical bacteria. For instance, Forsberg et al. 2012 identified multi-drug resistance cassettes in soil Proteobacteria with identical nucleotide sequences to ARGs found in a range of human pathogenic isolates, demonstrating horizontal gene transfer between environmental bacteria and clinical pathogens (Forsberg et al. 2012). These findings indicate that bacteria, including those which are phylogenetically distant, have the capacity to exchange genetic elements encoding multi-drug resistance (Forsberg et al. 2012).

1.1.1 Antimicrobial resistant gene accumulation and mobile genetic elements

The accumulation of ARGs, and subsequently the evolution of multi-drug resistance, is mediated by mobile genetic elements, which are composed of DNA segments encoding proteins and enzymes that facilitate the movement of DNA (Frost et al. 2005). The complete collection of these mobile genetic elements in a cell are referred to as the 'mobilome' and are often associated with ARGs, a subset of the mobilome referred to as the 'resistome' (Marano & Cytryn 2017; Nikaido 2009). Moreover, these elements often flank co-localised ARGs and are major hallmarks of multi-drug resistance (Forsberg et al. 2012).

The smallest mobile genetic elements are gene cassettes, circular elements which contain a gene, commonly an ARG, and a recombination site that allows them to integrate into an integron (Hall & Stokes 1993) (Fig 1.2). Integrons are two-gene mobile genetic elements that contain a gene to incorporate gene cassettes (Hall & Stokes 1993), and therefore function as 'gene capture systems' (Jackson et al. 2011). Mobile integrons can contain up to 10 gene cassettes and are often referred to as multi-resistance integrons (Stalder et al. 2012). Moreover, integrons have been significantly associated with multi-drug resistance in numerous Enterobacteriaceae species, which are the family of bacteria encompassing many taxa that infect the gastrointestinal system (Leverstein-van Hall et al. 2003). Insertion sequences are another simple mobile genetic element that facilitate the incorporation of ARGs through site-specific recombination on chromosomes and other DNA elements (Hartl et al. 1983; Ohtsubo & Ohtsubo 1977) (Fig 1.2). Both insertion sequences and integrons exist on bacterial chromosomes and large mobile genetic elements, including plasmids and transposons (Fluit & Schmitz 2004; Siguier et al. 2006), which are regularly identified in AMR-bacteria and associated with the mobilisation of ARGs (Dolejská & Papagiannitsis 2018; Forsberg et al. 2012; Jackson et al. 2011; Wang et al. 2017). When multi-drug resistance is encoded on integrons or accumulated by insertion sequences they are therefore co-selected with the transposon or plasmid that they are associated with (Stalder et al. 2012) (Fig 1.2).

DNA transposons are mobile genetic elements flanked by insertion sequences, which can facilitate the excision and integration of multiple genes into chromosomes, into plasmids and between them, allowing them to integrate multiple ARGs (Hartl et al. 1983) (Fig 1.2). Transposons can also incorporate integrons, enabling them to rapidly acquire multi-drug resistance (Liebert et al. 1999). Transposons vary broadly in their molecular components and mechanisms, and the biology of transposons has thoroughly been reviewed elsewhere (Feschotte & Pritham 2007). Additionally, transposons are frequently carried on and exchanged between extrachromosomal elements called plasmids, which are important features of prokaryote genomes (Liebert et al. 1999). Plasmids are independent, stable and self-replicating genetic molecules that often take the form of a circular, double-stranded DNA molecule (Hayes & Pritchard 1969). Network analyses have shown that plasmids are the primary vector of gene transfer between bacteria and have played a central role in bacterial evolutionary history (Halary et al. 2010). These larger mobile genetic elements consist of replicative genes, along with distinct accessory genes which can encode important survival

traits, such as virulence and resistance to heavy metals, biocides and antibiotics (Jackson et al. 2011). Plasmids can mobilise ARGs through recombination events that allow them to integrate genes associated with other autonomously replicating molecules, collectively known as replicons (Thomas & Nielsen 2005). This is facilitated by insertion sequences, which act as mobile recombination regions and make up 5-40% of plasmids larger than 20 kb (Jackson et al. 2011). Through homologous recombination, insertion sequences can facilitate the rearrangement of genetic elements between bacterial plasmids, a process referred to as plasmid reshuffling (Jackson et al. 2011; Siguier et al. 2006).

Plasmids are often linked to the horizontal transfer of ARGs and multi-drug resistance (Hughes & Datta 1983; Nikaido 2009), which has more recently been demonstrated *in vivo*. For example, although the authors did not specify the species of bird, an experimental study inoculated chicks with rifampin-resistant *E. coli* containing multi-drug resistance plasmids, which were then shown to horizontally transfer to rifampin-susceptible *E. coli* strains (Dheilly et al. 2012). Plasmids with a broad host range are particularly concerning as they facilitate the propagation of ARGs between distantly-related bacteria, including pathogenic strains (Smillie et al. 2011; Thomas & Nielsen 2005). Plasmids can also mobilise other genetic elements, including transposons and integrons (Jackson et al. 2011) (Fig 1.2).

Altogether, the interactions between different mobile genetic elements support the generation of multi-drug resistance. For example, a cascade of mobile genetic elements: ARG cassettes can be picked up by multi-resistance integrons and mobilised by transposons and plasmids (Marano & Cytryn 2017) (Fig 1.2). However, mobile genetic elements do not always interact in a linear fashion; for example, a single plasmid containing insertion sequences can integrate multiple ARGs through plasmid reshuffling and also incorporate multiple transposons and integrons, each with their own ARGs (Jackson et al. 2011; Siguier et al. 2006). Therefore, a combination of these mechanisms likely contributes to the development of multi-drug resistance.

The strong correlation between co-occurring mobile genetic elements and ARGs has been used presented by several studies and reviews as compelling evidence for the role of mobile genetic elements, particularly plasmids, in the dissemination and proliferation of AMR and multi-drug resistance (Dolejská & Papagiannitsis 2018; Forsberg et al. 2012; Nikaido 2009; San Millan 2018). However, to date, there has been no demonstration of the accumulation of

ARGs and the persistence of multi-drug resistance on mobile genetic elements within microbiota communities. Although it is clear that multi-drug resistance exists on mobile genetic elements that can be spread horizontally (San Millan 2018), the molecular mechanisms underpinning the accumulation of multi-drug resistance are yet to be assessed either *in vivo* or *in vitro*. A growing number of studies have conducted cross-sectional surveys that compare the sequence identity of ARGs associated with mobile genetic elements between multiple bacterial species within a host (e.g. Frye et al. 2011). Other studies have compared ARGs between host animals or individuals (e.g. Veldman et al. 2013), however, these provide limited evidence of *how* these ARGs are spread. It is also unclear how frequently mobile genetic element-associated multi-drug resistance is maintained within a bacterial host and subsequently how frequently it is transferred between bacteria. Understanding these mechanisms is crucial to understanding how multi-drug resistance is disseminated and how this contributes to the proliferation of AMR and the public health challenge that it presents.

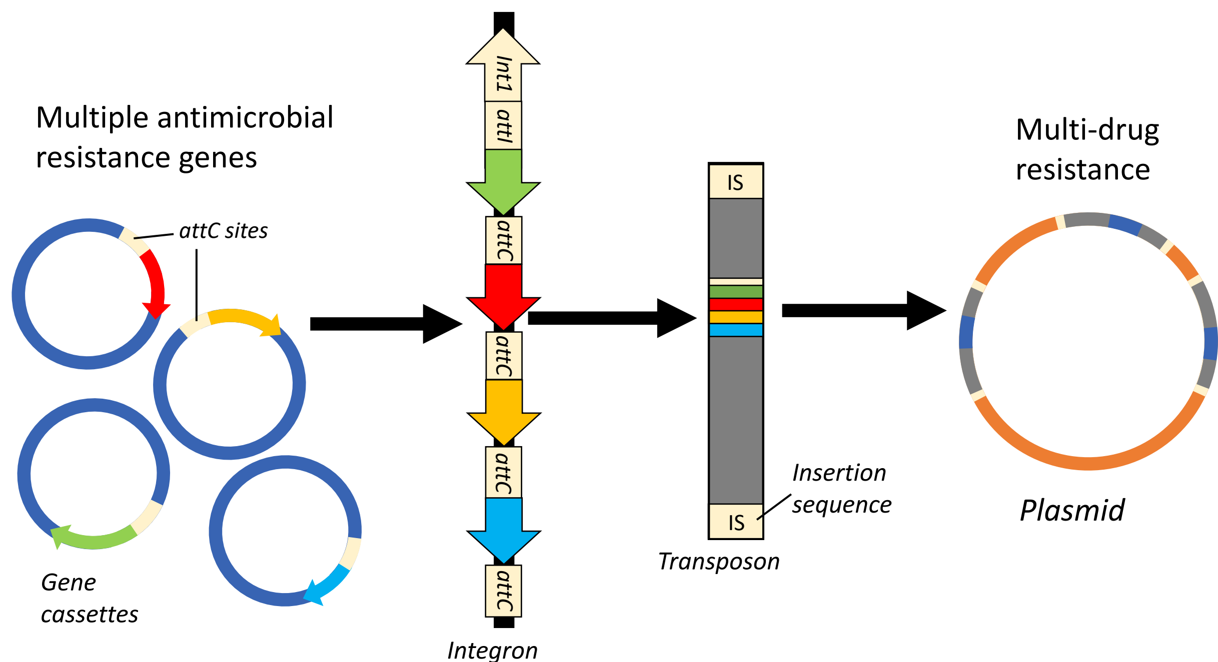


Figure 1.2. A cascade of mobile genetic elements can facilitate the accumulation of antimicrobial resistance genes (ARGs) and the development of multi-drug resistance (adapted from Marano & Cytryn 2017). Multiple gene cassettes containing ARGs can be integrated into integrons via complementary *attI* and *attC* site recombinases. Via the *Int1* site-specific recombinase, multi-drug resistance integrons can then be incorporated into and mobilised by transposons and plasmids. A multidrug resistance plasmid can contain multiple transposons, integrons and antimicrobial resistance genes.

1.1.2 Factors promoting antimicrobial resistant gene transfer

The transfer of ARGs is stimulated in the presence of antimicrobials and leads to the selection of multi-drug resistance bacteria in clinical settings (Dzidic & Bedeković 2003). Early investigations of AMR-bacteria transfer sought to confirm this connection, including one study which identified AMR transfer only when humans were treated with antibiotics (Anderson et al. 1973). However, a growing number of studies have since observed AMR conjugation in the absence of antimicrobials (Lerner et al. 2017). As previously discussed, Graham and Istock (1979) demonstrated AMR transfer between soil Proteobacteria in the absence of antibiotics to demonstrate horizontal gene transfer as a natural, ecological and evolutionary adaptative process. However, the factors promoting, or inhibiting, ARG transfer in the absence of antibiotics are not clear. Indeed, in a study of conjugation between Enterobacteriaceae *in vitro*, transfer frequency appeared to depend on the identity of both donor and recipient cells, as conjugation rates differed with each pairwise experiment between species and strains (Dionisio et al. 2002). However, by investigating gene transfer between two *E. coli* strains in the presence of a third strain, the authors found that this third 'amplifier' cell facilitated the conjugation and amplification of AMR plasmids from donor to recipient cells (Dionisio et al. 2002). The authors suggested that the interactions within diverse microbiota, such as within the human body, may therefore facilitate conjugation and thus the proliferation of ARGs (Dionisio et al. 2002). However, pairwise conjugation in the presence of the third 'amplifier cell' was only tested with a single combination of *E. coli* strains, providing limited evidence to suggest that this process applies to the wider microbiota.

Diverse communities of bacterial taxa that fill similar niches within the same broader ecological space may also facilitate the horizontal transfer of ARGs, as conjugation is most likely to occur in ecologically similar species (Smillie et al. 2011). By analysing over 10,000 genes, Smillie et al. (2011) suggested that the ecology of a bacterial cell, including spatial, functional and niche factors, are the strongest predictors of gene transfer (Smillie et al. 2011). By analysing the nucleotide similarity between these genes in over 2,000 bacterial genomes, Smillie et al. (2011) found that microbial inhabitants of similar ecological factors of similar ecological niches (fulfilling the same functional role within a given environment) showed evidence of higher gene exchange than between closely phylogenetically related species. Moreover, analysis of genes from pairs of bacteria showed that bacteria in ecologically

similar environments are more likely to share and maintain genes (Smillie et al. 2011). For example, bacteria from humans are 25-fold more likely to share transferred DNA than non-human bacteria. These trends were apparent even when the bacteria were geographically separated, such that bacteria with the same functional role shared more mobile genes than bacteria in close proximity (Smillie et al. 2011). Functional analysis of these genes further revealed that horizontal gene transfer is more common between bacteria from different ecological environments, including both human and non-human sample sites, when selective genes, such as those encoding AMR or pathogenicity, are involved in the transfer (Smillie et al. 2011). These findings suggest that animal hosts may have a multitude of selective factors that result in higher levels of gene transfer between bacteria (Smillie et al. 2011).

Phylogenetically related bacteria generally share ecological niche factors, such that a particular phyla is likely to encompass species that all inhabit similar environments or fill similar functional roles (Philippot et al. 2010). Additionally, horizontal gene transfer is significantly higher between bacteria of the same taxonomic group, than between taxonomic groups, at each phylogenetic level (Hu et al. 2016). Some bacterial phyla, including Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, are more likely to carry mobile ARGs, which likely influences and reflects their ability to transfer AMR (Hu et al. 2016). In particular, Proteobacteria have been shown to be significantly enriched in mobile ARGs, with a high abundance of ARGs observed in a large number of Proteobacterial species (Hu et al. 2016). Proteobacteria encompasses the Enterobacteriaceae family, which includes many clinically relevant pathogens and genera identified on the WHO priority list of bacteria, such as *Escherichia*, *Klebsiella* and *Salmonella* (Tacconelli et al. 2018). Additionally, Proteobacteria is the most phylogenetically diverse taxa and represents 34% of known bacteria (Euzéby 2021). As bacterial communities that are highly diverse and share similar ecological niches facilitate higher frequencies of horizontal gene transfer (Dionisio et al. 2002; Smillie et al. 2011), it is possible that communities rich in taxonomic groups such as Proteobacteria may constitute some of the most important species when considering the accumulation and proliferation of ARGs.

1.1.3 The gut microbiome as a 'melting pot' of antimicrobial resistance

Recent studies have indicated that, in particular, the gut acts as a favourable ecological niche for horizontal gene transfer (Lerner et al. 2017). The animal gut is a highly complex system

that supports a multitude of interactions both within the diverse and abundant microbial community and between these microbiota and their host (Shterzer & Mizrahi 2015). This community is highly dense, with each millilitre of gut content hosting greater than 10^{10} microorganisms (Shterzer & Mizrahi 2015), which equates to the total number of human cells within the body (San Millan 2018). The role of the human gut as a 'melting pot' for horizontal gene transfer (Shterzer & Mizrahi 2015) is supported by findings that bacterial genomes isolated from the gut have more similar gene contents than non-gut bacterial genomes at the same evolutionary distance (Lerner et al. 2017). Moreover, ARGs from non-pathogenic bacterial strains, from human saliva and fecal samples, have been found to share nucleotide identity to ARGs previously identified in major human pathogens, indicating that these genes can be horizontally transferred to and from transient bacteria (Sommer et al. 2009). Highly diverse microbial communities such as the gastrointestinal tract are therefore likely to facilitate dissemination of ARGs between pathogenic bacteria (Dionisio et al. 2002; Shterzer & Mizrahi 2015).

Observational studies of AMR in the gut have suggested that close proximity (Shterzer & Mizrahi 2015), high density and species richness (Francino 2016) of bacteria in the gut create ideal conditions for conjugation. This is supported by findings of higher ARG transfer rates *in vivo*, compared to *in vitro*, which may be due to the peristaltic mixing of the gut promoting high cell-cell contact (Dahl et al. 2007). Moreover, the gastrointestinal tract imposes stress on the microbiota, including competition between bacteria, acidity, bacterial and host metabolites, aerobic limitations and host defence mechanisms, which have each been shown to influence horizontal gene transfer (Zeng & Lin 2017). Within the gut, biofilms, which constitute dense, surface-associated microbial communities, may also facilitate interactions between bacteria that promote conjugation (Molin & Tolker-Nielsen 2003). The animal gut is a unique environment that provides physical and chemical stress, and hosts complex microbiota, including planktonic and biofilm communities (Molin & Tolker-Nielsen 2003). Multiple features of the gut are, therefore, likely to promote interactions and facilitate genetic exchange between bacteria within the microbiota community.

The role of the animal gut in promoting horizontal transfer is supported by studies which show that the frequency of ARG transfer is increased when bacteria are digested (Matsuo et al. 2010; Moliner et al. 2010; Thimm et al. 2001). The high frequencies of horizontally transferred ARGs and mobile genetic elements in the numerous animal models further

suggests that the gut may act to promote the evolution of host commensal and pathogenic bacteria and demonstrates this ‘melting pot’ of horizontal gene transfer (Dahl et al. 2007; Francino 2016; Johnsen et al. 2002; Moliner et al. 2010). Evidence for horizontal transfer between bacteria co-inhabiting the same host has been found in swine fecal samples, with many shared genes between *E. coli* and *Salmonella*, including one sample in which almost half of ARGs analysed were shared between these two species (Frye et al. 2011). While these investigations clearly illustrate the potential for horizontal gene transfer *in vivo*, they only investigate transfer between a limited number of bacterial species and therefore do not reflect ARG transfer in the context of the wider microbiome. A recent study by Fu et al. (2017) built upon these findings by demonstrating the transfer of multi-drug resistance plasmids from a lab strain of *E. coli* to 15% of indigenous microbiota in a zebrafish model. The authors demonstrated that, within the gut, ARGs not only proliferate clonally from donors but are also transferred with plasmids to the indigenous bacteria of the gut microbiota. This experimental study exemplifies a comprehensive approach to clearly identify conjugation and the dissemination of multi-drug resistance plasmids within the animal host (Fu et al. 2017). Overall, evidence of horizontal gene transfer from animal models suggests that the diverse microbiota of the animal gut may support conjugation of plasmids carrying multi-drug resistance (Shterzer & Mizrahi 2015). However, these studies provide limited information to demonstrate how the gut microbiome might facilitate the generation of multi-drug resistance on mobile genetic elements.

1.2 The role of wildlife in antimicrobial resistance

Numerous non-human animals have been shown to host bacteria that harbour AMR both in the presence and absence of antimicrobials (Dahl et al. 2007; Dheilly et al. 2012; Frye et al. 2011; Johnsen et al. 2002). This is evidenced by the high prevalence of ARGs in bacteria from companion and production animals, particularly those that are treated with antibiotic medicines (Marshall & Levy 2011). It has also been shown that production animals, such as chickens, are able to maintain AMR-bacteria and transmit ARGs to other individuals for many weeks, even in the absence of antibiotic treatment (Laetitia et al. 2011). Agricultural and companion animals have therefore been identified as possible reservoirs for bi-directional transmission of AMR to humans (Marshall & Levy 2011). However, for decades AMR-

bacteria have also been identified in wildlife species, illustrating the far-reaching dissemination of AMR that could potentially contribute to the proliferation of clinical resistance (Ramey & Ahlstrom 2020; Vittecoq et al. 2016).

1.2.1 Wildlife carriers of antimicrobial resistance

The presence of AMR-bacteria in wild animals has been well documented (Arnold et al. 2016; Bonnedahl & Järhult 2014; Greig et al. 2015; Radhouani et al. 2014) since it was first reported in pigeons and crows in Japan in the 1970s (Sato et al., 1978). Antimicrobial resistant bacteria have now been recorded across a range of taxa in every wildlife species that has been investigated, including insects, fish, reptiles, birds and mammals (Vittecoq et al. 2016) and the prevalence of AMR-bacteria in wildlife has been reportedly increasing over time (Vittecoq et al. 2016; Wallace et al. 2013). For example, a major review of AMR in wildlife found that almost 50% of *E. coli* samples reported from bird and mammal species exhibit AMR (Swift et al. 2019). Additionally, wildlife have also been found to carry bacteria encoding resistance to last-resort clinical antibiotics, including plasmid-associated colistin resistance genes (Wang et al. 2017). Concerningly, multi-drug resistance, including resistance to extended spectrum beta-lactamases, has been found in at least 80 different species of wildlife, mostly wild birds, across Europe, Africa, Asia, South America, North America, and Australia (Bonnedahl & Järhult 2014; Wang et al. 2017). Moreover, the prevalence of multi-drug resistance bacteria in wildlife, such as extended spectrum beta-lactamase-associated *E. coli,* is increasing (Guenther et al. 2011). Wildlife AMR-bacteria are often associated with proximity to humans (Furness et al. 2017; Kozak et al. 2009; Navarro-Gonzalez et al. 2018), however, it has also been identified in remote locations far from major human developments, such as the polar regions (Hernández & González-Acuña 2016) and in ocean-dwelling loggerhead turtles (Blasi et al. 2020).

Although AMR-bacteria are widespread in wildlife, differences in host-related ecological factors have been shown to impact the prevalence of these bacteria in a given animal species (Vittecoq et al. 2016; Williams et al. 2011). For example, many studies have reported higher carriage of AMR-bacteria in carnivorous and omnivorous species than in herbivores, which may reflect differences in exposure to AMR-bacteria (Vittecoq et al. 2016). Moreover, a study of two rodent species with similar distributions throughout the same habitat found that wood mice (*Apodemus sylvaticus*) carry a greater prevalence of AMR-*E. coli* than bank voles

(*Myodes glareolus*) (Williams et al. 2011). Although both species demonstrated overall increases in AMR-bacteria over time, the species exhibited distinct seasonal cycles of AMR prevalence, which peaked in different months for each species (Williams et al. 2011). Given that these species were exposed to no known source of antimicrobials, the authors suggest that these results illustrate that AMR carriage is influenced by seasonal foraging behaviours (Williams et al. 2011), which result in differences in exposure to AMR-bacteria. Therefore, numerous factors including ecology, diet and interactions with humans, may contribute to an individual host's carriage of bacterial AMR.

1.2.2 Evidence for the horizontal transfer of antimicrobial resistance in wildlife bacteria

The evolution of AMR in wildlife is unlikely due to direct exposure to antimicrobials (Dolejská & Papagiannitsis 2018). Although populations close to anthropogenic sources are more likely to carry bacteria harbouring ARGs, AMR-bacteria have also been found in populations in remote locations far from human activities (Blasi et al. 2020; Hernández & González-Acuña 2016). The increasing prevalence of AMR observed in wildlife therefore suggests that AMR is propagating within and between bacterial species in wildlife hosts, which can be spread to other individuals and populations (Dolejská & Papagiannitsis 2018; Wang et al. 2017). For example, Arnold et al. (2016) found similar patterns of AMR and ARGs in *E. coli* isolated from rodent residents and livestock from commercial farms. The authors found that few *E. coli* had shared genotypes, providing evidence for a resistome exchanged between the bacteria (Arnold et al. 2016). Moreover, mobile genetic elements are frequently identified in association with ARGs in wildlife, which further suggests that horizontal gene transfer facilitates the dissemination of these ARGs (Dolejská et al. 2009; Dolejská et al. 2007; Dolejská & Papagiannitsis 2018; Papagiannitsis et al. 2017). For example, in wild animal populations, higher prevalences of integrons carrying ARGs have been correlated to areas with greater exposure to humans, suggesting that these mobile genetic elements are important factors in the human-associated proliferation of bacterial AMR (Skurnik et al. 2006).

1.2.3 Transmission of antimicrobial resistance bacteria to and from wildlife

The association between the prevalence of bacterial AMR and proximity to humans suggests that AMR is propagated into wildlife from anthropogenic sources (Furness et al. 2017; Kozak

et al. 2009; Navarro-Gonzalez et al. 2018). For example, genetically similar AMR-Enterobacteriaceae have been identified in human and wildlife populations, demonstrating the potential for AMR dissemination (Hernández et al. 2013; Thompson 2019; Wang et al. 2017). This conclusion is further supported by numerous studies that have linked AMR to anthropogenic activity, including wastewater, terrestrial waste, agriculture and interactions with captive wildlife (Ahlstrom et al. 2018; Kozak et al. 2009; Messenger et al. 2014; Varela et al. 2015). Moreover, numerous environmental pathways are thought to contribute to the transmission of these sources into wild reservoirs, both directly through contact and indirectly, such as through soil and water (Arnold et al. 2016; Furness et al. 2017; Radhouani et al. 2014; Vittecoq et al. 2016). For example, genetically similar bacterial isolates have been identified in water samples and gulls that use those water sources (Atterby et al. 2017; Dolejská et al. 2009; Varela et al. 2015). This finding is supported by a 2016 review, which compared 177 studies of aquatic and terrestrial animals and found that the prevalence of AMR-bacteria was significantly higher in aquatic species (Vittecoq et al. 2016). However, few studies have provided definitive evidence to demonstrate the source of AMR-bacteria in wildlife bacteria, so the routes of transmission are often unresolved (Arnold et al. 2016) (Fig 1.3).

Wild animals, such as voles and mice, may have the potential to maintain bacterial AMR (Williams et al. 2011), which could occur through transmission of AMR-bacteria amongst hosts in the same population. Mallard's (*Anas platyrhynchos*) have been shown to maintain MDR *E. coli* for four weeks, within which time they transmit AMR between the group (Sandegren et al. 2018). Sandegren et al.'s study, however, was conducted in the laboratory setting using birds bred at a commercial farm (Sandegren et al. 2018), which provides limited information about the transmission ability of these birds in wild settings. Further research is needed to determine if AMR acquired by wildlife can be maintained and transmitted to other host individuals.

