Effects of dietary components on antibiotic resistance using mechanistic and plasmid based studies

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Declaration of Authorship

This thesis has not been submitted in whole or in part to this or any other university for any degree, and is original work of the author except where otherwise stated.

Signed: _____

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Comparison of plasmid DNA extraction methods from complex broiler caecal samples.

Abstract

Antibiotic resistance may have the potential to spread from animals to humans through the food chain. It has been observed that prebiotics such as mannan rich fraction (MRF) improve broiler growth and performance in a similar manner to antibiotic growth promotors. They have also been linked to a reduction in antibiotic resistance gene numbers. It was hypothesised that MRF has the ability to decrease resistance by reducing the variety or transfer of plasmids.

The most effective and efficient method to extract plasmids from the complex broiler caecum was investigated, and determined to be the exogenous plasmid isolation method. Analysis into the plasmids present in the broiler caecum was performed for a greater understanding of the genetic basis of this resistance. Plasmids were identified that matched to previously isolated plasmids from animal and human samples from locations worldwide. The microbiome and mobile resistome was assessed over time. A more stabilised microbiota was found to develop as the birds age, which may be better established to harbour the increased number of resistance plasmids identified in the older birds.

The effect of MRF on the metagenome of broilers was examined. No significant changes were observed in the microbiome, which we attributed to the already resident community of beneficial bacteria. Changes in the abundance of the resistome was observed at day 27, which may be the result of MRF, but high levels of variability were noted within the sampled groups. A study incorporating both metagenomic and plasmid based analysis concluded that MRF may have the ability to restore the microbiome of broilers after antibiotic treatment. A lower percentage of multi-drug

resistance plasmids and a reduced profile of resistance was observed in birds that had received MRF. Mannan rich fraction may have the ability to decrease the conjugative capability of the plasmids, and thus reduce the spread of antibiotic resistance

Chapter 1

Introduction

1.1 Antibiotic discovery and emergence of resistance

The discovery of the first antimicrobial, Salvarsan, in 1909 by Dr. Paul Ehrlich, altered the impact of infectious disease on human life[1]. Salvarsan was successfully used to treat syphilis until the discovery of penicillin by Sir Alexander Fleming in 1928, and its subsequent mass production and distribution in 1945[2]. Antibiotics are the only class of medicinal agents whose main target is not human tissue or its products[3]. Antibiotics have not only drastically reduced rates of morbidity and mortality from infectious diseases, but have also had a crucial role in accomplishing key advances in medicine and surgery, such as organ transplantations and cancer chemotherapy, in their ability to control infection[4]. Shortly after its introduction, penicillin resistance became a significant clinical problem[5].

However, this was not the beginning of bacterial resistance to antibiotics. Resistance has been detected in samples that are over 30,000 years old[6]. Most antibiotics in clinical use are produced by bacteria themselves. For example, *Actinomycetes* produce streptomycin, tetracycline, chloramphenicol, erythromycin and vancomycin antibiotics. This requires the bacteria to be resistant themselves to avoid succumbing to their own metabolites[7]. It was rather the overuse and misuse of antibiotics to treat non-bacterial infections, and inadequate antibiotic stewardship in clinical settings that has been attributed to the escalation of the development and spread of resistance[8].

The 'golden era' of antibiotic discovery spanned from the 1950s to the 1970s[9]. During this time it was believed that infectious diseases would soon become a controlled

public health issue as was the rapid rate of antibiotic discovery[10], with multiple new classes of antibiotics introduced over the two decades[11]. Subsequently, antibiotic discovery came to a halt, while resistance continued to exacerbate. In 2016, at least 700,000 deaths worldwide were caused by resistant infections[12].

1.2 Plasmid-mediated antibiotic resistance

Antibiotic resistance occurs through several mechanisms, the central ones being enzymatic degradation or alteration of the antibiotic, alteration of the target proteins for the antibiotic and changes in the membrane permeability to antibiotics[13]. Some bacteria are intrinsically resistant to certain classes of antibiotics[14]. However, it is acquired resistance that is of the greatest concern, whereby a previously susceptible population of bacteria becomes resistant to an antibiotic[15].

Plasmids are small, extrachromosomal pieces of DNA and are one of the main drivers in the spread of antibiotic resistance. They have the ability to self-replicate and many are conjugative, allowing them to easily transfer to other bacteria[16]. Broad host range plasmids also possess the ability to transfer to taxonomically distant species while stably maintaining the genes that they harbour[17]. Plasmids often carry antibiotic resistance genes, which can provide a benefit to the host bacterial cell under antibiotic pressure. However, these genes may also incur a high fitness cost, and so may not be continually maintained by the same host[18]. Multi-drug resistance occurs by the accumulation of resistance genes, each coding for resistance to a specific antibiotic, usually on plasmids[19]. Multi-drug resistance has grave consequences for health,

particularly if harboured by a pathogen, as the options for treatment are greatly limited[20].

Resistance to antibiotics which were previously only chromosomally encoded are being identified on plasmids. These include the *qnr* genes that confer resistance to quinolones, which were first detected only in 1994[21]. Similarly, the *mcr* genes were first identified in 2016; these genes confer resistance to the polymyxin colistin[22]. Even more concerning is that colistin is considered an antibiotic of last resort for the treatment of multi-drug resistant infections caused by Gram-negative bacteria[23].

1.3 Antibiotics in agriculture

The rapid rise in resistance cannot be confined to just a clinical context. Antibiotics are administered to animals for the treatment of disease, but also to prevent and control the spread of disease. In 2013, it was estimated most of the 100,000-200,000 tonnes of antibiotics manufactured every year goes to the agricultural, horticultural, and veterinary sectors[24]. It is important to note that the antibiotics which are used in agriculture have the same modes of action or belong to the same antibiotic classes as those used in human medicine[25].

In the 1950s, it was discovered that sub-therapeutic quantities of antibiotics could enhance the feed-to-weight ratio for poultry, swine, and beef cattle[26]. These antibiotic growth promotors (AGP) reduce normal microbial communities present in the animal gut, which compete with the host for nutrients. They also reduce the abundance of

harmful bacteria that may reduce performance by causing subclinical disease[27]. In combination, this results in an increase in growth. However, towards the end of the 1960s, plasmid-encoded oxytetracycline resistance was identified in the zoonotic pathogen *Salmonella* Typhimurium in farm animals[28]. In 1975, a study on a chicken farm using oxytetracycline as an AGP found not only the chickens but also the farm family to be colonised by resistant strains of *Escherichia coli*[29]. In 1993, Bates *et al*., linked the emergence of vancomycin resistant enterococci, which was causing huge clinical concern, with the use of the AGP avoparcin[30]. In 2005, Hershberger *et al.*, found that there was a significant reservoir of antibiotic-resistant enterococci among farm animals that were administered antibiotics[31].

Due to these concerns in the increases of antibiotic resistance, AGPs were banned by the European Union in 2006[32], and later in America in 2017[33]. However, Casewell *et al*., highlighted the important prophylactic activity of AGPs and associated their withdrawal with a deterioration in animal health. This included reports of increased diarrhoea, weight loss and mortality due to infections with *Escherichia coli* and *Lawsonia intracellularis* in pigs, and *clostridial necrotic* enteritis in broiler chickens[34]. Therefore, products with a similar growth-promoting and pathogen-reducing effect are required for the maintenance of animal health in the absence of AGPs.

1.4 Antibiotic resistance in poultry production and the risk to human health

Poultry meat is the main driver of growth in total meat production globally[35]. As the human population continues to increase, so too will the demand for poultry meat,

which is one of the most widely consumed protein sources for humans[36]. Large-scale intensive farming is expected to upscale to meet this demand, however this often involves housing a large quantity of birds in cramped conditions[37]. This allows for disease, but also resistance, to spread rapidly throughout the flock. Antibiotics are used therapeutically, prophylactically, metaphylactically or as growth promotors (in certain countries) within poultry production, with the antibiotic generally being administered to the entire flock[38]. This has been a factor that has contributed to the ability of modern production facilities to produce market ready chickens in six weeks[39].

There is a risk of resistance transfer from animals to humans through the food chain. Consumers may be exposed to resistant bacteria through the consumption of animal products. Foods from numerous different animal sources and at all stages of production contain abundant quantities of antibiotic resistant bacteria and genes[40]. Randall *et al*., found that the use of fluoroquinolones in broilers resulted in resistant *Campylobacter* that was linked to 10% of human *Campylobacter* infections in the same area[41]. Sorensen *et al*., demonstrated that glycopeptide-resistant *Enterococcus faecium*, that was ingested *via* chicken or pork, persisted in human stool for up to 14 days after ingestion[42]. Commensal bacteria in food animals may also serve as a reservoir for resistance-encoding plasmids, the proportion of which is enhanced by the use of antibiotics in agriculture. When ingested by humans, the animal commensals can transfer their resistance to bacteria in the human microbiome[43].

1.5 An alternative to antibiotic growth promotors

Prebiotics are feed supplements that provide benefits to the host. The non-digestible products stimulate specific changes in the composition or activity, or both, of the intestinal microbiota that confers benefits to the host[44]. Unlike normal sugars, they are not digested by the host, and act as an energy source for bacteria[45]. Prebiotics can be fermented by beneficial bacteria in the intestine and produce lactic acid, short-chain fatty acid or even some antibacterial substances against pathogenic species. This has the potential to benefit the intestinal microbiota while improving the integrity of intestinal epithelial cells. This results in increased absorption of nutrients and therefore, enhanced growth performance of animals[46]. Prebiotics are therefore regarded as an alternative to antibiotic growth promotors (AGPs).

Mannan-oligosaccharides (MOS) are yeast cell wall fragments that are derived from *Saccharomyces cerevisiae*[47]. MOS are extracted by a process of opening the yeast cell wall, followed by steps of centrifugation, spray drying and alkaline extraction to α-mannoproteins, which are concentrated by membrane ultrafiltration and spray drying[48]. MOS has been found to reduce pathogenic bacteria, enhance beneficial bacteria, increase villus height and decrease crypt depth, modulate immune response and improve performance in broiler chickens[49].

Disturbances to the intestinal microbiome have been shown to lead to susceptibility to infection in the host. Prebiotics have been demonstrated, by increasing the commensal bacteria in the microbiome, to increase the hosts ability to inhibit pathogens[50]. MOS

has been shown to enhance the abundance of beneficial bacteria in the intestines of broilers. This effect has mainly been seen in *Lactobacillus* and *Bifidobacteria* species[51]. Baurhoo *et al*. found that MOS boosted the population of *Bifidobacteria* spp. in the intestines of broilers by increasing goblet cells and mucin production[52].

Many Gram-negative bacteria, such as *Escherichia coli* and *Salmonella*, use mannose-specific fimbriae to attach to the intestinal epithelium of the host[53]. MOS provides competitive binding sites for the fimbriae on pathogenic bacteria, and thus prevents them from attaching to the gut wall. This prevents their stabilisation, colonisation and multiplication within the host and therefore their potential to cause disease[54]. As MOS is not enzymatically digested, the bacteria which become bound to MOS are likely to exit the intestinal tract without attaching to the epithelium[55]. Due to this, MOS is seen as a viable option for use in antibiotic-free farming as an alternative to growth promotors[54].

Mannan rich fraction (MRF) is the next generation of MOS technology, developed from particular sugars present in the cell wall of a specific strain of *Saccharomyces cerevisiae*[56]. It can be added to diets at lower inclusion rates while still providing the same benefits as MOS including enhancing nutrient utilisation, maintaining digestive function and enzyme activity, and controlling inflammation. A study by Smith *et al.,* found that MRF reduced *E. coli* adherence to intestinal porcine epithelial cells[57]. M'Sadeq *et al.,* discovered that MRF was effective in preventing performance decline from necrotic enteritis in broiler chickens[58]. The MRF is more purified than MOS,

allowing for greater attachment of pathogens[56], providing it with enhanced suitability as an alternative to in-feed antibiotics.

Chapter 2

A Comparison of Methods for the Extraction of Plasmids Capable of Conferring Antibiotic Resistance in a Human Pathogen from Complex Broiler Caecal Samples

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2.1 ABSTRACT

The direct extraction of plasmid DNA containing antibiotic resistance genes from complex samples is imperative when studying plasmid-mediated antibiotic resistance from a One Health perspective, in order to obtain a wide representation of all the resistance plasmids present in these microbial communities. There are also relatively few bacterial species from natural environments which can be cultured *in vitro*. Extracting plasmids from the cultivable fraction of these complex microbiomes may only represent a fraction of the total antibiotic resistance plasmids present. We compared different methods of plasmid extraction from broiler caecal samples, whose resistance could be expressed in a human pathogen - *Escherichia coli*. We found that kits designed for DNA extraction from complex samples such as soil or faeces did not extract intact plasmid DNA. Commercial kits specific for plasmid extraction were also generally unsuccessful, most likely due to the complexity of our sample and intended use of the kits with bacterial culture. An alkaline lysis method specific for plasmid extraction was ineffective, even with further optimisation. Transposon-aided capture of plasmids (TRACA) allowed for the acquirement of a small range of resistance plasmids. Multiple displacement amplification provided the broadest range of resistance plasmids by amplifying all extracted circular plasmid DNA, but the results were not reproducible across all samples. Exogenous plasmid isolation enabled the extraction of resistance plasmids from the microbial fraction by relying on the mobility of the plasmids in the sample. This was the most consistent method from which we obtained a range of resistance plasmids from our samples. We therefore recommend the use of the exogenous plasmid isolation method in order to reliably obtain the greatest representation of the total antibiotic resistance plasmidome in complex

samples. While this method has limitations, it is one which will vastly increase our current knowledge of antibiotic resistance plasmids present in complex environments and which are capable of transferring to a human and animal pathogen and environmental contaminant.

2.2 INTRODUCTION

The rapid rise of antibiotic resistance has led to further studies into mobile genetic elements. Plasmids have been shown to be central vectors of gene sharing amongst bacteria[1], and therefore play a key role in microbial evolution and the spread of antibiotic resistance, leading to the rise of multi-resistant pathogenic bacteria[2]. Bacterial plasmids allow resistance genes to transfer horizontally between taxa and between animals and humans[3]. It is the mobility of these antibiotic resistance plasmids that is causing the most concern, as it is probably the most common mechanism for the dissemination of resistance genes[4], and many plasmids have the ability to move from a non-clinical environment to clinical pathogenic or human commensal bacteria.

To study the antibiotic resistance plasmidome of a microbial population, there must be efficient methods of extracting the plasmid population directly from the sample being examined. However, plasmids make up only a small proportion of the total DNA present in complex samples[5], and the cultivable component of the sample is even smaller. Traditional culture-based methods are less than ideal for working with animal or environmental samples as only a small fraction of these bacteria can be cultured in a laboratory environment[5]. Therefore, a large proportion of the plasmids present in such samples are missed if relying solely on culture-based methods. Additionally, the use of metagenomics-based sequencing methods also has its limitations. The sequencing depth is usually insufficient to extract whole plasmids from the data, assembly is difficult due to the small size of the fragments, and genes present in low abundances are missed[6]. Also, plasmids often contain repeat sequences that are shared with genomic DNA, making assembly from short-read data difficult[7]. Therefore, there

is a need to determine what methods are capable of extracting these resistance plasmids directly from complex samples and which will provide a wide representation of the antibiotic resistance plasmid population present in the microbial environment.

In this study, we examined six methods of plasmid extraction and used broiler caecal samples as representatives of complex samples. The gastrointestinal tract of broilers hosts a complex microbial community of hundreds of bacterial species[8]. The plasmid DNA was transformed into *Escherichia coli* and selected on antibiotics to identify resistance plasmids. This allowed us to identify antibiotic resistance that could be expressed in a human pathogen, and further analyse the resistance mechanism in a well-characterised pathogen. There are also other plasmid extraction methods which have not been evaluated in this study but show good results. For example, Sentchilo *et al.,* (2013)[9] used a CsCl-EB method to isolate a variety of plasmids from activated sludge systems.

At present, there are no commercial kits designed to extract plasmid DNA directly from complex samples. Current plasmid extraction kits are intended to work with pure bacterial culture, which is less than ideal when dealing with complex environmental samples. Kits which are devised for use with complex samples such as soil or faeces target only genomic DNA. Alkaline lysis[10] is a widely used method for the extraction of plasmid DNA by separating it from chromosomal DNA based on the small size and supercoiled nature of plasmids. However, it is also only intended for use with bacterial culture, not with complex samples which contains other material as well as bacteria.

Exogenous plasmid isolation works by capturing the plasmids directly from the complex sample in biparental matings using a recipient bacteria. While this method allows for plasmids to be obtained directly from the sample, it relies strongly on the plasmid being stably maintained in the host, and on the conjugative ability of the plasmids present in the sample. Therefore, this method may give a misrepresentation of the total plasmids present in the sample, as the non-conjugative fraction may not be extracted with this method[11]. However, plasmids can become mobilised by a self-transmissible plasmid[12], and could therefore also be captured by this method. Additionally, the exogenous method can in general also isolate linear plasmids, which are frequently found in diverse microbial environments[11].

The Transposon-aided capture (TRACA) of plasmids allows for the acquirement of antibiotic resistance plasmids from complex samples[13]. It works by removing any contaminating chromosomal DNA from a total DNA sample, and then inserting a transposon onto the plasmids with a known selectable marker. Linear plasmids may not be captured by this method, as the *Tn5* origin of replication is not capable of replicating their extreme termini, and they could be degraded by the exonuclease unless specialised enzymes are used[14]. The main advantage of this method is that it has the capability to capture plasmids that do not have a selectable marker for *E. coli* and may not have the ability to replicate. It has been noted that this method favours the isolation of small plasmids, so it may give a misrepresentation of the total plasmid population[15].

The multiple displacement amplification method works by removing all sheared genomic DNA from a total DNA sample with plasmid-safe DNase. The remaining circular plasmid DNA is amplified by phi29 DNA polymerase, which has a rolling-circle mechanism. In short, by using random hexamers, phi29 allows for the unspecific amplification of the circular plasmid DNA present. The benefit of this method is that even when plasmids are present in very small numbers compared to the total DNA in the sample, this method allows for the generation of large amounts of plasmid DNA[5]. Similarly, this method also favours the selection of small plasmids[15], and like TRACA, disregards linear plasmids, some of which could be degraded by DNase treatment[11]. It should also be noted that large plasmids could be sheared during the extraction, by which they may also be degraded by the exonuclease treatment. Norman *et al.*, (2014)[16] described an electroelution step which could be applied prior to amplification to attempt to increase the number of large-sized plasmids obtained.

Our study compared these methods to identify which extracted the largest variety of antibiotic resistance plasmids (based on the banding patterns and resistance profiles of transformants or transconconjugants) present in the complex broiler caecal samples. We found that the exogenous isolation method best met these criteria, in both a time-efficient and consistent manner. While this method does not remove all bias, it does allow for the acquirement of antibiotic resistance plasmids which can be further phenotypically tested.

2.3 MATERIALS & METHODS

2.3.1 Samples

The broiler caecal samples were collected from a commercial poultry production unit in the United Kingdom. Samples were lyophilised and stored at -80°C. Each of the plasmid extraction methods were carried out with the same caecal sample (Sample A). All methods were also carried out with *Escherichia coli* NCTC 13400 containing plasmid pEK499 as a control.

2.3.2 Plasmid Extractions and Identifications

2.3.2.1 Culture Dependent Method

Caecal sample (0.01 g) was mixed with 0.1 mL of 0.85% NaCl. The 0.1 mL mix was spread on a Muller-Hinton (Merck) agar plate and incubated overnight at 37°C. All bacterial growth on the plate was scraped off, inoculated into 6 mL of non-selective Muller-Hinton broth and incubated overnight at 37°C with shaking at 225 rpm. Plasmid DNA was extracted from this bacterial culture using the NucleoSpin Plasmid Kit (Macherey-Nagel, Germany) according to the manufacturer's guidelines. The resulting DNA samples were visualised on a 1% agarose gel stained with 1X GelRed (Biotium) and run at 70 volts for 60 minutes.

2.3.2.2 Commercial DNA Extraction Kits

A. DNA was extracted from 0.05 g of caecal sample using the Mobio PowerSoil DNA Extraction Kit (now Qiagen), according to the manufacturer's guidelines.

B. DNA was extracted from 0.01 g of caecal sample using the Qiagen Plasmid Mini Kit, according to the manufacturer's guidelines.

C. DNA was extracted from 0.01 g of caecal sample using the Macherey-Nagel NucleoSpin Plasmid Kit, according to the manufacturer's guidelines.

Extracted DNA was visualised on a 1% agarose gel stained with 1X GelRed and run at 70 volts for 60 minutes. The DNA was electroporated into *E. coli* DH5α, selected on ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) or ciprofloxacin (4 mg/L), and incubated at 37°C overnight. Plasmid DNA was extracted from the transformants using the Machery-Nagel NucleoSpin Plasmid kit and digested with *EcoRI* restriction enzyme. Plasmids were visualised on a 1% agarose gel stained with 1X GelRed. Antibiotic susceptibility testing *via* the disk diffusion method was carried out on transformants according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2016)[17].