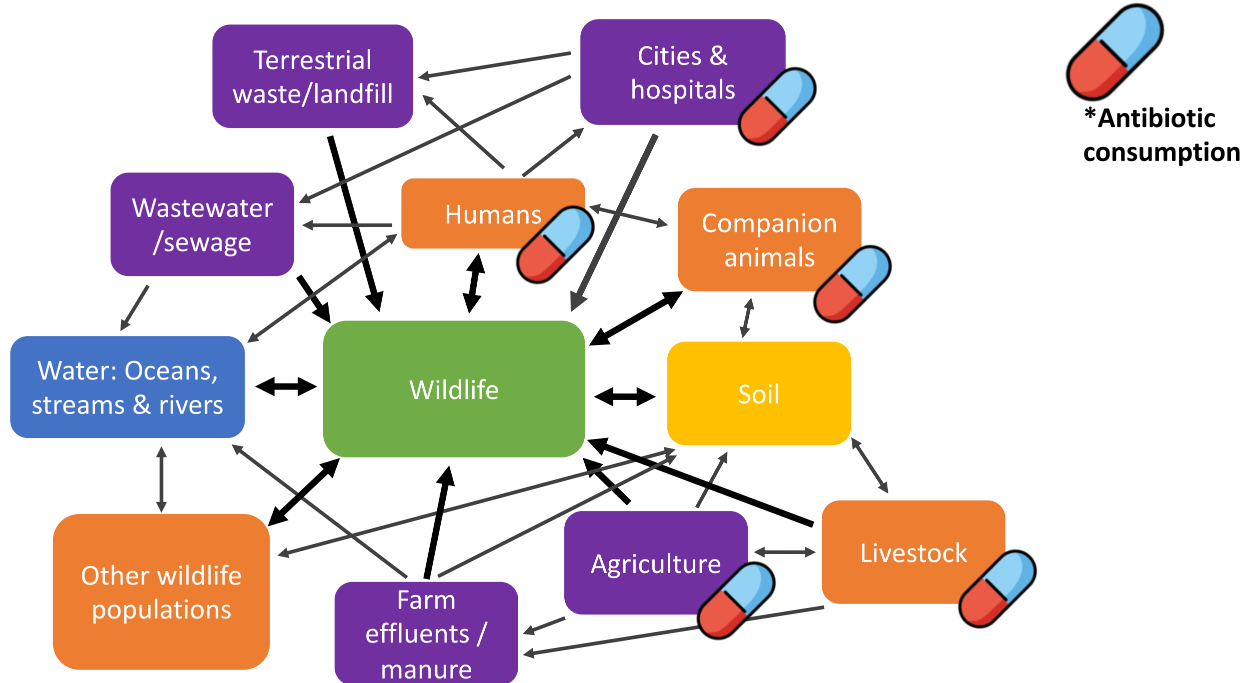


Figure 1.3. Potential transmission of antimicrobial resistance between wildlife, anthropogenic sources and environmental pathways (adapted from Arnold et al. 2016 and Dolejska & Papagiannitsis 2018).

1.2.4 The role of avian wildlife in the propagation of antimicrobial resistance

The differences in bacterial AMR carriage between animal species (Vittecoq et al. 2016; Williams et al. 2011) further suggests that some species may be more likely to acquire, maintain and subsequently spread AMR-bacteria (Atterby et al. 2017). For instance, bird and mammal species have been associated with a high prevalence of bacterial AMR, particularly those that interact closely with humans (Vittecoq et al. 2016). Avian species have been further suggested as a 'melting pot' of AMR due to the high prevalence of AMR-bacteria across the heterologous bird class (Bonnedahl & Järhult 2014; Marcelino et al. 2019), particularly amongst birds of prey, waterfowl and water-related birds (Guenther et al. 2011). For example, numerous European migrating gull species and Andean condors have been reported to carry AMR-bacteria (Fuentes-Castillo et al. 2019; Stedt et al. 2014). Given avian wildlife can be highly mobile and harbour a high prevalence of AMR-bacteria, it has been further speculated that bird species could act as vectors and play an important role in the dissemination of AMR over vast geographic distances (Bonnedahl & Järhult 2014; Fuentes-Castillo et al. 2019; Stedt et al. 2014). Moreover, as some wild birds can have high contact

with humans, they are postulated to act as a reservoir for clinically important AMR (Bonnedahl & Järhult 2014). However, as most studies of AMR in wildlife investigate bird hosts (Greig et al. 2015; Vittecoq et al. 2016), this avian focus may not reflect a unique ability to harbour, maintain and transmit ARGs to other species. Nonetheless, as avian species have been thoroughly examined in the context of AMR-bacteria, this makes them a logical study group to further investigate questions of ARG transfer.

In particular, gulls from the Laridae family have been well studied and identified as common carriers of AMR-bacteria (Guenther et al. 2011; Stedt et al. 2014). Many gull species have adapted to human urban environments and frequently scavenge at waste depots, wastewater treatment plants, agriculture and livestock areas (Stedt et al. 2014), which could act as sources of AMR (Ahlstrom et al. 2018; Atterby et al. 2016). In addition to these close associations with humans, carriage of clinical AMR-bacteria in gulls demonstrates their potential to act as bioindicators of AMR contamination in the environment (Stedt et al. 2014). Additionally, widespread extended spectrum beta-lactamase-*E. coli* have been found in Franklin's gulls, with a higher prevalence than the corresponding human population, indicating that the gulls may be disseminating AMR-bacteria (Hernández et al. 2013). Gull populations have also been shown to contain large numbers of AMR-*E. coli* and *Salmonella,* which also carry integrons and multiple ARGs (Dolejská et al. 2007; 2009). Due to the presence of mobile genetic element-associated multi-drug resistance in gulls, it has been suggested that horizontal gene transfer of ARGs in gull microbiomes plays an important role in the dissemination of resistance (Dolejská & Papagiannitsis 2018). Moreover, the close relatedness of AMR-bacterial isolates from gull, clinical, domestic animals and wastewater samples has led to speculations that gulls could have the potential to act as vectors and facilitate the circulation of AMR between anthropogenic and environmental compartments (Stedt et al. 2014; Varela et al. 2015). Avian species, such as gulls, are therefore an appropriate study species to investigate these AMR in wildlife.

1.2.5 Microbiome composition and antimicrobial resistance in avian wildlife

There is immense variation in microbiota diversity and composition between different host species, even those within the same animal class (Hammer et al. 2019). Additionally, while the specific composition of gut microbiota in some species such as humans is strongly correlated to host health, other taxa such as birds are less dependent on their microbiomes

(Hammer et al. 2019) and so may experience much greater fluctuations in bacterial species composition (Cao et al. 2020). Recent studies have also found that the diversity of avian microbiota is not correlated to host phylogenetic distance or diet type (Song 2020). Conversely, similar microbiota composition observed in different bird species may be a result of the geographic environment that the species co-inhabit (Cao et al. 2020). The gut microbiota of avian species is likely less structured than mammalian species, and composition may be more heavily influenced by environmental exposure to bacteria (Song 2020).

The microbiota of multiple bird species is composed primarily of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla, with Proteobacteria pre-dominant in many host individuals studied (Cao et al. 2020; Hammer et al. 2019; Song et al. 2020). Notably, the highly diverse Proteobacteria phyla (Euzéby 2021; Grond et al. 2018) is enriched in mobile ARGs and therefore may constitute species that facilitate the horizontal transfer of ARGs (Hu et al. 2016). In some avian species, such as migratory geese, Proteobacteria can constitute greater than 90% of a hosts microbiota (Cao et al. 2020). Even within a single species, the abundance of Proteobacteria can range so dramatically that some individuals host almost no Proteobacteria, while others are primarily composed of the Proteobacteria taxa (Cao et al. 2020; Hird et al. 2015; Teyssier et al. 2018). Avian species have relatively low microbiota diversity compared to humans and other animal taxa (Cao et al. 2020; Song et al. 2020) and Song et al. (2020) speculates that the decrease in resident microbiota associated with avian microbiomes may promote a higher proportion of transient environmental bacteria. This further indicates that avian microbiomes may be more likely to be exposed to pathogens and environmental Proteobacteria that could enhance the chance of ARGs horizontally transferred (Song et al. 2020). Given that avian species have been shown to harbour a high abundance of diverse Proteobacteria including AMR-associated and pathogenic species (Bonnedahl & Järhult 2014; Cao et al. 2020; Hu et al. 2016) and lack microbiome specificity (Song 2020), it is possible that birds have the ability to be colonised by a diverse range of Proteobacteria, thus allowing them to host opportunistic and pathogenic bacteria associated with AMR carriage. As ‘ecological sponges’, avian species may be able to indiscriminately accumulate AMR-bacteria from the environment (Mukerji et al. 2019), and therefore may facilitate elevated rates of cross-strain and interspecies horizontal gene transfer, thereby contributing to the proliferation of AMR.

1.3 Investigating bacterial and resistome dynamics in wildlife

1.3.1 The role of silver gulls in antimicrobial resistance

Populations of the Australian silver gull, *Chroicocephalus novaehollandiae,* have been expanding in urban settings along the coast of Australia for decades (Smith & Carlile 1993). Colonies of gulls congregate in high densities during breeding periods (Smith & Carlile 1992), bearing many juveniles with immature immune systems (Grond et al. 2017). Recently, Mukerji et al. (2019) identified widespread AMR-*E. coli* from silver gulls sampled across Australia. The authors demonstrated that the majority of isolates were clones of pathogenic *E. coli* previously identified in the human gut, suggesting that silver gulls act as 'ecological sponges' of AMR from anthropogenic sources (Mukerji et al. 2019). In particular, Big Island of the Five Islands Nature Reserve, off the coast of Wollongong, NSW Australia, accommodates 70% of silver gull's coastal breeding in New South Wales and has been hypothesised to be a reservoir of multi-drug resistance bacteria (Dolejská et al. 2016). AMR-bacterial isolates from gulls have demonstrated significant similarity to clinical isolates, suggesting that they were transmitted from nearby anthropogenic sources (Dolejská et al. 2016; Thompson 2019). Recent satellite tracking confirmed that these silver gulls frequent anthropogenic habitats, including landfill and wastewater treatment, where they engage in foraging activities (Browne 2020).

Enterobacteriaceae species such as *Salmonella* can host a broad array of ARGs and cause widespread infection in avian hosts (Tizard 2004) and have therefore been a focus of numerous AMR studies in gull hosts (Cummins et al. 2020; Dolejská et al. 2009; Dolejská et al. 2018; Palmgren et al. 2006). Recently, multi-drug resistant *Salmonella* isolates from silver gulls on Big Island were found to harbour more ARGs than phylogenetically related clinical isolates (Thompson 2019). This suggests that ARGs may be accumulating in AMR-bacteria within gull microbiomes, however, this is yet to be tested. Previous evidence from AMR-carrying bacteria in silver gulls on Big Island also suggests that ARGs are horizontally transferred between bacteria, which may underpin the accumulation of AMR within silver gull microbiomes (Dolejská et al. 2016). In 2016, Dolejska et al. identified carbapenemase-producing Enterobacteriaceae carrying a bla_{IMP} gene, which was found within an integron and carried by conjugative plasmids (Dolejská et al. 2016). The authors demonstrated that the same bla_{IMP} associated integrons were found in different plasmids and among different

species of bacteria and *E. coli* sequence types of significant genetic diversity (Dolejská et al. 2018). These results indicate that *bla_{IMP}*-associated integrons and mobile genetic elements may be involved in plasmid reshuffling and that these plasmids are frequently exchanged between bacterial species (Dolejská & Papagiannitsis 2018). It is, therefore, possible that multi-drug resistance carriage in silver gulls is a result of horizontal gene transfer amongst bacteria and subsequent AMR accumulation within the microbiome. However, the mechanisms of ARG accumulation and maintenance have not been investigated in wildlife, which limits our understanding of the potential of multi-drug resistance to propagate. Understanding the molecular mechanisms underlying these processes is crucial to understanding both the development of multi-drug resistance, and the role of wildlife in the spread of bacterial AMR.

1.3.2 Methods to investigate antimicrobial resistance dynamics in wildlife

To date, the high prevalence of AMR and multi-drug resistant genetic elements, as well as sequence comparisons between AMR-bacteria of wild animal and human hosts, provide the strongest evidence of AMR dissemination between animal species (Dolejská & Papagiannitsis 2018). Additionally, wildlife populations that frequently harbour plasmid-mediated resistance are speculated to act as vectors and reservoirs of AMR (Dolejská & Papagiannitsis 2018). However, whether wildlife is able to accumulate and maintain AMR-bacteria or if they are only temporary carriers needs to be determined in order to understand the ability of wildlife to act as reservoirs of AMR (Dolejská & Papagiannitsis 2018). Additionally, ARG transmission from a wildlife population into a human or other animal population remains to be demonstrated (Haydon et al. 2002). It is, therefore, unclear whether wildlife poses a risk of proliferating and transmitting clinically important AMR-bacteria into human populations.

The current understanding of AMR in wildlife is limited to cross-sectional surveys, composed of single time-point samples that identify AMR-bacteria and mobile genetic elements (Greig et al. 2015), often from a single pathogen species. For example, a recent paper investigated the role of wild and captive little penguins *(Eudyptula minor)* as bioindicators of AMR using polymerase chain reaction (PCR) screening for class 1 integrons and associated gene cassettes in microbial DNA extracted directly from faecal samples (Lundbäck et al. 2021). This method, which assumes class 1 integrons are the primary

determinants of AMR, misses a large cohort of mobile genetic elements and their associated ARGs (Jackson et al. 2011; Siguier et al. 2006). As integrons were not detected in the subsequent PCR screens of cultured *E. coli* and *Klebsiella pneumoniae*, which are two clinically relevant and commonly resistant pathogens, this reinforces the limitations of using these methods to assess AMR carriage. Traditional methods, such as those utilised by Lundbäack et al. (2021), therefore do not holistically assess the bacterial composition and resistome of samples.

Recent studies have, therefore, also utilised metagenomics and meta-transcriptomics, which assess genetic material recovered directly from a sample, and have greatly contributed to the knowledge of bacterial ARG pools in wildlife species (Cao et al. 2020; Marcelino et al. 2019). Metagenomics does not rely on culture-dependent isolation, which can only provide information on a small subset of the bacteria, but instead provides information on all bacteria species and their constituent genes (Yang & Zhang 2017). This method can, therefore, be used to analyse the entire composition of the microbiome and resistome, identifying bacterial species present and AMR carriage based on sequence analysis, which can then be compared within and between species (Cao et al. 2020; Marcelino et al. 2019). This is important because many studies focus solely on *E. coli*, *Salmonella* and *Enterococcus* species (Greig et al. 2015), yet other less common pathogens and non-pathogenic bacteria may also harbour ARGs (Sommer et al. 2009). For example, a recent study by Marcelino et al. (2019) used meta-transcriptomics to identify a diverse range of ARGs in various waterbird species (Marcelino et al. 2019). However, these analyses relied on pooled samples from 10 conspecific birds with no information on the co-occurrence of ARGs within a single host's microbiome (Marcelino et al. 2019), therefore, providing limited evidence to suggest if these bird species could act as a reservoir of multi-drug resistance. Moreover, this cross-sectional approach does not investigate the dynamic nature of AMR, which is an obvious gap in our understanding of how AMR is accumulated, propagated and spread.

A small number of longitudinal investigations in various environmental and animal microbiomes have been conducted to observe resistome dynamics through time, which have exhibited various trends in ARG prevalence. For example, a loss of AMR was exhibited after human patients were discharged from intensive care, however, this followed drastic increases in ARG carriage during admission (Buelow et al. 2014). Positive correlations between bacteria and ARGs have also been exhibited in environmental microbiome investigations

from cattle and swine feces (Alexander et al. 2011; Liu et al. 2016). However, these experiments were conducted in contained systems with constant a supply of antibiotics, and therefore illustrates the role of antimicrobial selection and not the microbiome in maintaining AMR. In contrast, two ontogenetic studies of human infants without previous medical or antibiotic exposure have shown that infants maintain their AMR carriage over time, with some ARGs persisting through the first year of life (Loo et al. 2020; von Wintersdorff et al. 2016). Overall, dynamic investigations demonstrating the evolution of AMR-bacteria are limited and there are clear gaps in our knowledge of AMR dynamics, notably in wildlife microbiomes, which are crucial to understand the proliferation of AMR.

1.4 Summary and Thesis Aims

Horizontal gene transfer is critical for the evolution of AMR-bacteria as it allows functional ARGs to be proliferated between diverse bacterial species without direct exposure to antimicrobial selective pressures. This natural mechanism has allowed clinically relevant pathogenic bacteria to repeatedly overcome commercial antimicrobials, resulting in a health threat that is nearing crisis. Beyond anthropogenic settings, AMR-bacteria are frequently found in wildlife populations, however, studies that have investigated the role of wildlife hosts in the proliferation of AMR are limited. Wild birds, in particular, have been found to host bacteria with a diverse range of ARGs. Moreover, it is speculated that avian species, including *Laridae*, may act as bioindicators of environmental contamination of AMR, likely due to the close interactions of some populations with anthropogenic sources. In addition, the diverse microbiome of the animal gut, particularly of avian species, likely provides conditions that facilitate the transfer of ARGs. Nonetheless, studies that have investigated the role of the wildlife gut microbiome in the proliferation of clinically relevant AMR are limited. Most studies of AMR in wild hosts constitute cross-sectional surveys that identify ARGs carried on mobile genetic elements, usually from a single bacterial species. To bridge this gap, longitudinal metagenomic analyses can be used to explicate if multi-drug resistance is accumulated and whether it has the ability to persist within the microbiome and thus proliferate and spread to other animal hosts.

In this thesis, metagenomic analysis of multi-species microbiota samples, which were subcultured for Enterobacteriaceae, was undertaken to examine bacterial and AMR diversity

and composition in 60 samples from 23 silver gull chicks. This thesis forms the first longitudinal metagenomic investigation to examine the dynamic nature of the microbiome and AMR in a wildlife host. Specifically, this thesis was conducted to examine whether silver gull chicks play a role in the propagation of AMR. The aims of this thesis were to:

1. Optimise the DNA extraction process for multi-species microbiota samples to prepare DNA samples to be sequenced for metagenomic analysis.
2. Describe bacterial and resistome diversity and composition within multi-species microbiota samples to understand the ability of silver gull chick microbiomes to host AMR.
3. Examine ontogenetic shifts in bacterial and ARG composition to assess if ARGs are accumulated and persist within the silver gull chick microbiome.

Understanding the patterns of ARG accumulation and persistence is necessary to understand the role of wildlife in the evolution of AMR-bacteria. These findings may subsequently advise management of the potential sources and transmission pathways of AMR to help mitigate the spread and proliferation of AMR in pathogenic bacteria.

2 METHODS

2.1 Study Design

An ontogenetic study of silver gull chick microbiomes was undertaken to investigate patterns of AMR accumulation and persistence in a wildlife host. Studying the microbiome of hatchlings offers a naïve, undeveloped study site to investigate novel changes in ARG composition. Unlike mammals, who begin microbiome development in utero, wild avian microbiomes are thought to be established only after hatching (Grond et al. 2017; van der Wielen et al. 2002) and are primarily influenced by exposure to the environment and their parents (Grond et al. 2018; Kohl 2012). While adults may have previously hosted numerous AMR-bacteria, chick microbiomes are unlikely to have a history of AMR, so can be utilised to observe resistome and bacterial associations. Additionally, chicks are more susceptible to transient bacteria and pathogens (Grond et al. 2017; Johnsen et al. 2002) and in silver gulls host more human pathogens, such as *Salmonella*, than adult microbiomes (Browne 2020; Dolejská et al. 2016; Thompson 2019).

This study examined 60 multi-species microbiota community samples derived from cloacal samples collected from 23 silver gull chicks, sampled on Big Island (34.4902° S, 150.9286° E) of the Five Islands Nature Reserve, off the coast of Wollongong, NSW Australia, during the breeding seasons of 2018 and 2019. These samples were produced by sub-culturing cloacal samples for Enterobacteriaceae, a family of Proteobacteria which constitute a large proportion of pathogenic and clinically relevant bacteria (Hu et al. 2016). This subpopulation was examined to investigate phylogenetically related taxa that may contribute to the accumulation and proliferation of AMR (Dionisio et al. 2002; Smillie et al. 2011). Additionally, these samples had previously been screened for *Salmonella* (Thompson 2019; Browne 2020; Hoye et al. unpublished data), which was used as an indicator of bacterial AMR. Chicks with a history of *Salmonella* infection were therefore chosen to further investigate AMR carriage and temporal dynamics.

Initially, 75 samples from 24 chicks, each with two to four longitudinal samples, were chosen based on this previous identification of *Salmonella* to investigate the effect of *Salmonella* infection on the microbiome (Appendix 6.1) (Thompson 2019). Seventeen chicks were chosen that had a positive *Salmonella* sample at any time in the season, and that had greater than two samples. Thirteen of these chicks had samples that tested negative to *Salmonella* prior to samples that tested positive, of which six had negative samples after the positive sample. Another 10 chicks were chosen which had a positive sample before consecutive negative samples. This design provided 13 instances to observe microbiome composition before the *Salmonella* infection and 16 instances to observe after. Seven chicks that did not have *Salmonella* at any time during the sampling season were also included as a non-*Salmonella* reference group. These chicks were chosen based on the date of their first sample, weight and intervals between samples that were similar to the chicks with a positive *Salmonella* sample. Using this design, samples were grouped as '*Salmonella* in Chick' or 'No *Salmonella* in Chick' based on the infection history of the respective chick (Appendix 6.1).

Of the 75 samples, six were previously sequenced and processed through bioinformatic pipelines at the Australian Genome Research Facility (AGRF). Of the remaining 69 samples, one sample was not included as extracts did not produce sufficient DNA yield or quality. After 68 samples were sent to AGRF, 14 samples could not be analysed as two samples opened during transport and 12 did not meet quality checks due to insufficient yield,

contamination or significant degradation. This reduced the number of chicks to 23, and the total number of samples to 60 (Appendix 6.1). This final design provided eight instances to observe microbiome composition before the positive *Salmonella* result, eight instances to observe after, and six that did not have detectable *Salmonella* at any time during the season (Appendix 6.1).

2.2 Sample collection and processing bacterial cultures

The 75 microbial samples from 2018 and 2019 used in this study were collected, cultured and stored as communities per the following methods prior to the start of this project. As these samples were used in retrospect, the author of this thesis undertook fieldwork and sample processing for the 2020 breeding season, using these same methodologies. However, samples from the 2020 breeding season were not used in the analyses for the current thesis.

The samples used in this study were collected on Big Island in 2018 (September – November) and 2019 (October – November), under University of Wollongong Animal Ethics Committee approval # AE17-20. At the beginning of the breeding season, fenced plots were constructed around nesting grounds, and nests were marked so that they could be identified throughout the sampling period. Hatchlings in marked nests were caught and marked with a coloured band, to be replaced with an Australian Bird and Bat Banding Scheme aluminium band once chicks were large enough, which allowed them to be resampled. Chicks were sampled each week (when they could be found in designated plots), and cloacal swabs were taken, as they are non-lethal and are considered to represent diversity in the gut more reliably than faecal samples (Grond et al. 2018). Additional measurements (tarsus length, head-bill length and mass) and blood samples were also taken. Age was recorded for hatchlings of approximately 0-2 days old.

Each microbial community was derived from a single cloacal swab collected from an individual chick using sterile rayon swabs. Swabs were placed in Amies medium (without charcoal) (COPAN© Diagnostics) stored on ice for transportation and then stored at 4°C. Cloacal swabs were then cultured for enteric bacteria. Briefly, each swab was placed in 1 mL Lysogeny broth (LB), vortexed for 30 seconds and 10 µL of broth was 16-streaked onto MacConkey agar (Micromedia), to select for gram-negative lactose fermenting

Proteobacteria, specifically Enterobacteriaceae (Elazhary et al. 1973). Plates were incubated overnight at 37°C. Complete cultures of multi-species microbiota were then harvested and placed in 1 mL of a 10% glycerol and 1% casamino acids milli Q solution, vortexed for 30 seconds and stored at -80°C.

Samples were also processed with an additional selection for *Salmonella*. Briefly, 500 ul of the 1 mL LB (above) was added to 10 mL selenite F enrichment broth (Micromedia). Following overnight incubation at 37°C, 10 µL was 16-streaked onto MacConkey agar (Micromedia) and again incubated overnight at 37°C. These cultures were then harvested and stored at -80°C in 1 mL of a 10% glycerol and 1% casamino acids milli Q solution.

2.3 *Salmonella* screening

Samples were previously screened for the presence of *Salmonella* by Thompson (2019) and Browne (2020). Briefly, a single suspected *Salmonella* colony was harvested from the MacConkey agar culture (above). Selection was based on their phenotypic profile, appearing flat, colourless and with a defined edge. These colonies were then screened using a triple sugar iron (TSI) slant (Micromedia) according to the manufacturer's instructions. The colonies were also plated on Brilliant Green agar to obtain pure isolates for species identification using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (Bruker). To examine carriage of ARG, these isolates were also subject to whole-genome sequencing, as described in Thompson (2020) and by Hoye et al. (unpublished data).

2.4 Extracting DNA from microbiome samples for metagenomics

DNA was extracted from cultures of multi-species microbiota samples stored in glycerol for each sample using the ISOLATE II Genomic DNA Kit (Bioline™, Meridian Bioscience®). Before DNA extraction, these multi-species microbiota samples stored in glycerol were re-cultured in LB to produce adequate starting material for DNA extraction. Briefly, the cultured samples in LB broth were pelleted and resuspended in lysis buffer and proteinase K and then incubated at 56°C until complete lysis was obtained. An additional lysis buffer was added,

and the sample was incubated at 70°C for 10 mins. After the addition of ethanol, the sample was transferred to a spin column, and the DNA is bound to the column by filtering the sample via centrifugation. A series of two washes were then filtered through the column via centrifugation, and the column was dried with a third centrifugation step. Finally, the DNA was eluted with a pre-heated (70°C) buffer.

To assess the purity of DNA extracted, nucleic acid concentration (ng/µL) and nucleic acid purity was measured using a NanoDrop™ (ThermoFisher Scientific), which uses ratios of absorbance readings at 260, 280 and 230 nm. For pure DNA, the A260/A280 ratio is expected to be approximately 1.8, whilst pure RNA is expected to read at approximately 2.0 (Matlock 2015). The 260/230 ratio is a secondary measure of nucleic acid purity to detect other contamination, which should be higher than the 260/280 ratio, and is expected to be in the range of 2.0-2.2 for pure DNA (Matlock 2015). Three readings were measured for each DNA extract. Additionally, select samples were electrophoresed in agarose gels to assess the quality of DNA and identify DNA degradation. When visualised on an agarose gel, whole genomic DNA presents at a high molecular weight, while degraded DNA and contaminating RNA are present at a low molecular weight.

2.4.1 DNA extractions of pilot samples

Six of the 75 multi-species microbiota samples (two chicks, each with three samples) were used for the pilot metagenomic analysis. The samples were plated onto LB agar from stored glycerol stocks of multi-species microbiota samples and grown overnight at 37°C. These cultures were then harvested, and genomic DNA was extracted as per the ISOLATE II Genomic DNA Kit.

2.4.2 Trials to optimise the DNA extraction protocol

The six pilot samples had been identified as highly degraded in the quality control report from AGRF. Therefore, to optimise the DNA extractions of the remaining 69 multi-species microbiota samples, a number of protocols adapted from the ISOLATE II Genomic DNA kit were trialled.

The Bioline protocol was optimised using practice multi-species microbiota samples that were not used for further metagenomic analysis. An overview of the initial methods trialled, and the corresponding results are summarised in Appendix 6.2. Initially, glycerol stocks of multi-species microbiota samples were resuspended in PBS (diluted 1:40), serially diluted, and 100 µL of each dilution was plated onto LB and grown overnight at 37°C. Plates with a high coverage of bacteria were selected for extraction. In the first trials, the original resuspension and 1:10 and 1:100 dilutions were used for the DNA extractions (Appendix 6.2: Extracts 1-10). Due to the heterogeneous nature of the bacteria communities, a 'hard to lyse' (HTL) buffer supplemented with lysozyme was used and incubated at 37°C before the 56°C incubation with proteinase K. The plated cultures produced a large bacterial pellet which was difficult to resuspend with a small volume, so double the volume of reagents was used. As these trials showed no visible evidence of cell lysis after both incubation steps, they were incubated overnight at 37°C (Appendix 6.2: Extracts 1-2). To increase cell lysis in the next two trials, an additional dose of lysozyme was added before the overnight lysis at 37°C (Appendix 6.2: Extracts 3-4). Next, syringe lysis was trialled to reduce incubation time and increase lysis (Appendix 6.2: Extracts 5-10). As these trials did not show evidence of improved lysis, the next two trials incorporated a 3 h incubation at 56°C with both the HTL buffer and proteinase K (Appendix 6.2: Extracts 11-12).

All previous trials exhibited evidence of incomplete lysis of the bacterial pellet, likely due to high quantities of bacteria in each sample. Therefore, to increase DNA concentration and reduce incubation time, resuspensions of glycerol stocks were diluted to 1:50 and 1:100. Additionally, a Ribonuclease (RNase) A digest was trialled to reduce RNA contamination, firstly from an old 2010 stock, which was added before the second lysis buffer (Appendix 6.2: Extracts 13-14). To test the effectiveness of the RNase, a 2016 stock (Appendix 6.2: Extracts 15) was trialled with single volumes of the reagents. An agarose gel was run to examine the quality of the extracted DNA, which was visually degraded.

A series of treatments were then trialled to review previous methods and explore further solutions to prevent DNA degradation and optimise bacterial cell lysis. These included lysis with the HTL buffer compared to the provided GL buffer, with and without RNase, and in liquid and plated cultures, all with single volumes of the reagents (Appendix 6.2: Extracts 16-23). The liquid cultures were 1:100 resuspensions of the multi-species microbiota samples glycerol stocks, grown in LB overnight. The plated cultures were 1:100 dilutions of the 1:40

glycerol stocks, as for previous cultures. Unlike previous trials that had been incubated with the HTL buffer, these trials were also incubated at 37°C prior to the 56°C proteinase K incubation. An agarose gel was run to confirm the quality of the extracted DNA. The success of treatments using HTL buffer, RNase and liquid cultures of 1:50 diluted resuspensions was confirmed with another two glycerol samples (Appendix 6.2; Extracts 24-25).

2.4.3 Follow-up DNA extractions for metagenomic analysis

The optimised method was then used to extract DNA from the remaining 69 multi-species microbiota samples included in the study design, however, 25 extracts were either of low concentration or exhibited evidence of degradation when electrophoresed (Appendix 6.3 & 6.4: Extracts 1-69; Appendix 6.4 a). It was evident from previous optimisation steps and from handling the samples that differences in the concentration of the original glycerol stocks of multi-species microbiota samples were a major factor contributing to the quality of extracts. These 25 multi-species microbiota samples were therefore re-extracted using liquid cultures diluted 1:100 for glycerol stocks that initially appeared concentrated and 1:25 for those that appeared dilute. Of these, 20 extractions exhibited no or little degradation and sufficient yield (Appendix 6.4 b-c; Appendix 6.3 & 6.4: Extracts 70-94).

The remaining 5 samples were re-extracted from liquid cultures of 1:10 and 1:25 resuspensions for glycerol stocks that appeared dilute, and 1:200 and 1:400 resuspensions for glycerol stocks appeared concentrated, which were prepared the previous day and frozen overnight. Additionally, five samples that yielded sufficient but lower concentrations of DNA were also subject to re-extraction to increase DNA yield. However, all extracts were of very low concentration and quality (Appendix 6.3: Extracts 95-102). DNA was re-extracted for these samples without freezing the bacterial pellet; however, the extracts were again of low concentration and quality (Appendix 6.3: Extracts 103-120). New enzymes and buffers were trialled to assess if low concentrations and quality of extracts were due to the low efficacy of reagents, which could cause incomplete lysis. The use of new reagents did not greatly improve the extracts (Appendix 6.3 & 6.4: Extracts 121-138; Appendix 6.4d), however, due to time constraints, two samples with sufficient concentrations from this batch were included in the extracts sent for metagenomic analysis (Appendix 2.3: Extracts 129 & 136).

A salt precipitation step was then undertaken to de-contaminate eight extracts with low A260/A230 values, which is indicative of guanidine contamination (Matlock 2015). Extracts were topped up to 200 µL with dH2O, and 20 µL Sodium Acetate was added to the sample before adding pre-cooled (–20°C) 100% ethanol equal to 1.5x the original sample volume. Extracts were then incubated for 2 h at –20°C, before centrifugation at 14,000×g and 4°C for 15 min. The supernatant was removed, before the addition of 200 µL pre-cooled (–20°C) 70% ethanol, and samples were pipetted to resuspend the DNA before another centrifugation at 14,000×g and 4°C for 10 mins. The supernatant was then removed, and samples were left to dry overnight in a fume hood. Finally, the DNA was resuspended in 100 µL elution buffer. To measure DNA yield and quality, readings were obtained from two Nanodrop™ Spectrophotometers, and 10 µL of each sample was electrophoresed on agarose. The gel image was then used to calculate the nucleic acid concentration of extracts based on the intensity of the agarose gel using ImageJ/Fiji (Schindelin et al. 2012). A line of best fit was calculated using the DNA Hyperladder, with the known ng plotted against the intensity reading for each band, and the linear equation was used to estimate nucleic acid concentration. As sample 18-163 did not exhibit evidence of DNA on the agarose gel, salt precipitations were re-trialled using the two supernatants collected in the first precipitation, however, no DNA was detected using the Nanodrop™ or when electrophoresed on an agarose gel.

As samples 18-213 and 18-258 could not be extracted using the methods trialled, DNA from two additional multi-species microbiota samples from the same chicks were extracted (Appendix 6.3 & 6.4: Extracts 140-141). Additionally, 18-163 was re-cultured and re-extracted to compensate for the extract lost in the salt precipitation (Appendix 6.3: Extract 139). Of these three additional extracts, two exhibited sufficient yields and quality (Appendix 6.3 & 6.4: Extracts 139-141; Appendix 6.4f). Overall, DNA was successfully extracted from 68 multi-species microbiome samples (Appendix 6.3 & 6.4).

2.6 Metagenomic sequencing and bioinformatics

2.6.1 Pilot metagenomic analysis

Library preparations and sequencing were undertaken at the Australian Genome Research Facility (AGRF) using the Illumina Nextera XT DNA Library Preparation kit (Illumina®, Inc.) with 1 ng input and unique-dual indexing, as per the manufacturer's instructions. Illumina high-throughput sequencing was then completed by AGRF using the NovaSeq 6000 (Illumina®, Inc.) with an SP-500 cycle kit and Illumina’s 1% PhiX spike control, generating 250 bp paired-end reads to a raw depth of 16 million reads/sample and approximately 8 Gb per sample. Image analysis was performed in real-time by the NovaSeq Control Software (NCS) v1.6.0 and Real-Time Analysis (RTA) v3.4.4 (Illumina®, Inc.). The sequence data was then generated using the Illumina bcl2fastq 2.20.0.422 pipeline. The raw read sequences were processed by trimgalore 0.4.4 (https://github.com/FelixKrueger/TrimGalore) to identify and remove sequencing adapters and low-quality stretches of base quality <=20. The filtered sequences were processed using Bracken (Lu et al. 2017) for profiling the composition of microbial communities using the NCBI Genome and Nucleotide Database, which contains bacterial, fungal and viral genomes and contigs. For quality control, the database also includes human, plant and other vector sequences.

2.6.2 Follow-up metagenomic analysis

Library preparation and sequencing for the follow up metagenomic analysis was also undertaken by the AGRF. Sequencing libraries for each sample were completed using the Illumina DNA Preparation (M) Kit (Illumina®, Inc.) with 1 ng input as per the manufacturer’s instructions. Library inserts of ~350 bp were generated and tagged with a unique-dual index for multiplexing. The finished libraries were diluted to 5 nM and quantified using Illumina’s NEBNext Library Quant Kit.

Pooled libraries were loaded onto the NovaSeq 6000 and clustered at 350 pM. Sequencing was conducted using the SP-500 Cycle Kit (Illumina®, Inc.) and Illumina’s 1% PhiX spike as a standard, at a read-length of 150 bp paired-ends. Image analysis was performed in real-time by the NovaSeq Control Software (NCS) v1.7.0 and Real-Time Analysis (RTA) v3.4.4, which performs real-time base calling on the NovaSeq instrument computer (Illumina®,

Inc.). Two sequencing lanes were run to produce a read depth of ~22 (± 3) million reads per sample and ~7 (± 0.9) Gb per sample. Low-quality sequences (<Q30) were removed to allow a call accuracy of at least 99.9% (Illumina 2011). The sequence data was then generated using the Illumina bcl2fastq 2.20.0.422 pipeline (Illumina®, Inc.). The raw read sequences were processed by trimgalore 0.4.4 (https://github.com/FelixKrueger/TrimGalore) to identify and remove sequencing adapters and low-quality stretches of base quality <=20. Data produced from the two sequencing lanes were merged to produce one fastq file, which was then processed using kracken2 (v2.0.8) and Bracken (v2.5) (Lu et al. 2017) for profiling the composition of microbial communities. Kracken2 and Bracken identify species present by comparing sequence data to genomes and contigs from the NCBI Genome and Nucleotide Database.

ResFinder 4.1 (Bortolaia et al. 2020) was used to identify resistance genes in the metagenomic dataset, using a nucleotide identity threshold of 90% and >60% coverage in length. The ResFinder repository contains acquired resistance genes gathered from published papers and databases with resistance to Aminoglycosides, Beta-lactams, Colistins, Fluoroquinolones, Fosfomycins, Fusidic acids, Glycopeptides, MLS (Macrolide, Lincosamide and Streptogramin), Nitroimidazolse, Oxazolidinones, Phenicols, Pseudomonic acids, Rifampicins, Sulphonamides, Tetracyclines, Trimethoprims and Disinfectants. The database does not include ARGs generated by de novo mutation, thus only identifying ARGs from the mobile resistome.

2.7 Data processing and statistical analysis

The metagenomic data on species composition listed >9000 species for the pilot samples and >22,000 species for the follow-up samples, constituting mostly bacterial and viral species, and also included archaea and eukaryotes. The dataset also provided the relative and absolute abundance values for each species. To remove incorrectly identified species, the relative abundance dataset was filtered to include only species above a minimum threshold based on the relative abundance of sequence reads. Although species listed in the dataset included archaea and eukaryotes, the culture of raw samples prior to DNA extraction is unlikely to have supported the replication of eukaryotic cells. Therefore, the value equal to the most

abundant eukaryote species, which was a relative abundance of 0.000309, was identified as a minimum threshold. All taxa with a relative abundance below this threshold were removed from further analyses, generating a dataset of 329 bacterial and viral species across the 60 microbiota samples. Thirty of the viral taxa in this dataset were not detected in any sample in the pilot analysis and were rare across samples in the follow-up analysis. These taxa were therefore recorded as absent (zeroed) in the six pilot samples.

As samples cultured Enterobacteriaceae (Elazhary et al. 1973) were used in the metagenomic analysis, the relative abundances in the dataset do not reflect abundances within the silver gull microbiota. The subculturing of samples through MacConkey agar, and further growth steps in LB broth, would alter competitive interactions that could promote or inhibit the growth of certain species, and which would further be dependent on the composition of each multi-species community. These processing steps actively exclude certain species, and the relative abundance values produced would not accurately represent the *in vivo* relative abundances of the cultured Proteobacteria. These abundance values were therefore transformed into a presence-absence dataset. Additionally, as only 19 chicks had two or more longitudinal samples, four chicks with single samples were excluded from statistical analyses.

To investigate ontogenetic microbiome changes across chicks and samples, an age estimate of the chick at the time of sampling was calculated for samples included in the metagenomic dataset. In addition to the chicks used in this study, 607 chicks were also sampled in 2018 and 2019. Chicks with known hatch dates (n = 335) were used to define a linear relationship between age (to 30 days) and tarsus length ($R^2 = 0.8533$, $p < 0.05$, Appendix 6.6). This relationship was used to calculate the approximate age of chicks used in this study on the day they were first banded and sampled (even if those samples were not part of this study). This initial age, together with the time between sampling events, was then used to define chick age at all subsequent sampling events.

General linear mixed models ('lmerTest' package R 4.0.2) were used to assess correlations between (1) bacterial species and genera richness; (2) species/genera richness and the number of pathogen species; (3) genera richness and the number of pathogenic genera; (4) species richness and age; (5) the number of ARGs and age; and (6) the number of ARGs and species richness. All models accounted for repeated measures on individual chicks and the Poisson distribution of the richness data. Correlation plots and bacterial and ARG bar plots were

generated in R 4.0.2 using ‘ggplot2’ (Wickham 2020). Heatmaps were produced using the ‘pheatmap’ package in R 4.0.2 (Kolde 2019).

A permutational multivariate analysis of variance (PERMANOVA) was performed on Jaccard distances for bacterial and ARG presence-absence datasets to test for effects of age on bacterial and resistome composition, accounting for repeated measures on individual chicks, using the ‘vegan’ package (Oksanen et al. 2020) package in R 4.0.2. The effect of chick on bacterial and resistome composition was assessed with additional PERMANOVAs also conducted in ‘vegan’ (R 4.0.2). Principal Coordinate Analyses (PCoAs) based on Jaccard distances were also performed using ‘vegan’ and ‘ggplot2’ (R 4.0.2) to assess the differences in bacterial and resistome composition between samples.

Procrustes correlation was performed on Jaccard distances for bacterial and ARG presence-absence datasets to assess correlations between bacterial and resistome composition using the ‘vegan’ package in R 4.0.2. The analysis was performed for the whole data, and with additional Procrustes analyses on samples from nine chicks in the first week of life (age 1-6 days), and nine chicks in their final days before fledging (age 41-56 days). To identify associations between specific bacterial genera and ARGs pairwise Spearman’s rank correlations were calculated on bacterial genera and ARG presence-absence datasets, using the Matrix package in R 4.0.2 (Bates et al. 2021). Correlations with ρ coefficient values above 0.75 and p-values below 0.05 were considered significant associations. Correlations between species and ARGs with significant associations were visualised in ‘pheatmap’ (R 4.0.2).

3 RESULTS

3.1 Optimisation of DNA extractions of multi-species microbiota samples

To optimise the DNA extractions of glycerol samples of multi-species microbiota community samples, a number of protocols adapted from the ISOLATE II Genomic DNA kit (Bioline™, Meridian Bioscience®) were trialled. The first extracts produced a seemingly high nucleic acid concentration, yet A260/A280 ratios were above 1.8, indicating RNA contamination (Fig 3.1.1a-c). Additionally, for the diluted sample, A260/A230 ratio was outside of the expected range (Fig 3.1.1b). The next two trials were conducted to improve bacterial cell lysis; however, the extracts did not exhibit improved A260/A280, and A260/A330 ratios (Fig 3.1.1b-c; Fig 3.1.2b-c), and trials treated with syringe lysis measured much lower nucleic acid concentrations (Fig 3.1.2a).

Trials treated with RNase exhibited A260/A280 ratios close to 1.8, indicating that RNase successfully reduced RNA contamination (Fig 3.1.3a). For most samples, A260/A230 ratios were also within the expected range, further confirming these results (Fig 3.1.3b). Additionally, RNase treated extracts exhibited much lower nucleic acid concentrations (Fig 3.1.3c). The above results suggest that high nucleic acid concentrations, in combination with high A260/A280 readings, are indicative of RNA contamination. Although the results of the RNase treatment suggest high DNA purity (as indicated by A260/A230 ratios), the complementary agarose gel indicated that this DNA was heavily degraded (Fig 3.1.3d), indicating incomplete lysis.

A series of treatments to optimise the lysis reaction were then conducted to facilitate the extraction of undegraded genomic DNA. In concordance with the previous extraction method, RNase treated extracts exhibited A260/A280 readings closer to 1.8 than extracts without RNase (Fig 3.1.4a). Additionally, extracts prepared directly from plated cultures treated with RNase exhibited A260/A280 readings closer to 1.8 than those prepared from liquid cultures (Fig 3.1.4a). These results were confirmed with A260/A230 readings, which were within the expected range for samples prepared as liquid cultures treated with RNase

(Fig 3.1.4b). Nucleic acid concentrations varied between treatments but were clearly lower for RNase treated extracts, as expected (Fig 3.1.4c). Nucleic acid concentrations were also lower for extracts from liquid cultured samples (Fig 3.1.4c) and samples treated with HTL buffer exhibited higher nucleic acid concentrations than those treated with GL buffer (Fig 3.1.4c). Moreover, all liquid culture samples exhibited evidence of genomic DNA, as determined by the presence of a molecular weight band on the agarose gel, indicating that the lower density liquid cultures helped decrease DNA degradation (Fig 3.1.4c). Plated cultures treated with RNase also exhibited evidence of undegraded DNA (Fig 3.1.4d), unlike previous cultures (Fig 3.1.3d). Overall, these results indicated that the use of liquid cultures and the addition of RNase help to support complete lysis and produce high purity and quality DNA. Notably, the liquid cultured extracts treated with HTL buffer and RNase produced a highly pure, high quality and concentrated DNA sample, reflecting the success of these methods when used in conjunction.

Using this optimised protocol adjusted to different initial dilutions, DNA was successfully extracted from 68 multi-species microbiome samples at a sufficient concentrated and quality, to be used for metagenomic analysis.

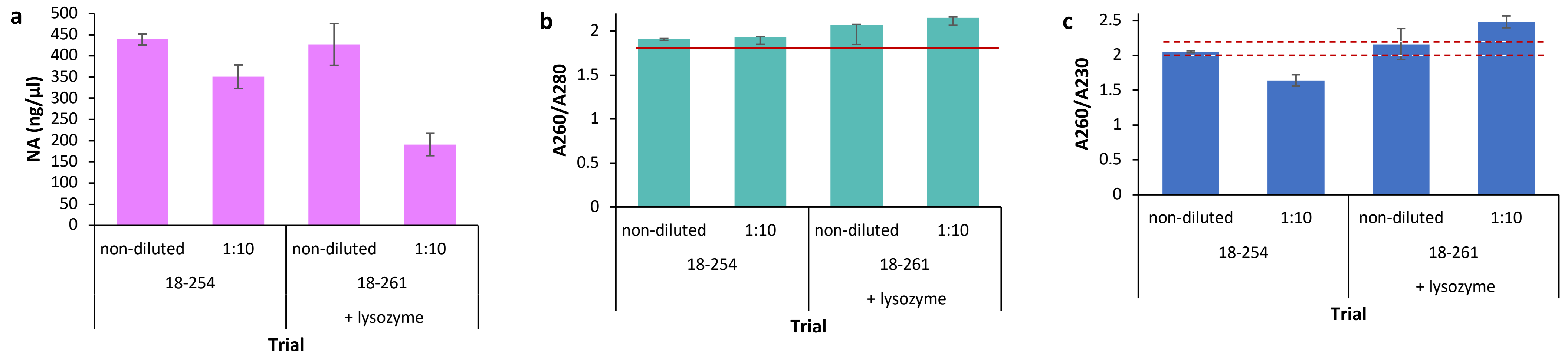


Figure 3.1.1. DNA quality assessments for DNA extraction trials treated with and without additional lysozyme. **(a)** Nucleic Acid (NA) concentration. (**b)** A260/A280 ratio. Solid line represents the optimum A260/A280 value for pure DNA. **(c)** A260/A230 ratio. Dotted lines represent the expected A260/A280 range for pure DNA. Presented are non-diluted and 1:10 dilution treatments of each microbiota culture. Given are mean ± SD of Nanodrop™ readings.

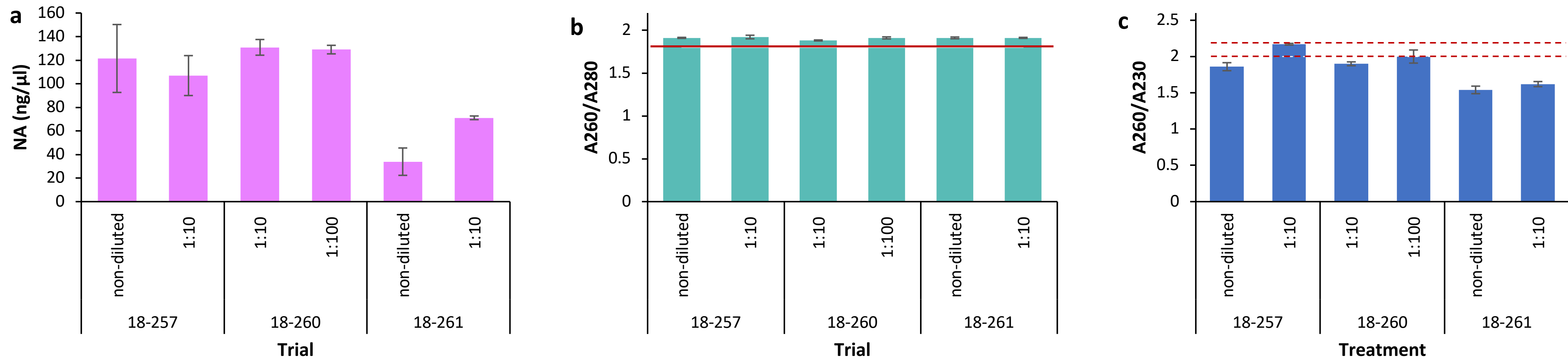


Figure 3.1.2. DNA quality assessments for DNA extraction trials processed with syringe lysis. **(a)** Nucleic Acid (NA) concentration; **(b)** A260/A280 ratio. Solid line represents the optimum A260/A280 value for pure DNA; **(c)** A260/A230 ratio. Dotted lines represent the expected A260/A280 range for pure DNA. Presented are non-diluted and 1:10 dilutions (or 1:10 and 1:100 dilutions) of each treatment. Given are mean ± SD of Nanodrop™ readings

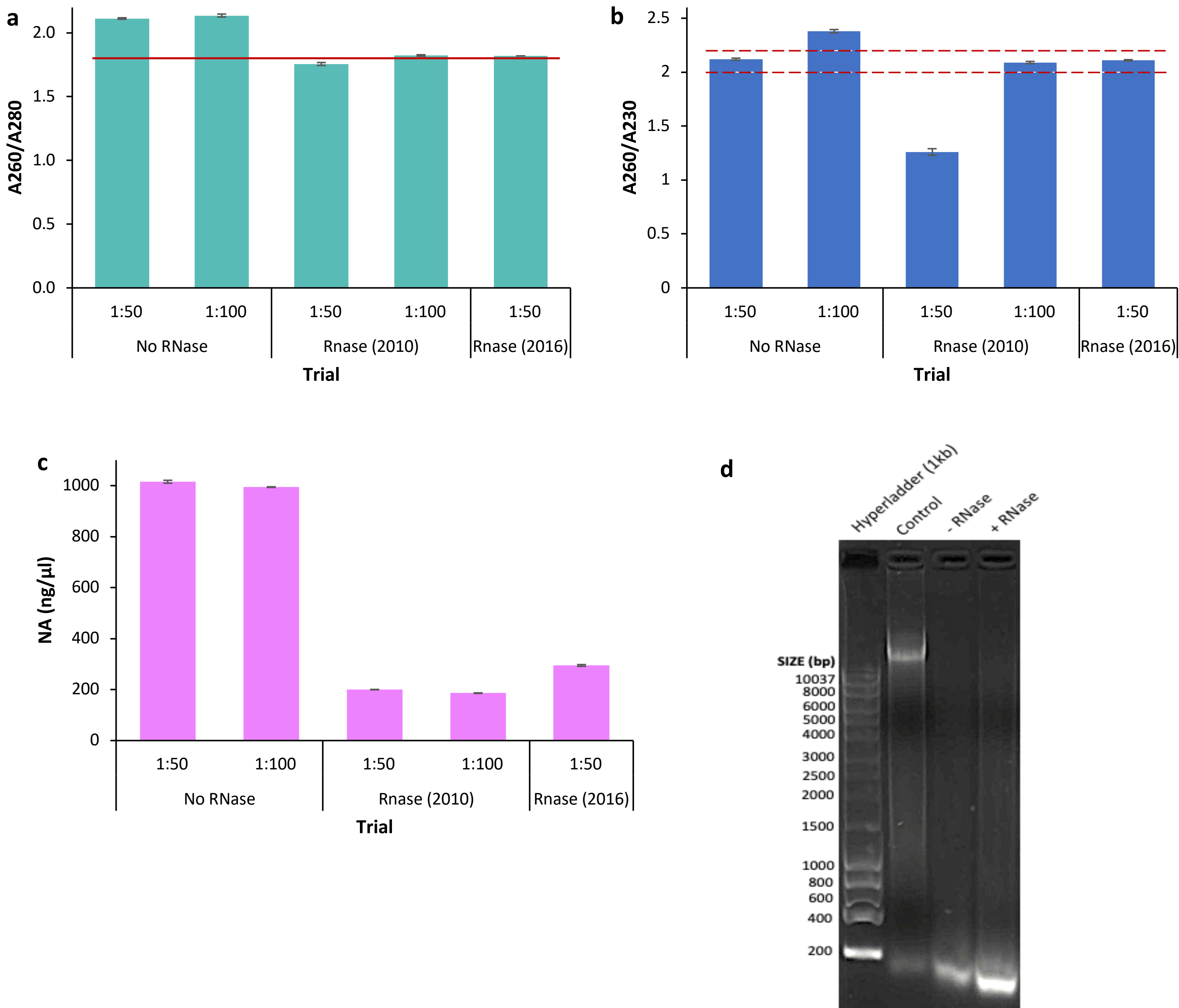


Figure 3.1.3. DNA quality assessments for DNA extraction trials using multi-species microbiota sample 18-261 processed with and without RNase. **(a)** Nanodrop™ A260/A280 readings. Solid line represents the optimum A260/A280 value for pure DNA. **(b)** Nanodrop™ A260/A230 readings. Dotted lines represent the expected A260/A280 range for pure DNA. **(c)** Nanodrop™ nucleic acid (NA) concentration readings. Given are mean ± SD. **(d)** Agarose gel image of electrophoresed samples prepared from bacterial suspensions diluted 1:50, with or without RNase (2016). Control sample is a high-quality genomic extract of *Streptococcus pyogenes* DNA previously used for PCR and sequencing, used for comparison.