2.3.2.3 Alkaline Lysis Method

Plasmid DNA was extracted using an alkaline lysis method[10]. The caecal sample (0.03 g) was resuspended in 100 µL ice-cold resuspension buffer (50 mM glucose, 25 mM TrisCl (pH 8.0), 10 mM EDTA (pH 8.0)). Bacterial cells were lysed with 200 µL lysis

solution (0.2 N NaOH, 1% (wt/vol) sodium dodecyl sulfate (SDS)) for 4 minutes and neutralised with 150 µL of chilled 3 M potassium acetate, pH 4.8. The samples were centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant containing the plasmid was mixed with an equal volume of isopropanol and incubated at -20°C for 15 minutes. Samples were centrifuged at 14000 rpm for 30 minutes at 25°C. The supernatant was removed and 500 µL of 70% ethanol was added to the pellet and centrifuged at 14000 rpm for 5 minutes at 25°C. The pellet was resuspended in 50 µL MilliQ water. Extracted DNA was visualised on a 1% agarose gel stained with 1X GelRed and run at 70 volts for 60 minutes. The DNA was electroporated into *E. coli* DH5α, selected on ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) or ciprofloxacin (4 mg/L), and incubated at 37°C overnight. Plasmid DNA was extracted from the transformants using the Machery-Nagel NucleoSpin Plasmid kit and digested with *EcoRI* restriction enzyme. Plasmids were visualised on a 1% agarose gel stained with 1X GelRed run at 70 volts for 60 minutes. Antibiotic susceptibility testing *via* the disk diffusion method was carried out on transformants according to CLSI guidelines[17].

2.3.2.4 Exogenous Plasmid Isolation

Plasmid DNA was exogenously isolated in biparental matings[18]. Caecal sample (0.01 g) was added to 0.9 mL of non-selective Tryptic Soy Broth (TSB) (Sigma Aldrich) and incubated at 20°C with shaking at 50 rpm overnight. The supernatant containing the bacterial fraction (0.8 mL) was centrifuged at 2800 ×g for 10 mins at room temperature (RT). The pellet was resuspended in 80 µL of TSB. This comprised the donor culture. A culture of rifampicin resistant *E. coli* DH5α was grown overnight at

28°C and shaking at 180 rpm. The bacterial content was pelleted by centrifugation at 2800 ×g for 5 minutes at RT, washed in 140 µL LB broth (Duchefa-Biochemie) and resuspended in 140 µL LB broth. This comprises the recipient culture. Donor and recipient culture (50 µl each) were mixed and centrifuged at 2800 ×g for 5 mins at RT. The supernatant was removed and the pellet resuspended in 50 µl of TSB. This was applied to a 0.2 µm filter on an LB agar plate and incubated at 28°C for 20 hours. The filter was removed from the plate and the cells resuspended in 0.85% NaCl by vortexing. A volume of 100 µL was plated on Eosin Methylene Blue (EMB) agar (Sigma) with rifampicin (100 mg/L) and one of the following antibiotics: ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) or ciprofloxacin (4 mg/L). Plates were incubated at 28°C for 1-2 days until transconjugant colonies appeared. Plasmids were extracted from each of the colonies using the Macherey-Nagel NucleoSpin Plasmid Kit and digested with *EcoRI* restriction enzyme. Plasmids were visualised on a 1% agarose gel stained with 1X GelRed run at 70 volts for 60 minutes. Antibiotic susceptibility testing *via* the disk diffusion method was carried out on transconjugants according to CLSI guidelines[17].

2.3.2.5 Transposon-Aided Capture of Plasmids (TRACA)

Transposon-Aided Capture of plasmids (TRACA) was carried out as previously described[13]. TRACA is based on the insertion of a transposon with a known origin of replication and antibiotic resistance marker into the plasmids, which can then subsequently be "captured". Bacterial cells were separated from the caecal samples by adding 0.1 g of caecal sample to 0.9 mL of non-selective TSB and incubating at 20°C and 50 rpm overnight. The supernatant (0.8 mL) was centrifuged at 2800 ×g for 10

mins at RT and DNA was extracted by performing alkaline lysis (as previously described) on the pellet.

The removal of sheared chromosomal DNA prior to performing the TRACA reaction ensures that the transposon is only inserted onto plasmid DNA. DNA was treated with Plasmid-Safe DNase (Epicentre), according to the manufacturer's guidelines. Amplification of the 16S rRNA genes by PCR was performed to ensure the ratio of plasmid:chromosomal DNA in the sample was reversed. This was carried out using the following primers[19]:

Forward

5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA

G-3' and Reverse

5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC

TAATCC-3'; and under the following conditions: 95°C for 3 minutes; 35 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and finally 72°C for 5 minutes.

TRACA was performed using the EZ-*Tn5* <R6Kγori/KAN-2> Insertion Kit (Epicentre), according to the manufacturer's guidelines. The 50 µL reaction was diluted with 450 µL sterile water and purified with Vivaspin 500 MWCO 100,000

Protein Concentrator Spin Columns (GE Healthcare Life Sciences) which reduced the reaction volume to 10 µL. 5 µL was electroporated at 1.8 kV into 100 µL TransforMax EC100D pir-116 Electrocompetent *E. coli* (Epicentre). The transformed cells were spread onto LB agar plates with 50 mg/L kanamycin to select for EZ-*Tn5*. Plasmid DNA was extracted from TRACA clones using the Qiagen HiSpeed Plasmid Midi kit and visualised on a 1% agarose gel stained with 1X GelRed, run at 70 volts for 60 minutes. Bands of plasmid DNA (B1 and B2) were harvested from a 1% agarose gel stained with SYBR Safe using the Cleaver Scientific runVIEW system run at the same conditions as before. The harvested DNA bands were electroporated into *E. coli* DH5α, selected on ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) or ciprofloxacin (4 mg/L), and incubated at 37°C overnight. Transformants were obtained on ampicillin, tetracycline and ciprofloxacin with DNA from band 2 and on ciprofloxacin with DNA from band 1. Plasmid DNA was extracted from the transformants using the Machery-Nagel NucleoSpin Plasmid kit and digested with *EcoRI* restriction enzyme. Plasmids were visualised on a 1% agarose gel stained with 1X GelRed. Antibiotic susceptibility testing *via* the disk diffusion method was carried out on transformants according to CLSI guidelines[17].

2.3.2.6 Multiple Displacement Amplification

The multiple displacement amplification method utilises the rolling circle amplification mechanism of phi29 DNA polymerase to obtain large amounts of plasmid DNA from a complex sample. Plasmid DNA was extracted from the caecal sample by following protocol B from Kav *et al.*, (2013)[5], which was adapted from Hansen & Olsen (1978)[20]. The caecal sample (0.225 g) was resuspended in 8.1 mL of

25% sucrose, 50 mM Tris (pH 8). Lysozyme (10 mg/ml in 250 mM Tris (pH 8)) (0.6 mL) was added and the reaction was incubated on ice for 5 minutes. Ethylenediaminetetraacetic acid (EDTA) (3 ml of 250 mM, pH 8) was added and incubated on ice for 5 minutes. Sodium dodecyl sulfate (SDS) (6 mL of 10%) was added and mixed by inversion. Samples were incubated for eight cycles of heat pulsing and mixing (15 seconds at 55°C, 15 seconds at RT). NaOH (3 mL of 3 M) was added and mixed by inversion for 3 minutes. Tris (6 mL of 2 M, pH 7.0) was added and mixed by inversion. SDS (7.92 mL of 10%) was added, followed immediately by 7.5 mL of 5 M NaCl. Samples were incubated at 4°C overnight. Samples were centrifuged at 3000 ×g for 30 minutes at 4°C and the supernatant transferred to a new tube. 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropanol were added and samples incubated overnight at 4°C.

As with TRACA, sheared chromosomal DNA was removed with plasmid-safe DNase prior to amplification to ensure only circular plasmid DNA was amplified. Removal of chromosomal DNA and amplification of plasmid DNA was carried out as described previously by Kav *et al.*, (2013)[5]. A 50 µL reaction composed of 20 µL DNA, 24 µL MilliQ water, 1 µL ATP, 2.5 µL reaction buffer and 2.5 µL plasmid-safe DNase was incubated at 37°C overnight and deactivated at 70°C for 30 minutes. Amplification of the 16S rRNA genes by PCR as previously described was performed to ensure the ratio of plasmid:chromosomal DNA was reversed in the sample, *i.e.* high plasmid to low chromosomal DNA ratio. If bands were visible the assay was repeated. 0.1 volumes of 3 M sodium acetate (pH 5.2) and 0.6 volumes of isopropanol were added and incubated overnight at 4°C. Samples were centrifuged at 14000 rpm 4°C for 30 min. The supernatant was removed and 70% ethanol added. Samples were mixed and

centrifuged at 14000 rpm 4°C for 15 min. The supernatant was removed and the pellet resuspended in 10 µl MilliQ water.

Plasmid DNA was amplified by adding 1 µl of 10 µM Exo-Resistant Random Primer (Thermo Scientific), 2 µl phi29 DNA Polymerase Reaction Buffer (New England Biolabs) and 8.2 µl of MilliQ water to 5 µl of the purified treated DNA. Samples were incubated at 95°C for 5 min and immediately chilled on ice for 5 min. 1.6 µl phi29 DNA polymerase (New England Biolabs), 0.02 µl of inorganic pyrophosphatase (from yeast) (New England Biolabs) and 2 µl of dNTPs (10 mM) (Thermo Scientific) were added and incubated at 30°C for 16 hours.

Amplified plasmid DNA (5 µL) was electroporated at 1.8 kV into 15 µL of *E. coli* DH5α cells. Transformants were plated on LB agar plates with one of the following antibiotics: ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), cefotaxime (16 mg/L), colistin (16 mg/L) or ciprofloxacin (16 mg/L). Plasmids were extracted using the Qiagen HiSpeed Midi kit and digested with *EcoRI* restriction enzyme. Plasmids were visualised on a 1% agarose gel stained with 1X GelRed run at 70 volts for 1 hour. Antibiotic susceptibility testing *via* the disk diffusion method was carried out on transformants according to CLSI guidelines[17].

2.4 RESULTS

2.4.1 Culture Dependent Method

All cultivable bacteria grew on a non-selective rich medium and the DNA was extracted using a commercial plasmid extraction kit. Several bands were visible on an agarose gel (Fig 1), however when transformation was carried out it failed to yield any transformants on antibiotic plates. This could indicate that the plasmids present in the cultivable fraction did not harbour any resistance genes to the antibiotics tested. The plasmid pEK499 in *E. coli* was used as a pure bacterial culture control, and was successfully extracted using this method (Fig S1).

2.4.2 Commercial DNA Extraction Kits

The MoBio kit resulted in a single band of DNA located near the top of the agarose gel (Fig 2). Initially we thought that this band was genomic DNA or large plasmids. However, as no transformants were obtained after electroporation on any antibiotic plates (ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) and ciprofloxacin (4 mg/L)) we concluded that this was genomic DNA. We also used this kit with 5 ml of *E. coli* culture harbouring our control plasmid pEK499, which resulted in a very bright band (Fig S2). It appears that as the DNA is at such a high concentration, and pEK499 is a large plasmid which diffuses slowly through the agarose gel, it is likely present along with genomic DNA.

The Qiagen Plasmid Mini kit and the Machery-Nagel NucleoSpin Plasmid kit are both designed for the extraction of plasmids from bacterial culture. Both kits work well for this purpose, which can be seen in Fig S2, where they both extracted our control plasmid pEK499 from *E. coli* culture. However, when we used these kits with our caecal sample, we did not obtain clear bands of plasmid DNA. The NucleoSpin kit resulted in a smear on the gel (Fig 2), and yielded transformants on ciprofloxacin and tetracycline selective plates only. After subjecting these tranformants to a further plasmid extraction, digestion and antibiotic susceptibility testing, they had the same banding pattern and resistance profile. The Qiagen plasmid kit did not appear to retrieve any DNA from our samples (Fig 2) and did not yield any transformants on any antibiotic selective plates (ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) and ciprofloxacin (4 mg/L)).

Plasmid DNA extracted from the caecal sample using the Macherey-Nagel NucleoSpin Plasmid kit was transformed into *E. coli* DH5α. Transformants grew on ciprofloxacin (4 mg/L) and tetracycline (16 mg/L) plates only (Fig 3). Antibiotic susceptibility testing *via* a disk diffusion assay provided the resistance profile of the resulting transformants (Table 1).

2.4.3 Alkaline Lysis Method

A smear of DNA on a gel was detected after performing alkaline lysis directly on the caecal sample (Fig 4). This was the best result, even after reducing the amount of sample used (0.03 g), adding additional bead beating steps at varying time lengths,

and the addition of varying concentrations of RNase A, proteinase K and lysozyme at different time points and incubation temperatures. We obtained transformants on ciprofloxacin (4 mg/L), which had similar banding patterns (Fig 5) and resistance profiles to the transformants selected on ciprofloxacin obtained with the NucleoSpin kit. The extracted DNA was electroporated into *E. coli* DH5α, selected on ciprofloxacin (4 mg/L), extracted from the transformants and digested with *Eco*RI (Fig 5). The method was repeated with control plasmid pEK499 (Fig S3).

2.4.4 Exogenous Plasmid Isolation

The exogenous plasmids were obtained by the recipient in biparental matings, and selected on ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) and ciprofloxacin (4 mg/L). Transformants were isolated from the plates containing ampicillin, tetracycline (with two colony morphologies; big colonies (BC) and small colonies (SC)) and kanamycin. This method isolated plasmids obtained from the caecal sample and control plasmid pEK499 (Fig 6; Fig S4).

2.4.5 Transposon-Aided Capture of Plasmids (TRACA)

TRACA allowed for the acquisition of plasmids from the caecal samples by inserting a transposon with a selectable resistance marker and transforming the DNA into *E. coli*. The two largest bands of plasmid DNA were extracted directly from the gel (Fig 7) (B1- lower band; B2- higher band) and electroporated into *E. coli*. Transformants were selected on ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L), colistin (16 mg/L) and ciprofloxacin (4 mg/L), with ampicillin, tetracycline and

ciprofloxacin plates yielding transformants (Fig 8). We found that all but one of the transformants tested had a similar banding pattern and resistance profile to the plasmids extracted using the alkaline lysis and NucleoSpin kit.

2.4.6 Multiple Displacement Amplification

The multiple displacement amplification method allows for unspecific but selective amplification of circular DNA after DNase digestion (Fig 9), through which we acquired antibiotic resistance plasmids from a caecal sample. This method gave us the largest range of plasmids from our caecal samples. That is, the greatest number of antibiotic plates (ampicillin (32 mg/L), tetracycline (16 mg/L), kanamycin (25 mg/L) and ciprofloxacin (16 mg/L)) which yielded transformants and each antibiotic plate transformants had a different banding pattern after digestion with *EcoRI* (Fig 10) and resistance profile (Table 1). However, the results shown are from a different caecal sample (Sample B) as the method was unsuccessful for sample A.

Antibiotic susceptibility testing *via* a disk diffusion method gave the resistance profile of the plasmids (Table 1). This, along with the banding patterns of the digested plasmids on agarose gels, allowed for the identification of the variety of plasmids obtained from each extraction method. It also allowed for a comparison of the plasmids acquired using the different methods from the same sample, to determine if the same or different plasmids were obtained. There was no single antibiotic that selected for transformants using all methods. However, plasmids with identical antibiotic resistance patterns were identified using the different methods.

Transformants isolated using the exogenous method had four different antibiotic susceptibility patterns, suggesting the presence of at least four different plasmids. The exogenous transformants had the widest range of resistance, with three transformants resistant to four different classes of antibiotics. T_B1_Cip transformant had the same resistance profile as M_Kan and M_Cip selected transformants. Based on visual analysis of the banding patterns (Fig 11) and antimicrobial susceptibility patterns (Table 2) combined, the plasmids identified in MN_Cip, MN_Tet, A_Cip, T_B2_Cip and T_B2_Tet are probably the same plasmids or highly similar. Further analysis methods, such as sequencing, are required to confirm that these plasmids are identical. The remaining transformants had unique resistance profiles.

2.5 DISCUSSION

Plasmids isolated from complex samples have previously been examined using methods such as gradient gel resolution of PCR products, quantification of incompatibility groups using qPCR[21] or Southern blotting[22]. Recently, the study of plasmids involves the extraction of plasmid DNA followed by various sequencing approaches[15]. However, if multiple plasmids are present in a sample or if they are at low copy number, these won't be identified *via* sequencing due to the depth of current metagenomic sequencing technologies. Similarly, assembly is difficult with short-reads, especially if plasmids are present in low copy numbers or if the reads match to genomic DNA[23]. We performed this work to identify a method suitable for the extraction of plasmids harbouring antibiotic resistance genes from complex broiler caecal samples, which could be transformed into a human pathogen, in this case *Escherichia coli*. This would then allow for further analysis, sequencing and assembly of the plasmid in a well-defined bacterium. The variety of resistance plasmids obtained was determined by analysis of the banding patterns (shown in figures) and resistance profiles (shown in tables) of the transformants or transconjugants obtained.

In order to carry out studies on the overall resistance plasmid population present in a complex sample, the method to extract plasmid DNA must be optimised to give as best a representation as possible of the total resistance plasmids present. The first method we performed was a culture-dependent method on non-selective media. Another way of performing a more specific culture-dependent extraction would be to use selective agars. This would assist in the identification of which bacterial species a certain plasmid may have come from. However, this would introduce a bias to the

results, as one would be choosing which agars and, hence, which bacteria to select. The main disadvantage of a culture-dependent method for complex samples is that only a small number of environmental bacteria can currently be cultured in the laboratory[24]. This means that by using only a culture-based method, it would greatly limit the number of plasmids isolated. Therefore, the representation of results from a culture-dependent study would give a limited view of the total resistance plasmidome. Our experiment did not yield any transformants on antibiotic plates, indicating that the plasmids present in the cultivable fraction did not harbour any resistance genes to the antibiotics tested.

The MoBio PowerSoil DNA Isolation kit is specifically designed to extract DNA from complex soil samples. This kit did not extract any plasmids harbouring antibiotic resistance genes from our caecal sample as we failed to obtain any transformants, indicating it is more suitable for studies analysing chromosomal DNA or fragmented DNA, rather than intact plasmid DNA. The Qiagen Plasmid Mini kit and the Machery-Nagel NucleoSpin Plasmid kit are both designed for the extraction of plasmids from bacterial culture. It seems that the caecal samples are too complex for these kits. The sample blocked the spin columns used in these kits, therefore little to no DNA was retrieved. Minimal success resulted with these commercial plasmid extraction kits, as few antibiotic resistance plasmids were obtained. It thus appears that these kits were not capable of dealing with the complexities associated with our samples.

The alkaline lysis method[10] is a common method of plasmid extraction, on which most commercial plasmid extraction kits are based. A benefit of using this method is that

the chemicals used and their concentrations in the solutions can be decided upon and adapted for individual needs. There are standard protocols available for constituting resuspension, lysis and neutralisation buffers, but, for example, additional enzymes can be added to the buffers. This type of adaptation is difficult with commercial kits as most do not share the components of their buffers. This is also why the alkaline lysis method and two commercial plasmid extraction kits were tested in this study, as they cannot be directly compared. However, this method still yielded few antibiotic resistance plasmids, even after additional modifications (as mentioned in methods section) to the protocol.

The exogenous method of plasmid isolation is based on the capture of conjugative plasmids directly from a complex sample, *via* a recipient bacteria in biparental matings[18]. This method captured resistance plasmids with different resistance profiles and was also the most consistent and not overly time-consuming. The disadvantages of this method are that it relies on the mobility of plasmids in the donor sample and the donor sample comprises an overnight culture of the total bacterial community. Therefore, the non-mobile plasmids present in the sample may not be captured with this method and the bacteria not capable of growth at the specific conditions will not be included as donors. However, many resistance plasmids are conjugative, and others can be mobile when assisted by a conjugative plasmid also residing in the same bacterial cell[25]. We are suggesting this method, not as a solution to all plasmid analysis problems, but rather as a first step in optimising the analysis of antibiotic resistance plasmids from complex samples.

TRACA allowed for the acquisition of resistance plasmids from our sample but with similar banding patterns and resistance profiles. Therefore, it seems the expense associated with this method is not justified given the small variety of plasmids captured. Three of the four plasmids isolated using TRACA were also isolated using the alkaline lysis method or the Macherey-Nagel NucleoSpin Plasmid kit.

The multiple displacement amplification method allowed plasmids with the greatest range of resistance profiles to be obtained from our complex caecal samples. However, it should be noted that there were also difficulties and inconsistencies with this method. While good results were achieved using this method on Sample B (shown in results), many difficulties arose while carrying out the method on both Sample A and the control sample. The DNase step can be variable and time consuming, working well after one or two treatments at some times and not working after several more at other times. This also led to further downstream complications, as the more DNase treatments the sample was subjected to, the more salt that was present in the sample. This caused difficulties when performing electroporation, where salt concentration must be low. Therefore, the plasmid DNA isolated from both Sample A and control plasmid pEK499 did not transform into *E. coli*.

Plasmids now encode resistance to almost all classes of antibiotics currently in clinical use[25]. Therefore, the study of plasmids is crucial to fight the battle against antibiotic resistance that we are currently facing. Our comparative study shows the advantages and disadvantages of six methods for the extraction of plasmids harbouring antibiotic resistance genes from complex broiler caecal samples, which can be applied to other

complex environmental samples. This will assist researchers with the selection of the best method to use in their plasmid studies. Different Gram-negative bacteria other than *E. coli* could be used for similar studies and the isolated plasmid DNA could be transformed into a Gram-positive bacterium to further broaden the study. The exogenous plasmid isolation method was the best for obtaining a range of multi-drug resistance plasmids in a realistic timeframe with consistent results. However, even this method only resulted in a small range of resistance plasmids being isolated.

2.6 CONCLUSION

Overall, the multiple displacement amplification method provided the greatest range of resistance plasmids from the investigated caecal samples. However, due to the inconsistencies of the results and the difficulties experienced with this method, it is not the ideal protocol to use when working with a large volume of samples under short deadlines. The commercial kits, alkaline lysis method and TRACA did not provide a wide range of resistance plasmids from our sample compared to the others tested. Therefore, the exogenous plasmid isolation method resulted in the widest range of resistance plasmids with ease of application and consistency across samples. While this method relies on the conjugative ability of the plasmids present, it is both an efficient (plasmids can be obtained in a short time-frame) and effective (a good range of plasmids can be acquired) method which worked with all of the caecal samples tested. Therefore, we recommend the exogenous plasmid isolation method when extracting antibiotic resistance plasmids of clinical relevance from a large number of complex samples.

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Author Contributions: SD: Performed all experiments, data analysis and manuscript preparation; RM: Data analysis and manuscript preparation; FW: Study design, data analysis and manuscript preparation.

Funding: This project was sponsored by a PhD studentship (Sarah Delaney) from Alltech.

Ethics Statement: Ethical approval was not required for this study. The sampling was performed by Alltech from a commercial farm. These sampling techniques were in line with national regulations about animal welfare ethics. All the animals were monitored throughout the study.

Conflict of Interest Statement: Alltech played no role in the study design or interpretation of the results. We declare no conflict of interests in this study.

2.7 TABLES

Table 1. Disk diffusion results of resistant transformants obtained from each of the extraction methods. Red= Resistant, Yellow= Intermediate, Green= Susceptible; determined according to the CLSI guidelines (2016). MN= Machery Nagel kit, A= Alkaline Lysis, E=Exogenous Isolation, T=TRACA, M=MDA. Amp = selected on ampicillin, Tet = selected on tetracycline, Kan = selected on kanamycin, Cip = selected on ciprofloxacin. B1 = Lower band on gel, B2 = Higher band on gel; both extracted and electroporated into *E. coli* DH5α. (BC) And (SC) refer to the two different colony morphologies, big or small colonies, present on the same antibiotic plate.

Sample		Ampicillin	Tetracycline	Kanamycin	Cefotaxime	Nalidixic Acid	Ciprofloxacin	Imipeniem
Commercial Kits (A)	MN_Cip	I	R	I	S	R	R	S
	MN_Tet	I	R	I	S	R	R	S
Alkaline Lysis (A)	A_Cip	I	R	I	S	R	R	S
Exogenous Isolation (A)	E_Amp	R	R	R	R	R	R	S
	E_Tet_BC	R	R	R	S	S	R	S
	E_Tet_SC	R	R	I	S	S	S	S
	E_Kan	R	R	R	I	R	R	S
TRACA (A)	T_B1_Cip	R	R	R	S	R	R	S
	T_B2_Cip	I	R	I	S	R	R	S
	T_B2_Amp	R	S	S	S	I	R	S
	T_B2_Tet	I	R	I	S	R	R	S
MDA (B)	M_Amp_BC	R	R	I	I	R	R	S
	M_Amp_SC	R	R	S	S	R	R	S
	M_Tet_BC	R	R	R	R	R	R	S
	M_Tet_SC	R	R	S	S	S	S	S
	M_Kan	R	R	R	S	R	R	S
	M_Cip	R	R	R	S	R	R	S

Table 2. Transformants with identical resistance profiles from a disk diffusion assay. Along with a similar banding pattern, this indicates the strains are probably harbouring the same plasmids.

Red= Resistant, Yellow= Intermediate, Green= Susceptible; determined according to the CLSI guidelines (2016).

MN= Machery Nagel kit, A= Alkaline Lysis, T=TRACA. Tet = selected on tetracycline, Cip = selected on ciprofloxacin. B2 = Higher band on gel; extracted and electroporated into *E. coli* DH5α.

Sample		Ampicillin	Tetracycline	Kanamycin	Cefotaxime	Nalidixic Acid	Ciprofloxacin	Imipeniem
Commercial Kits (A)	MN_Cip	I	R	I	S	R	R	S
	MN_Tet	I	R	I	S	R	R	S
Alkaline Lysis (A)	A_Cip	I	R	I	S	R	R	S
TRACA (A)	T_B2_Cip	I	R	I	S	R	R	S
	T_B2_Tet	I	R	I	S	R	R	S

2.8 FIGURES

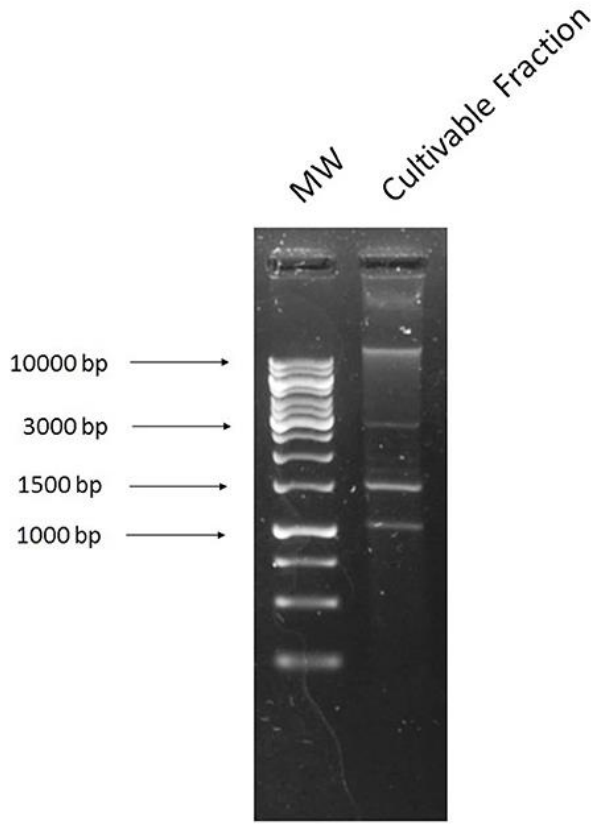

Fig 1. Agarose gel image of the plasmids extracted using the culture-dependent method from the broiler caecal sample, which was grown on non-selective Mueller-Hinton media and extracted with the Macherey-Nagel NucleoSpin Plasmid kit.

1 = 1 kb ladder; **2** = DNA extracted from the cultivable fraction of the caecal sample.

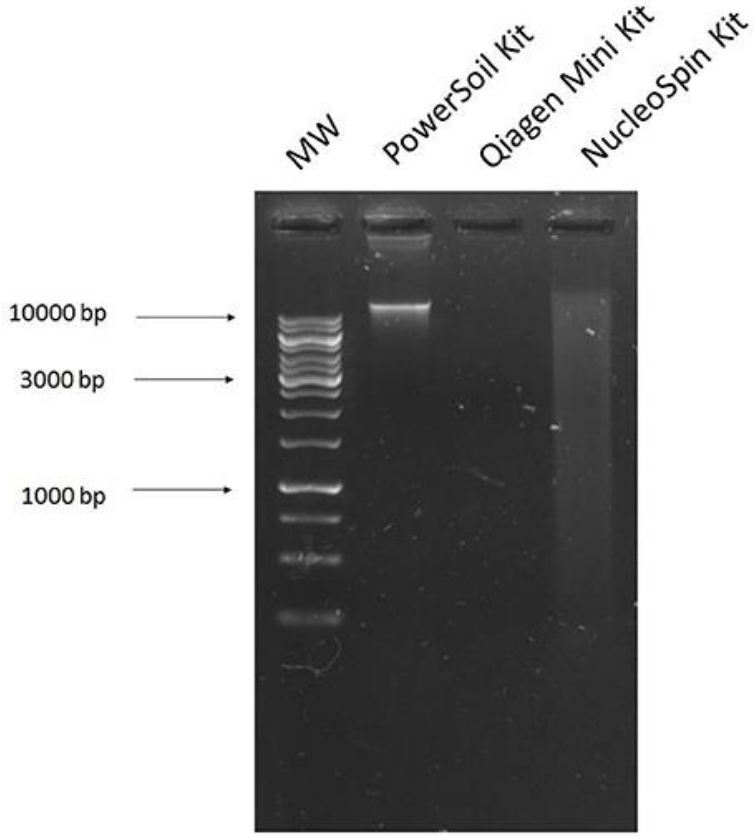

Fig 2. Agarose gel image of DNA extracted from caecal samples using commercial kits.

1= 1 kb ladder; **2** = DNA extracted from the caecal sample using the MoBio PowerSoil DNA Isolation Kit; **3 =** DNA extracted from the caecal sample using the Qiagen Plasmid Mini Kit; **4** = DNA extracted from the caecal sample using the Macherey-Nagel NucleoSpin Plasmid Kit.

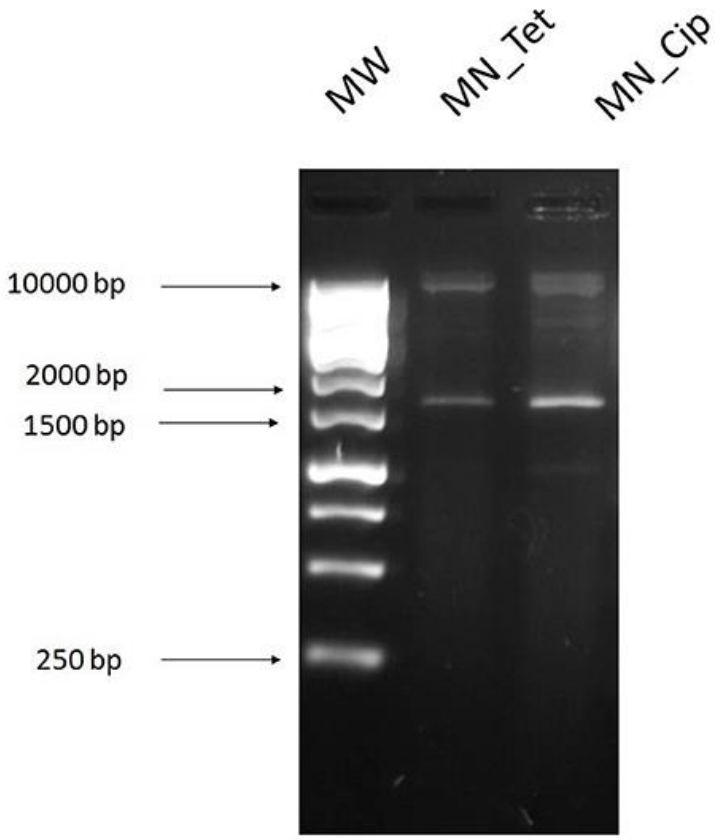

Fig 3. Agarose gel image of digested plasmid DNA extracted from transformants, which were obtained by electroporating the DNA from the direct extraction with the Macherey-Nagel NucleoSpin Plasmid kit into *E. coli* and digested with *Eco*RI restriction enzyme.

1= 1 kb ladder; Digested plasmid DNA extracted from transformants selected on agar plates containing **2** = tetracycline 16 mg/L (MN_Tet), **3** = ciprofloxacin 4 mg/L (MN_Cip).

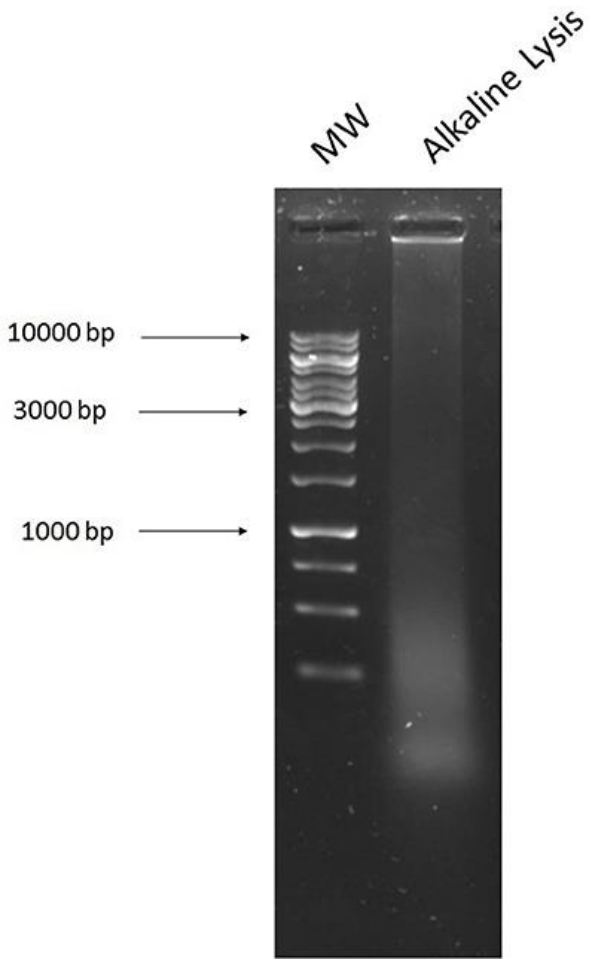

Fig 4. Agarose gel image of DNA extracted from the caecal sample using the alkaline lysis method.

1= 1 kb Ladder; **2**= DNA extracted from the caecal sample using the alkaline lysis method.

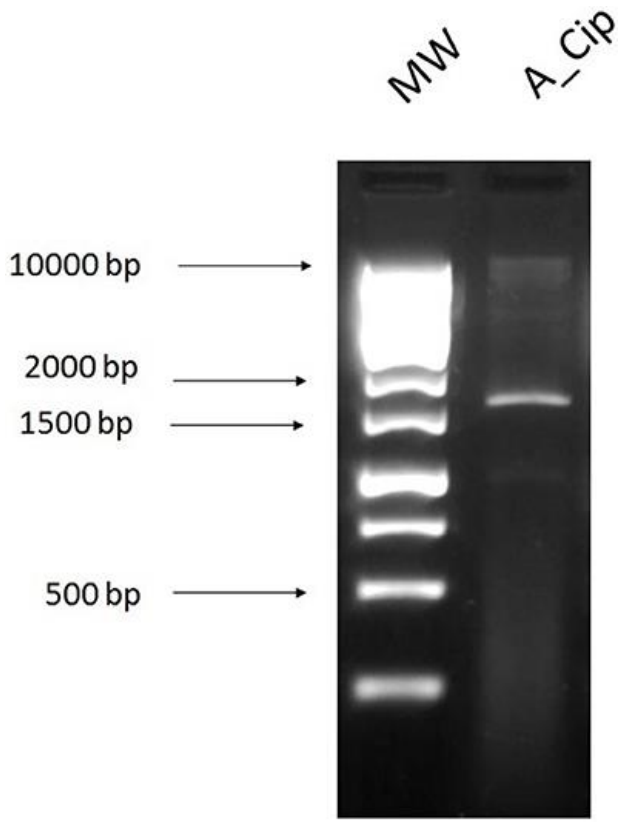

Fig 5. Agarose gel image of digested plasmids which were obtained by transforming *E. coli* with plasmid DNA extracted using the alkaline lysis method and selected on ciprofloxacin 4 mg/L (A_Cip) and digested with *Eco*RI.

1= 1 kb ladder; **2**= digested plasmid DNA extracted from the transformant (alkaline lysis method) and selected on ciprofloxacin 4 mg/L (A_Cip).

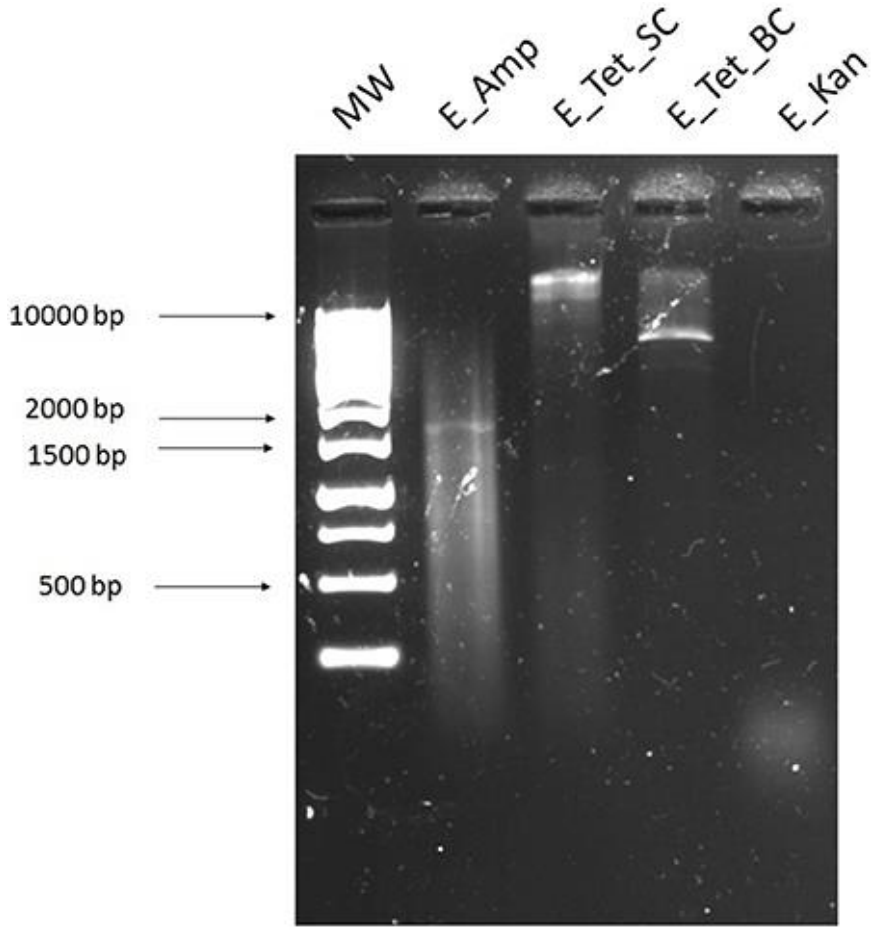

Fig 6. Agarose gel image of exogenously isolated plasmids from the caecal sample digested with *EcoRI* restriction enzyme.

1=1 kb ladder and DNA extracted from the caecal sample using the exogenous plasmid isolation method. Digested plasmid DNA extracted from transformants selected on agar plates containing **2**= ampicillin 32 mg/L (E_Amp); **3**= tetracycline (SC) 16 mg/L (E_Tet_SC); **4**= tetracycline (BC) 16 mg/L (E_Tet_BC); and **5**= kanamycin 25 mg/L (E_Kan). BC and SC refer to the two different colony morphology types, big or small colonies, on the same antibiotic plate.

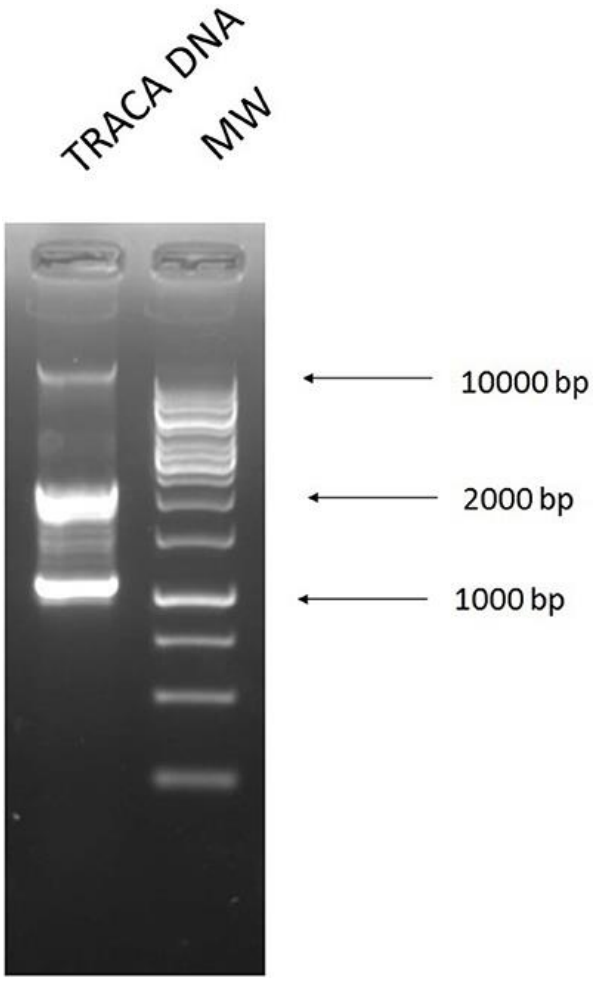

Fig 7. DNA extracted from caecal sample using the TRACA method of plasmid isolation.

1= DNA extracted from transformants selected on kanamycin 50 mg/L after TRACA reaction and **2**= 1 kb ladder.

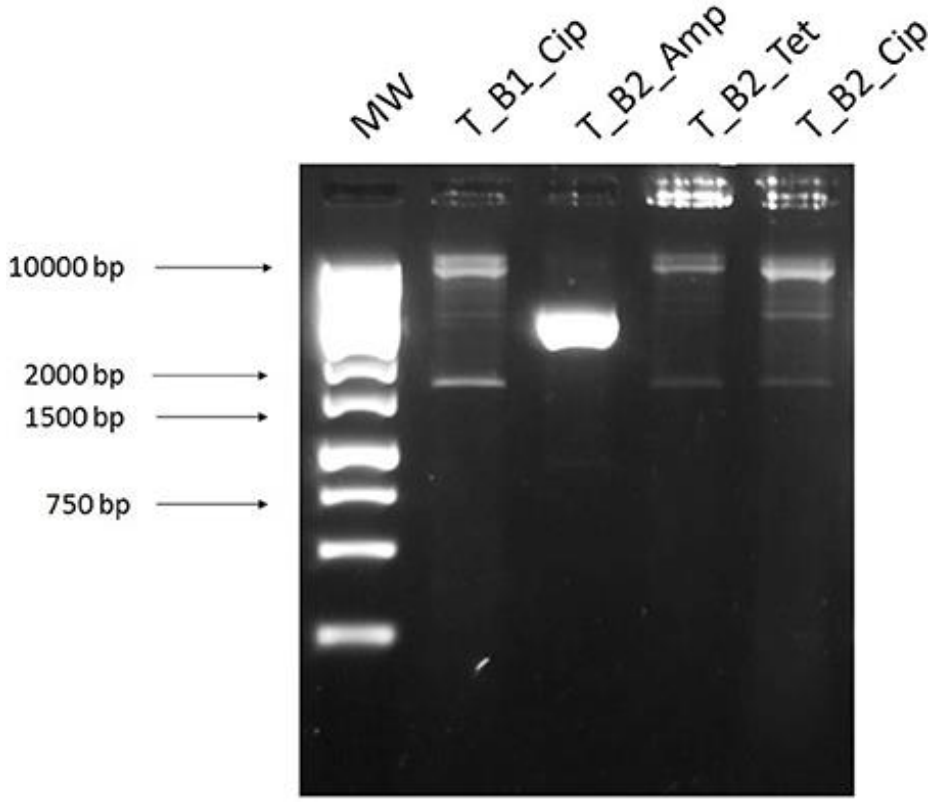

Fig 8. Digested plasmid DNA extracted from *E. coli* transformed with plasmid DNA from TRACA clones and selected on antibiotics.