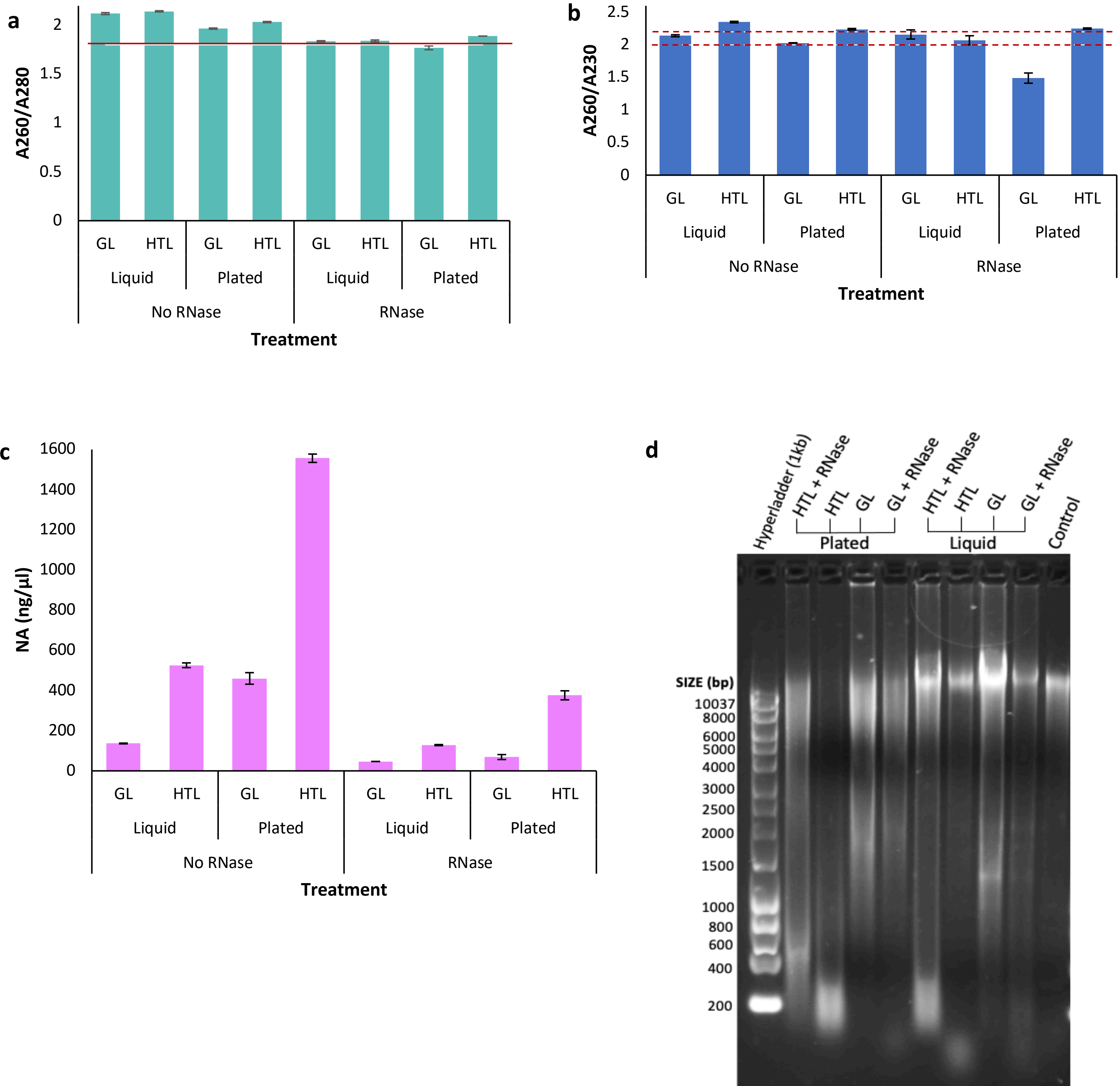


Figure 3.1.4. DNA quality assessments for DNA extraction trials using multi-species microbiota sample 18-257 grown in plates or liquid culture, processed with and without RNase, and with 'hard to lyse' (HTL) or GL buffer. **(a)** Nucleic Acid (NA) concentration. **(b)** A260/A280 ratio. Given are mean ± SD. Solid line represents the optimum A260/A280 value for pure DNA. **(c)** A260/A230 ratio. Dotted lines represent the expected A260/A280 range for pure DNA. **(d)** Agarose gel image of electrophoresed samples prepared from bacterial suspensions diluted 1:50, with or without RNase (2016). Control sample is a high-quality genomic extract of *Streptococcus pyogenes* DNA previously used for PCR and sequencing, used for comparison.

3.2 Microbiome and resistome diversity and ontogenetic investigations

Sixty samples, from chicks ranging from 1–56 days old, were subjected to metagenomic analysis. Across samples, 328 bacterial and viral species were detected and collectively accounted for 97.0–99.6% of the relative abundance of sequence reads in each sample. The remaining 22,366 species, including eukaryotes and archaea, were never detected above the minimum threshold. Of the 328 species identified, 62% (203) were bacterial taxa, and 38% (125) were viral taxa, however bacteria accounted for 75.1-99.9% of sequence reads, with an average relative abundance of 96% (Fig 3.2.1a).

Viral taxa were present in at least one sample in all chicks but made up a minority of sequence reads (Fig 3.2.1a). Of the viral taxa, 98% (123/125) belonged to the Caudovirales order, a group of tailed bacteriophages (Ackermann 1998). More than 60% (77/125) of viral taxa were phages of *Escherichia*, 13% (16/125) were phages of *Salmonella* and another 14% (18/125) were Enterobacteriaceae phages. The viral diversity observed in the dataset was therefore reflective of bacterial host diversity across samples.

3.2.1 Bacterial species composition

A total of 203 bacterial species from 36 genera were identified in the metagenomic dataset. As expected from our selective culturing methods, 98% (199/203) of bacterial taxa detected belonged to the Proteobacteria phyla and of these, 99% (197/199) were of the class Gammaproteobacteria. Within the Gammaproteobacteria class, the orders Enterobacterales and Pseudomonadales constituted 57% (113/199) and 41% (82/199) of taxa, respectively. The most speciose family was Enterobacteriaceae of the order Enterobacterales, which accounted for 39% (80/203) of all taxa. At the genera level, *Pseudomonas*, order Pseudomonadales, was the richest genera, constituting 41% (82/203) of all bacterial species detected, however 29% (24/82) of these were taxa of unknown species. Numerous genera of the order Enterobacterales were also species rich, including *Enterobacter* and *Citrobacter*, which constituted 12% (25/203) and 10% (21/203) of species, respectively. Approximately half (11/25 & 11/21) of species in these two genera were of unknown identity. The Enterobacterales genera *Serratia*

(11/203), *Klebsiella* (9/203) and *Escherichia* (5/203) constituted 5%, 4% and 2% of bacterial taxa, respectively, the majority of which were unknown species.

Relative abundance was used to examine the contribution of bacterial families and genera to the proportion of sequence reads within samples. Enterobacteriaceae was the most abundant family in all but two samples (Fig 3.2.1b). Across all samples, the remaining sequence reads were primarily composed of Hafniaceae, Pseudomonadaceae, Morganellaceae and Yersiniaceae (Fig 3.2.1b). *Escherichia* was the most abundant genus in 82% (49/60) of samples, and at least one sample from all chicks was dominated by *Escherichia* (Fig 3.2.1c), contributing up to 97.7% of sequence reads. However, in seven chicks, the dominant genera changed between samples. Three samples from different chicks were dominated by *Hafnia*, and one sample was dominated by *Salmonella*, three by *Klebsiella,* including two from the same chick, and four by *Enterobacter*, also with two from the same chick. These genera, as well as *Serratia*, *Proteus*, and *Shigella*, were common across samples (Fig 3.2.1c).

Additionally, *Salmonella* was identified in all samples, contrary to detection by traditional methods. Four *Salmonella* species were detected including *S. enterica*, which was detected in all 60 samples and constituted the majority of *Salmonella* sequence reads. Salmonella classifications developed in the original design were therefore not included in these analyses.

Despite *Pseudomonas* constituting the most speciose genera, 88% (72/82) of *Pseudomonas* species were found only in samples 18-19-T1 and 19-14-T1 (Appendix 6.8). These two samples also contained the highest relative abundances of *Pseudomonas*, which is clearly complemented by the high within-sample species richness (Fig 3.2.1c). However, the high species richness of this genera was novel, and other genera highly abundant within samples did not exhibit the same inflation in species richness (Fig 3.2.2b). For example, other dominant genera all contained less than 10 species: *Escherichia* (5), *Salmonella* (4), *Klebsiella* (9) and *Hafnia* (3). Additionally, the PCoA analysis showed that these two samples were the largest outliers, exhibiting the greatest dissimilarity from other samples in the ordination (Appendix 6.9). Therefore, to correct for what is likely overestimation in *Pseudomonas* species, as a result of low-resolution in the ResFinder database, 72 species found only in samples 18-19-T1 and 18-14-T1 were removed from the dataset. Further analyses were conducted with this corrected dataset, however, the removal of these two samples did not change the outcome of the statistical analyses. After this correction, the total

number of species was reduced to 131, with 95% (125/131) of the class Gammaproteobacteria and 86% (113/131) of the order Enterobacterales.

Of the 131 bacterial species identified in this study, 34% (45/131) were identified as pathogenic and 87% (114/131) of taxa were of a genera that contain pathogenic species, based on a previously published list of bacterial pathogens (Li et al. 2015). Of the 36 genera detected in these samples, 21 had representatives on this pathogen list. All pathogen species were Gammaproteobacteria, primarily of the Enterobacterales order (41/45), with a few species of Pseudomonadales (3/45) and a single species of the Xanthomonadales order. Additionally, 12 of the pathogenic genera identified were of the 21 genera on the WHO Priority List of Antibiotic Resistant Bacteria, including *Klebsiella*, *Citrobacter*, *Escherichia*, *Enterobacter*, *Serratia*, *Pseudomonas, Proteus*, *Salmonella*, *Shigella*, *Enterococcus*, *Morganella* and *Providencia* (Tacconelli et al. 2018).

The number of bacterial taxa within a given sample ranged between 7 and 55 species, from 5 to 18 genera, and such that the sample with the highest number of species (19-11-T4) represented 42% (55/131) of the total diversity seen across all samples. Within a given sample, the number of pathogenic species ranged from 4 to 24, and the sample with the highest number of pathogens (18-06-T1) contained over half (24/45) of the total number of pathogenic species. Additionally, there were numerous rare species and genera found in a single sample, including the genera *Pluribacter* (species *gergoviae*) and *Kluyvera* (species *intermedia*) (Fig 3.2.3). Seven unknown species of *Citrobacter*, five unknown species of Enterobacter and two species of *Lelliottia*, including species *nimipressuralis* and one unknown species, were also unique to a single sample.

a

Proportion of Taxa

Sample

Kingdom: k__Bacteria, k__Viruses

b

Proportion of Taxa

Sample

Family: f__Aeromonadaceae, f__Alcaligenaceae, f__Burkholderiaceae, f__Chthonomonadaceae, f__Enterobacteriaceae, f__Enterococcaceae, f__Erwiniaceae, f__Hafniaceae, f__Morganellaceae, f__Pectobacteriaceae, f__Pseudomonadaceae, f__Streptomycetaceae, f__Tissierellaceae, f__Xanthomonadaceae, f__Yersiniaceae

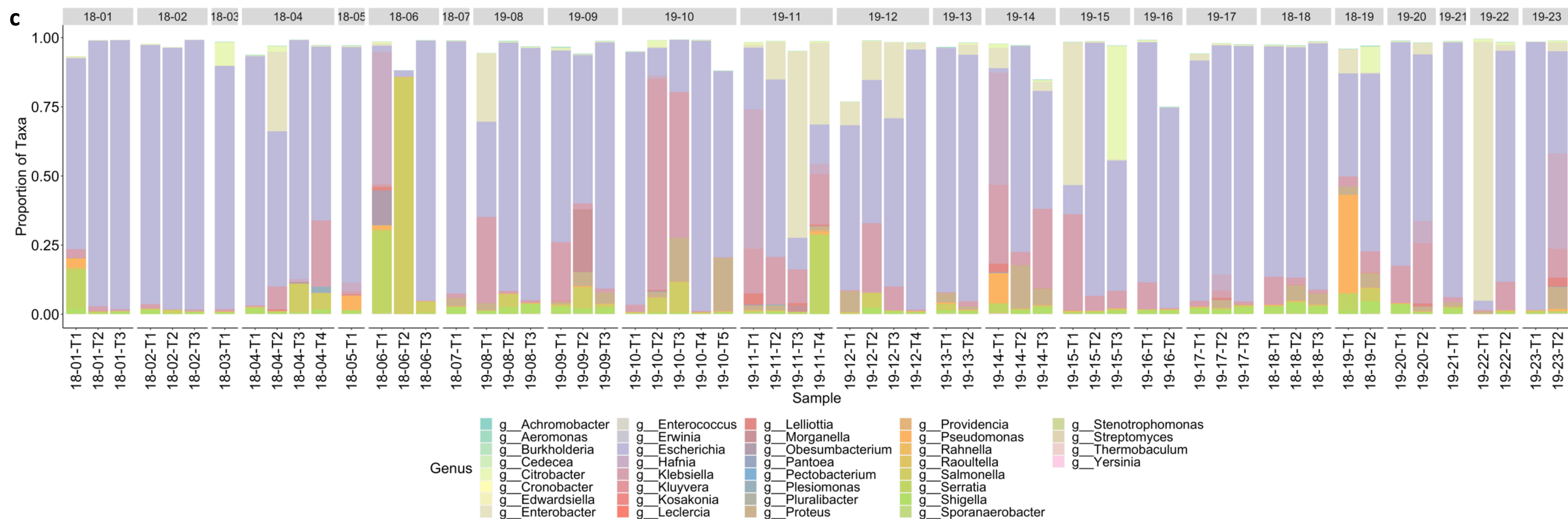


Figure 3.2.1. Relative abundance of bacterial taxa of **(a)** bacterial kingdoms; **(b)** bacterial families; **(c)** bacterial genera from longitudinal multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are arranged in sequential order by chick.

Most samples exhibited similar patterns of both family and genera richness (Fig 3.2.2a-b). Enterobacteriaceae was the most speciose family and contributed a diverse range of speciose genera across all samples (Fig 3.2.2a; Fig 3.2.2b). Multiple bacterial genera exhibited similar species richness across samples, such as *Achromobacter*, *Klebsiella*, *Salmonella* and *Stenotrophomonas* (Fig 3.2.3). For example, 3 to 4 species of *Shigella* were detected in 95% (57/60) of samples (Fig 3.2.3). *Escherichia* was present in all samples, ranging from 2 to 5 species per sample (Fig 3.2.3). Conversely, some genera exhibited large variations in species richness. For example, *Enterobacter* was present in 97% (58/60) of samples, ranging between 1 and 19 species per sample (Fig 3.2.4). Similarly, *Citrobacter* genera were present in 92% (55/60) of samples, with a maximum of 18 species per sample. In contrast, numerous genera of low species richness were present in only two or three samples, such as Enterococcus (Fig 3.2.3). *Serratia* was present in only 12% (7/60) of samples, which contained between one and eleven *Serratia* species. *Pseudomonas* exhibited low species richness across samples, with one to five species in 92% (55/60) of samples (Fig 3.2.2a-b). After removal of excess *Pseudomonas* species, samples 18-19-T1 and 19-14-T1 contained 10 and 7 *Pseudomonas* species, respectively (Fig 3.2.3).

Bacterial composition was analysed using a PCoA, which revealed the dissimilarity of bacterial composition between chicks (Fig 3.2.4). Bacterial composition was loosely clustered by chick (adonis, $R^2 = 0.44$, $p = 0.001$), however, the ordination exhibited large overlaps between samples of different chicks, indicative of the similarities in bacterial species composition between samples (Fig 3.2.2a; Fig 3.2.2b). Outliers of the PCoA were characterised by samples of high species richness and diversity (Fig 3.2.2), including four samples from four different chicks rich in *Pseudomonas* and *Serratia* species (18-14-T1, 18-06-T1, 19-11-T4, 19-19-T1) and one sample rich in *Enterobacter* (18-22-T1). Conversely, the largest outlier, sample 18-06-T2, was the least diverse sample (Fig 3.2.2; Fig 3.2.3) and was dominated by *Salmonella* (Fig 3.2.1).

Across samples, species and genera richness were correlated ($F_{1, 53} = 102$, $p = <0.0001$), such that the addition of more genera also added more species (Fig 3.2.5a), with an average of 3.6 species per genera across all samples. The number of pathogenic species per sample was also positively correlated to species richness and genera richness ($F_{1, 50} = 123$, $p = <0.0001$; $F_{1, 54} = 28$, $p = <0.0001$; Fig 3.2.5b). Moreover, the number of pathogenic genera in samples was positively correlated to genera richness ($F_{1, 53} = 23$, $p = <0.0001$).

Figure 3.2.2. Number of species by **(a)** Family and **(b)** Genera of multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are arranged in sequential order by chick.

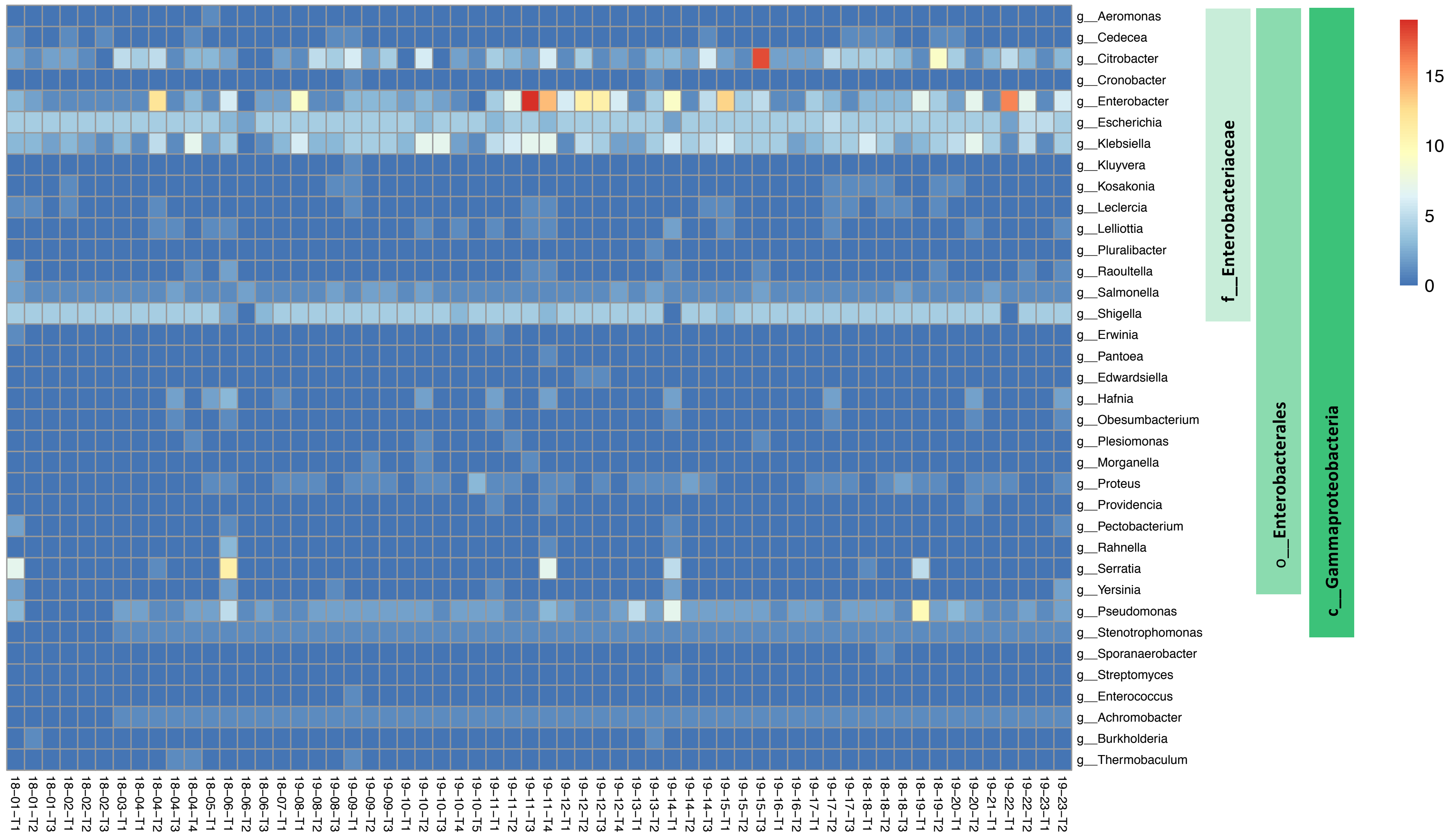


Figure 3.2.3. Heatmap of the number of species by genera of multi-species microbiota samples collected from silver gull chicks a Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are arranged in sequential order by chick, and genera are arranged by bacterial family.

Figure 3.2.4. Principle Coordination Analysis (PCoA) based on Jaccard distance of multi-species microbiota samples from silver gull chicks as dissimilarities in bacterial composition. Longitudinal samples were collected from silver gull chicks at the Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are coloured and grouped by chick.

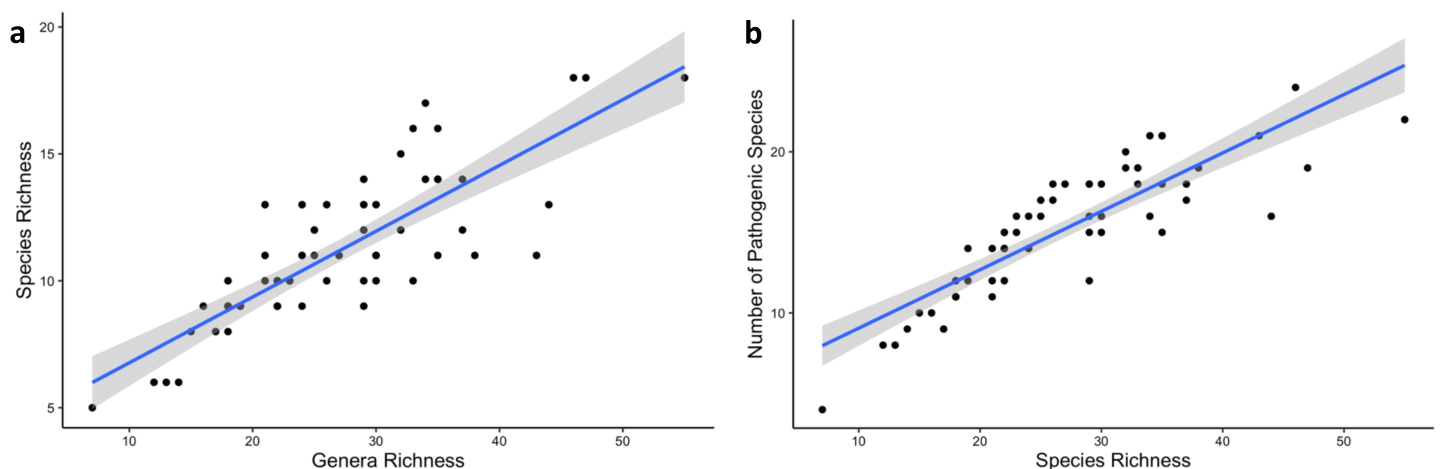


Figure 3.2.5. Correlations between **(a)** species richness by genera richness and **(b)** the number of pathogens by species richness of multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis.

3.2.2 Bacterial dynamics

The richness of species and genera were analysed to investigate ontogenetic changes in bacterial diversity. Species richness within chicks was dynamic, either increasing, decreasing or fluctuating with consecutive samples (Fig 3.2.6) and both species richness and genera richness were not correlated to age ($F_{1, 53} = 5$, $p = 0.025$; $F_{1, 53} = 0.28$, $p = 0.60$). However, there was a slight overall decrease in species richness over time, such that prior to fledging (days 40-56), chicks had on average 24 species across 10 genera, compared to 30 species across 10 genera in the first few days of life (Fig 3.2.6). Additionally, an increase in species richness from the first to the second sample was observed in 7 of 9 hatchlings that were first sampled before the age of 7 days (Fig 3.2.6). Bacterial composition was also not correlated to age (adonis, $R^2 = 0.021$, $p = 0.54$) (Fig 3.2.5).

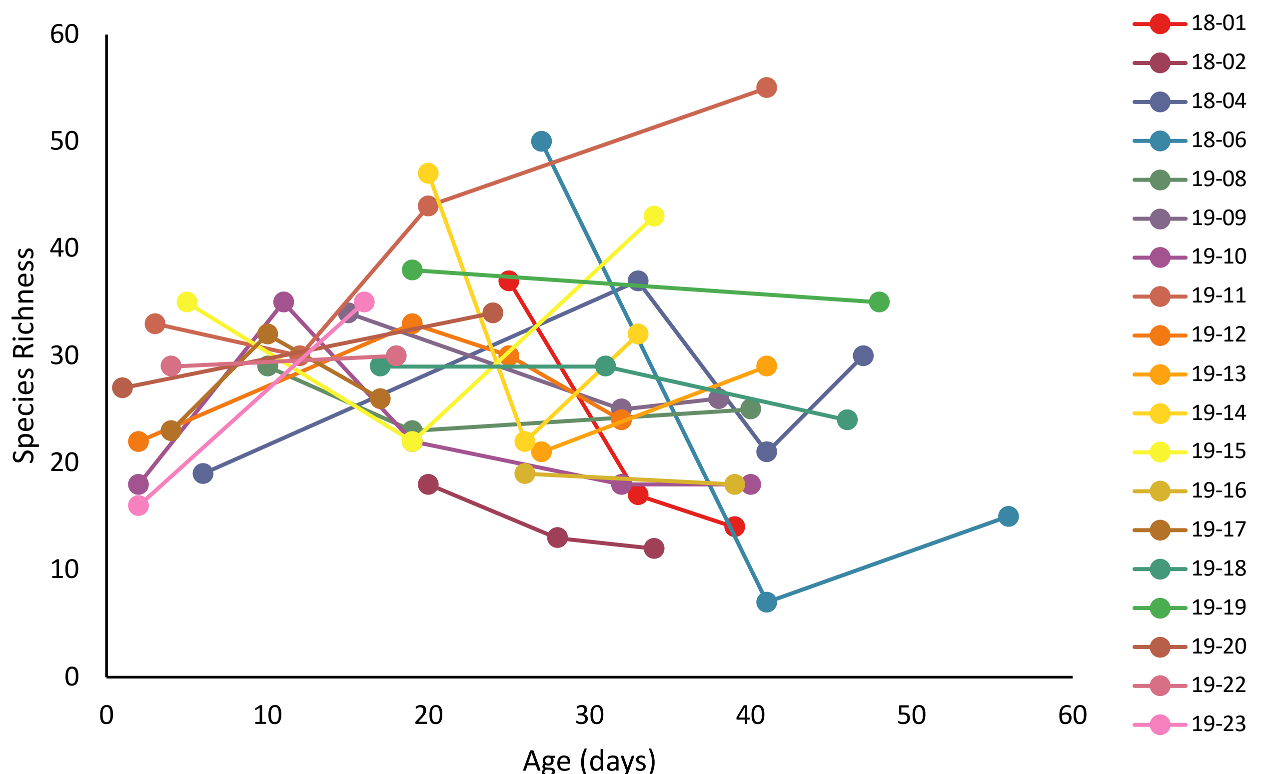


Figure 3.2.6. Species richness by chick age of longitudinal multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are coloured by chick.