1= 1 kb ladder and bands of plasmid DNA extracted from a 1% SYBR safe gel and transformed into *E. coli*. Digested plasmid DNA extracted from transformants selected on agar plates containing: **2=** B1 ciprofloxacin 4 mg/L (T_B1_Cip); **3=** B2 ampicillin 32 mg/L (T_B2_Amp); **4**= B2 tetracycline 16 mg/L (T_B2_Tet) and **5**= B2 ciprofloxacin 4 mg/L (T_B2_Cip).

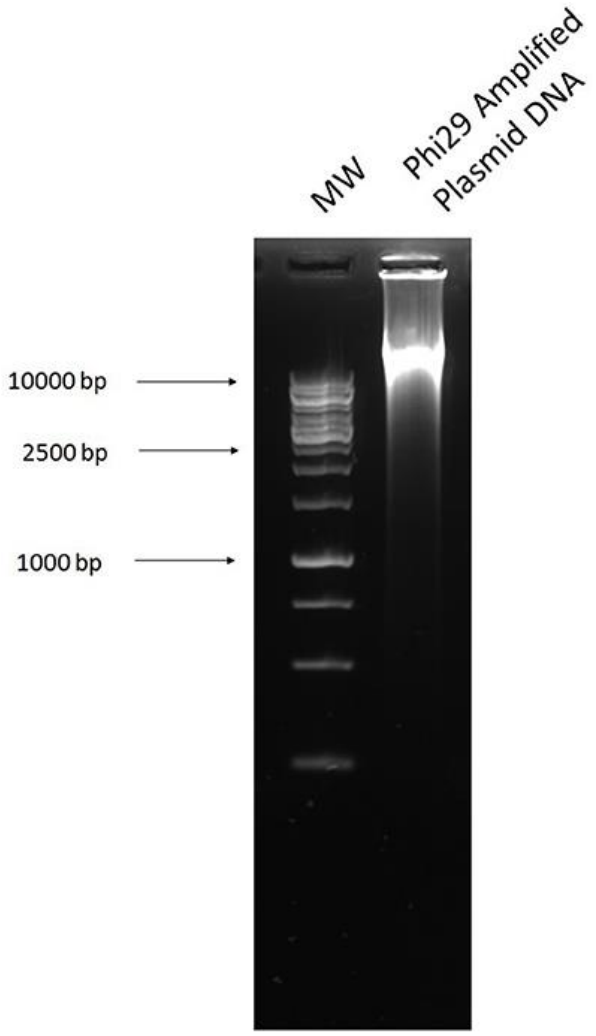

Fig 9. Plasmid DNA from the caecal sample after amplification with phi29 polymerase.

1= 1 kb ladder and **2**= Plasmid DNA amplified with Phi29 DNA polymerase.

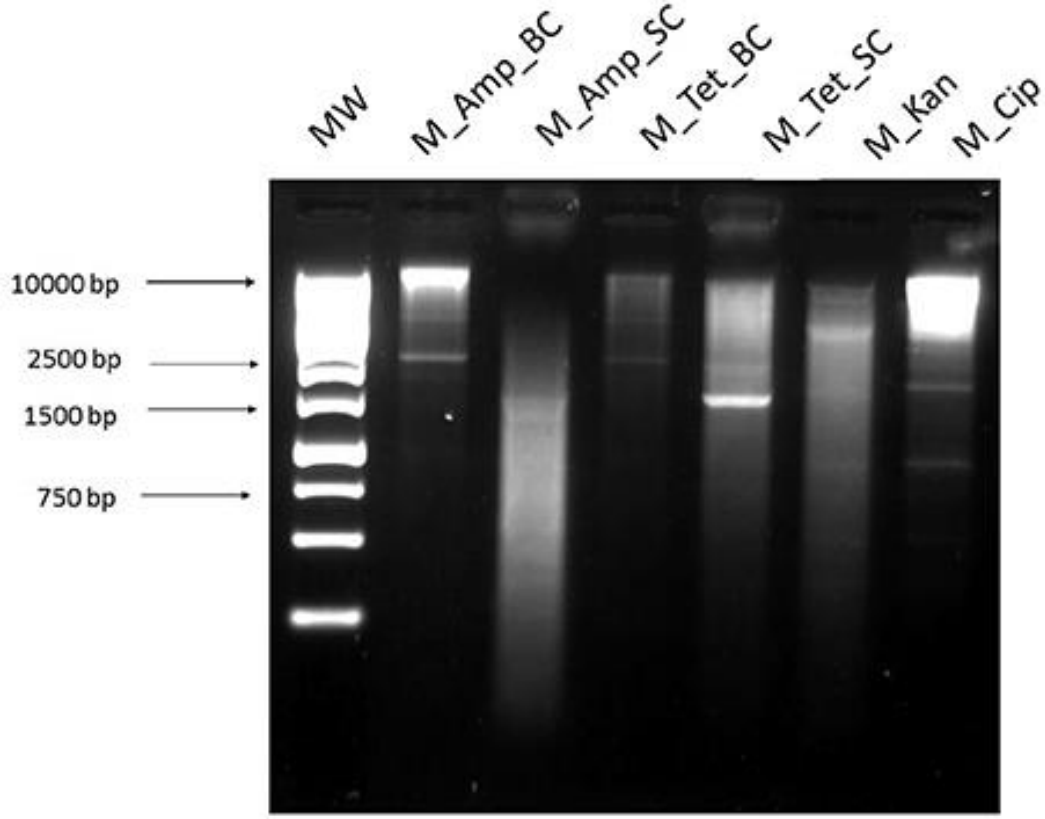

Fig 10. Digested plasmid DNA extracted from *E. coli* transformants after electroporation with the phi29 polymerase amplified DNA.

1= 1 kb ladder; Plasmid DNA extracted from transformants selected on agar plates containing: **2=** ampicillin 32 mg/L (M_Amp_BC); **3=** ampicillin 32 mg/L (M_Amp_SC); **4=** tetracycline 16 mg/L (M_Tet_BC); **5=** tetracycline 16 mg/L (M_Tet_SC); **6=** kanamycin 25 mg/L (M_Kan); **7=** ciprofloxacin 16 mg/L (M_Cip). BC and SC refer to the two different colony morphology types, big or small colonies, on the same antibiotic plate. There were no transformants on colistin or cefotaxime selective plates.

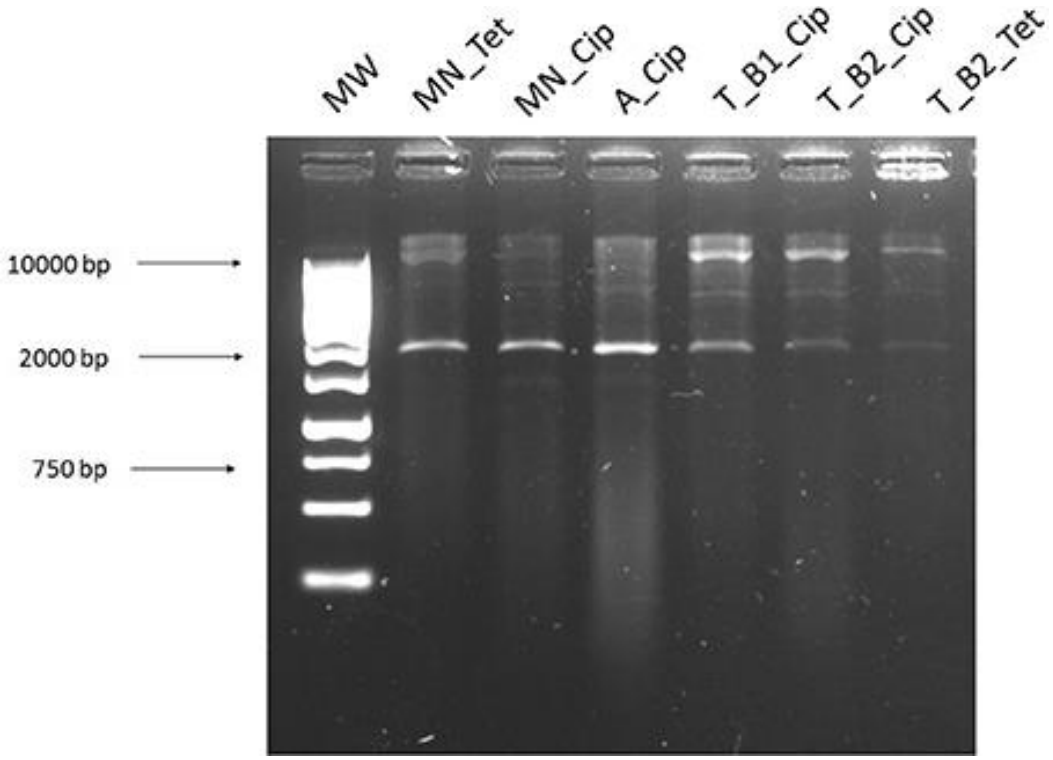

Fig 11. Transformants from different isolation methods with identical banding patterns after plasmid digestion. Along with similar resistance profiles, this indicates the strains are probably harbouring the same plasmid. **1=** 1 Kb Ladder; **2=** MN_Tet; **3=** MN_Cip; **4=** A_Cip; **5**= T_B1_Cip; **6=** T_B2_Cip; **7=** T_B2_Tet.

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2.10 SUPPLEMENTARY DATA

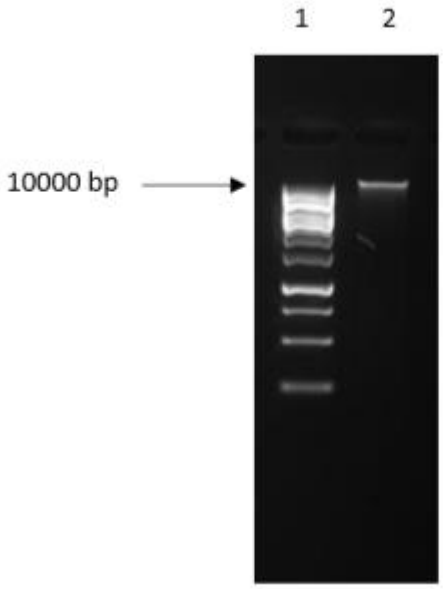

Fig S1. Agarose gel image of the pEK499 plasmid extracted from the cultured *E. coli* using the culture dependent method.

1= 1 Kb ladder; **2**= DNA extracted from *E.coli* harbouring the pEK499 plasmid.

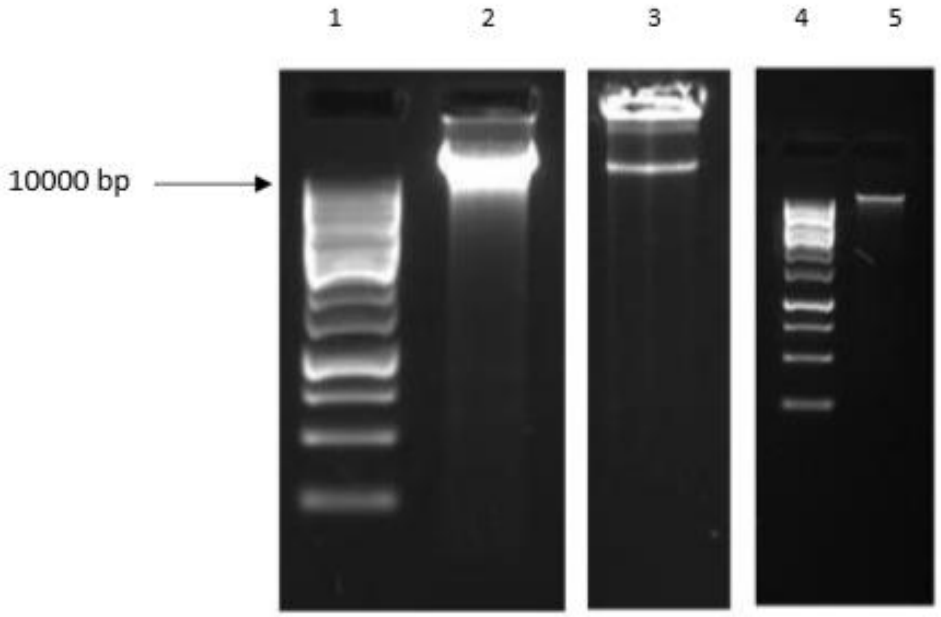

Fig S2. Agarose gel image of the control plasmid pEK499 extracted using commercial kits.

1= 1 Kb ladder; **2**= control plasmid pEK499 extracted using the MoBio PowerSoil DNA Isolation kit; **3**= control plasmid pEK499 extracted using the Qiagen Plasmid Mini kit; **4**= 1 Kb ladder; **5**= control plasmid pEK499 extracted using the Macherey-Nagel NucleoSpin Plasmid kit.

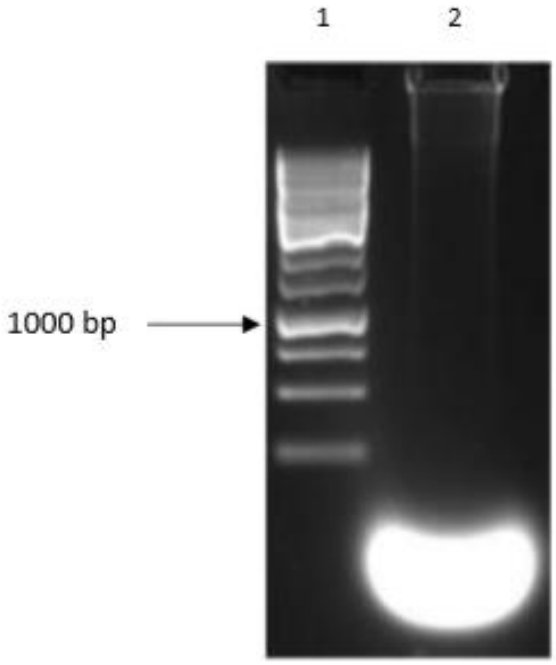

Fig S3. Agarose gel image of pEK499 extracted using the alkaline lysis method.

1= 1 Kb ladder; **2**= control plasmid pEK499 extracted using the alkaline lysis method.

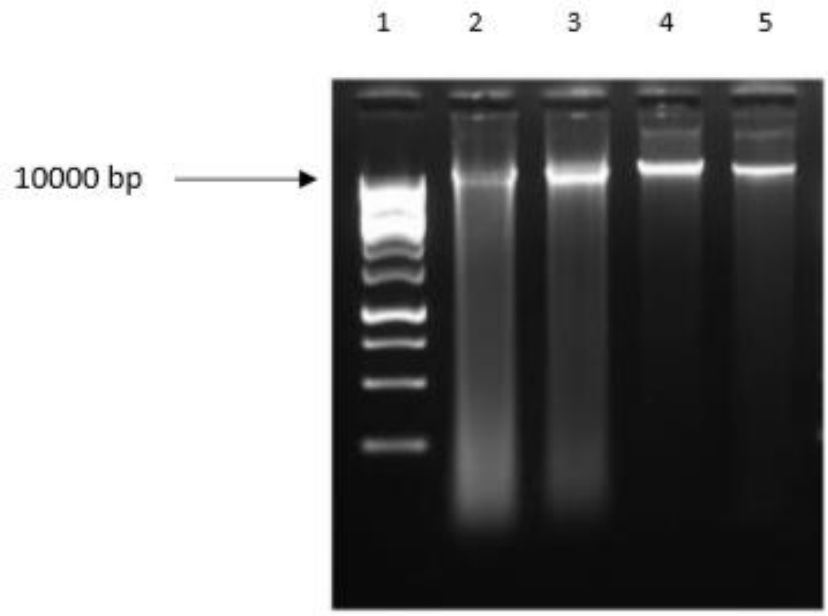

Fig S4. Agarose gel image of pEK499 isolated using the exogenous method.

1= 1 Kb ladder; Control plasmid pEK499 extracted using the exogenous plasmid isolation method- plasmid DNA extracted from transformants and selected on agar plates containing: **2**= ampicillin 32 mg/L; **3**= tetracycline 16 mg/L; **4**= kanamycin 25 mg/L; and **5**= ciprofloxacin 4 mg/L.

Chapter 3

Characterisation of multi-drug resistant plasmids isolated from the caecum of broiler chickens

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3.1 ABSTRACT

Plasmids are well-known for their involvement in increasing the genetic diversity and adaptability of prokaryotes. This is through their ability to replicate independently of the chromosome and their capacity to self-transfer. This has also contributed to the rapid development and spread of antibiotic resistance. We isolated five antibiotic resistance plasmids from the caecum of broiler chickens using the multiple displacement amplification method. These plasmids were sequenced using Oxford Nanopore MinION technology. The plasmid sizes ranged from 42,654 bp to 151,806 bp. All of the plasmids carried antibiotic resistance genes, while three possessed conjugative machinery. The plasmids were highly similar to other plasmids isolated worldwide, from chicken, pig and human samples. This highlights the importance of the 'One Health' initiative, and the interlinking spread and dissemination of antibiotic resistance between humans and animals.

3.2 INTRODUCTION

Antibiotic resistance poses a serious threat to the health of humans, animals, and the environment worldwide. The development and spread of antibiotic resistance has been attributed to certain factors including the excessive use of antibiotics both in humans and animals, the availability of antibiotics over-the-counter in certain countries, release into the environment of non-metabolized antibiotics or their residues through manure, poor sanitation and increased international travel[1]. Bacteria acquire resistance to antibiotics *via* chromosomal mutations or through the acquisition of mobile genetic elements such as plasmids[2].

Plasmids are key drivers in the spread of antibiotic resistance. Not only do they have the ability to obtain and maintain resistance genes, they also have the ability to spread to other bacteria. The majority of plasmids have the capability to replicate within the species of at least one genus, and are therefore readily disseminated between species of that genus. However, broad-host-range plasmids boast the ability to replicate within the species of many genera[3]. Accessory genes, such as antibiotic resistance genes, are frequently associated with small mobile elements such as transposons, which facilitates intracellular mobilisation amongst plasmids[4].

The widespread use of antibiotics in agricultural settings as therapeutics, prophylaxis, metaphylaxis and growth promotion has created a selective pressure and driven the increase of resistant bacteria present in food-producing animals[5]. Antibiotic resistant pathogens often lead to treatment failure in the animals, leading to economic losses.

However, they could also be regarded as a source of resistant bacteria that may represent a risk to human health[6]. Several studies have documented the animal-to-human spread of antibiotic resistance. This is through direct or indirect contact with animals, contaminated food and water, or manure application[7]. Previous work has linked the consumption of food harbouring resistant bacteria with antibiotic resistant infections in humans[8]. Jensen *et al.,* identified the *satA* gene, which confers resistance to streptogramin- a treatment for vancomycin resistant *Enterococcus faecium*, in both human and animal *E. faecium* isolates[9]. Ho *et al.,* found that the *aacC2* gene in isolates from food-producing animals in Hong Kong was also present in urinary tract infection isolates[10]. Bertrand *et al*. tracked the $bla_{CTX\text{-}M\text{-}2}$ gene in *Salmonella enterica* from poultry flocks, to poultry meat, through to human isolates in Belgium[11]. In animals, it is the resistant zoonotic enteropathogens including *Salmonella enterica* and commensals such as *Escherichia coli* that are most likely to be transferred through the food chain to humans[12]. New advances in molecular technologies have allowed for further investigations into the epidemiology of such transfer events[13].

Sequencing of plasmid DNA has previously been a rather troublesome and unsuccessful endeavour. The small fragments of DNA and repeat regions of DNA characteristic of plasmids have led to the difficulties in assembling reads from high-throughput short-read sequencing, such as those generated using Illumina technologies, meaning that complete plasmid sequences may not be accurately reconstructed[14]. This is because plasmids frequently contain many small mobile repeat structures such as insertion sequences and transposable elements, that extend beyond the current insert size of paired-end short-read sequencing (~300–500 bp), preventing complete plasmid assembly[15]. This therefore hindered the localisation of resistance

genes to specific plasmids. PacBio long-read sequencing technology is capable of spanning repetitive sequences and closing gaps from short-read data, however it incurs high costs that are prohibitive to many laboratories[16]. The Oxford Nanopore MinION sequencer is a relatively new, rapid, long-read sequencing technology. The main benefits include lower costs when compared to other technologies, making it more accessible for many; but more importantly is its sensitive detection abilities from limited starting material. High concentrations of plasmid DNA can be difficult to obtain, particularly from environmental samples, where plasmids are often present in low-copy numbers[17]. While single-read error rates have been noted to be higher for MinION than those for Illumina short-reads, the generation of consensus sequences from multiple reads allows for a higher accuracy to be attained[18].

While this is only a recently developed technology, some studies have already employed MinION sequencing to characterise plasmids carrying antimicrobial resistance (AMR) genes from clinical isolates. Power *et al.,* characterized an IncL/M-like plasmid containing a bla_{OXA-48}-encoding gene from a clinical isolate of *Klebsiella pneumoniae*[19]. Liao *et al*., obtained 12 chromosomes and 36 plasmids from three *Acinetobacter nosocomialis*, five *A. pittii,* and four *Staphylococcus aureus* isolates from clinical samples[20]. Lemon *et al.,* utilised MinION sequencing to identify AMR genes from extended-spectrum-beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates[18]. There have also been some reports of the utilisation of MinION sequencing for examining samples of animal origin. Taylor *et al*., characterised plasmids from *Salmonella enterica subsp. enterica* serovar Bareilly isolated from shrimp and *Escherichia coli* O157:H7 isolated from ground beef[21]. Hadziabdic *et al*., sequenced bla_{NDM-1}-carrying IncA/C2 plasmids from

Salmonella corvallis isolates from chicken faecal samples[22]. However, the work undertaken here is to our knowledge the first report of plasmids from the caecum of broiler chickens sequenced using MinION sequencing.

In this study, we describe the sequences of five antibiotic resistance plasmids, four of which were multi-drug resistant. They were isolated from the caecum of a broiler chicken, which are raised for meat production, and sequenced using MinION technology. We identified the resistance genes present and compared them to similar plasmids that have been isolated in previous studies.

3.3 MATERIALS AND METHODS

3.3.1 Samples

The broiler caecal sample was obtained from a commercial poultry production unit in the European Union. Samples were lyophilised and stored at -80°C before analysis.

3.3.2 Plasmid Isolation, Antibiotic Susceptibility Testing and Plasmid Extraction

Plasmids were isolated from a caecal sample using a protocol for direct extraction, the multiple displacement amplification (MDA) method as previously described[23]. Plasmids were then maintained in an antibiotic-susceptible *Escherichia coli* DH5α host. Antibiotic susceptibility testing to ampicillin, tetracycline, kanamycin, cefotaxime, ciprofloxacin and imipenem was performed *via* a disk diffusion method according to CLSI guidelines[24]. Recipient *E. coli* isolates showing a multi-drug resistance phenotype were selected. Plasmid DNA was extracted using the Qiagen HiSpeed Midi kit. Plasmid DNA concentrations and purity were checked using an Invitrogen Qubit Fluorometer (dsDNA high-sensitivity assay kit) and a DeNovix DS-11 spectrophotometer. Plasmids were visualised on a 1% agarose gel stained with 1X GelRed (Biotium) and run at 70 volts for 1 hour.

3.3.3 Plasmid Sequencing

Plasmids were sequenced using an Oxford Nanopore MinION sequencer. The 1D genomic DNA protocol (SQK-LSK108) was followed for a barcoded run for 48 hours. Adapters were trimmed using PoreChop (v0.2.1, https://github.com/rrwick/Porechop). Albacore (https://github.com/dvera/albacore) basecalled and demultiplexed the reads. The reads were mapped against the reference strain *E.coli* 12_MG1655 in GraphMap[25] to remove bacterial host DNA. Reads were assembled using Unicycler[26]. Then, the raw reads were mapped back to the contigs from the assembly using GraphMap. Contigs with low and uneven coverage were discarded. The remaining contigs were aligned against each other using LastZ[27] to remove duplicates due to barcode leakage. The remaining contigs were polished using Nanopolish[28] and annotated with RAST[29]. Raw reads were aligned with Graphmap to the CARD[30] database. Contigs with less than 10% coverage compared to the coverage of closed replicons were removed and antibiotic resistance genes were annotated. Plasmid maps were generated using SnapGene (https://www.snapgene.com/).

The sequences are deposited in the European Nucleotide Archive (ENA) under the primary accession PRJEB38985 and secondary accession ERP122449.

3.4 RESULTS

3.4.1 Plasmids isolated from sample A

The *E. coli* containing the isolated plasmids from sample A was resistant to ampicillin, tetracycline, cefotaxime and kanamycin following a disk diffusion assay. The extracted plasmid DNA displayed four distinct bands on an agarose gel (Fig 1). This extracted plasmid DNA was sequenced using the MinION sequencer. A total of 40,418 reads were obtained with a mean length of 5,212 bp. The extracted plasmid DNA comprised three plasmids; pBC01.1, pBC01.2 and pBC01.3.

The first of the plasmids (pBC01.1), an IncF plasmid, was 151,806 bp in length with 49% GC content. It carried the resistance genes MacA, MacB, TetR, TetA, TetD, *aph(3'')-I* and *aph(6)-Ic*. It also contained conjugative machinery (Fig 2). MacA and MacB are membrane fusion proteins that form an antibiotic efflux complex with TolC and are associated with macrolide resistance. TetR is a tetracycline resistance repressor protein. TetA and TetD are tetracycline resistance proteins that confer resistance by efflux. The *aph(3'')-I* and *aph(6)-Ic* genes are phosphotransferases that confer resistance to the aminoglycoside streptomycin and are also described as *strA* and *strB* genes[31]. The total plasmid DNA sequence had 99.64% identity and 97% query coverage with *Salmonella enterica subsp. enterica* serovar Kentucky str. CVM29188 plasmid pCVM29188_146. This plasmid was previously identified in a chicken breast sample in the United States of America (GenBank accession no. CP001122)[32]. TetD was not present on the pCVM29188_146 plasmid and was unique to the pBC01.1 plasmid.

The IncI1 plasmid pBC01.2 was 110,152 bp in length with 51% GC content. It harboured the antibiotic resistance genes *aac(6')-Ib, aph(3'')-Ia, aadA5, dfrA17, sul2* and $bla_{\text{CTX-M-1}}$. The plasmid also contained conjugative machinery (Fig 3). The genes *aac(6')-Ib, aph(3'')-Ia* and *aadA5* each confer aminoglycoside resistance to amikacin and kanamycin, streptomycin and spectinomycin respectively. The *dfrA17* gene is a trimethoprim resistance gene. The *sul2* gene confers sulphonamide resistance and $bla_{\text{CTX-M-1}}$ is an ESBL producing gene. While $bla_{\text{CTX-M-15}}$ is the dominant ESBL type in humans, the ESBL $bla_{\text{CTX-M-1}}$ is the most common type in livestock[33,34] and $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ being the most common ESBL-types in poultry[35]. The plasmid had 99% identity to *Escherichia coli* plasmid pC49-108 with 100% query coverage. This plasmid has previously been isolated from a chicken faecal sample in Switzerland (GenBank accession no. KJ484638)[36]. The genes *aac(6')-Ib, aph(3'')-Ia* and *sul2* were present only in pBC01.2 and not in pC49-108.

The third IncFIB plasmid, pBC01.3 (Fig 4), identified in this sample was 97,991 bp long with 48% GC content. It carried a class A beta-lactamase, $bla_{\text{TEM-215}}$. This plasmid matched to a section of the *Klebsiella pneumoniae* strain ST11 plasmid pKP12226 (267,645 bp) with 98.98% identity and 83% query coverage, a plasmid isolated from a human patient with bacteraemia in South Korea (GenBank accession no. KP453775)[37]. Although the bla_{TEM} gene identified in pKP12226 plasmid was $bla_{\text{TEM-1}}$, not $bla_{\text{TEM-215}}$. The antibiotic resistance phenotype of the *E. coli* containing the three plasmids could be accounted for by the AMR genes present on at least one of the three plasmids.

The transformant sample A was selected on tetracycline (16 mg/L). It resulted in the selection of pBC01.1 which harboured *tet* genes, but also of the plasmids pBC01.2 and pBC01.3. Neither of these plasmids carried any determinants for tetracycline resistance, showing that the tetracycline allowed for the co-selection of aminoglycoside, macrolide, beta-lactam, trimethoprim and sulphonamide resistance.

3.4.2 Plasmids isolated from sample B

Escherichia coli containing the plasmids isolated from sample B was resistant to ampicillin, tetracycline, kanamycin and ciprofloxacin. The total extracted plasmid DNA had three distinct bands on an agarose gel (Fig 1). A total of 57,747 reads were attained with a mean length of 4,511 bp. Two plasmids, pBC02.1 and pBC02.2, were identified within the sequenced DNA.

Plasmid pBC02.1 (Fig 5) was 135,664 bp in length with 49% GC content and belonged to the IncF incompatibility group. The plasmid harboured the *aph(6), aph(3'')-I, tetR, tetB* and $bla_{\text{TEM-215}}$ resistance genes; and conjugative machinery. Each of the AMR genes, except *tetB* were also detected on plasmids isolated from sample 1. This indicates that while the genes are contained on different plasmids within the two samples, certain AMR genes are mobile and common to more than one plasmid. It had 98.72% identity to *Escherichia coli* plasmid pH2291-144 with 75% query coverage. pH2291-144 was isolated from a faecal sample from a healthy human in Switzerland (GenBank accession no. KJ484628)[32]. This plasmid did not contain the aminoglycoside phosphatases present in our sample, and it contained $bla_{\text{TEM-1}}$ instead

of *bla*$_{\text{TEM-215.}}$ However, it contained a different streptomycin resistance gene *aadA1*. Thus, while the genes are different the resistance phenotype was the same.

The other phage-like plasmid from sample B, pBC02.2 (Fig 6), was 42,654 bp in length with 49% GC content and belonged to the incompatibility group IncN. It contained *aph(3')-Ia, mef(B) and cmlA* antibiotic resistance genes. These genes confer resistance to streptomycin, and both macrolides and chloramphenicol *via* efflux. It also contained a *qacE* quaternary ammonium compounds resistance gene, and cobalt-zinc-cadmium and copper resistance genes. It matched to *Escherichia coli* strain HYEC7 plasmid pHYEC7-110 with 99.56% identity and 62% query coverage. This plasmid was identified in a pig faecal sample from China and contained all of the resistance genes and IncN present in plasmid pBC02.2 (GenBank accession no. KX518744)[38]. Neither of these two plasmids contained a known plasmid mediated quinolone resistance gene, although the *E. coli* was phenotypically resistant to ciprofloxacin. However, as these plasmids contained many hypothetical genes any one of these could be a novel quinolone resistance gene which requires further investigation.

While the plasmids from the broiler caecal samples were not an exact match to the plasmids previously identified, fragments were identical across plasmids. Based on these results, it show that these fragments are highly mobile. They have been seen in both animal and human gut microbiomes, and plasmids in locations worldwide.

3.5 DISCUSSION

We obtained five plasmids from two transformants from a broiler caecal sample. Three plasmids were isolated from the first transformant, sample A. Sample B harboured two plasmids. Antibiotic resistance genes were identified on all of the plasmids. Both samples harboured multi-drug resistance plasmids. The ciprofloxacin resistance detected from the disk diffusion assay could be attributed to either a chromosomal mutation or a novel plasmid-mediated gene. An interesting thing to note, pBC02.2 contained a *qacE* gene. Previous studies have found a strong correlation between the presence of the *qacE* gene with resistance to some antibiotics[39]. There was also the co-occurrence of both heavy metal and antibiotic resistance determinants on pBC02.2. Copper and zinc are used in agriculture to support animal health and growth[40]. However, heavy metals are known to function as co-selecting agents in the spread of antibiotic resistance in human pathogens[41].

In sample A, two of the plasmids contained genes for conjugation, indicating their potential ability to transfer to other bacteria. Non-mobilisable plasmids have been known to transfer alongside self-transmissible plasmids[42], leading to the possibility that all three plasmids could have the ability to disseminate further. This is equally plausible for sample B, where pBC02.1 had conjugative genes but not pBC02.2.

Two of the plasmids identified in sample A matched to previously identified plasmids found in chicken meat[32] and chicken faecal samples[36]. This shows the plasmid can persist through the gastrointestinal tract, into faeces and on chicken meat which could

be consumed by humans. The other plasmid present in the broiler caecal sample, pBC01.3, matched to a plasmid previously isolated from a patient with bacteraemia[37]. From a One Health perspective, the appearance of a highly similar plasmid in both a human and animal sample raises the question as to the route of transmission of the plasmid. It confirms that the plasmid can reside in the chicken gut as well as having the ability to persist in human pathogenic bacteria. The presence of antibiotic resistance plasmids in such cases can seriously limit the treatment options available. In sample B, one of the plasmids, pBC02.1, matched to a plasmid isolated from a faecal sample taken from a healthy human. While it is impossible to conclude as to how the human obtained this plasmid, it highlights that antibiotic resistance plasmids can reside within the commensal bacteria of humans. The other plasmid from this sample, pBC02.2, showed high similarity to a plasmid from a pig faecal sample. These results show that the same plasmid can persist in various gastrointestinal environments.

From all the plasmids sequenced, they were found to originate from *E. coli*, *S. enterica* and *K. pneumoniae*, which are all known to be pathogenic to humans. Our experimental design for the direct extraction of the plasmids means the original host is unknown, but the *S. enterica* and *K. pneumoniae* plasmids were stabily maintained in *E. coli*. The sequenced plasmids match to plasmids previously isolated globally; in America, Switzerland, South Korea and China. The presence of these plasmids in broilers from the European Union raises the concern as to how these plasmids have disseminated worldwide, and their potential to transfer between animals and humans. It also demonstrates the ability of sections of mobile DNA containing antibiotic resistance genes on plasmids to move between plasmids; and that these plasmids move

between chicken faeces, chicken meat and animal and human gut microbiota even in different host bacteria. Thus the ability of fragments of DNA to move between plasmids is as important as the movement of plasmids between bacteria.

3.6 CONCLUSION

Our results have highlighted the possibility of animal-to-animal and animal-to-human transfer of plasmids, and their ability to disseminate globally, driving the spread of antibiotic resistance. This study also demonstrated the presence of several plasmids containing the same AMR gene or genes conferring the same AMR phenotype within one chicken. Almost all of these plasmids were multi-drug resistant plasmids and may be selected due to the use of any number of antibiotics or in one case even quaternary ammonium compounds, which are frequently used as disinfectants. This study highlights the importance of analysing the depth and variety of plasmids present within a complex sample in addition to the national or global surveillance of AMR within animals.

3.7 FIGURES

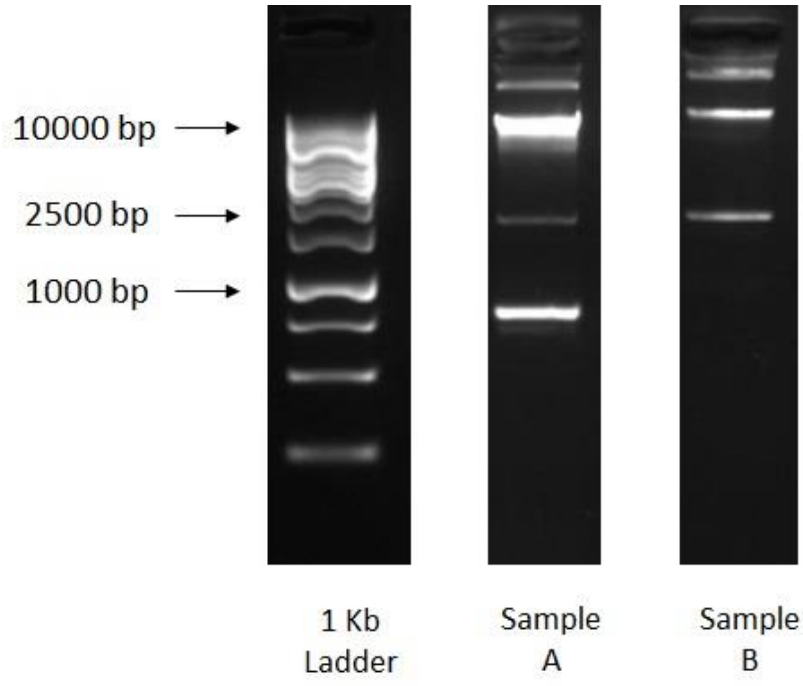

Fig 1. Agarose gel image of plasmid DNA extracted from *E. coli* transformants.

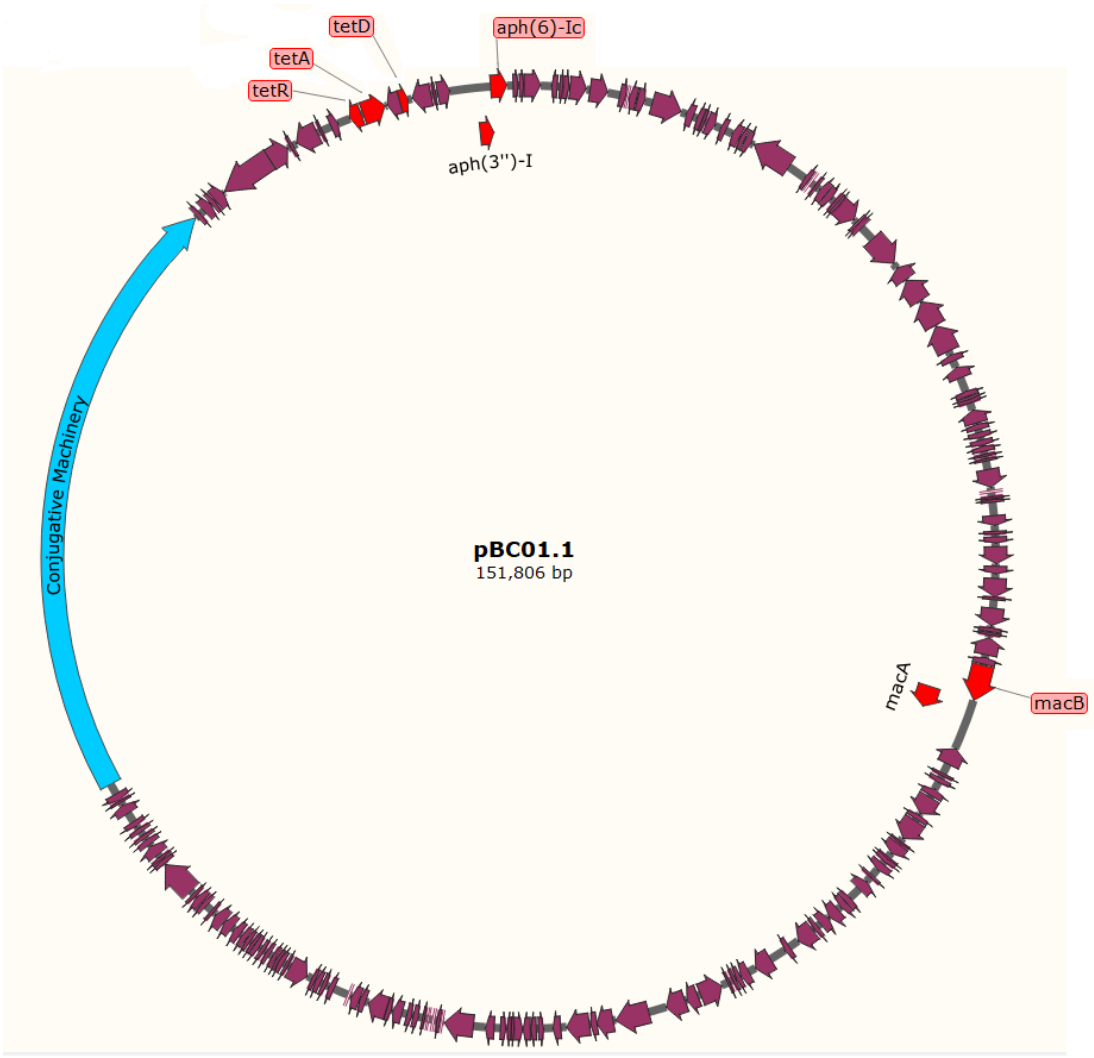

Fig 2. Plasmid pBC01.1 isolated from sample A. Antibiotic resistance genes are highlighted in red, conjugative genes in blue.