3.2.3 Resistome composition

Across all multi-species microbiota samples, 115 acquired ARGs were identified with resistance to 15 antimicrobial phenotypes, including 13 antibiotic classes. The majority of ARGs were Beta-lactam resistance (*bla*) genes, which accounted for 44% (59/115) of genes observed. Aminoglycoside resistance (*aac*/*aad*/*ant*/*aph*) and Quinolone resistance (*qnr/Oxq*) genes also made up a significant proportion of all genes observed, accounting for 10% (12/115) each.

The number of ARGs within a given sample ranged between 2 and 40, with the same ARG detected numerous times in some samples, such that the sample with the highest number of ARGs (19-20-T2) represented 30% (35/115) of all ARG diversity found in the dataset.

Resistance to Beta-lactams and Aminoglycoside were the most prominent phenotypes across samples, which were detected in 90% (54/60) and 87% (52/60) of samples, with up to 11 and 8 ARGs in a single sample, respectively (Fig. 3.2.7; Fig. 3.2.8). Tetracycline and Sulphonamide resistance was also common, present in 87% (52/60) and 77% (46/60), with up to 4 and 3 ARGs per sample, respectively. Quinoline resistance and Fosfomycin resistance genes were less common and present in 63% (38/60) and 57% (34/60) of samples, with up to 6 ARGs per sample. Conversely*, mdf* (board-spectrum resistance) and *sit* (disinfectant resistance) gene types, which comprised a single gene, were present in all samples (Fig. 3.2.8). Other genes prominent across samples included *tet(A)* (48/60), *aph(3")-lb* and *aph(6)-ld* (41/60), *sul2* (40/60), *blaTEM-1B* (31/60), *fosA* (30/60), *OxqA* (29/60), and *sul1* (31/60).

The number of ARGs in gene types varied between samples, with high variances in gene richness of *bla* (Beta-lactam resistance) genes, *fos* (Fosfomycin resistance) genes, and *Oqx* (Quinoline resistance) genes (Fig. 3.2.8). Conversely, numerous resistance phenotypes were novel to a small number of samples, including Rifampicin (*ARR-3* gene) and Lincomycin (*lnu(G)* gene) resistance which were unique to one sample, Lincosamide (*lnu(F)* and *lnu(C)* genes) resistance identified in two samples and Trimethoprim (*dfrA12* and *dfrA17* genes) resistance in three samples (Fig. 3.2.8). Additionally, 43% (55/115) of ARGs were unique to one sample in the dataset, including 33 *bla* (Beta-lactam resistance) genes, 7 *qnr* (Quinolone resistance) genes, 3 Aminoglycoside resistance (*aac* and *aph*) genes, and the *fosA6* (Fosfomycin resistance) and *formA* (disinfectant resistance) genes.

Figure 3.2.7. (*Legend below*)

Figure 3.2.7. (Figure above) Number of ARGs by **(a)** Resistance Phenotype and **(b)** Gene Type of multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are arranged in sequential order by chick.

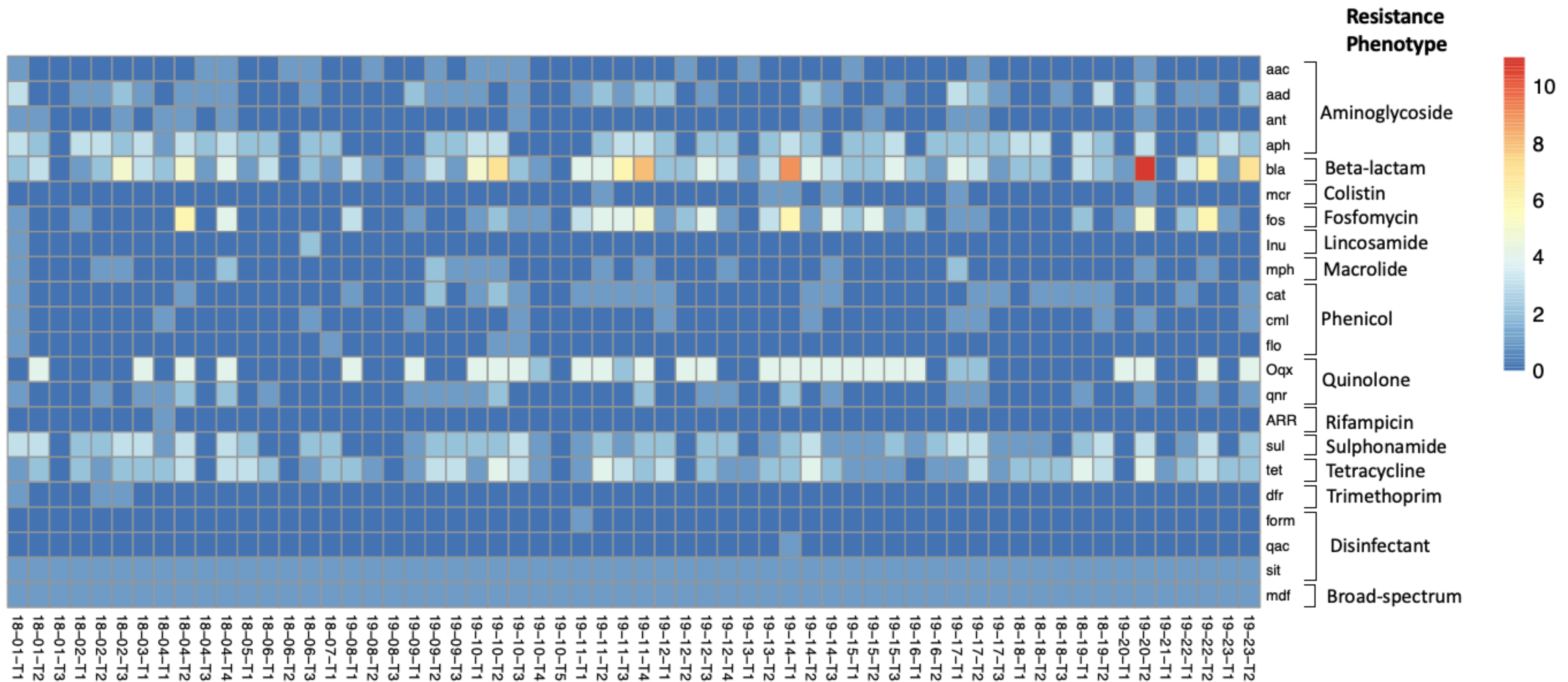


Figure 3.2.8. Heatmap of the number of antimicrobial resistance genes by gene type in multi-species microbiota samples collected from silver gull chicks a Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are arranged in sequential order by chick, and gene types are arranged by phenotypic resistance.

Resistome composition of samples was loosely clustered by chick (adonis, $R^2 = 0.37$, $p = 0.045$; Fig 3.2.9). However, the ordination revealed a large overlap between samples of different chicks, indicative of the high flux of ARGs between samples (Fig 3.2.9). In this ordination, outliers were characterised by low ARG richness (Fig 3.2.7b), and the largest outliers were three samples with identical resistome composition (18-01-T3, 19-08-T3 & 19-10-T5). These three identical resistomes were solely composed of the *mdf(A)* and *sitABC* genes, which were the only two ARGs present in all samples (Fig 3.2.7; Fig. 3.2.8). The remaining outliers contained 3–7 ARGs from 1–3 phenotypic resistance groups of the most common classes (Aminoglycosides, Beta-lactams, Tetracylines and Phenicols) (Fig 3.2.7b; 3.2.8).

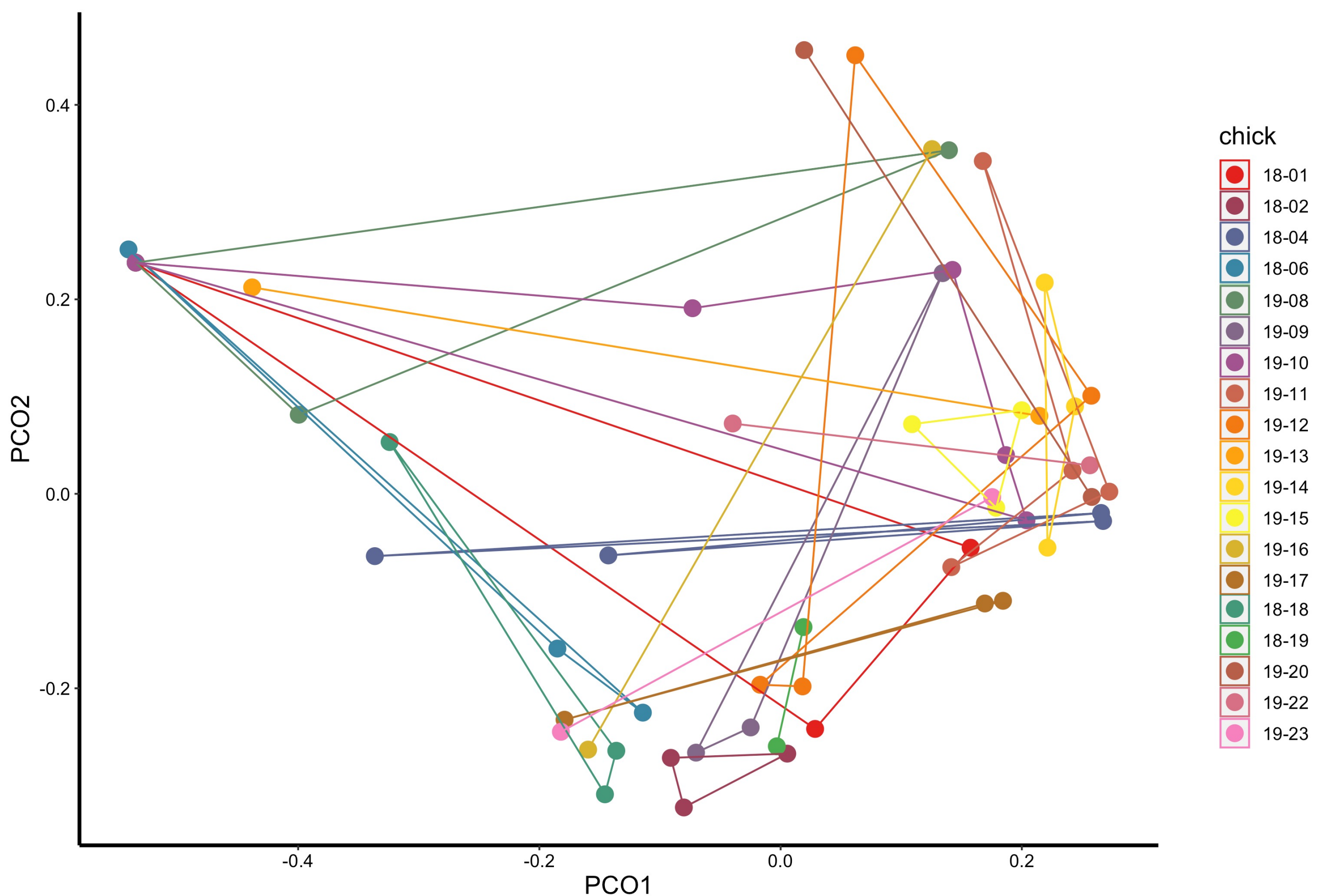


Figure 3.2.9. Principle Coordination Analysis (PCoA) based on Jaccard distance of multi-species microbiota samples from silver gull chicks as dissimilarities in resistome composition. Longitudinal samples were collected from silver gull chicks at the Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are and grouped coloured by chick.

3.2.4 Resistome dynamics

ARG richness was not significantly correlated to age ($F_{1, 53} = 50$, $p = 0.55$), with large fluctuations between samples in most chicks (Fig 3.2.10). While some chicks showed increasing ARG richness over time (19-20, 19-22, 19-23), others showed decreased ARG richness with consecutive samples (18-01, 19-10, 19-17). Additionally, an increase in ARG richness from the first to the second sample was observed in 6 of 9 hatchlings that were first sampled in before the age of seven days (Fig 3.2.10). Resistome composition was also not correlated to age ($R^2 = 0.029$, $p = 0.109$) (Fig 3.2.9).

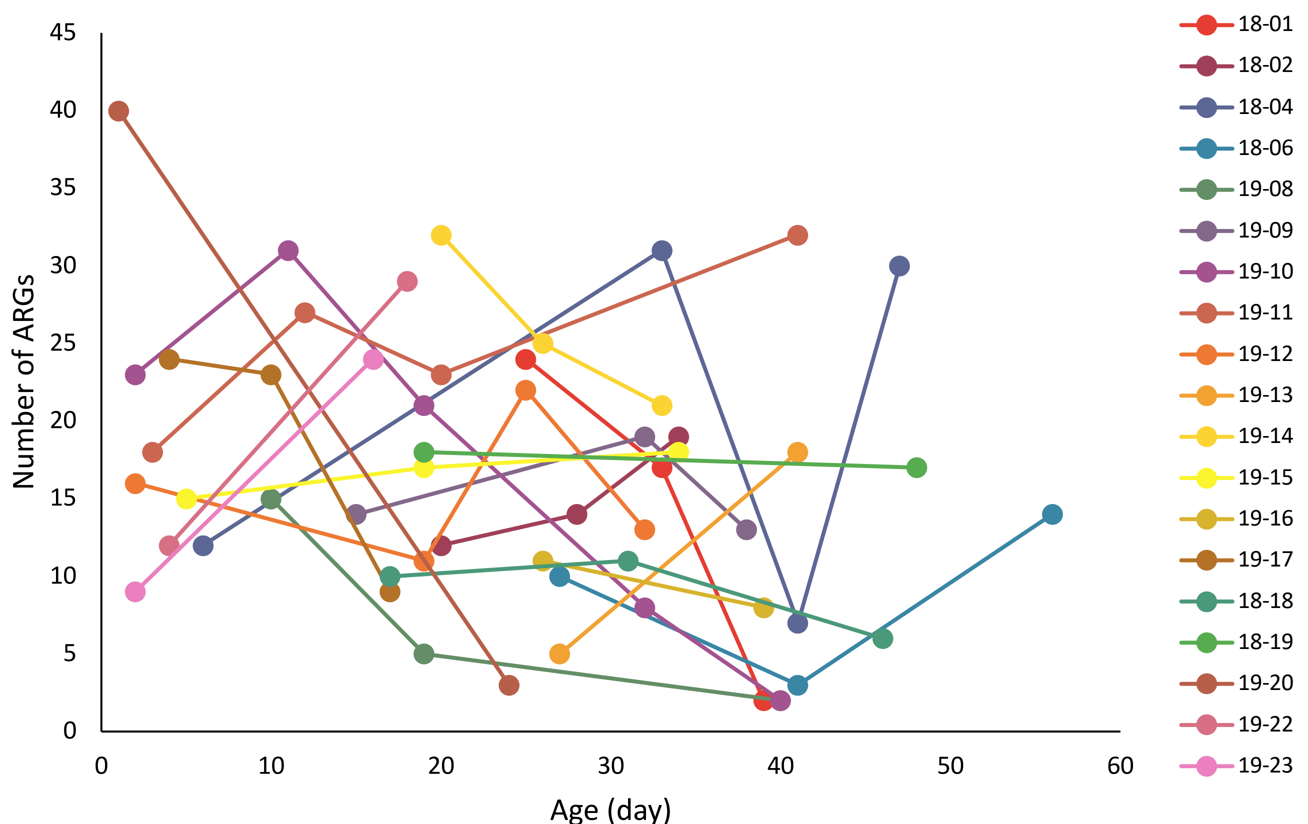


Figure 3.2.10. The number of antimicrobial resistance genes (ARGs) by chick age of longitudinal microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are coloured by chick.

3.2.5 Bacterial-resistome associations

ARG richness was correlated to species and genera richness ($F_{1, 53} = 40$, p = <0.0001; $F_{1, 54} = 7$, p = 0.0054), with most samples of higher species richness also containing higher ARG richness (Fig 3.2.11). For example, sample 19-10 increased in both species richness and ARG richness between the first and second samples, however, both species and ARG richness decreased in subsequent samples (Fig. 3.2.11). This is further supported by the loss and regain of very similar ARG and bacterial species profiles in consecutive samples in chick 18-04 (Fig. 3.2.11; Fig 3.2.7a; Fig 3.2.2). However, some chicks exhibited opposite trends, for example, chicks 18-02 and 18-06, which increased in ARG richness with consecutive samples despite decreasing in bacterial species richness (Fig. 3.2.11).

Additionally, associations between bacterial and resistome compositions were assessed with Procrustes analyses. Bacterial and resistome composition were significantly correlated ($M^2 = 0.91$, p = 0.012), indicating that samples with similar bacterial compositions also had similar resistome compositions (Fig 3.2.12). The majority of samples were clustered by bacterial composition in the Procrustes ordination (Fig 3.2.12), which reflects the clustering of the majority of samples also exhibited in the bacterial composition PCoA (Fig 3.2.5). Additionally, the Procrustes ordination exhibited two distinct clusters of bacterial-resistome correlations (Fig 3.2.12), indicating that two groups of samples with similar bacterial compositions also exhibited similar resistome compositions. Procrustes were also conducted by age, for nine samples after hatching and before fledging, to assess bacterial-resistome correlations change with time. However, no association was found in the first six days after hatching ($M^2 = 0.91$, p = 0.87) or the days prior to fledging ($M^2 = 0.69$, p = 0.11) (Appendix 6.10).

Pairwise spearman's correlation between bacterial and resistome compositions for all samples were then conducted to assess specific bacterial-ARG associations (Fig 3.2.13). The strongest correlations were between species and ARGs found in a single sample (Fig 3.2.13). These included associations with four *bla* genes which were each correlated with numerous bacterial species, despite being found only in a single sample, thus reflecting the broad associations derived for these rare ARGs. However, as these ARGs are found only in a single sample, it is unlikely that they are correlated with numerous bacteria in that sample. Other significant correlations were also based also on co-occurrence between a small number

of samples. For example, *Klebsiella variicola* was found in 47 samples, and was correlated with *tetG* and *blaSHV-132*, despite these genes only occurring in two samples. In contrast, *Enterobacter sp.ODB01* co-occurred with *blaACT-9* in 8 out of the 9 samples they were each found in. Additionally, all *blaACC-4* genes, found in 7 samples, co-occurred with *Hafnia paralvei*, which was found in a total of 11 samples. However, as the majority of these correlations reflect the co-occurrence of bacterial species and ARGs in less than four samples, it is unlikely that they reflect the bacterial-resistome compositional associations identified in the Procrustes.

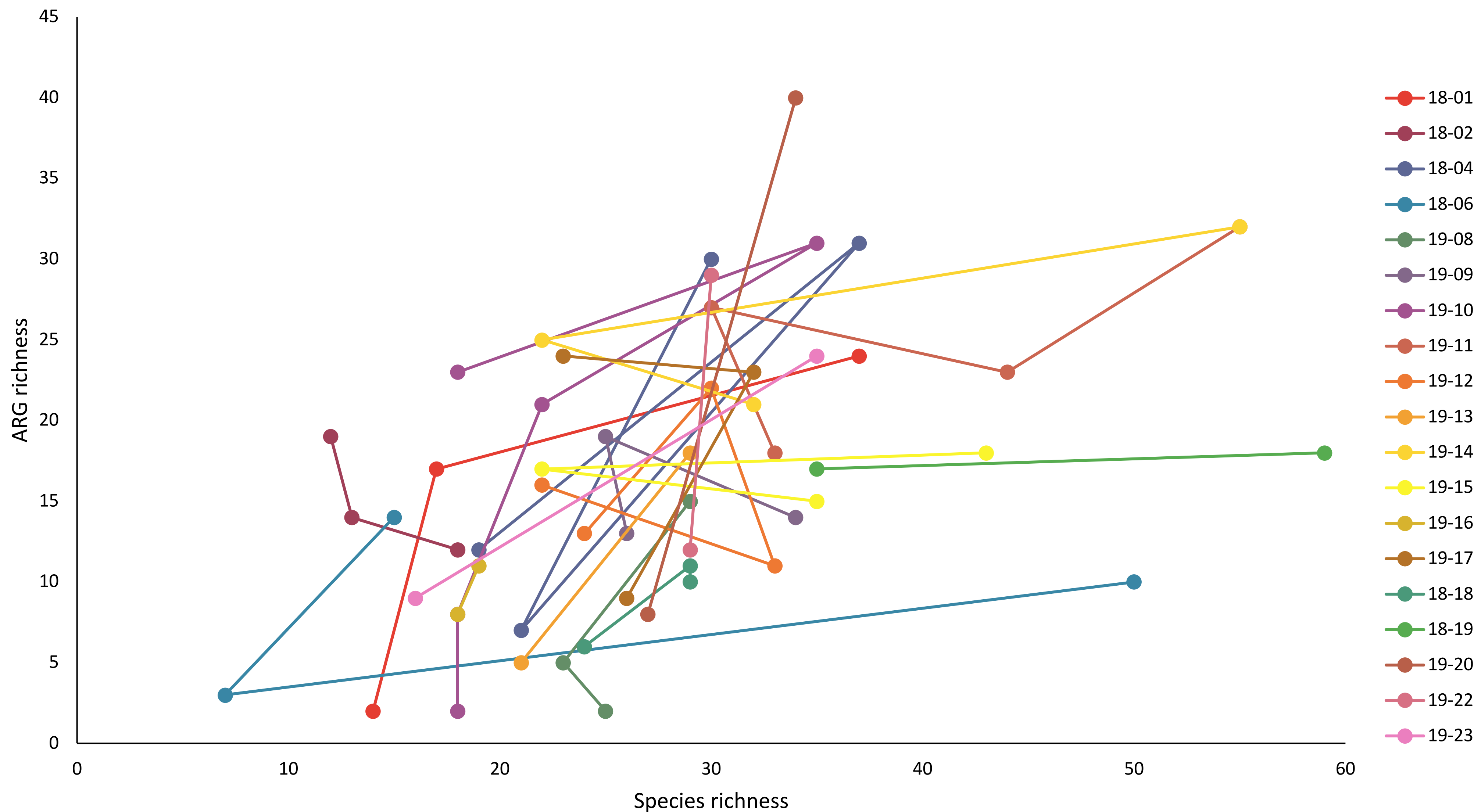


Figure 3.2.11. Correlations between species richness and the number of antimicrobial resistance genes (ARGs) in multi species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Samples are coloured by chick.

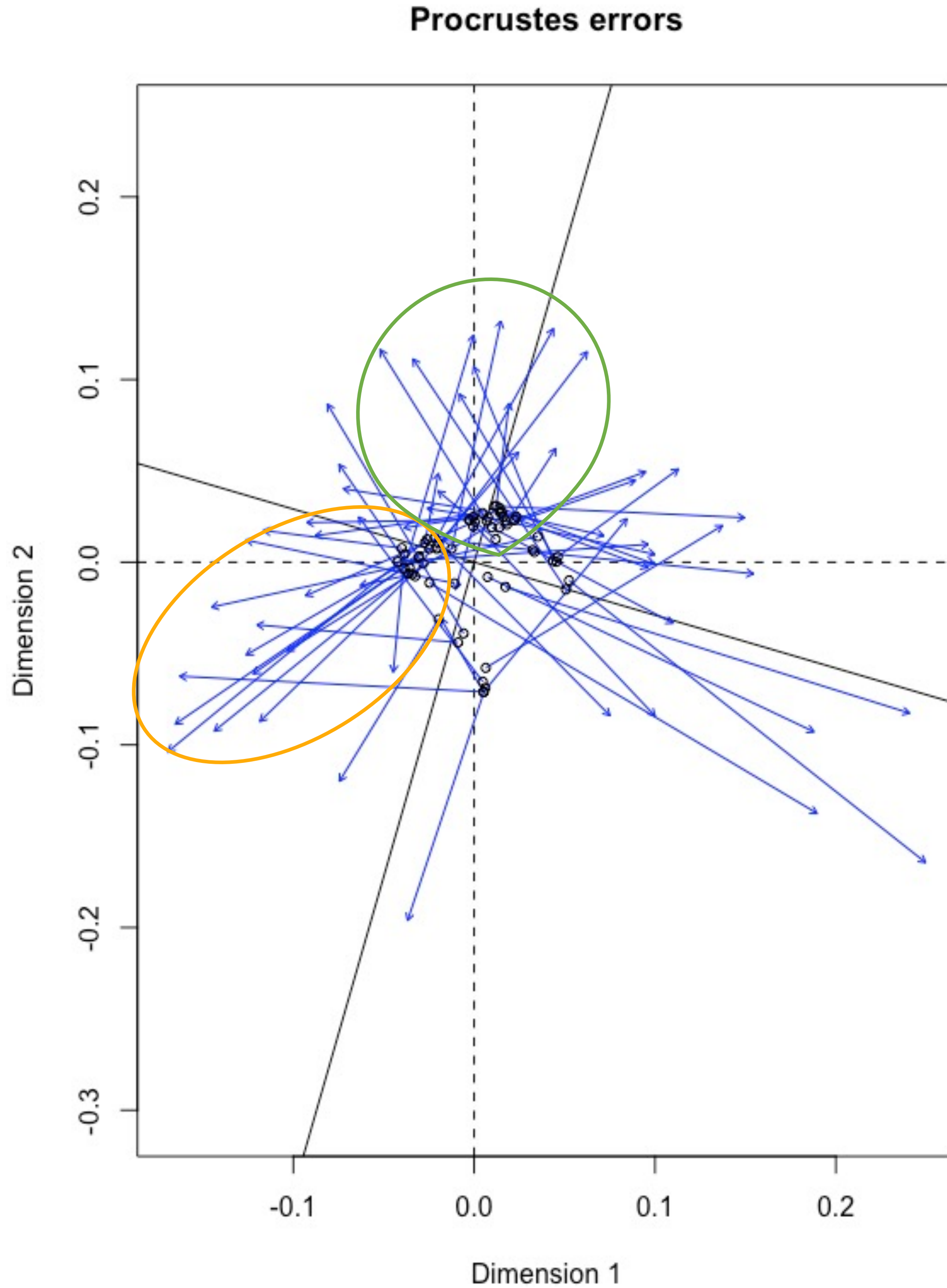


Figure 3.2.12. Procrustes correlation between bacterial and resistome composition of multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Circles represent samples in the bacterial composition ordination, and arrows represent the correlation of samples to their corresponding resistome composition. Green and orange outlines show clusters of bacterial/resistome correlations.

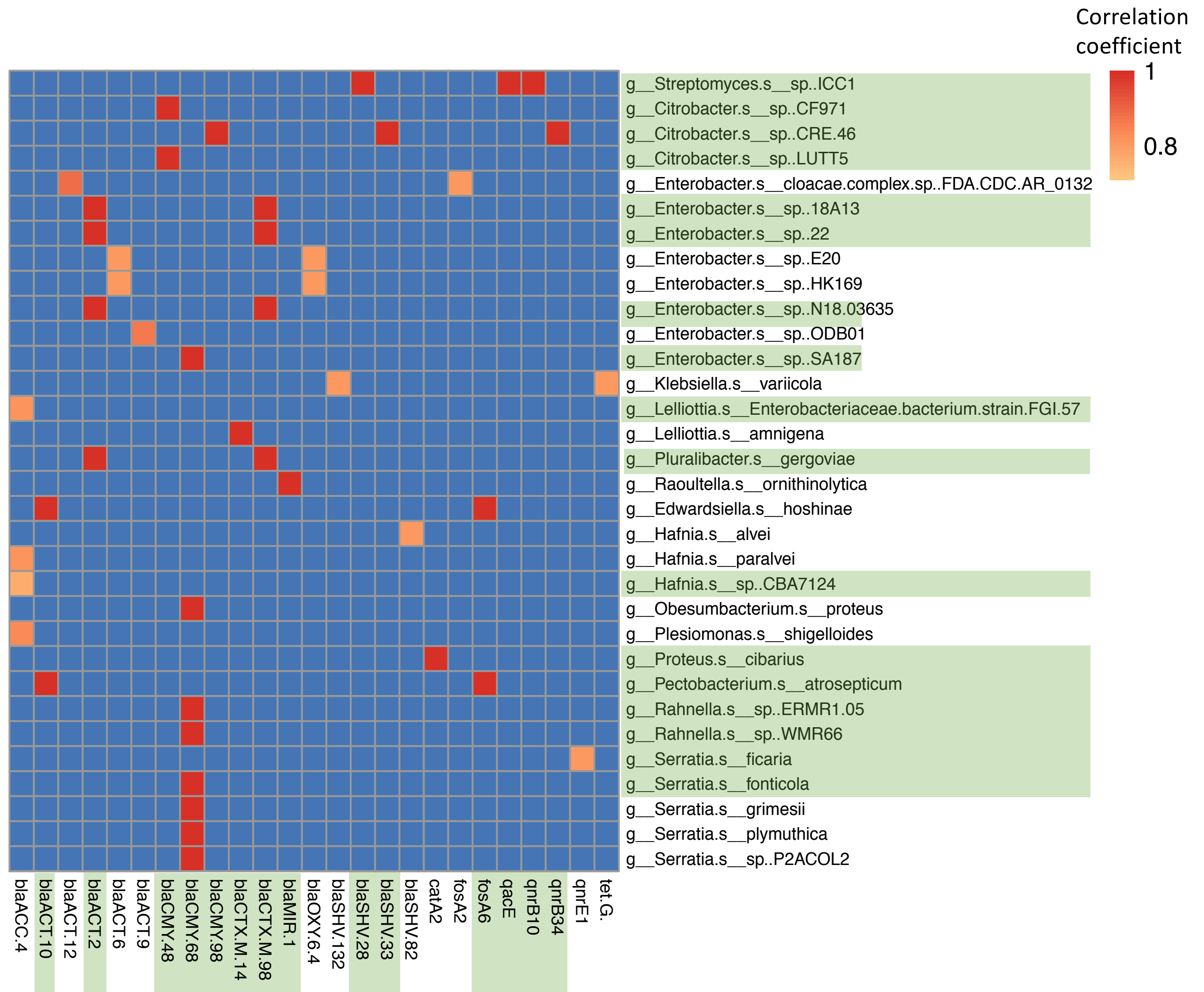


Fig 3.2.13. Spearman's correlations (ρ = 0.75–1, $p < 0.05$) between bacterial species and antimicrobial resistance genes (ARGs) in multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, which were corrected for low-resolution *Pseudomonas* species after bioinformatic analysis. Species and ARGs highlighted in green were found in a single sample across the dataset.

4 DISCUSSION

Resistance to antimicrobials has proliferated in recent decades, and the reduced efficacy of antibiotic therapeutics to treat clinical bacterial infections is indicative of an impending public health crisis (Spellberg et al. 2013). The evolution of AMR-bacteria is predominantly underpinned by the horizontal transfer of ARGs and their accumulation on mobile genetic elements, which allows AMR to be propagated between bacterial species, thus promoting the proliferation of AMR (Davies & Davies 2010; Nikaido 2009). To better understand the pathways of AMR propagation and transmission requires a One Health approach, looking beyond the human and human-associated spheres, including the role of wildlife (Djordjevic & Morgan 2019). There is a growing body of evidence to suggest that avian species, especially gulls, host a large amount of AMR and multi-drug resistance bacteria (Arnold et al. 2016; Bonnedahl & Järhult 2014; Thompson 2019). However, speculations on the function of avian hosts as reservoirs with the capability to disseminate AMR rely on cross-sectional observations of AMR-associated mobile genetic elements from a small number of bacterial species (Dolejská & Papagiannitsis 2018; Mukerji et al. 2020; Mukerji et al. 2019). A longitudinal metagenomic investigation of silver gull chicks was, therefore, conducted to investigate the role of avian wildlife in the evolution of AMR-bacteria. This thesis firstly aimed to optimise the DNA extraction of multi-species microbiota from silver gull chick samples for metagenomic analysis. The metagenomic results were then utilised to examine bacterial and resistome composition and diversity to assess the ability of silver gulls to host AMR. Finally, ontogenetic shifts were examined to investigate whether AMR could be accumulated and maintained in the gut microbiome of silver gull chicks.

4.1 Optimisation of DNA extractions

The first aim of this thesis was to optimise the DNA extraction process for multi-species microbiota samples from silver gull chicks for metagenomic analysis. After trialling a number of treatments, the ISOLATE II Genomic DNA Bacteria protocol (Bioline) was successfully optimised to extract DNA from multi-species microbiota samples. As the multi-species microbiota samples varied in concentration and composition, a major challenge was to culture enough bacteria to grow a good representative sample while not overwhelming the kit with too

much starting material. Overall, the findings of this work confirmed the use of a 'hard to lyse' buffer supplemented with lysozyme, RNase to reduce RNA contamination, and cultures grown in liquid LB to produce a lower concentration of the sample for extraction. Additionally, altering the concentration of resuspensions for liquid culture was crucial for glycerol stocks of multi-species microbiota samples with high or low concentrations. This protocol was then used to successfully extract 54 DNA samples for metagenomic analysis.

4.2 Metagenomic investigations of silver gull chick microbiomes

While there is good evidence to suggest that avian wildlife, particularly gulls, can act as bioindicators of environmental AMR contaminants from humans (Atterby et al. 2017; Thompson 2019), it is necessary to discern whether AMR can be accumulated and maintained within avian hosts in order to determine whether they have the ability to then transmit AMR to other individuals and species. The current study, which formed a longitudinal metagenomic analysis, therefore, aimed to describe the diversity of bacteria and ARGs in a subset of Proteobacteria from the gut microbiome of silver gull chicks to understand their ability to host AMR. Ontogenetic shifts in bacterial and resistome composition were also examined to determine whether AMR may be accumulated and maintained. This investigation was conducted to explicate whether silver gull chicks may play a role in the proliferation of AMR.

4.2.1 Bacterial and resistome composition and diversity

Microbiome diversity, including bacterial and AMR carriage, was examined to understand the ability of silver gull microbiomes to host AMR. When sub-cultured for Enterobacteriaceae, a diverse bacterial community of 30 genera and 125 species of Gammaproteobacteria, including 14 Enterobacteriaceae genera, were identified in 60 gut microbiota samples from 23 silver gull chicks. Comparatively, in a metagenomic analysis of 10 waterbird species, Cao et al. (2020) found that the total genera richness of avian microbiomes (including in birds of the orders Charadriiformes, Anseriformes, Pelecaniformes, Gruiformes) varied greatly between species, averaging ~35 genera across all bird species, and with a maximum of ~90 genera within a single host species. The high diversity of microbiota from just one class described in the current study therefore indicates that silver gull microbiomes contain a high diversity of Enterobacteriaceae and other

Gammaproteobacteria, however, the portion of the total microbiome that these constitute remains unknown. As the bacteria identified in this study are only a subset of the silver gull gut microbiome, the diverse cohort of Enterobacteriaceae may further be reflective of greater diversity in the Gammaproteobacterial class, and likewise a highly diverse community of Proteobacteria. Proteobacteria have been previously identified as the most phylogenetically diverse phyla in avian gut microbiomes, even when constituting a minor portion of the microbiota (Teyssier et al. 2018). Moreover, the average proportion of Proteobacteria in avian microbiomes is much higher than in mammals (Song et al. 2020), and Proteobacteria, predominantly Gammaproteobacteria, can dominate the gut microbiota in individuals of multiple bird species (Cao et al. 2020; Hammer et al. 2019; Song et al. 2020). The high diversity of Gammaproteobacteria exhibited in the current thesis therefore supports previous studies which found that avian microbiomes host a higher proportion of Proteobacteria than other host species, such as humans.

Given that the initial sample selection was based on the presence or absence of *Salmonella* infection in chicks throughout the sampling period, the detection of *Salmonella* species in all 60 samples examined here was unexpected. These original experimental design categories were therefore not biologically relevant variables for statistical analyses in the current study. Further investigations are therefore needed to determine why *Salmonella* was not identified in all samples through the culture-based phenotypic characterisation methods initially used. Future studies may need to utilise other methods, such as PCR based screening, to identify *Salmonella* in these multi-species microbiota samples.

The highly diverse Gammaproteobacterial community identified within silver gull chick microbiomes in the current study also hosted a high number of pathogenic and AMR-associated genera. The number of pathogenic species was much higher than in a previous investigation using the same published list of pathogens, which identified 59 opportunistic pathogens across 10 migratory waterbird species with a maximum of 35 pathogens in a single avian species (Li et al. 2015). Comparatively, in the current study, 45 pathogenic species of Gammaproteobacteria were identified within a single host species and with a maximum of 24 pathogens in a single sample. Moreover, all speciose genera identified in the current study were of pathogenic and AMR relevance, the majority of which were Enterobacteriaceae taxa. *Pseudomonas*, which contains multiple pathogenic species, was originally identified as the most speciose genera, however 92% of these species were from two samples with high

abundances of *Pseudomonas*, which likely caused an inflation in the number of species identified, indicative of the low-resolution of the genera in the bioinformatic analysis. After *Pseudomonas* was adjusted by the removal of 76 species found only in these two samples, the corrected datasets had little effect on the results and did not change the significance of statistical analyses, with *Pseudomonas* genera remaining the second most speciose genera. However, as *Pseudomonas* and other speciose genera contained a large proportion of unknown species further analysis should be undertaken to investigate the unknown species identities. *Escherichia*, predominantly *E. coli*, dominated relative abundance in most samples and was also one of the most speciose genera in the analyses across all samples. Correspondingly, *E. coli* has also been previously identified as the most prevalent pathogenic bacteria in multiple avian species (Cao et al. 2020). The current work also observed a high abundance and species count of *Klebsiella* across most samples, including the clinically relevant *K. pneumoniae*, which was present in all chicks in 98% (59/60) of samples. Similarly, previous studies have identified *E. coli* and *K. pneumoniae* as two important species harbouring and exchanging mobile ARGs (Hu et al. 2016). Additionally, Hu et al. (2016) found that these two species shared the highest number of ARGs, based on sequence similarities, which exemplifies the ability of phylogenetically related pathogens of different genera to exchange AMR. The findings of the current thesis therefore emphasise the ability of silver gull microbiomes to host a large number of bacteria capable of carrying and exchanging AMR within the diverse community of Gammaproteobacteria in silver gull chicks.

Many of the Enterobacteriaceae species identified in this study are also associated with multi-drug resistance. For example, a previous study, on the same silver gull population, identified carbapenemase-producing *S. enterica*, *E. coli*, *Escherichia fergusonii*, *K. pneumoniae, Enterobacter cloacae* and *Proteus mirabilis* and *Citrobacter freundii* (Dolejská et al. 2016), and these bacterial species were identified in 92-100% of samples in the current study. Dolejská et al. (2016) found that more than half of the carbapenemase-producing isolates were able to be conjugated between bacteria and were associated with plasmids, thereby reflecting their ability to spread and potentially proliferate AMR. The Enterobacteriaceae species found in the current study are also associated with extended spectrum beta-lactamases (Guenther et al. 2011), which have been previously identified in avian species on every populated continent (Bonnedahl & Järhult 2014), including numerous Laridae species (Atterby et al. 2017; Bonnedahl et al. 2009; Hernández et al. 2013; Wang et al. 2017). The

current thesis therefore supports the idea that avian microbiomes, particularly gulls, have the ability to host a high prevalence of clinically relevant MDR bacteria. Moreover, the Enterobacteriaceae species identified in the current study correspond to those commonly identified in clinical settings in Australia, especially *Escherichia coli*, *Klebsiella* and *Enterobacter* which are considered AMR priority species (MedicineWise 2016). The bacterial species identified in the current study may therefore be indicative of environmental contamination of AMR (Dolejská et al. 2016; Thompson 2019). This suggests that the silver gull chick microbiome has the ability to be colonised by AMR-associated Proteobacteria from the environment (Song et al. 2020), however future studies should conduct comparative genomic analyses to confirm these links to clinical and environmental bacteria.

Identification of a large number of pathogenic and AMR-associated bacteria also confirms the utility of a subculturing step to examine the Gammaproteobacterial sub-population of the microbiome in order to investigate AMR interactions. However, due to this subculturing step, statistical analyses in this thesis were limited to presence-absence datasets. Relative abundance was examined to understand the contribution of bacterial taxa to the dataset, but not as a reflection of the conditions in the gut microbial community of silver gull chicks. The MacConkey subculture utilised in the current study promotes the growth of aerobic gram-negative enteric bacteria, specifically Enterobacteriaceae (Elazhary et al. 1973), which was the target taxa for this study as it encompasses many pathogenic and AMR-associated species (Hu et al. 2016). Consequently, this selection step also inhibits the growth of non-culturable and non-aerobic bacteria (Elazhary et al. 1973), which thus limits the bacterial interactions that can be observed in the metagenomic analysis. This thesis therefore does not examine bacterial and resistome associations across the entire microbial community and may miss AMR interactions that occur between distantly related bacteria of the gut. Future studies using uncultured metagenomic samples should therefore be utilised to investigate phylogenetically broader community interactions. However, as phylogenetically related species are more likely to engage in interspecies interactions, particularly with reference to AMR (Hu et al. 2016), this subpopulation brings focus to bacterial associations that are more likely to be involved in horizontal gene transfer. Additionally, the high number of bacterial taxa identified in this thesis, including many pathogens and AMR-associated bacteria, indicates that this Gammaproteobacterial subpopulation represents a diverse portion of the

microbiome, which likely encompasses a large portion of bacterial interactions in the gut microbiome.

The number of ARGs identified across silver gull chicks was also much greater than those identified by a previous study using ResFinder, which identified a maximum of 44 ARGs within a single species (Marcelino et al. 2019), compared to the 115 unique ARGs identified in silver gull chicks in the current study. The high AMR carriage of Proteobacteria in silver gull microbial communities is supported by previous findings that the majority of ARG-carrying contigs originated from Proteobacteria in avian microbiome samples (Cao et al. 2020). Additionally, Cao et al. (2020) identified more antibiotic resistance proteins in avian samples than in human samples, despite lower overall bacterial phylogenetic diversity. In the current study, the high diversity of AMR-associated Gammaproteobacteria and samples enriched in ARGs therefore suggests that avian species may host a greater diversity of AMR-associated bacteria than other host taxa. This further supports similar findings of high ARG richness in *Salmonella* from this silver gull population compared to isolates from humans (Thompson 2019).

Beta-lactam, Aminoglycoside, and Quinoline resistance genes were the most common ARGs identified in the current study, and these antibiotics are the most commonly used clinical (ACSQHC 2016) and veterinary (Zlitni et al. 2020) agents in Australia. The AMR phenotypes and AMR-associated Gammaproteobacteria identified in the current study therefore likely reflect environmental contamination of AMR-bacteria (Dolejská et al. 2016; Thompson 2019). These results further support the idea that silver gull chick microbiomes act as bioindicators of AMR contamination from humans, with the ability to host a diverse community of AMR-associated bacteria from the environment (Mukerji et al. 2019).

The diversity of bacterial species in the current study may be explained by the concept of 'Diversity Begets Diversity' (DBD), which theorises that biodiversity spurs the evolution of further diversity due to the generation of novel niches resulting from interspecies interactions (Whittaker 1972). The DBD theory has been applied to the microbiome context by Madi et al. (2020), who assessed species/genera ratios as a proportion of their microbiota communities, using sequencing data from the Earth Microbiome Project. The authors found that DBD is strong in low-diversity microbiomes but is limited in high diversity microbiomes, as is predicted by niche filling theories (Madi et al. 2020). In the current thesis,

species richness was directly correlated to genera richness, such that that samples with more genera also had more species, with an average of 3.6 species per genera across all samples. As higher species richness in samples can be attributed to higher genera richness, this may reflect the DBD phenomena amongst Gammaproteobacteria populations in the silver gull microbiome. Therefore, it is possible that, in the relatively low overall diversity of avian microbiomes, the high diversity of the Proteobacterial phyla may facilitate the establishment of a high diversity of species, including AMR-associated bacteria. The results of the current thesis also suggest that the DBD phenomena may be extrapolated to pathogenic species and ARGs. The number of pathogens and the number of ARGs were also correlated to species and genera richness in the current thesis, indicating that more taxonomically diverse samples contain more pathogens and ARGs. The high phylogenetic diversity in the Proteobacteria phyla may, therefore, beget the diversity of AMR-associated Gammaproteobacterial species, which in turn begets the diversity of ARGs. The current study therefore demonstrates how the composition of avian microbiomes may allow them to host a large diversity of AMR-associated bacteria and subsequently act as bioindicators of environmental contamination of AMR.

4.2.2 Bacterial dynamics

The longitudinal results presented here are the first to examine the dynamic nature of Gammaproteobacterial subpopulations of wildlife microbiomes. Bacterial species richness within individual chicks fluctuated with consecutive samples, such that richness was not correlated to age. These fluctuations were further supported by the bacterial composition PCoA, which illustrated large variations in bacterial species composition within chicks and between samples.

The large variations in Gammaproteobacterial diversity and composition between samples and chicks seen in the current thesis is supported by previous findings that microbiota composition is highly variable between individuals within a species (Cao et al. 2020; Teyssier et al. 2018). Given this highly variable nature of avian microbiomes, it is not surprising that variations would also occur within chicks over time, as found in the current study. These variations may be specific to avian species, which lack resident microbiota and

therefore taxonomic structure within their microbiomes (Song et al. 2020). Moreover, this lack of microbiome specificity in avian species may allow a higher proportion of transient bacteria to colonise the microbiome (Song et al. 2020), which could explain the large proportion of Proteobacteria observed in some avian microbiomes (Cao et al. 2020), and moreover the fluctuations in Gammaproteobacterial species observed in silver gull microbiomes in the current study. The variations and fluctuations in microbiome diversity and composition may therefore be a result of differences in environmental exposure (Song et al. 2020), including through food. As gulls are altricial and born underdeveloped, chicks are reliant on parental food investments in the first month of life before fledging (Royle & Hamer 1998). Moreover, as opportunists, silver gulls utilise a range of feeding habitats, including natural and anthropogenic locations, and habitat preferences differ between individual gulls (Browne 2020). As the food source of chicks differs, varying between natural foods, such as fish, and human refuse (Browne 2020), chicks may be differentially exposed to AMR. Furthermore, this may even change over time for the same chick (Browne 2020). Previous GPS tracking investigations by Browne (2020) did not find differences in habitat usage between adult gulls that had chicks with and without *Salmonella* infection. However, as *Salmonella* was identified in all samples in the current study, this indicates that *Salmonella* infection alone is not indicative of AMR exposure and carriage. Future investigations should therefore utilise metagenomic analysis, such as in the current study, combined with GPS tracking of adult gulls to determine whether the foraging patterns of adults affect the microbiome composition of chicks.

This thesis is the first study to follow host individuals to investigate ontogenetic changes in wild avian microbiomes. The initial increase seen in bacterial species richness in the first few days of life in the current thesis is reflective of the early colonisation of the gut microbiome, which predominantly occurs after hatching when chicks are exposed to the environment (Grond et al. 2017; Kohl 2012). The lack of correlation between age and bacterial diversity in the current thesis is further indicative of the large fluctuations in microbiome diversity. However, the slight decrease in species richness with age across chicks suggests that the Gammaproteobacterial community within the microbiome of younger chicks may be more diverse than older chicks, with the ability to host a higher proportion of transient opportunistic bacteria. This complements a previous study which found that the relative abundance of Proteobacteria was strongly reduced with age, which indicates that young

chicks may host more opportunistic bacteria than older chicks and adults due to the naïve immune system of juveniles (Hennessy et al. 2020).

The results of the current thesis and previous investigations of ontogenetic microbiome development do not, however, examine microbiome changes in gulls into adulthood, so it is unclear how these results reflect the long-term development of the avian microbiome. Teyssier et al. (2018) found that cloacal diversity significantly decreased with age in tit nestlings between the ages of 8 and 15 days old, which was suggested to be a result of the stabilisation of the microbiome (Teyssier et al. 2018). In contrast, Grond et al. (2017) found that bacterial richness was much higher in shorebird adults than in chicks, which would suggest that adult microbiomes are overall more diverse than chicks. This has been demonstrated in mammalian studies, for example in calves, which found that microbiome diversity increases ontogenetically as the juvenile microbiome develops and stabilises as the animal matures (Hennessy et al. 2020). However, a longitudinal study of catfish intestinal microbiomes illustrated that alpha diversity fluctuated between four sampling events in the first 193 days of life, with a slight stabilisation with time (Bledsoe et al. 2016). Moreover, each of these studies investigated microbiome changes by averaging diversity estimates from multiple individuals, and therefore do not account for the large variations seen between individuals, especially avian hosts (Bledsoe et al. 2016; Grond et al. 2017; Teyssier et al. 2018). Future long-term microbiome studies should therefore be conducted to determine if the silver gull microbiome does stabilise as the host individual matures and whether this is characterised by an increase or decrease in resident microbiota. Such investigations could further determine whether the maturation of the avian microbiome is accompanied by an overall decrease in Proteobacteria and AMR-associated bacteria, as suggested by the results of previous studies and the current thesis.

4.2.3 Resistome dynamics

Ontogenetic changes in resistome composition were analysed to examine the ability of silver gull microbiomes to accumulate and maintain AMR and the results of this thesis form the first known insight into resistome dynamics in a wild avian host. Resistome composition of samples was loosely clustered by chick, which was expected as samples from the same chicks were not independent. However, within chicks, resistome communities were dynamic, with large variations in richness measures and bacterial composition. These analyses suggest that

the resistome in avian chick microbiomes is in constant flux and can vary greatly over a chick's ontogenetic development.

Previous literature examining resistome dynamics in environmental and animal microbiomes is scarce and inconsistent. The majority of these longitudinal investigations confound the impact of antibiotic selection on AMR proliferation and thus do not examine the ability of the microbiome itself to accumulate and maintain AMR (Alexander et al. 2011; Buelow et al. 2014; Liu et al. 2016). However, studies in human infants have shown that AMR can increase over time, with some ARGs persisting for the first year of the child's life (Loo et al. 2020; von Wintersdorff et al. 2016). Conversely, in the current study, although ARGs increased with consecutive samples in some chicks, most chicks showed evidence of AMR fluctuation, and ARGs did not tend to persist over time. Moreover, the fluctuations in ARG richness over time, and the initial increase in ARG richness in samples less than 7 days old, indicates that, like bacterial composition, the resistome is a product of environmental exposure (Song et al. 2020). This is supported by a previous study on human microbiomes, which found that infants exhibit similar ARG profiles to their siblings, but are drastically different to their mothers, indicating that the resistome is majorly influenced by environmental exposure (Moore et al. 2015). The current thesis, therefore, shows that, like bacterial composition, resistomes in avian microbiomes fluctuate over time, and are therefore not maintained within the silver gull microbiome.

4.2.4 Bacterial and resistome associations

The results of this thesis show that bacterial and resistome diversity and composition are associated within the Gammaproteobacterial population of silver gull microbiomes. Bacterial and resistome composition were correlated, indicating that resistomes were shaped by the bacteria present within samples. This further indicates that ARGs are not randomly associated with any bacterium, which suggests that there is minimal sharing of ARGs amongst the bacterial community. The majority of samples were clustered by bacterial composition in the Procrustes ordination, and were associated with similar resistome compositions. This is indicative of the similarities in both bacterial and resistome composition and diversity seen across samples. To further investigate bacterial-resistome associations, pairwise Spearman's correlations were conducted across samples. However, the bacterial species-ARG correlations identified were indicative of novel associations in a small number of samples and

therefore did not reflect the bacterial-resistome associations exhibited across samples in the Procrustes. However, these results emphasise the tight correlation between individual ARGs and bacteria, which supports the idea that resistomes are shaped by the specific bacteria present within the microbiome.

Procrustes analyses were also conducted on samples collected from chicks in the first week of life, and the days before fledging. However, these analyses were not significant, likely due to the small number of samples in these age groups (n = 9). Future investigations with more samples from new hatchlings and near fledglings should be conducted to discern whether bacterial-resistome composition associations change over time.