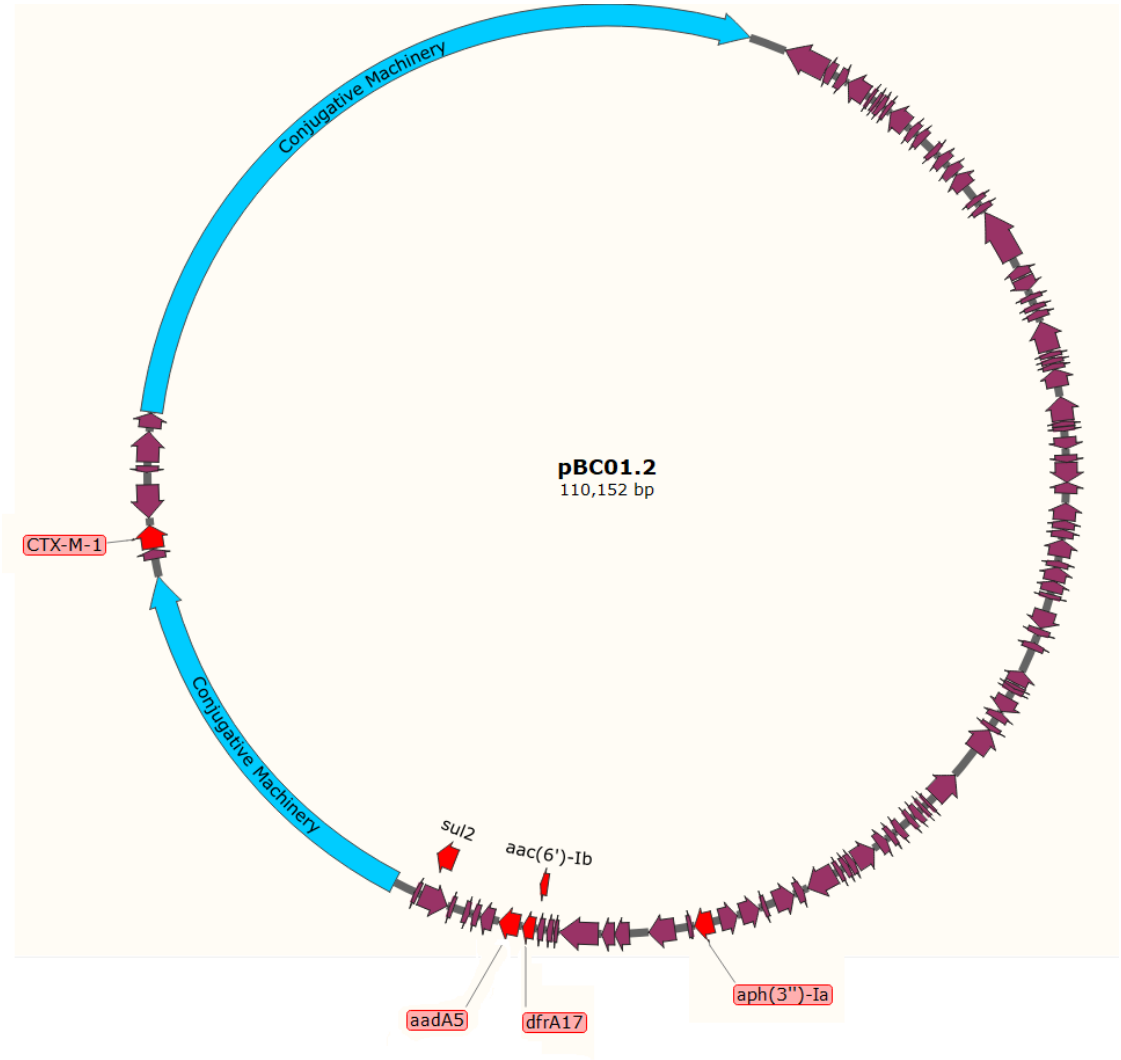

Fig 3. Plasmid pBC01.2 isolated from sample A. Antibiotic resistance genes are highlighted in red, conjugative genes in blue.

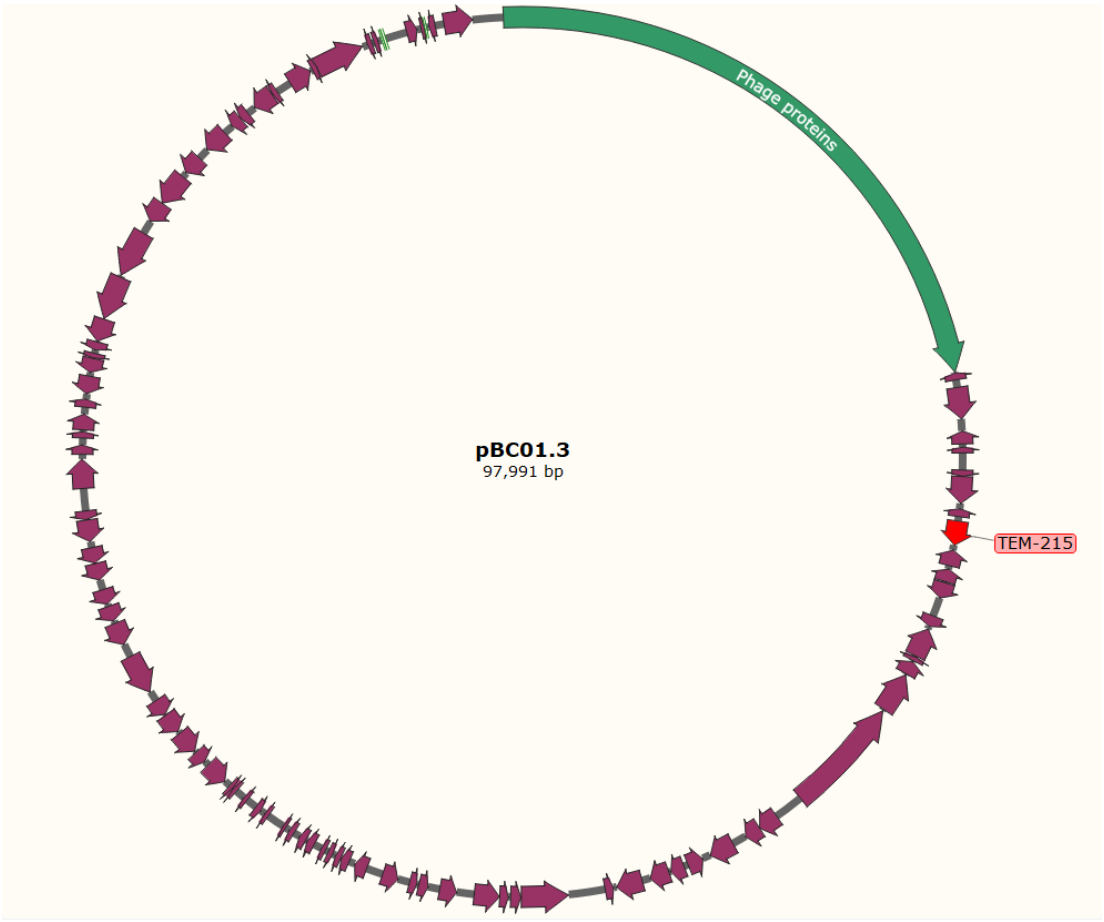

Fig 4. Plasmid pBC01.2 isolated from sample A. Antibiotic resistance genes are highlighted in red, phage proteins indicated in green.

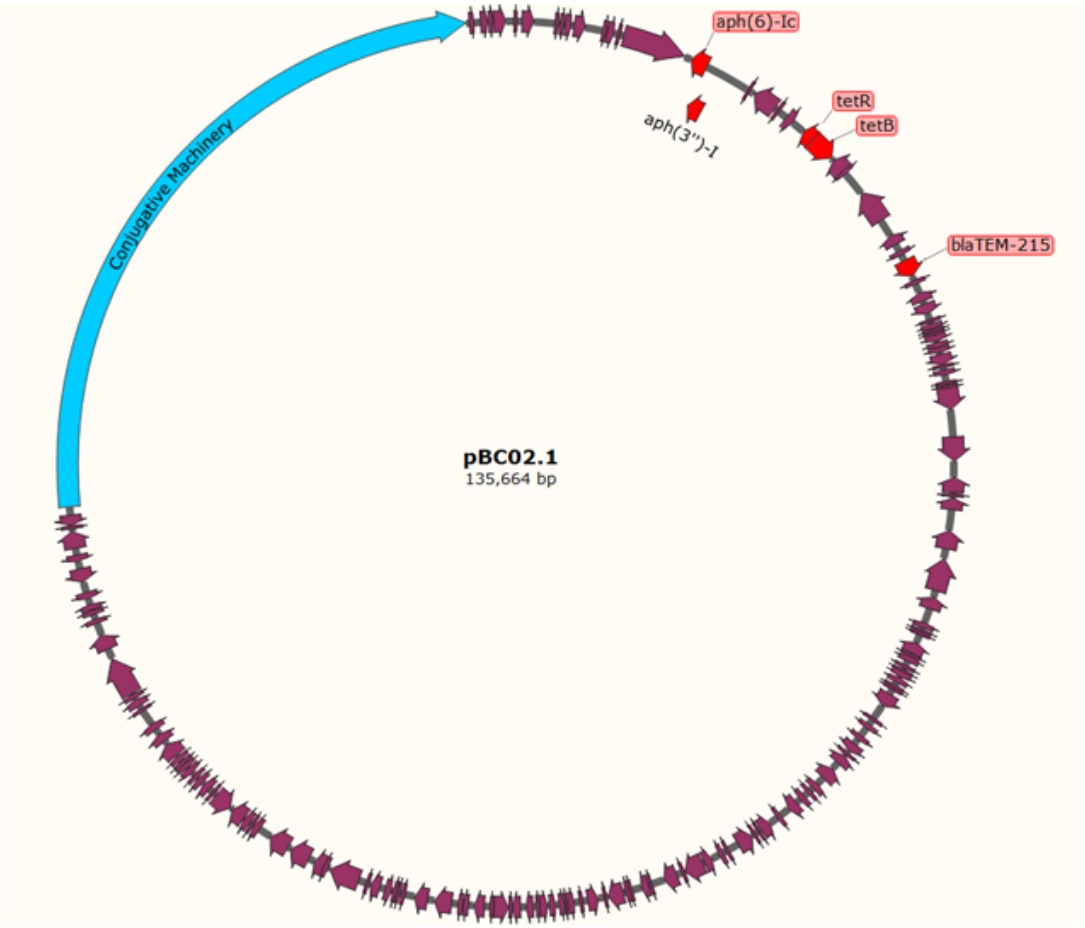

Fig 5. Plasmid pBC02.1 isolated from sample B. Antibiotic resistance genes are highlighted in red, conjugative genes in blue.

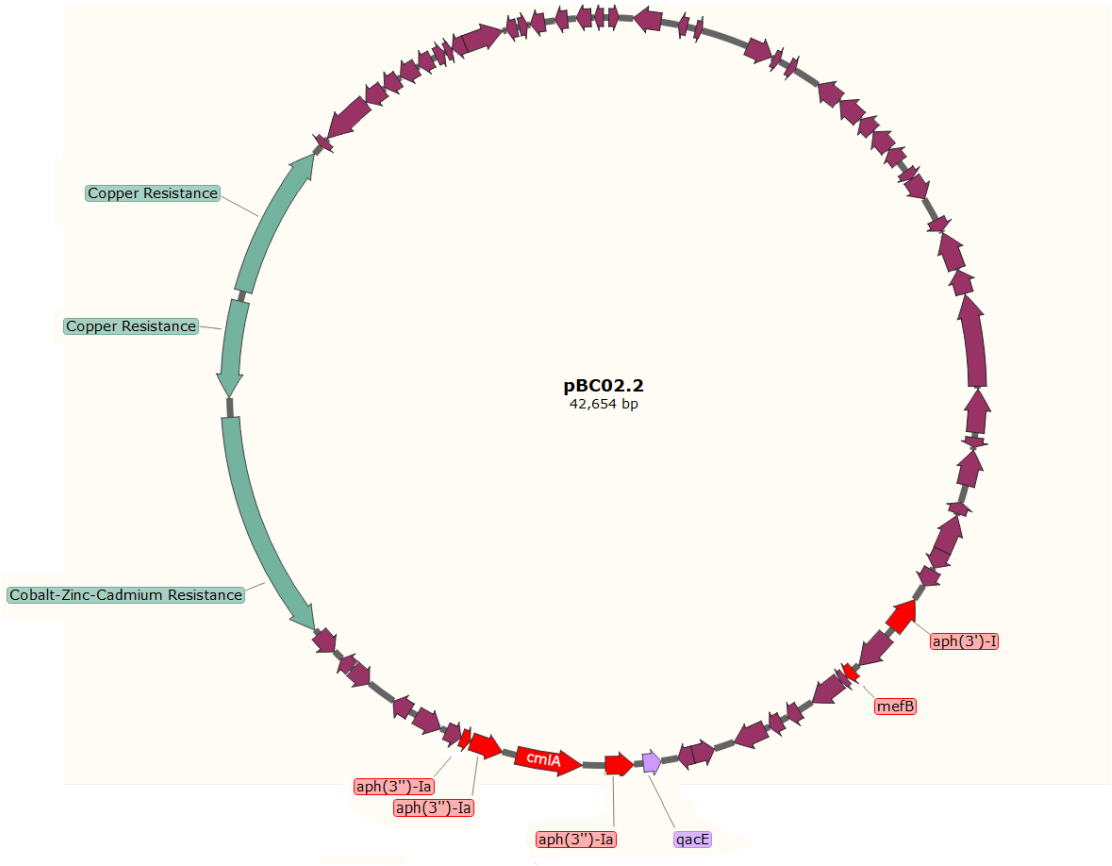

Fig 6. Plasmid pBC02.2 isolated from sample B. Antibiotic resistance genes are highlighted in red, heavy metal genes in green and quaternary ammonium compounds genes in purple.

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Chapter 4

Microbiome and antibiotic resistance plasmid variation within chickens over time

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4.1 ABSTRACT

The caecum plays host to the largest number of microorganisms within the broiler gastrointestinal tract. These microbial communities provide numerous benefits to the host, including playing a role in nutrition and immunity. We examined the caecal microbiome of broiler chickens over time. The phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were the most abundant; *Clostridia* and *Bacteroidia* the most abundant classes; and *Faecalibacterium* and *Bacteroides* were the most abundant genera. However, significant differences in the bacterial communities can be seen between birds at day 21 and 35, but also between individual birds from each group. We observed a stabilisation of the microbial communities within the caecum over time. Antibiotic resistance is an ever-growing concern worldwide, and we examined the presence of conjugative resistance plasmids within the broiler caecum. Plasmids harbouring resistance to ampicillin, tetracycline, trimethoprim, cefotaxime and chloramphenicol were detected at both time-points. Over half of the plasmids from day 35 were multi-drug resistant. The shifts in dominance within the bacterial communities over time may have contributed to the increase in resistance observed at the later time-point. These plasmids could be captured and maintained by a human pathogen, and may have the potential to spread *via* the food chain.

4.2 INTRODUCTION

Poultry is one of the most commonly consumed protein sources worldwide with over 60 billion chickens produced annually[1]. The microbial population present in the gastrointestinal tract of chickens are thought to have a number of advantages for the host. These include influencing host nutrition *via* nutrient assimilation, adding metabolic potential, vitamin and amino acid production, influencing gut development and physiology, and prevention of colonisation by invading pathogens[2]. Poultry have a shorter gastrointestinal tract and a faster digesta transit, which selects for a highly diverse intestinal microbiome in comparison with other food-producing animals[3]. The diversity of the bacterial communities in the chicken gastrointestinal tract is to a great extent influenced by the age of the birds and location in the digestive tract[4]. Between days 15 and 22, microbiota maturation occurs and has been found to remain in a stable status[5]. However, the variation in the resistome remains unknown. The majority of these bacteria reside in the distal intestine which includes the caeca, where densities approach 10^{11} to 10^{12} cells/g, noted to be the highest density recorded for any microbial environment[6]. Bacteria can also be introduced to the caeca by reflux from the urodeum and cloaca[7].

It is estimated that less than 20% of bacterial taxa which inhabit the poultry gastrointestinal tract have been recovered by cultivation[8]. Culture-independent methods have allowed for more detailed information on microbial community composition and diversity[9]. High-throughput sequencing technologies have allowed for the identification of highly complex and diverse communities in the gastrointestinal tract with greater depth and coverage. Targeted 16S rRNA gene

amplicon sequencing is one of the main DNA-based methods currently used for the analysis of bacterial community profiling[10].

Poultry production is predicted to produce around 130 million tons of chicken meat in 2020 (OECD/FAO[11]) to meet the demands of an increasing global population[12]. This is achieved through intensive farming, where antibiotics are used extensively. Antibiotics have been vital for decreasing the rates of morbidity and mortality from infectious diseases in both humans and animals since their discovery. However, the increasing rate of the development of antibiotic resistance has become a serious issue worldwide in both the areas of medicine and agriculture[13]. With treatment options limited, especially in the case of infections resistant to antibiotics of last-resort, it is resulting in reduced clinical efficacy, increased treatment costs and higher mortality rates[14]. In agriculture, antibiotics are given therapeutically to treat infections in sick animals. However, within intensive farming systems, it can be uneconomic to treat individual animals, which results in the treatment of an entire group, usually through the feed or water[15]. Antibiotics are also used for metaphylactic reasons to control disease, and prophylactically to prevent disease. In some countries, antibiotics are also administered at low and sub-therapeutic doses to improve feed efficiency and promote animal growth of the animal[16]. It is this overuse of antibiotics that has contributed to the rapid increase in the rates of resistance in food-producing animals[17]. These resistant bacteria can then be transmitted to humans through the food chain[18]. However, changes in microbial community and bacterial resistome in the poultry gastrointestinal tract remain largely unknown.

All commensal, pathogenic and environmental bacteria form a reservoir of antibiotic resistance genes, of which pathogenic bacteria can acquire these genes by horizontal gene transfer. This has allowed for antibiotic resistance to spread from commensal and environmental bacteria to pathogens[14]. Plasmids have the ability to transfer genes to different species, genera, and kingdoms, dependent on the plasmid host range[19]. It has also been noted that plasmids lacking conjugative machinery could be mobilised by self-transmissible plasmids that are also present in the donor cell[20], and therefore may also be obtained using this method. It is well-known that *Escherichia coli* is a part of the commensal flora of humans, but is also an opportunistic pathogen, and some can be highly virulent[21]. By using *E. coli* as the donor strain, it allowed for the determination of the resistance profile of plasmids that could be transferred to, and maintained in, a human pathogen.

This study aimed to 1) characterise the changes in the structure and diversity of the bacterial communities; and 2) compare the conjugative plasmids harbouring antibiotic resistance genes at two growth stages in the caecum of broiler chickens. This work provides an insight in to the changing diversity of bacterial communities and plasmid-mediated antibiotic resistance in animals entering the food chain. We hypothesise that changes in the bacterial community effects the mobile resistome present in the chicken caecal microbiome.

4.3 MATERIALS & METHODS

4.3.1 Samples

Broiler caecal samples were obtained from a commercial poultry production unit in the European Union. The samples were collected at two time-points, day 21 and day 35 post-hatch. Samples were lyophilised and stored at -80°C before analysis.

4.3.2 Microbiome Sequencing

Twelve random caecal samples were chosen for sequencing, six from each time-point. Total DNA was extracted from 0.05 g of each caecal sample with the Mobio PowerSoil DNA Extraction Kit (now Qiagen), following the manufacturer's instructions. The concentration and purity of the extracted DNA was measured by spectrophotometry (DeNovix DS-11 spectrophotometer). The samples were prepared and sequenced as described by Do *et al.*, 2019[22]. The library was prepared according to the 16S Metagenomic Sequencing Library guidelines (Illumina-a. 16S Metagenomic Sequencing Library Preparation) and then pooled in the MiSeq v3 reagent cartridge. An Illumina chastity filter (Illumina-b. Miseq Reporter Software Guide (15042295)) filtered the sequenced data, with the cluster of reads that had no more than 1 base call and a chastity value of less than 0.6 in the first 25 cycles passing the filter. BaseSpace-the Metagenomics workflow (16S Metagenomics app vesion 1.0.1.0 with Isis v2.5.35.6, Greengenes data base 13.5) (DeSantis *et al.*, 2006[23]; Illumina-c. 16s Metagenomics App) was used to demultiplex reads. It was also used to generate FASTQ files, with the 3' portion of non-index reads with low quality scores being

trimmed by QualityScoreTrim. The RDP Naïve Bayesian classifier[24] provided taxonomic level classification. The sequences are deposited in the European Nucleotide Archive (ENA) under the primary accession PRJEB37133 and secondary accession ERP120433.

4.3.3 Microbiome Data Analysis

Statistical analysis of the microbiome data was performed using Calypso (http://cgenome.net/calypso/)[25]. The data were normalized to render it suitable for statistical analysis. Samples with less than 1000 sequence reads were removed. Rare taxa, which had less than 0.001% relative abundance were also removed. Principal component analysis (PCA) and rarefaction analyses were carried out using default settings. The microbial community composition was quantitatively visualized by bar charts and heat maps. The relative abundances of phylum, class and genus taxonomic levels were compared between time-points by ANOVA. The calculated P-values (ANOVA) were adjusted for multiple testing and false discovery rate. Shannon index was used to estimate the bacterial alpha diversity and Chao1 to estimate richness.

4.3.4 Exogenous Plasmid Isolation

Plasmids harbouring antibiotic resistance genes were isolated from the caecal samples (n=34) using the exogenous plasmid isolation method, as previously described[26]. Briefly, plasmids from the 'donor' caecal samples were transferred to the 'recipient' *Escherichia coli* DH5α *via* biparental mating. Exogenous transconjugants were selected on Eosin Methylene Blue (EMB) agar (Sigma) with rifampicin (100 mg/L)

and ampicillin, tetracycline, gentamicin, colistin, cefotaxime or ciprofloxacin at breakpoint concentrations according to CLSI guidelines (2018)[27]. From each antibiotic selective plate with growth after exogenous isolation, a transconjugant from each was selected at random. If the same plate appeared to have bacteria with different features (colour, morphology), both were selected.

4.3.5 Plasmid Analysis

Plasmids were extracted from the putative recipient *E. coli* strains using the Macherey-Nagel NucleoSpin Plasmid kit following the low-copy number protocol according to the manufacturer's guidelines. The extracted plasmids were visualised on a 1% agarose gel stained with GelRed (Biotium), run at 70 volts for 60 minutes. Antibiotic susceptibility testing was performed on the exogenous transconjugant strains in duplicate *via* the disk diffusion method according to CLSI guidelines (2018)[27] against 9 antibiotics from 8 different classes.

4.4 RESULTS & DISCUSSION

4.4.1 16S rRNA gene amplicon sequencing

The number of quality controlled reads in each sample passing filters were between 4,378 and 207,046, which were used for further analysis. 31 phyla, 65 classes, 128 orders, 285 families and 867 genera were included. Rarefaction analysis was used to identify the quality of the sequenced data representing the diversity of the bacterial communities, which showed a sufficient sequencing depth was reached (Fig 1). One sample from the day 21 time-point had less than 1000 sequence reads, and was therefore excluded from further analysis.

4.4.2 Microbial Community Composition

The bacterial community was analysed by comparison of the 16S rRNA amplicon sequences. *Firmicutes* was the most predominant phyla, with up to 61.63% of all classified reads from the day 21 time-point and up to 48.96% from day 35 Fig 2(a). This was followed by *Bacteroidetes* (up to 36.73% from day 21 and 42.26% from day 35), *Proteobacteria* (up to 33.89% from day 21 and 16.67% from day 35) and *Actinobacteria* (up to 23.26% from day 21 and 14.56% from day 35). The predominance of *Firmicutes* followed by *Bacteroidetes* and *Proteobacteria* has been previously identified in chicken faecal analysis[28,29], however, variation in bacterial composition occurred between the two time-points (Fig 2(a)). Surprisingly, we identified higher proportions of *Actinobacteria* than previous studies into the poultry caecal microbiome. Xiao *et al*., found *Actinobacteria* to be almost absent in the

caecum, but found them to be dominant in the ileum[30]. Even studies noting *Actinobacteria* as a predominant phyla report much lower precentages compared to our findings of 23.26% and 14.56%. Xiong *et* al., reported 1.3% of *Actinobacteria* from broiler faecal samples[31], Wei *et al*., report 3.2% from turkey caecal samples[32] and Thomas *et al*., report a still quite low 6.77% from chicken intestinal samples[33]. As these results are non-concurring, the increased proportions may be due to factors such as broiler breed, geographical location, feed or housing conditions[34]. A higher proportion of *Firmicutes*, *Proteobacteria* and *Actinobacteria* were present in the day 21 group, while a larger amount of *Bacteroidetes* were present in the day 35 group.

The relative abundance of the top 20 classes is displayed in Fig 2(b). *Clostridia* was the most abundant with up to 63.77% from day 21 and 46.24% from day 35 of all classified reads. The following dominant classes were *Bacteroidia* (up to 22.92% from day 21 and 39.53% from day 35), *Actinobacteria* (up to 23.44% from day 21 and 14.69% from day 35) and *Epsilonproteobacteria* (up to 19.97% from day 21 and 6.33% from day 35). Again, the variation between groups can be seen in Fig 2(b), with a higher proportion of *Bacteroidia* in the day 35 group compared to day 21.

The heat-map (Fig 3) shows the relative abundances of the top 20 detected genera, ranging from most abundant (red) to least (blue). *Faecalibacterium* was the most dominant (up to 30.82% from day 21 and 15.38% from day 35), followed by *Bacteroides* (up to 4.75% from day 21 and 27.79% from day 35), *Bifidobacterium* (up to 26.87% from day 21 and 15.95% from day 35) and *Megamonas* (up to 12.81% from day 21 and 19.19% from day 35). The variation between groups can clearly be seen at

genus level, with the day 21 group dominated by *Faecalibacterium*, whereas *Bacteroides* is the most dominant from the day 35 group. The human pathogens *Campylobacter* spp., which cause foodborne gastroenteritic disease; and *Helicobacter* spp., which are associated with stomach cancer and duodenal ulcers[35]; were both detected within the top 20 genera. Broilers are considered to be the primary vector for transmission of *Campylobacter* to humans[36]. *Helicobacter* is known to reside in the poultry gut, with contamination of consumer meat products with the bacteria likely to occur during the slaughtering process[35].

As well as the variations between the two time-points, there were also differences among the microbiota of the chickens within the same time-point group at all taxonomic ranks. It has been demonstrated previously that there is a large individual variation in the microbiota amongst chickens of the same breed, with identical diets and under tightly controlled experimental conditions[37]. For example, at phylum level from the day 21 group, the number of classified reads for *Proteobacteria* varied from 33.89% in one bird to 3.89% in another. This is also observed at class rank, with the percentage for *Epsilonproteobacteria* ranging from 19.97% in one bird to 0.02% in a different bird within the same day 21 group. Likewise, at genus level, *Bifidobacterium* ranged from 26.87% in one bird to 0.59% in another. Similar variations, albeit to a much lesser extent, can be seen within the chickens from the day 35 group. These results demonstrate the changes in the broiler microbiome, even over a short period of time, and perhaps the establishment of a more stabilised, less variable microbiota as the bird ages. However, this stabilisation is later than 3 weeks as previously described[8].

ANOVA was used to compare the taxa abundance at phylum, class and genus levels between the two time-points (Fig 4). When P <0.05, the difference in relative abundance was considered significant. Pair-wise comparisons were performed by t-test and annotated as *: p<0.05, **: p<0.01, ***: p<0.001. Four phyla showed a significant variance between day 21 and day 35 (*Firmicutes*, *Bacteroidetes*, *Cyanobacteria* and *Chloroflexi*), while 5 classes (*Clostridia*, *Bacteroidia*, *Betaproteobacteria*, *Nostocophycideae* and *Anaerolineae*) and 9 genera (*Faecalibacterium*, *Bacteroides*, *Megamonas*, *Blautia*, *Flavobacterium*, *Sutterella*, *Ruminococcus*, *Oscillospira* and *Dysgonomonas*) also showed a significant difference. Within both the phyla and class, variations occurred in two of the most abundant taxa, indicating a significant change in the microbiome composition over time. Broilers are typically administered a starter diet from days 0-21 and followed by a finisher diet until slaughter (between days 30-50)[38]. This may be a contributing factor towards the changes observed between groups.

The relative abundance of operational taxonomic units (OTUs) at phylum, class and genus taxonomic levels were plotted using principal component analysis (PCA) (Fig 5). Day 21 has a large intra-cluster distance along the PC1 axis at phylum level (Fig 5). This displays the variation among the samples in this group. A similar pattern is observed at class and genus levels, also with a large intra-cluster distance along the PC2 axis. Data from the day 35 time-point had a smaller intra-cluster distance, indicating less variation between samples. The clusters from both time-points have a clearly defined inter-cluster distance with no overlap of the clusters at any taxonomic rank. This indicates that the microbiome across birds is more stable at day 35 than day 21, and that there is significant variation in microbiome development from day 21 to

day 35. Interestingly, Sergeant *et al*., found *Megamonas* to be the most dominant genera in their study, from caecal samples taken from broilers at day 42[39]. We found *Megamonas* to be the third most prevalent genera in our study and was mainly detected in the older birds at day 35. This suggests that the variations in the microbial communities continue to occur as the birds age. While our findings are mostly concurrent with previous studies regarding the most dominant taxa in the broiler caecum, variations are still seen from study to study. Sakaridis *et al*., report *Tenericutes* as the third most prevalent phyla present[40], whereas it was found in much lower proportions in our samples, as the seventh most prevalent. Numerous factors are recognised to have an effect on the diversity of the poultry microbiome in commercial facilities, including diet, stocking density, geographical location, bird environment, and pathogen presence[41].

4.4.3 Alpha Diversity, Richness and Evenness

The microbial alpha diversity was analysed using Shannon index (Fig 6). A significant difference was identified at phylum, family and genus taxonomic levels, where $P < 0.05$, demonstrating a few strongly dominating taxa. This was not seen at class or order taxonomic ranks, which had a more equal OTU distribution at these levels. The Shannon indices were higher for samples collected at day 35 for all taxonomic ranks. The bacterial community richness was assessed using Chao1 (Fig 7). Again, the Chao1 indices were higher from the day 35 time-point than day 21, highlighting the differences between the two groups. The evenness of the microbial community is displayed in Fig 8. No significant differences were noted for any of the taxonomic

ranks, with P >0.05. Evenness indices were similar at class and order taxonomic levels, but were slightly higher for day 35 at phylum, family and genus levels.

4.4.4 Mobile Resistomes

A total of 43 antibiotic resistant transconjugants from 15 birds at day 21 (Fig 9) and 52 antibiotic resistant transconjugants from 20 birds at day 35 (Fig 10) were selected for further analysis. No bird was free from antibiotic resistance plasmids.

From the day 21 time-point, 98% of all transconjugants tested were resistant to ampicillin, 72% to tetracycline, 47% to trimethoprim, 23% to cefotaxime and 9% to chloramphenicol. 39.5% were multi-drug resistant, harbouring resistance to three or more different classes of antibiotics (Table 1). The most frequently isolated resistance plasmids conferred resistant to ampicillin or tetracycline. One transconjugant displayed intermediate resistance to imipenem and one transconjugant displayed intermediate resistance to gentamicin. No transconjugants were resistant to ciprofloxacin or kanamycin.

From the day 35 group, all transconjugants were resistant to ampicillin, 81% were resistant to tetracycline, 44% to trimethoprim, 29% to cefotaxime, 15% to gentamicin, 13% to chloramphenicol, 9.5% to kanamycin, 8% to ciprofloxacin and 2% to imipenem. 51.9% were multi-drug resistant (Table 2). Resistance patterns were similar to those seen at day 21, again with the most resistance to ampicillin and

tetracycline. It could be that these are the same plasmids from day 21 that are being maintained, giving the bacteria harbouring them a survival benefit within the caecal microbiome. However, at day 35, resistance to kanamycin, gentamicin, ciprofloxacin and imipenem that was absent at the earlier time-point was observed. A higher percentage of transconjugants carried multi-drug resistance plasmids also. It appears that as the birds age, they obtain a greater variety of resistance plasmids.

All of the plasmids isolated conferred resistance to at least one antibiotic. Over half of all transconjugants at day 35 were multi-drug resistant and almost 40% from day 21. From the day 21 group, transconjugants were found with resistance to 5 of the 9 tested antibiotics; whereas from the day 35 group, resistance to all the tested antibiotics was observed. This could possibly be attributed to the changes in the microbiome. The highly variable microbiome present at day 21 may present less favourable conditions for the bacteria to obtain and maintain resistance plasmids. As the microbial communities stabilise as the bird ages, it may favour harbouring plasmids which would give them a survival advantage, and they may be better established to deal with any fitness cost associated with this.

All of the plasmids isolated at day 21 have a similar resistance profile, mainly displaying resistance to beta-lactam, tetracycline and trimethoprim antibiotics. It is possible that the same plasmid or group of plasmids disseminated throughout the birds from this group. Chickens can ingest bacteria carrying resistance plasmids from litter, feed or water[7]. Kolar *et al*., found similarly high levels of resistance to tetracycline (97%) and ampicillin (51%) in *E. coli* strains isolated from poultry[42]. A similar

resistance profiling study also found high resistance to tetracycline in broilers on farms where no antibiotics had been used[43]. The authors concur that the plasmid-mediated resistance determinants originated from non-sampled sources, such as farm workers, farm run-off or wildlife. This reiterates the importance of One Health and identifying all of the potential sources of resistance, whether human, animal or environmental. Intensive farming practices such as overcrowding[44] in high-throughput commercial facilities has also created an environment ideal for bacterial and plasmid transfer throughout an entire flock of birds.

A greater variety of plasmids were identified in the day 35 group, with resistance to all tested antibiotics. Perhaps one of the most notable changes in the microbiome from day 21 to 35 was the shift from a *Faecalibacterium* dominated to a *Bacteroides* dominant microbiota at genera level. This may correlate with the changes observed in plasmid profile between the two groups. *Bacteroides* spp. resistant to tetracycline, beta-lactams, aminoglycosides, metronidazole and the macrolide-lincosamide-streptogramin (MLS) group of antibiotics, with all their resistance determinants located on transmissible genetic elements, have all been previously reported[45]. *Bacteroides* spp. are opportunistic pathogens with highly promiscuous conjugation systems enabling other bacteria to obtain their resistance determinants[46]. This may explain the increase in resistance plasmids and our ability to readily capture these plasmids in our *E. coli* host. This worryingly also highlights the ability of multi-drug resistance plasmids to be transferred and maintained in human pathogenic bacteria. Studies such as Jakobsen *et al.*, have already indicated the risk of transfer of resistance from animals to humans through the food chain and its potential to cause infection[47]. The presence of high amounts of multi-drug resistance plasmids we found in broilers,

which conferred resistance to antibiotics listed on the WHO's list of critically important antimicrobials for human medicine[48], would greatly limit treatment options in such circumstances. Interestingly, sample E had a significantly higher percentage of *Bifidobacterium* (26.87%) (Fig 3) compared to the other samples (between 15.95%-0.59%). The corresponding exogenous transconjugant did not harbour a multi-drug resistance plasmid, and displayed resistance only to ampicillin. *Bifidobacteria* are considered beneficial as they are capable of producing positive impacts for host health[49]. Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and mannan-oligosaccharides (MOS) are administered as prebiotics, to increase the proliferation of *Bifidobacteria* which utilise them as substrates[50]. *Bifidobacteria* are also given as probiotics, to improve intestinal microbiota[51]. Our results demonstrate the possibility that a larger population of *Bifidobacterium* is associated with reduced antibiotic resistance in the broiler caecum, and the potential for prebiotics and probiotics to assist in reducing the spread of resistance.

4.5 CONCLUSION

The broiler caecum was dominated by the phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. This is consistent with previous studies into the chicken microbiome. However, we found notable variations between the two time-points, highlighting the changes in the microbial communities over time. We also saw high levels of variation within the microbiome of chickens from the same group, which was more evident at day 21. This may indicate that the microbiome becomes more stabilised as the birds age, even beyond the currently reported stabilisation age. We identified multi-drug resistance plasmids within the broiler caecum at both time-points. There was a higher level and more varied resistance present at day 35, which may be attributed to the stabilisation of the microbiome, and therefore may be better established to harbour these plasmids that may incur high fitness costs. The shift from a *Faecalibacterium* to a *Bacteroides* dominant microbiota over time may also have contributed to the increase in resistance. If a particular group of bacteria appear to harbour high levels of resistance determinants, the use of pre- or probiotics may reducing the numbers of these bacteria by increasing beneficial bacteria. We have demonstrated that these plasmids can be captured and maintained by a human pathogen. These multi-drug resistance plasmids may have the ability to transfer to humans through the food chain, and limit the available treatment for infections.

4.6 TABLES

Table 1. Resistance profile of exogenous transconjugant strains harbouring antibiotic resistance plasmids from caecal samples taken at day 21[a,b,c,d].

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	IMP	C
	1 AMP BC	R	S	S	S	S	S	R	S	S
	1 AMP SC	R	S	S	I	S	S	R	S	S
	1 TET	R	R	S	S	S	S	R	S	S
	1 GEN	R	S	S	S	S	S	S	S	S
A	2 AMP BC	R	R	S	R	S	S	S	S	S
A	2 AMP SC	R	R	S	R	S	S	S	S	S
A	2 TET BC	R	R	S	R	S	S	S	S	S
A	2 TET SC	R	R	S	R	S	S	S	S	S
A	2 CEF	R	R	S	R	S	S	S	S	S
	3 AMP	R	R	S	S	S	S	S	S	S
	3 TET	R	R	S	S	S	S	S	S	S
	4 AMP	R	R	S	S	S	S	R	S	S
	4 TET	S	R	S	S	S	S	S	S	S
	5 AMP	R	R	S	S	S	S	R	S	S
	5 TET	R	R	S	S	S	S	R	S	S
	6 AMP	R	R	S	R	S	S	S	S	S
	6 TET	R	R	S	S	S	S	R	S	S
	6 GEN	R	S	S	S	S	S	S	S	S
	7 AMP	R	R	S	S	S	S	R	S	S
	7 TET	R	R	S	S	S	S	R	S	S
	7 GEN	R	R	S	R	S	S	R	S	S
B	8 AMP BC	R	S	S	S	S	S	R	S	S
B	8 AMP SC	R	S	S	S	S	S	R	S	S
B	8 TET	R	R	S	S	S	S	S	S	S
	9 AMP	R	R	S	S	S	S	R	S	S
	9 TET	R	R	S	R	S	S	S	S	S
C	10 AMP	R	S	S	S	S	S	R	S	I
C	10 GEN	R	S	S	S	S	S	S	S	S
	11 AMP LAWN	R	R	S	S	S	S	R	S	S
	11 AMP COLONIES	R	R	S	R	S	S	S	S	S
	11 TET LAWN	R	R	S	I	S	S	R	S	S
	11 TET COLONIES	R	R	S	S	S	S	R	S	S
	12 AMP	R	R	S	I	S	S	R	S	R
	12 TET BC	R	R	S	I	S	S	S	S	R

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	IMP	C
	12 TET SC	R	R	S	I	S	S	S	I	R
	12 CEF	R	R	S	R	S	I	R	S	S
D	13 AMP	R	S	S	S	S	S	R	S	S
D	13 TET	R	R	S	I	S	S	S	S	R
D	13 GEN	R	R	S	S	S	S	S	S	S
	14 AMP BC	R	S	S	S	S	S	S	S	S
	14 AMP SC	R	S	S	S	S	S	S	S	S
	14 TET	R	R	S	I	S	S	S	S	S
E	15 AMP	R	S	S	S	S	S	S	S	S

[a]M-S= corresponding microbiome sample.

[b]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, IMP=Imipenem, C=Chloramphenicol.

[c]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

[d]Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony) or had both a lawn of growth (LAWN) with some distinct colonies (COLONIES).

Table 2. Resistance profile of exogenous transconjugant strains harbouring antibiotic resistance plasmids from caecal samples taken at day 35[a,b,c,d].

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	IMP	C
	1 AMP	R	R	R	R	I	R	S	I	S
	1 TET BC	R	R	S	S	S	S	R	S	R
	1 TET SC	R	R	I	R	S	S	I	S	S
	2 TET	R	R	R	R	R	R	S	S	R
F	3 AMP	R	R	S	S	I	S	R	S	R
F	3 TET	R	R	S	S	S	S	S	S	S
F	3 GEN	R	R	S	S	S	S	S	S	S
	4 COL PINK	R	S	I	I	S	S	R	S	S
	4 COL PURPLE	R	I	S	I	S	S	R	I	S
G	5 TET	R	R	R	R	R	R	S	S	S
	6 AMP BC	R	R	S	R	S	S	R	S	S
	6 AMP SC	R	R	S	R	R	S	S	S	S
	6 TET	R	R	S	R	S	S	S	S	S
	6 COL	R	R	I	R	S	S	S	S	S
	7 AMP	R	R	R	R	S	S	R	R	S
	7 TET	R	R	I	S	S	R	R	S	S
	7 COL	R	R	I	I	S	S	R	I	S
	8 AMP	R	R	R	R	R	I	R	S	S
	8 TET	R	R	S	S	S	S	R	S	S
H	9 AMP BC	R	S	I	S	S	R	S	S	S
H	9 AMP SC	R	R	I	I	S	S	R	I	S
H	9 TET	R	R	S	S	S	S	R	S	S
H	9 GEN	R	S	S	S	S	R	S	S	S
	10 AMP PINK	R	R	S	I	S	S	S	I	R
	10 AMP PURPLE	R	S	S	I	S	S	S	S	S
	10 CEF	R	R	S	R	S	S	S	S	S
I	11 AMP BC	R	R	S	I	S	S	S	S	S
I	11 AMP SC	R	R	S	I	S	S	S	I	R
I	11 TET	R	R	I	S	S	S	S	I	R
I	11 GEN	R	R	I	R	S	R	R	S	S
	12 AMP	R	R	I	I	S	S	S	S	S
	12 TET BC	R	R	S	I	S	S	S	I	S
	12 TET SC	R	R	S	S	S	S	S	S	S
	13 AMP	R	S	S	I	S	S	R	S	S
J	14 AMP BC	R	R	S	S	S	S	R	S	S
J	14 AMP SC	R	R	S	I	S	S	R	S	S
J	14 TET BC	R	R	I	I	S	S	R	I	S
J	14 TET SC	R	R	I	I	S	S	S	S	S
J	14 GEN	R	R	I	R	S	R	R	S	S
J	14 CEF	R	R	S	R	S	I	R	S	S
K	15 AMP	R	R	I	S	S	S	S	I	S

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	IMP	C
K	15 TET	R	R	I	I	S	S	S	S	S
K	15 CEF	R	R	I	I	S	S	S	I	S
	16 AMP BC	R	R	I	R	S	S	R	I	S
	16 AMP SC	R	S	S	S	S	S	R	S	S
	16 TET BC	R	R	S	I	S	S	R	S	S
	16 TET SC	R	R	S	I	S	S	R	I	S
	17 AMP	R	S	S	S	S	S	S	S	S
	18 TET	R	R	S	S	S	S	S	S	R
	19 AMP BC	R	S	S	S	S	S	S	S	S
	19 AMP SC	R	S	S	S	S	S	S	S	S
	19 TET	R	R	I	S	S	S	S	I	S

[a]M-S= corresponding microbiome sample.

[b]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, IMP=Imipenem, C=Chloramphenicol.

[c]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

[d]Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony) or different colours on EMB agar (pink/purple).