Bacterial species and ARG richness were also significantly correlated, such that the change in the number of ARGs was attributed to changes in the number of bacterial species. This supports the idea that increases in ARGs are predominantly due to the colonisation of additional bacterial species harbouring ARGs, and not due to the horizontal transfer and subsequent accumulation of AMR, as one might expect. Moreover, microbiome diversity and composition of individual chicks varied greatly with consecutive samples, with each chick exhibiting different patterns of bacterial and resistome composition over time. In some chicks, the increase in the number of ARGs accompanied the increase in bacterial species richness, but which were then lost with the reduction of bacteria in consecutive samples. These results show that AMR within silver gull microbiomes is not maintained, and ARGs tend to be lost with the bacteria they are carried by. In contrast, evidence of potential AMR accumulation was exhibited in two samples, 18-02 and 18-06, which both increased in ARG richness despite decreases in bacterial species richness with consecutive samples. Future research is therefore needed to explore whether AMR accumulation is possible, and if these ARGs are able to persist in the silver gull microbiome. However, as the number of ARGs are correlated with species richness in all other chicks in the current study, it is unlikely that AMR accumulation frequently occurs in the silver gull chick microbiome. Additionally, the fluctuations and variations exhibited between samples could further be explicated through measurements of beta diversity for bacterial species and ARGs, which should be conducted in future investigations of this dataset.

Although metagenomic analysis is unable to directly link identified ARGs to their bacterial carriers, the bacterial-resistome associations in the current study provide a broader conceptual understanding of interactions within the silver gull microbiome, which cannot be achieved through traditional single-species studies. Therefore, metagenomic investigations, such as the current thesis, serve as a powerful preliminary tool to identify trends in bacterial and resistome composition, generating a wholistic understanding of the microbiome and AMR. Such studies can therefore direct future work to comprehensively analyse specific AMR-bacteria, for example speciose genera identified in the current study such as *Klebsiella* and *Enterobacter*, using techniques such as whole genome sequencing.

4.2.5 General discussion

The results of this thesis, for the first time, provide insights into the dynamics of AMR in a wild avian host. The high bacterial diversity and variable composition of bacterial communities described in this study, and seen in previous investigations (Cao et al. 2020; Song et al. 2020; Teyssier et al. 2018), supports the idea that the gut microbiome of avian species has the ability to host a large range of bacterial species from the environment, but are only transiently infected (Song et al. 2020). Additionally, the data presented here show that it is unlikely that ARGs are accumulated or persist in the avian microbiome. This is evidenced by the bacterial and resistome dynamics results of this thesis, which show that ARGs fluctuate between samples, without evidence of AMR accumulation or subsequent persistence in most chicks. Moreover, the current work showed that ARGs within chicks are not correlated with age, further reflective of these fluctuations. Additionally, the bacterial-resistome associations within samples indicate that the resistome is a result of bacterial composition, and ARGs reside with the bacteria they are brought into the microbiome with, without evidence of shared ARGs or horizontal gene transfer. As the number of ARGs were attributed to the number of bacterial species, this emphasises that more ARGs are brought into the microbiome through additional species, rather than through AMR proliferation within the microbiome.

This thesis supports the idea that silver gull chicks may act as ‘ecological sponges’ and bioindicators of AMR contamination due to their ability to host a high diversity of transient pathogenic and AMR-associated bacteria (Mukerji et al. 2019; Thompson 2019). This is

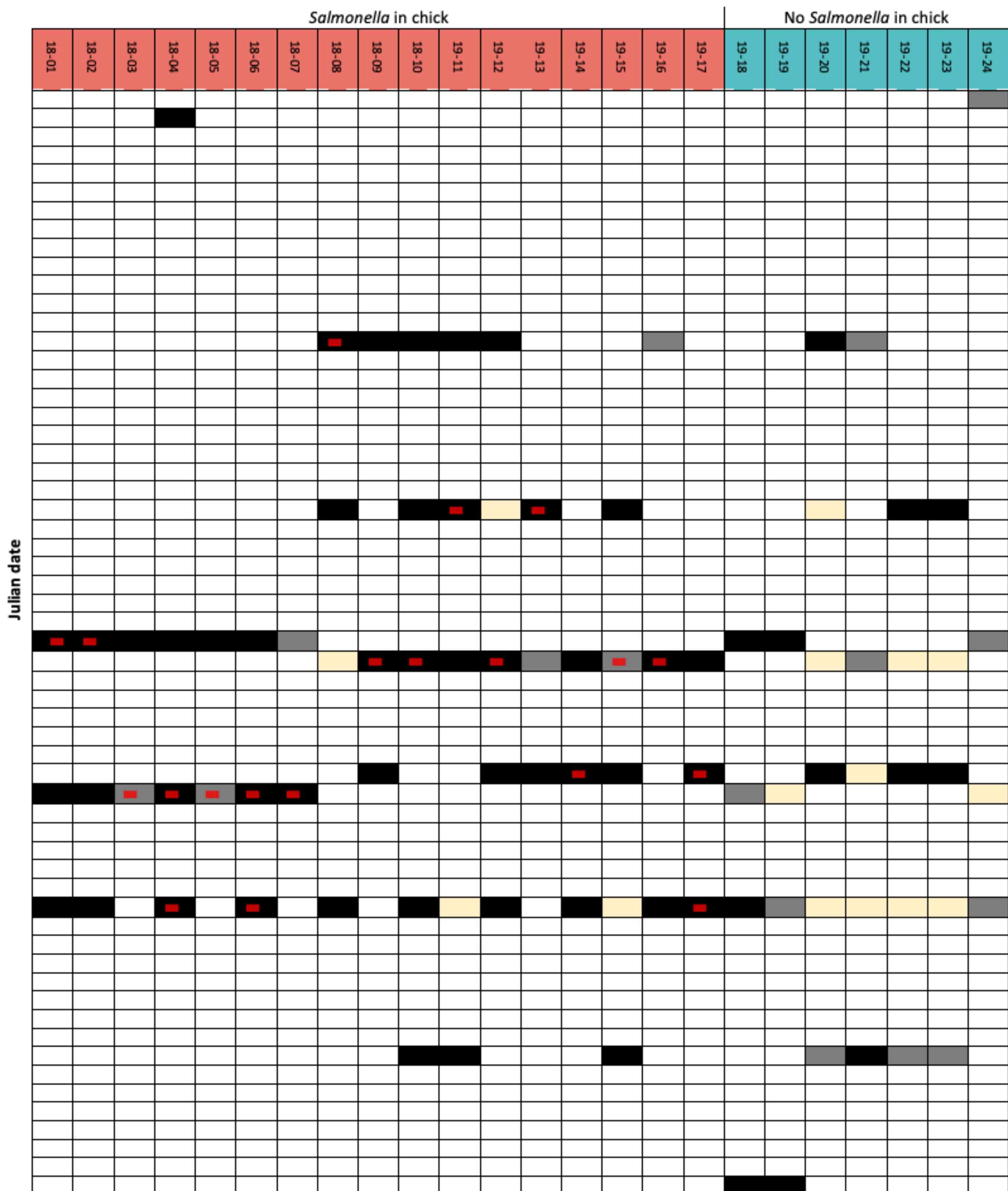
further supported by previous studies which elucidate the ability of silver gull chicks to host MDR bacteria (Dolejská et al. 2016; Dolejská et al. 2018), including bacterial isolates that are closely related to clinical bacteria (Thompson 2019). The current thesis further suggests that MDR within AMR-bacteria is not accumulated within the silver gull chick microbiome and is acquired with bacteria that colonise the gut. The diversity of bacterial pathogens and AMR profiles seen in these samples may, therefore, reflect a high level of AMR contamination in the environment, and future studies should be conducted to investigate the sources of such contamination. Moreover, as a larger number of ARGs have been previously identified in *Salmonella* from silver gull chicks than from corresponding clinical isolates (Thompson 2019), future studies should investigate whether AMR is propagated within other environmental or wildlife reservoirs.

The work presented in this thesis provides strong evidence to suggest that silver gull chicks do not accumulate or maintain AMR in their microbiomes, and it is therefore unlikely that these gulls are disseminating AMR to other individuals or have the ability to spread them back into humans. Rather than contributing to the proliferation of AMR, silver gull chicks seem to reflect AMR-bacterial contamination through a diverse transient microbiota community. There is evidence to suggest that the ability of silver gull chick microbiomes to accumulate a diverse range of AMR-bacteria is widespread in other bird species (Atterby et al. 2017; Marcelino et al. 2019; Wang et al. 2017). This ability may be distinct to avian wildlife, due to their low microbiome diversity, their lack of microbiota structure, and their ability to host a high proportion of Proteobacteria, compared to other species, especially mammals (Cao et al. 2020; Song et al. 2020). It is possible that this ability of avian species to host a high diversity of Proteobacteria begets the diversity of AMR-associated bacteria, which in turn begets the diversity of ARGs. However, adult microbiomes may be more stable than in juveniles (Grond et al. 2017; Hennessy et al. 2020), and this matured microbiota may therefore not be as easily colonised by transient pathogens, however this postulation needs to be examined further. Furthermore, it is possible that other wildlife populations may constitute AMR reservoirs, and further research should be conducted to discern the role of a range of wildlife species in the evolution of AMR-bacteria. Additionally, wildlife with direct exposure to antibiotics may, like humans and other animals, contribute to the proliferation of AMR (Marshall & Levy 2011). Therefore, wildlife populations with exposure to antibiotics should be considered in future investigations of AMR-bacterial evolution in wildlife.

5 CONCLUSIONS

This longitudinal metagenomic study of silver gull chick microbiomes was conducted to investigate the role of an avian wildlife microbiome in the evolution of AMR-bacteria. Wildlife is a crucial component of the One Health approach to tackle the AMR health crisis, and this study was designed to fill the gap in our understanding of wildlife's role in the proliferation of AMR. This thesis therefore investigated the ability of silver gull chicks to accumulate and maintain ARGs by conducting an ontogenetic metagenomic analysis. The findings of this work support the idea that microbiomes of avian species have the potential to host a large number of AMR-associated bacteria through transient infection by a high diversity of Gammaproteobacterial species. This provides support for previous postulations that avian microbiomes act as 'ecological sponges' of AMR-bacteria and reflect AMR contamination in the environment. However, it appears that the resistome is not formed by the accumulation of ARGs, and AMR is lost with the bacteria it is carried by. These results indicate that the silver gull chick gut microbiome does not facilitate the evolution of AMR-bacteria and further does not act as a reservoir contributing to the proliferation of AMR. However, future investigations are required to examine the sources of AMR in this silver gull population, and other potential environmental and wildlife reservoirs of AMR should be considered. Overall, this metagenomic investigation demonstrates a novel and effective approach to examine a dynamic ecological process, thus generating a wholistic picture of the role of silver gull microbiomes in the evolution and proliferation of AMR.

6 APPENDIX



Appendix 6.1. Longitudinal study design of 24 silver gull chick samples by Julian date. Cloacal samples were collected on Big Island of the Five Island Nature Reserve during the 2018/2019 breeding seasons, which were then sub-cultured for Enterobacteriaceae and extracted for metagenomic analysis. The '*Salmonella* in Chick' group includes chicks with at least one sample with previous phenotypic detection of *Salmonella*. The 'No *Salmonella* in Chick' group includes chicks with no prior detection of *Salmonella*. Samples included in the study are represented by black cells. Grey cells represent samples included in the original study design, but not included in the final metagenomic analysis. Cream cells represent samples collected from chicks but not included in the study design. Cells with red bars represent samples with *Salmonella*.

Appendix 6.2. Summary of trails for the optimisation of DNA extractions from silver gull multi-species gut microbiota samples (N = 6) using the ISOLATE II Genomic DNA Kit (Bioline, Meridian Bioscience). The protocols described for each extract includes the procedure for culture preparation, including the dilution of resuspension, and the treatments for the kit extraction, including lysis procedure and reagents (N = 25). NanoDrop™ readings to measure yield, (NA) and quality (A260/A280 and A260/A230**)** are summarised for each extract.

Date	Extract no.	Sample ID	Culture	Dilution	Lysis buffer/incubation	Reagent treatments	NA (ng/µl)	A260/A280	A260/A230
24/09/20	1	18-254	Plated	non-diluted	HTL*/overnight + 1 h	2x reagents	438.93	1.91	2.05
24/09/20	2	18-254	Plated	1:10	HTL/overnight	2x reagents	350.97	1.93	1.64
30/09/20	3	18-257	Plated	non-diluted	HTL/syringe + 1 h	2x reagents	121.43	1.91	1.86
30/09/20	4	18-257	Plated	1:10	HTL/syringe + 1 h	2x reagents	106.93	1.92	2.17
30/09/20	5	18-260	Plated	1:10	HTL/syringe + 1 h	2x reagents	130.87	1.88	1.90
30/09/20	6	18-260	Plated	1:100	HTL/syringe + 1 h	2x reagents	128.97	1.91	2.00
30/09/20	7	18-261	Plated	non-diluted	HTL/syringe + 1 h	2x reagents	33.90	1.91	1.54
30/09/20	8	18-261	Plated	1:10	HTL/syringe + 1 h	2x reagents	71.10	1.91	1.62
06/10/20	9	18-261	Plated	non-diluted	HTL/overnight + 1 h	2x reagents, x4 lysozyme	426.90	2.07	2.16
06/10/20	10	18-261	Plated	1:10	HTL/overnight + 1 h	2x reagents, x4 lysozyme	190.80	2.15	2.48
21/10/20	11	18-261	Plated	1:50	HTL/3 h	2x reagents	1015.17	2.11	2.12
21/10/20	12	18-261	Plated	1:100	HTL/3 h	2x reagents	994.17	2.14	2.38
29/10/20	13	18-261	Plated	1:100	HTL/3 h	2x reagents + RNase (2010)	186.07	1.82	2.09
29/10/20	14	18-261	Plated	1:50	HTL/3 h	2x reagents + RNase (2010)	199.83	1.76	1.26
29/10/20	15	18-261	Plated	1:50	HTL/3 h	RNase (2016)	294.63	1.82	2.11
16/11/20	16	18-257	Plated	1:100	GL**/3 h		460.17	1.97	2.03
16/11/20	17	18-257	Plated	1:100	GL/3 h	RNase (new)	68.80	1.77	1.49
16/11/20	18	18-257	Plated	1:100	HTL/1 + 2 hr		1556.43	2.03	2.24
16/11/20	19	18-257	Plated	1:100	HTL/1 + 2 hr	RNase (new)	376.07	1.89	2.25
16/11/20	20	18-257	Liquid	1:100	GL/3 h		136.37	2.12	2.14
16/11/20	21	18-257	Liquid	1:100	GL/3 h	RNase (new)	46.73	1.84	2.16
16/11/20	22	18-257	Liquid	1:100	HTL/1 + 2 h		525.73	2.14	2.35
16/11/20	23	18-257	Liquid	1:100	HTL/1 + 2 h	RNase (new)	128.30	1.84	2.07
18/11/20	24	18-244	Liquid	1:50	HTL/1 + 2 h	RNase (new)	135.73	1.83	2.04
18/11/20	25	18-259	Liquid	1:50	HTL/1 + 2 h	RNase (new)	148.47	1.84	2.10

* HTL: 'hard to lyse' buffer **GL: ISOLATE II provided lysis buffer (Bioline, Meridian Bioscience)

Appendix 6.3. DNA extracts included in the metagenomic analysis for the current study (N = 54). DNA was extracted from silver gull multi-species gut microbiota samples (N = 69) using the ISOLATE II Genomic DNA Kit (Bioline, Meridian Bioscience). The culture resuspension dilution for DNA extraction and NanoDrop™ readings to measure yield (NA) and quality (A260/A280 and A260/A230) are summarised for each extract. Included samples were of sufficient yield and quality for metagenomic analysis.

Date	Extract no.	Sample ID	Dilution	NA (ng/µl)	A260/A280	A260/A230
1/12/20	1	19-138	1:50	163.30	1.84	1.90
1/12/20	2	19-167	1:50	103.07	1.84	1.94
1/12/20	3	19-171	1:50	122.33	1.82	1.88
1/12/20	4	19-210	1:50	131.43	1.84	2.01
1/12/20	5	19-250	1:50	137.17	1.85	1.96
1/12/20	7	19-307	1:50	132.00	1.83	1.91
1/12/20	8	19-322	1:50	102.70	1.82	1.90
1/12/20	9	19-327	1:50	119.33	1.82	1.84
1/12/20	10	19-347	1:50	93.90	1.81	1.85
1/12/20	11	19-380	1:50	150.23	1.84	2.02
1/12/20	12	19-400	1:50	89.63	1.81	1.95
1/12/20	13	18-086	1:50	124.70	1.74	1.43
1/12/20	14	18-097	1:50	119.03	1.81	2.01
1/12/20	15	18-102	1:50	119.63	1.84	1.91
1/12/20	16	18-119	1:50	95.70	1.84	2.05
1/12/20	17	18-136	1:50	125.93	1.83	1.89
1/12/20	20	18-165	1:50	100.57	1.84	1.97
1/12/20	22	18-190	1:50	131.37	1.85	1.96
1/12/20	23	18-220	1:50	122.47	1.85	1.98
1/12/20	25	18-255	1:50	101.57	1.83	1.98
4/12/20	27	19-071	1:50	98.77	1.77	1.83
4/12/20	30	19-140	1:50	100.93	1.81	2.16
4/12/20	31	19-182	1:50	62.03	1.77	1.84
4/12/20	32	19-196	1:50	99.57	1.8	2
4/12/20	33	19-233	1:50	88.07	1.77	1.86
4/12/20	37	19-292	1:50	127.53	1.84	2.01
4/12/20	38	19-334	1:50	104.53	1.82	2.04
4/12/20	45	19-449	1:50	105.4	1.82	2.07
4/12/20	48	19-485	1:50	156.27	1.86	2.07
11/12/20	50	18-095	1:50	116.93	1.83	2.23
11/12/20	53	18-210	1:50	126.00	1.84	2.30
11/12/20	56	18-246	1:50	123.27	1.71	1.65
11/12/20	57	18-247	1:50	121.90	1.83	2.31
11/12/20	58	19-094	1:50	134.40	1.82	2.29
11/12/20	60	19-148	1:50	121.13	1.83	2.26
11/12/20	61	19-191	1:50	197.30	1.84	2.17
11/12/20	62	19-248	1:50	108.67	1.82	2.27
11/12/20	64	19-359	1:50	127.80	1.85	2.24
11/12/20	65	19-381	1:50	186.80	1.83	2.25
11/12/20	68	19-477	1:50	142.77	1.83	2.22

23/12/20	70	18-104	1:100	82.20	1.78	1.51
23/12/20	75	18-240	1:25	126.23	1.83	1.97
23/12/20	77	19-084	1:25	111.53	1.83	1.95
23/12/20	78	19-115	1:25	138.37	1.83	1.97
23/12/20	81	19-284	1:100	177.33	1.82	1.87
23/12/20	83	19-288	1:100	134.07	1.82	1.85
23/12/20	84	19-346	1:100	121.63	1.84	2.11
23/12/20	85	19-356	1:100	157.73	1.85	2.10
23/12/20	86	19-397	1:100	104.93	1.82	2.18
23/12/20	87	19-418	1:100	129.47	1.81	1.92
23/12/20	88	19-419	1:100	115.67	1.85	2.15
23/12/20	89	19-431	1:25	125.53	1.82	2.00
23/12/20	92	19-469	1:100	135.83	1.85	2.22
23/12/20	93	19-481	1:25	79.90	1.82	2.26
18/1/21	140	19-378	1:100	140.6	1.85	2.03

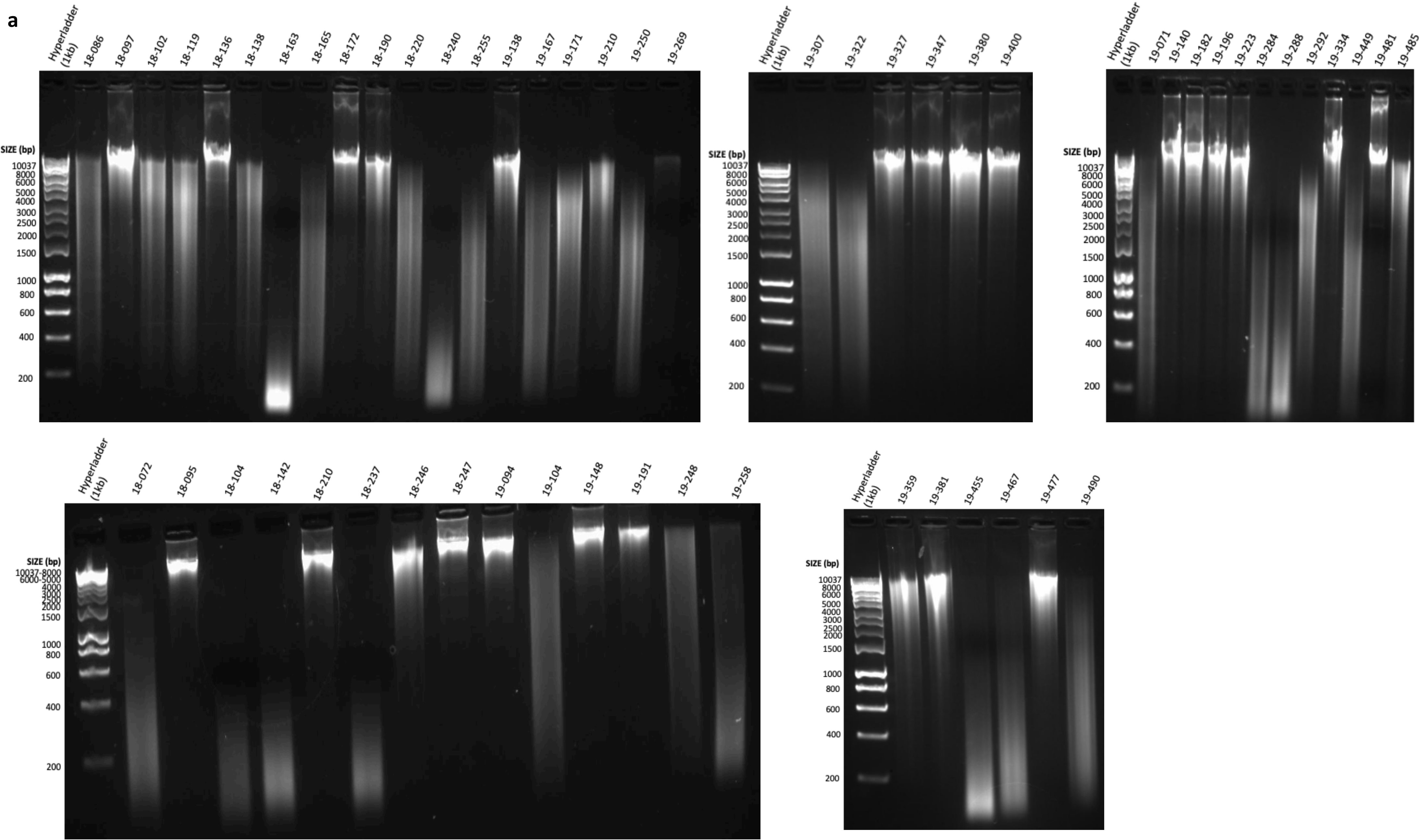
Appendix 6.4. DNA extracts excluded from the metagenomic analysis in the current study (N = 89). DNA was extracted from silver gull multi-species gut microbiota samples (N = 69) using the ISOLATE II Genomic DNA Kit (Bioline, Meridian Bioscience). The culture resuspension dilution for DNA extraction and NanoDrop™ readings to measure yield (NA) and quality (A260/A280 and A260/A230) are summarised for each extract. Included samples were of sufficient yield and quality for metagenomic analysis.

Date	Extract no.	Sample ID	Dilution	NA (ng/µl)	A260/A280	A260/A230	Reason Excluded
1/12/20	1	19-138	1:50	163.30	1.84	1.90	Low quality (AGRF QC*)
1/12/20	6	19-269	1:50	9.90	1.23	0.39	Low concentration
1/12/20	18	18-138	1:50	138.30	1.79	1.58	Low quality (AGRF QC)
1/12/20	19	18-163	1:50	28.90	1.74	1.35	Low concentration/ Degraded DNA
1/12/20	21	18-172	1:50	70.47	1.82	1.70	Low quality (AGRF QC)
1/12/20	24	18-240	1:50	46.87	1.81	1.69	Low concentration/ Degraded DNA
4/12/20	26	19-070	1:50	1.87	0.70	0.23	Low concentration
4/12/20	28	19-089	1:50	12.73	1.57	1.27	Low concentration
4/12/20	29	19-115	1:50	2.73	0.72	0.2	Low concentration
4/12/20	34	19-284	1:50	55.4	1.6	1.13	Low concentration/ Degraded DNA
4/12/20	35	19-287	1:50	5.77	1.08	0.36	Low concentration
4/12/20	36	19-288	1:50	30	1.5	0.97	Low concentration/ Degraded DNA
4/12/20	39	19-346	1:50	0.3	0.16	0.06	Low concentration
4/12/20	40	19-356	1:50	1.53	0.84	0.42	Low concentration
4/12/20	41	19-397	1:50	-0.37	-1.73	-0.11	Low concentration
4/12/20	42	19-418	1:50	3.73	1.2	0.55	Low concentration
4/12/20	43	19-419	1:50	0.83	0.47	0.18	Low concentration
4/12/20	44	19-431	1:50	0.83	0.48	0.13	Low concentration
4/12/20	46	19-469	1:50	7.4	1.54	1.5	Low concentration
4/12/20	47	19-481	1:50	19	1.81	3	Low concentration
11/12/20	49	18-072	1:50	71.07	1.81	2.22	Low quality (AGRF QC)

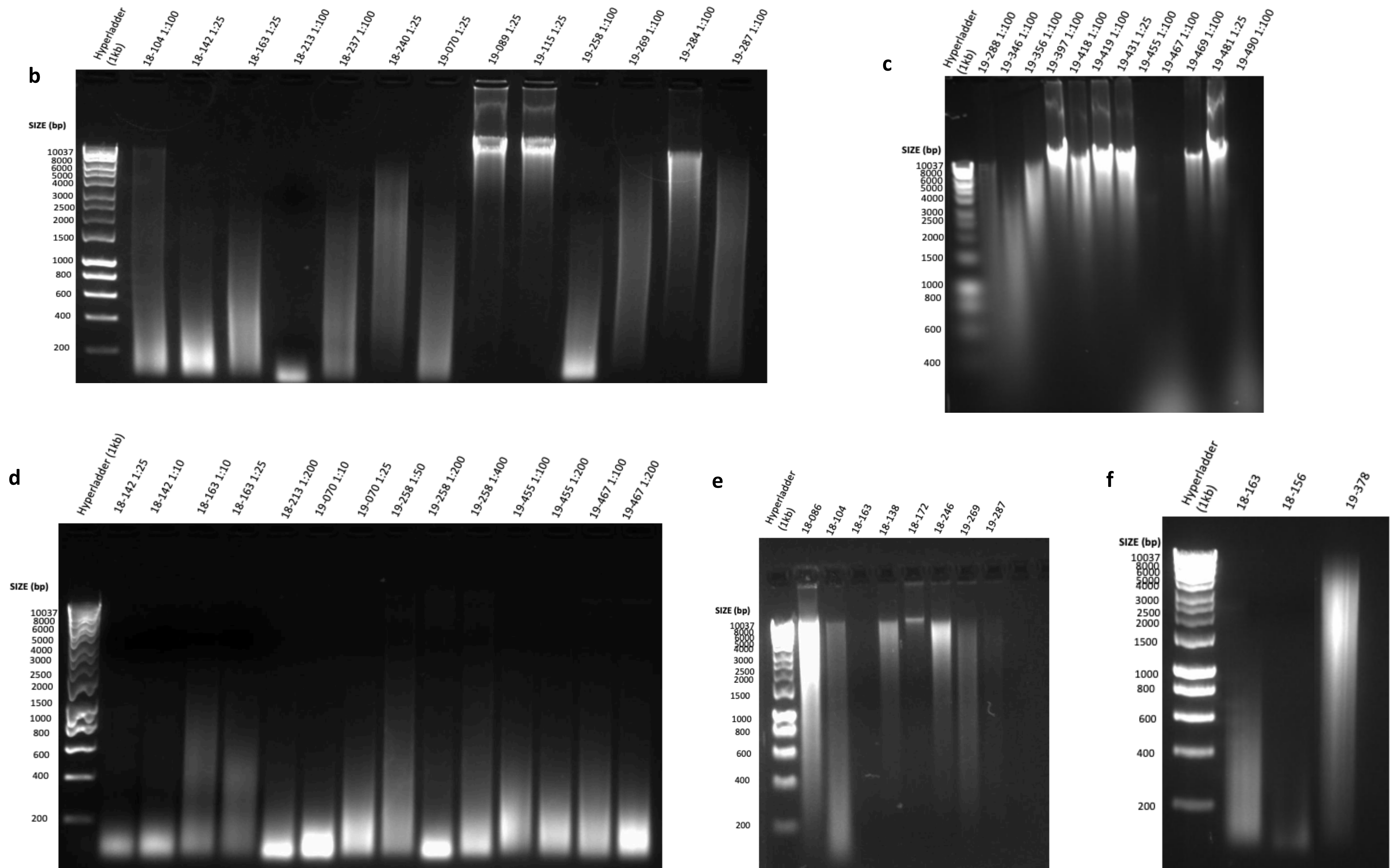
11/12/20	51	18-104	1:50	16.23	1.64	1.54	Low concentration/ Degraded DNA
11/12/20	52	18-142	1:50	25.60	1.69	1.48	Low concentration/ Degraded DNA
11/12/20	54	18-213	1:50	4.25	1.38	1.29	Low concentration
11/12/20	55	18-237	1:50	21.60	1.77	1.95	Low concentration/ Degraded DNA
11/12/20	59	19-104	1:50	96.90	1.81	2.37	Low quality (AGRF QC)
11/12/20	62	19-248	1:50	108.67	1.82	2.27	Opened in transport
11/12/20	63	19-258	1:50	50.17	1.81	2.48	Low concentration/ Degraded DNA
11/12/20	66	19-455	1:50	44.53	1.80	2.32	Low concentration
11/12/20	67	19-467	1:50	63.53	1.83	2.35	Degraded DNA
11/12/20	68	19-477	1:50	142.77	1.83	2.22	Low quality (AGRF QC)
11/12/20	69	19-490	1:50	87.10	1.81	2.24	Opened in transport
23/12/20	71	18-142	1:25	71.80	1.80	1.82	Low quality (AGRF QC)
23/12/20	72	18-163	1:25	72.50	1.76	1.53	Degraded DNA
23/12/20	73	18-213	1:100	13.43	1.63	0.96	Low concentration/ Degraded DNA
23/12/20	74	18-237	1:100	105.23	1.82	2.04	Low quality (AGRF QC)
23/12/20	76	19-070	1:25	95.47	1.82	2.05	Low quality (AGRF QC)
23/12/20	79	19-258	1:100	35.70	1.75	1.21	Low concentration/ Degraded DNA
23/12/20	80	19-269	1:100	152.47	1.82	1.73	Low quality (AGRF QC)
23/12/20	82	19-287	1:100	113.37	1.79	1.54	Low quality (AGRF QC)
23/12/20	90	19-455	1:100	53.37	1.76	1.67	Low concentration/ Degraded DNA
23/12/20	91	19-467	1:100	57.67	1.82	2.07	Low concentration/ Degraded DNA
23/12/20	94	19-490	1:100	63.90	1.78	1.77	Degraded DNA
24/12/20	95	18-142	1:25	10	2.88	-1.95	Low concentration
24/12/20	96	18-163	1:25	37	1.87	10.41	Low concentration
24/12/20	97	18-213	1:400	10	1.51	-98.77	Low concentration
24/12/20	98	18-237	1:25	57.00	1.81	3.93	Low concentration
24/12/20	99	19-070	1:25	56.00	1.78	3.95	Low concentration
24/12/20	100	19-258	1:200	-1.6	2.25	0.17	Low concentration
24/12/20	101	19-455	1:200	4.2	1.14	1.73	Low concentration
24/12/20	102	19-467	1:200	6	1.54	-1.02	Low concentration
31/12/20	103	18-163	1:25	25.6	1.67	8.43	Low concentration
31/12/20	104	18-237	1:10	37.6	1.71	4	Low concentration
31/12/20	105	18-237	1:25	72.8	1.78	3.01	Other extract with higher concentration
31/12/20	106	19-070	1:10	70.9	1.73	1.52	Other extract with higher concentration
31/12/20	107	19-070	1:25	52	1.75	1.94	Low concentration
31/12/20	108	19-258	1:100	32.1	1.76	11.48	Low concentration
31/12/20	109	19-455	1:100	43.1	1.77	2.89	Low concentration
31/12/20	110	19-467	1:100	49.2	1.79	4.19	Low concentration
31/12/20	111	19-490	1:100	60.2	1.77	3.21	Low concentration
31/12/20	112	18-142	1:10	20.3	1.51	2.4	Low concentration
31/12/20	113	18-142	1:25	27.3	1.65	2.38	Low concentration
31/12/20	114	18-163	1:10	58.1	1.78	3.53	Low concentration
31/12/20	115	18-213	1:200	8.6	1.12	0.63	Low concentration
31/12/20	116	18-213	1:400	2.4	0.97	-0.28	Low concentration
31/12/20	117	19-258	1:200	24	1.62	16.16	Low concentration
31/12/20	118	19-455	1:200	46.3	1.72	3.03	Low concentration
31/12/20	119	19-467	1:200	57	1.76	3.59	Low concentration

31/12/20	120	19-490	1:200	49.3	1.75	3.71	Low concentration
8/1/21	121	18-142	1:25	19.5	1.6	3.5	Low concentration/ Degraded DNA
8/1/21	122	18-142	1:10	29.3	1.59	2.33	Low concentration/ Degraded DNA
8/1/21	123	18-163	1:10	60.8	1.79	3.08	Low concentration/ Degraded DNA
8/1/21	124	18-163	1:25	40.3	1.7	4.52	Low concentration/ Degraded DNA
8/1/21	125	18-213	1:100	1.7	0.52	-0.43	Low concentration
8/1/21	126	18-213	1:50	1.6	0.52	-0.44	Low concentration
8/1/21	127	19-070	1:10	50.3	1.73	3.25	Low concentration/ Degraded DNA
8/1/21	128	19-070	1:25	84.4	1.77	2.71	Degraded DNA
8/1/21	129	19-467	1:100	99.6	1.8	2.74	Low quality (AGRF QC)
8/1/21	130	18-213	1:200	32.3	1.65	8.25	Low concentration
8/1/21	131	18-213	1:400	0.2	0.1	-0.02	Low concentration
8/1/21	132	19-258	1:100	3	0.82	1.3	Low concentration
8/1/21	133	19-258	1:200	27.9	1.68	6.08	Low concentration/ Degraded DNA
8/1/21	134	19-258	1:400	51.4	1.75	4.78	Low concentration/ Degraded DNA
8/1/21	135	19-258	1:50	59.9	1.76	3.12	Low concentration/ Degraded DNA
8/1/21	136	19-455	1:100	81.1	1.79	2.85	Low quality (AGRF QC)
8/1/21	137	19-455	1:200	75.5	1.75	2.71	Other extract with higher concentration
8/1/21	138	19-467	1:200	64	1.75	2.92	Other extract with higher concentration
18/1/21	139	18-163	1:25	153.50	1.46	0.66	Low quality (AGRF QC)
18/1/21	141	18-156	1:50	8.8	1.7	0.78	Low concentration

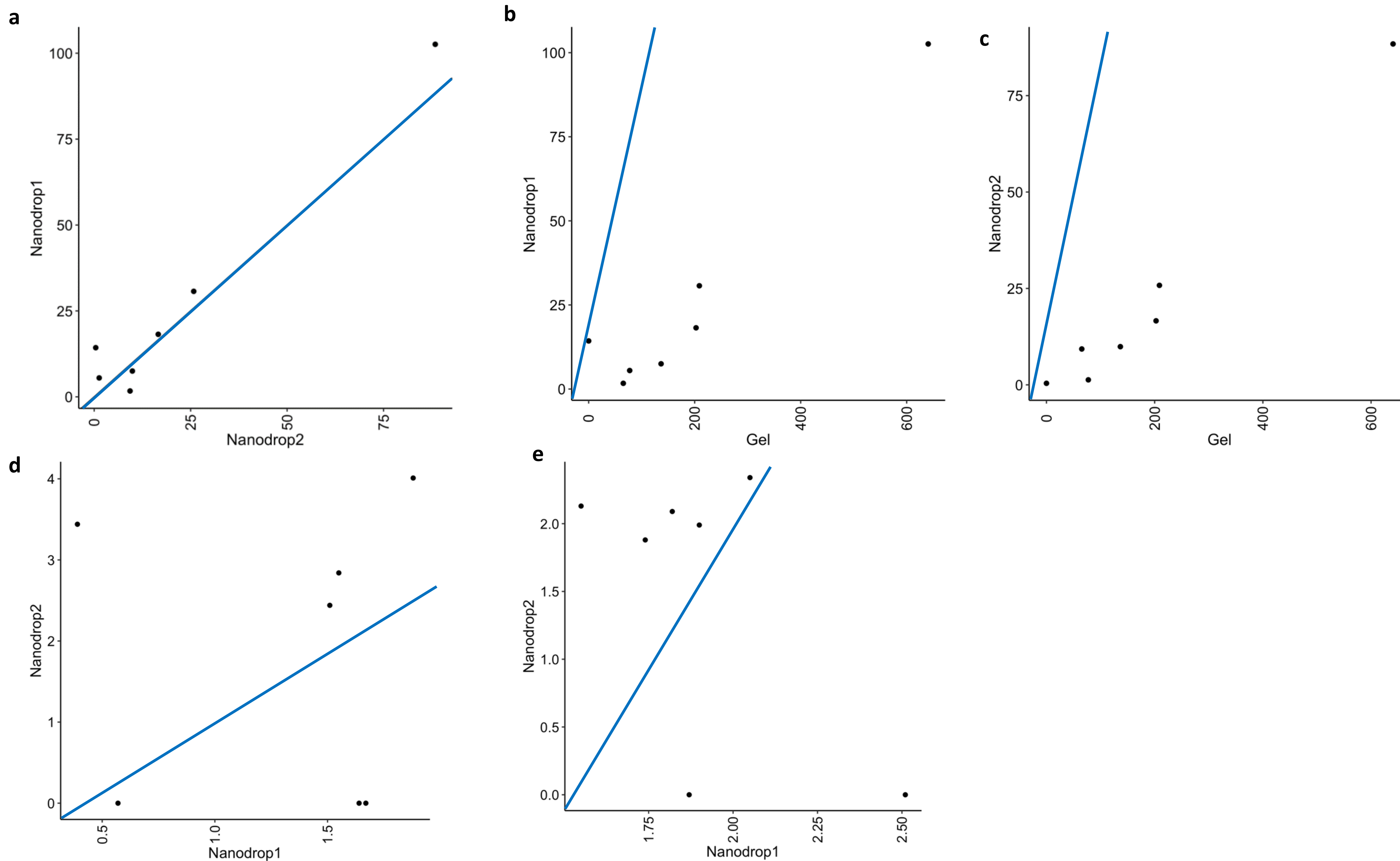
*AGRF QC: Sample did not meet quality control (QC) checks when sent for sequencing at the Australian Genome Research Facility (AGRF)



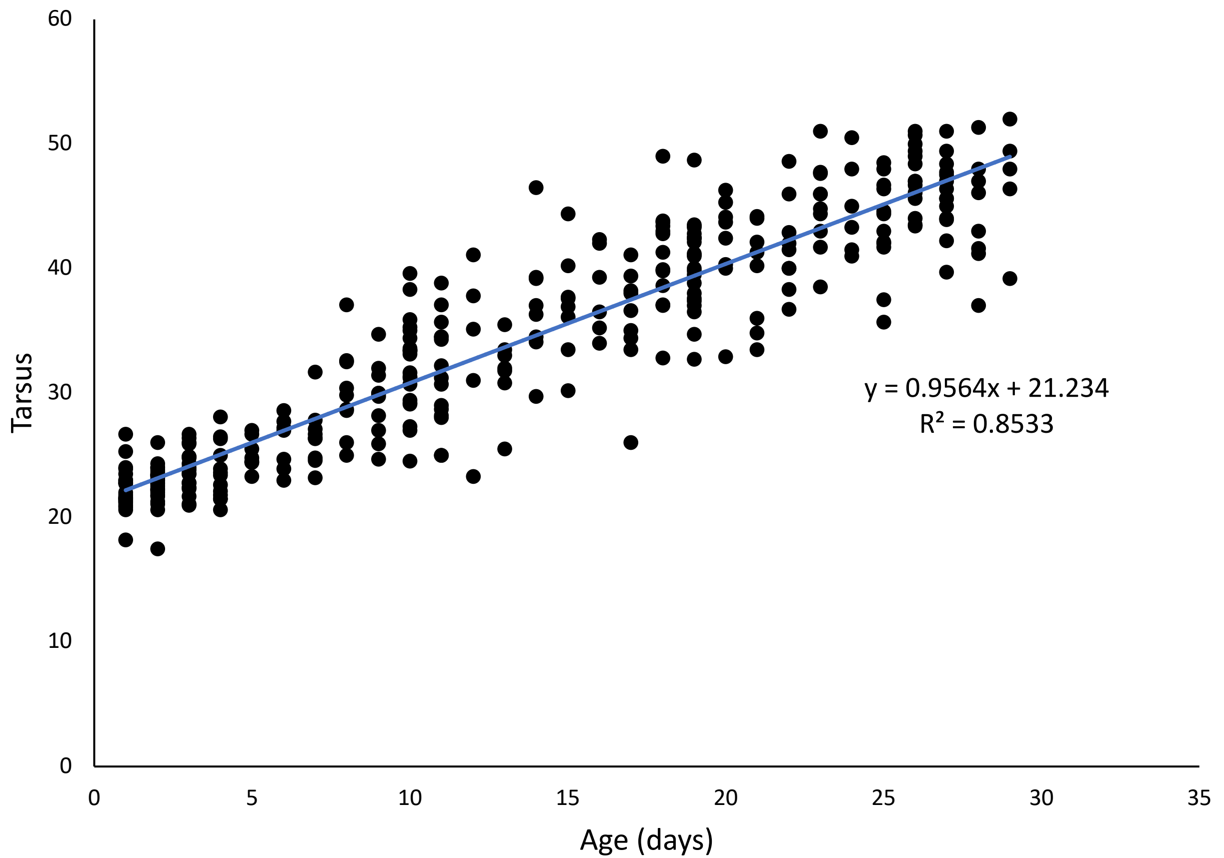
(**Appendix 6.5.** Continued on next page)



Appendix 6.5. Agarose gels of electrophorised DNA extracts from silver gull gut microbiota samples, extracted using the optimised protocol for ISOLATE II Genomic DNA Kit (Bioline, Meridian Bioscience). **(a)** First DNA extractions (N = 57) using the optimised protocol; **(b-c)** Extractions prepared from resuspensions diluted 1:25 or 1:100; **(d)** Extractions conducted with new reagents; **(e)** Extractions decontaminated with a salt precipitation; **(f)** Final extractions to replace missing samples.

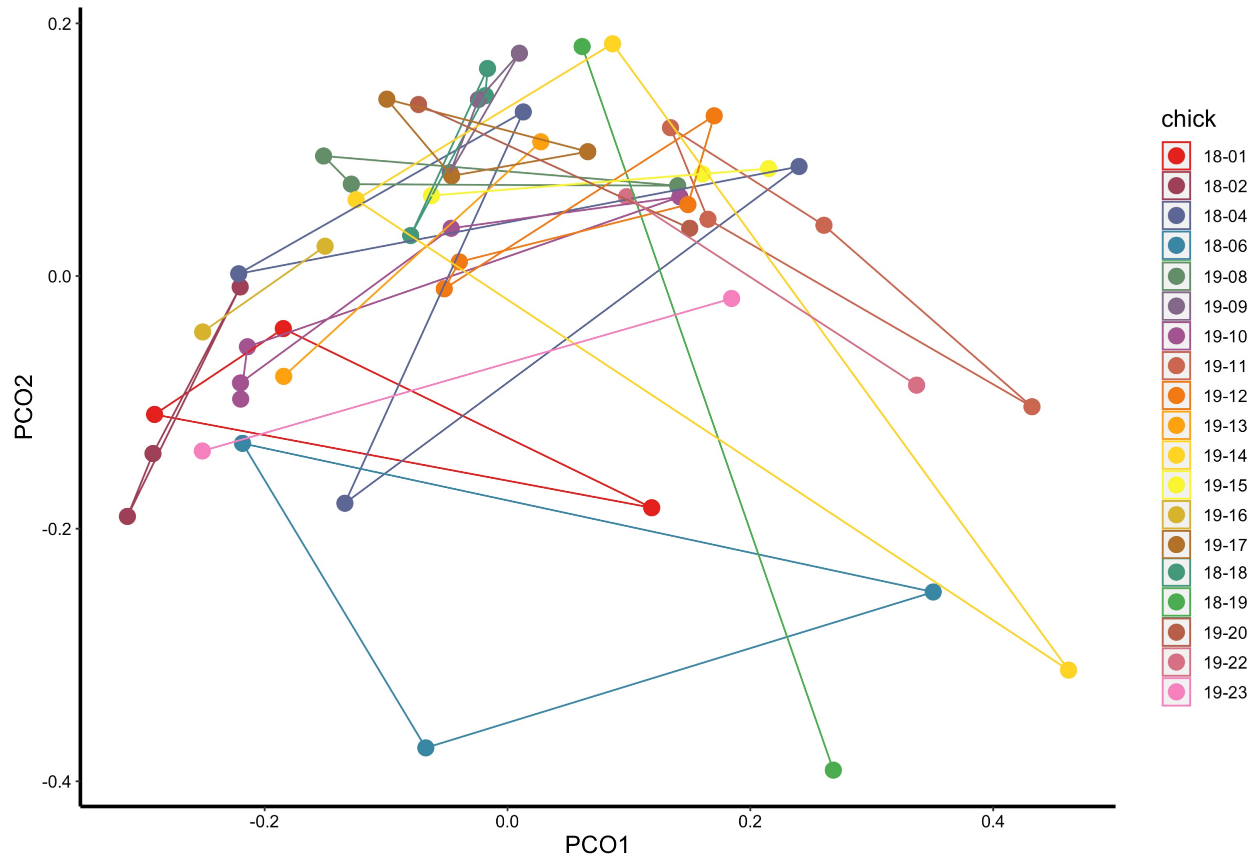


Appendix 6.6. Comparison of yield and quality measurements from extracts after salt precipitation: **(a-c)** Concentration (NA ng/µl) comparing two Nanodrop™ readings and ImageJ calculations using band intensity of Agarose gels; **(d)** A260/A280 measurements comparing both Nanodrop™ readings; **(d)** A260/A230 measurements comparing both Nanodrop™ readings. Lines show (expected) linear relationship, sloped 1.

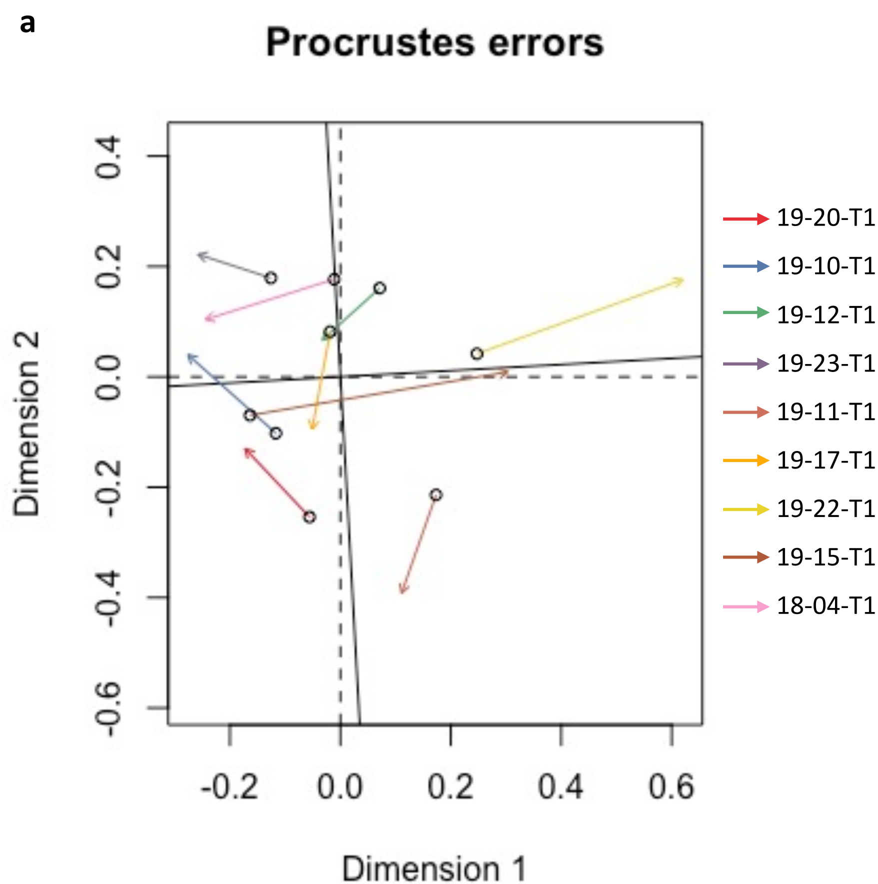


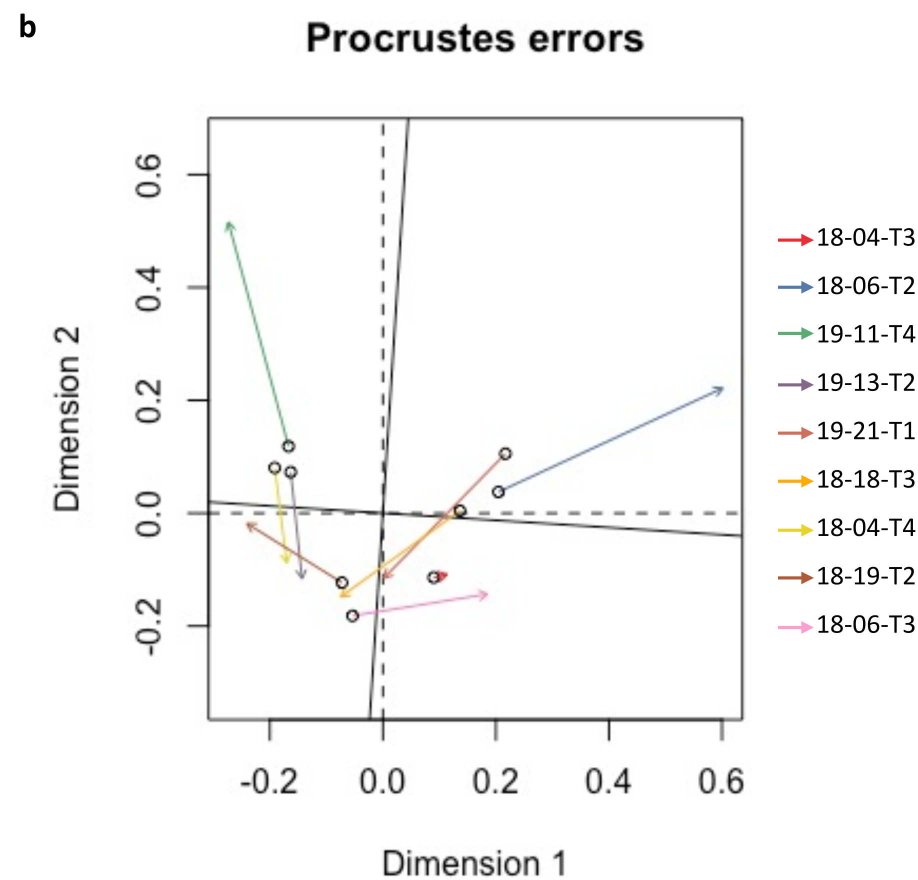
Appendix 6.7. Linear fit of tarsus measurements plotted against known age for samples of chicks aged less than 30 days. Samples and tarsus (a measurement of a bird's elbow to wrist) of chicks were collected on Big Island of the Five Islands Nature Reserve in the breeding season of 2018/2019. The linear equation was used to calculate an age estimate for the first samples of all chicks included in the metagenomic analysis.

Appendix 6.8. Relative abundance of bacterial taxa of **(a)** bacterial Families and **(b)** bacterial Genera from longitudinal multi-species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, before correction for low-resolution *Pseudomonas* species in samples 19-14-T1 and 18-19-T1. Samples are arranged in sequential order by chick.



Appendix 6.9. Principle Coordination Analysis (PCoA) based on Jaccard distance of multi-species microbiota samples from silver gull chicks as dissimilarities in bacterial composition. Longitudinal samples were collected from silver gull chicks at the Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). Presented are metagenomic samples subcultured for Enterobacteriaceae, before correction for low-resolution *Pseudomonas* species in samples 19-14-T1 and 18-19-T1. Samples are coloured and grouped by chick.





Appendix 6.10. Procrustes correlation between bacterial and resistome composition of multi species microbiota samples collected from silver gull chicks at Five Islands Nature Reserve, Australia, during the 2018 and 2019 breeding seasons (N = 60). **(a)** Samples of hatchlings, chick age 1-6 days (n = 9); **(b)** Samples before fledging, chick age 41-56 days (n = 9). Presented are metagenomic samples subcultured for Enterobacteriaceae.(Ackermann 1998; Martiny et al. 2015)

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