4.7 FIGURES

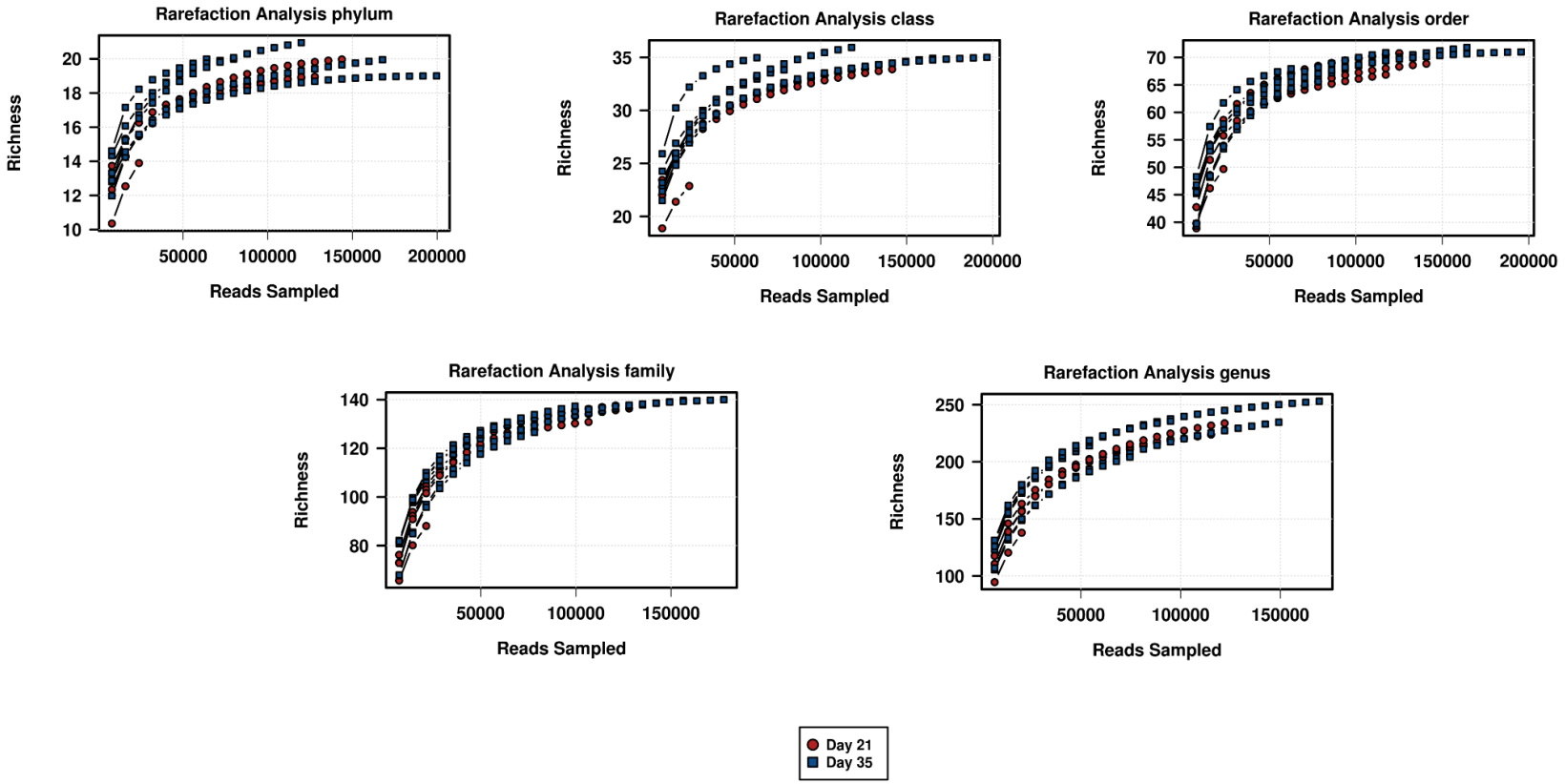

Fig 1. Rarefaction curves at all taxonomic ranks.

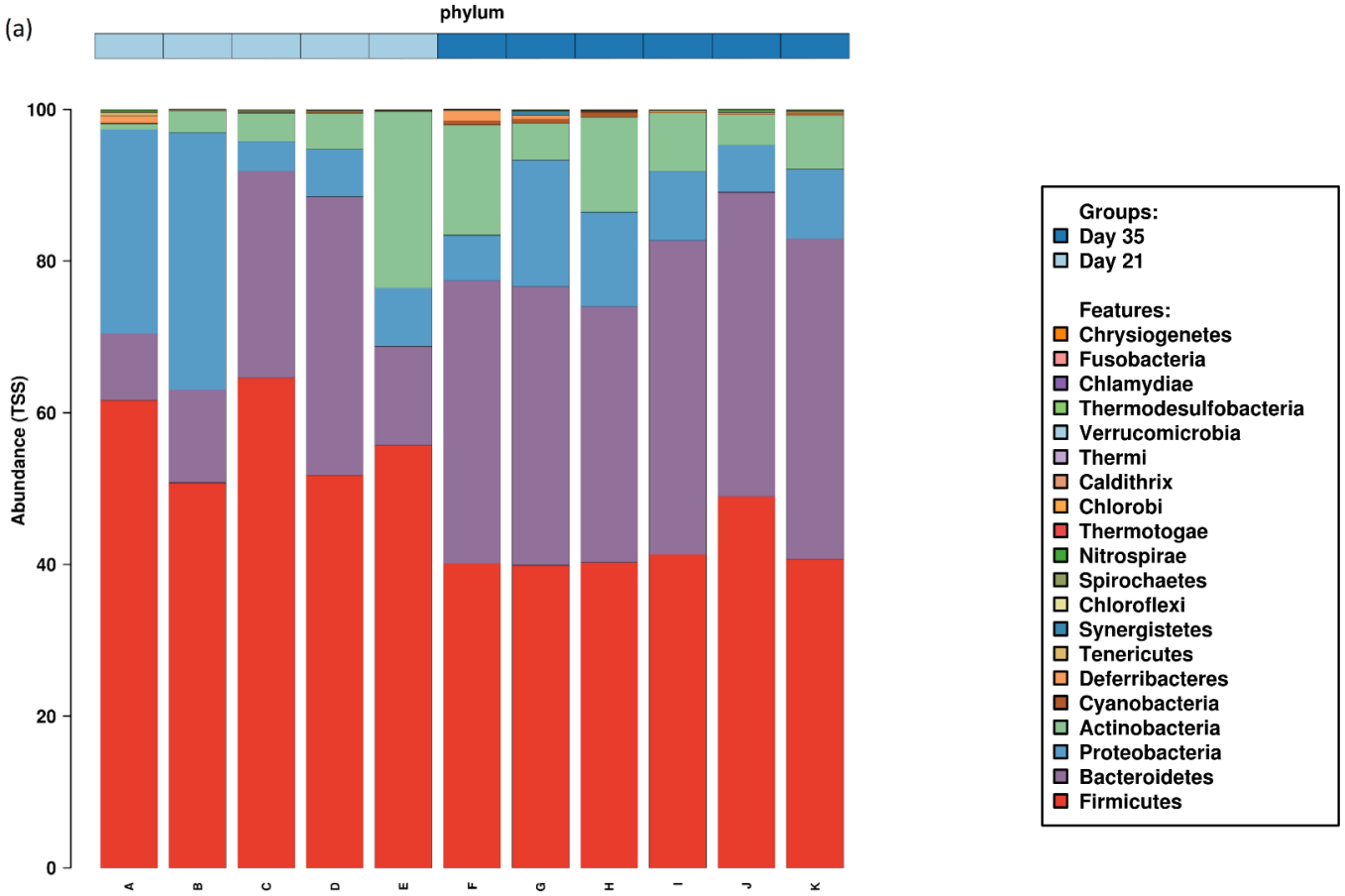

Fig 2(a). Relative abundance of microbial communities at phylum taxonomic rank.

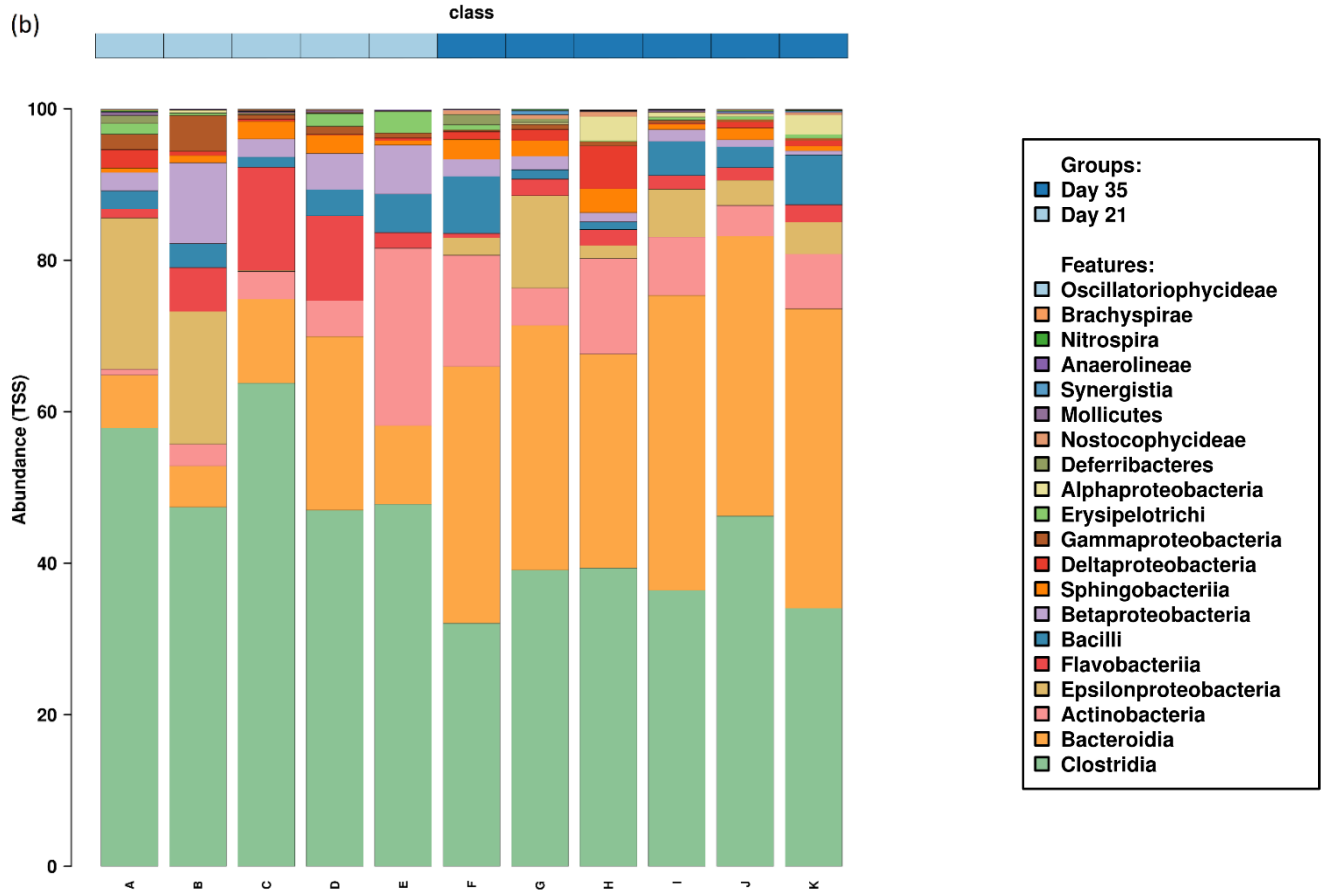

Fig 2(b). Relative abundance of microbial communities at class taxonomic rank.

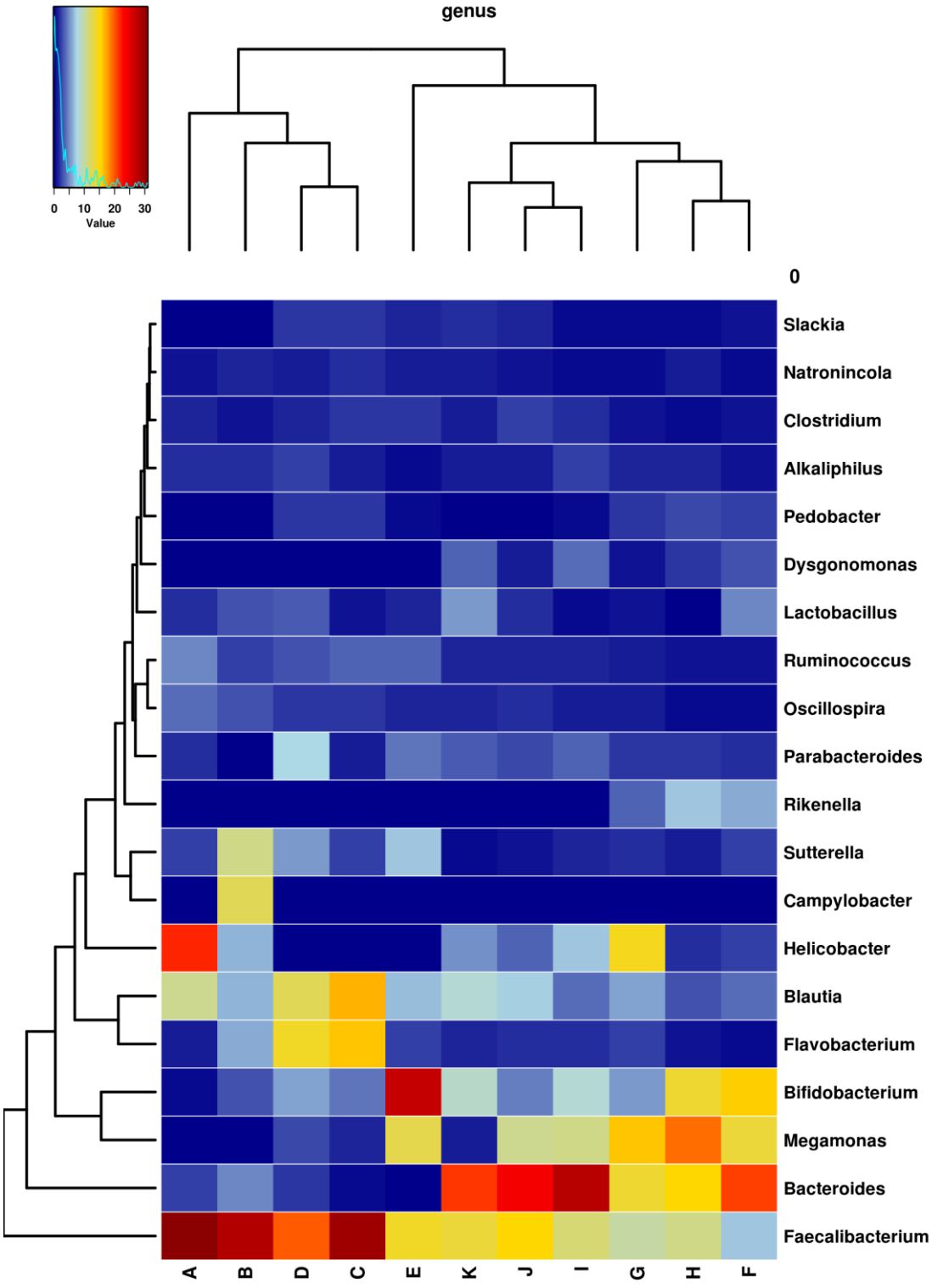

Fig 3. Heat map showing the relative abundance of the top 20 genera present, ranging from highest abundance (red) to least (blue).

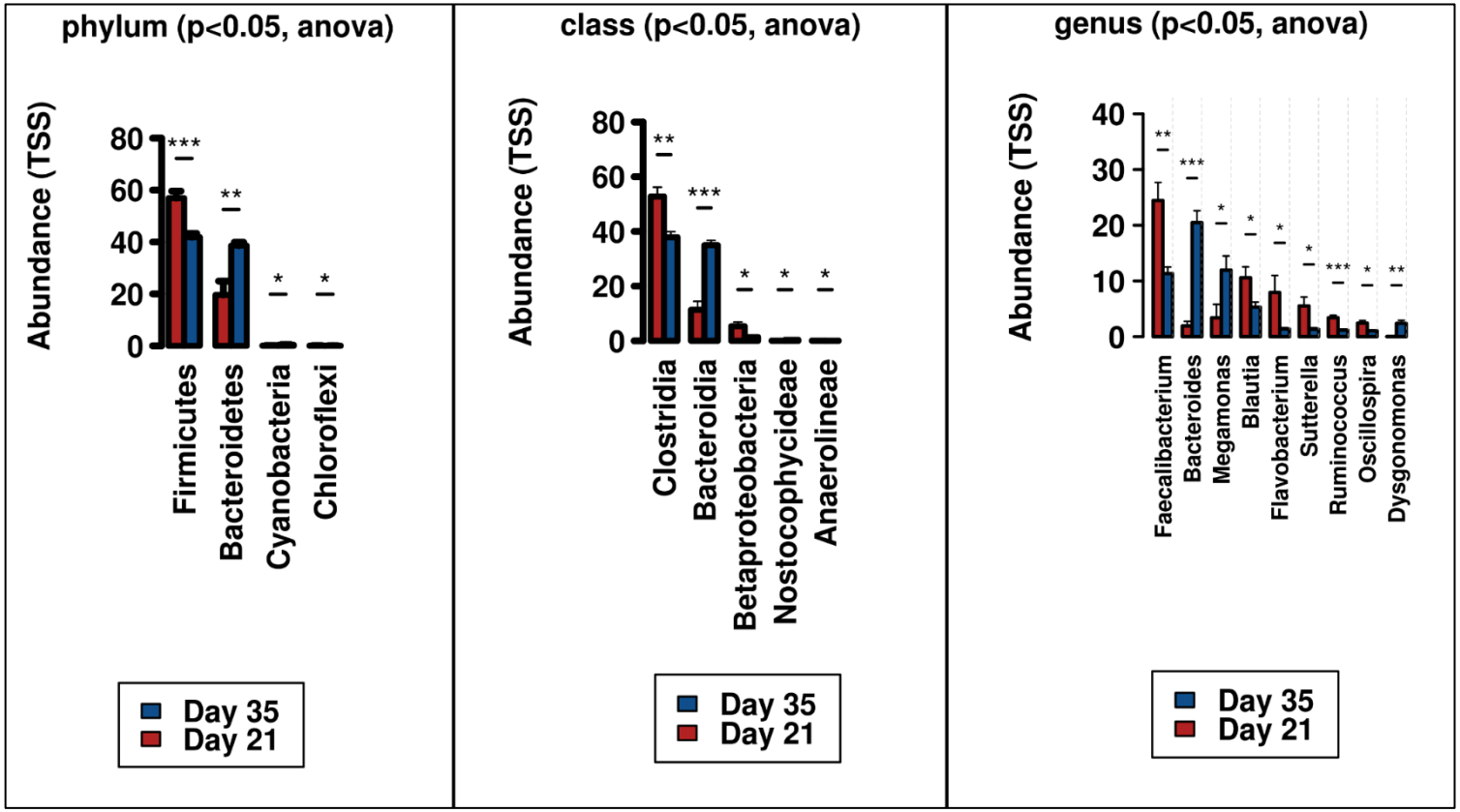

Fig 4. Comparison of taxa abundance across sample groups using ANOVA.

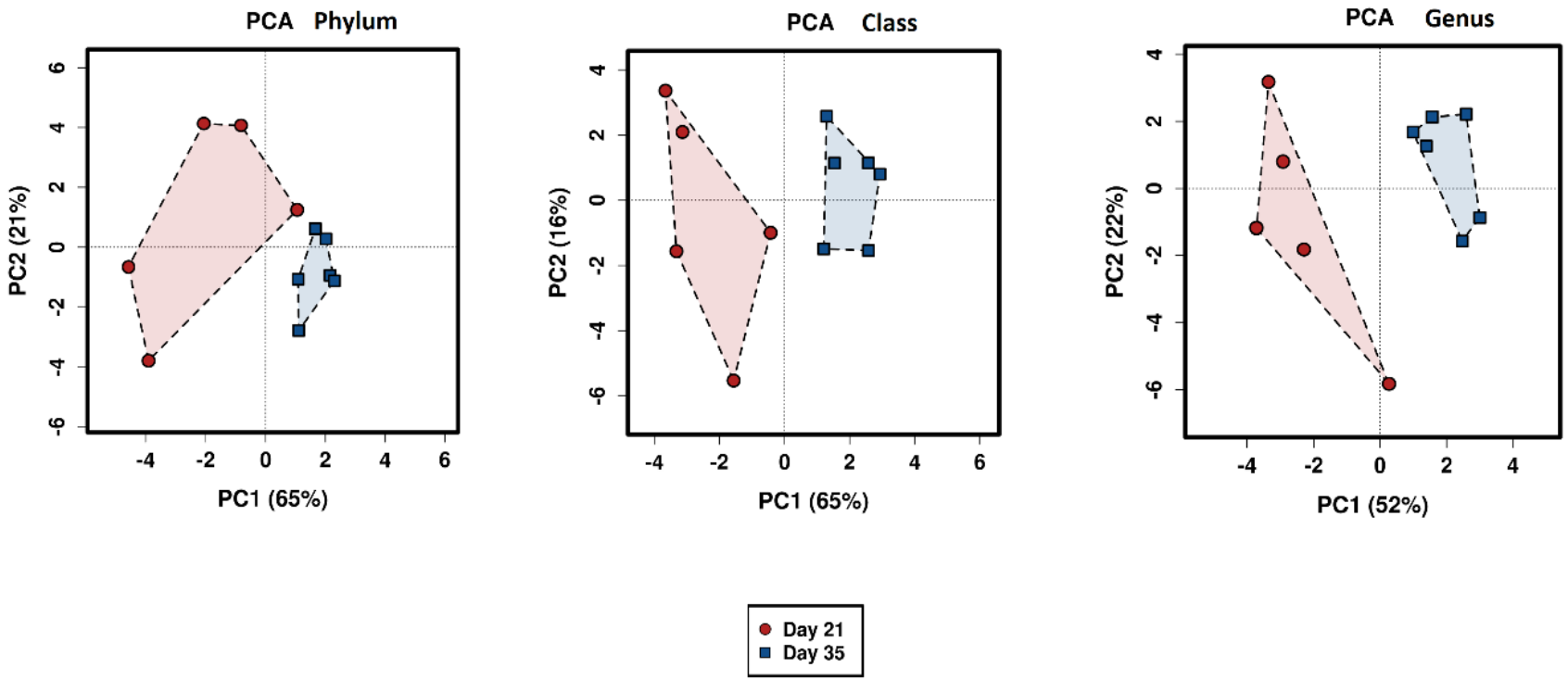

Fig 5. Principal component analysis of sample groups at phylum, class and genus taxonomic levels.

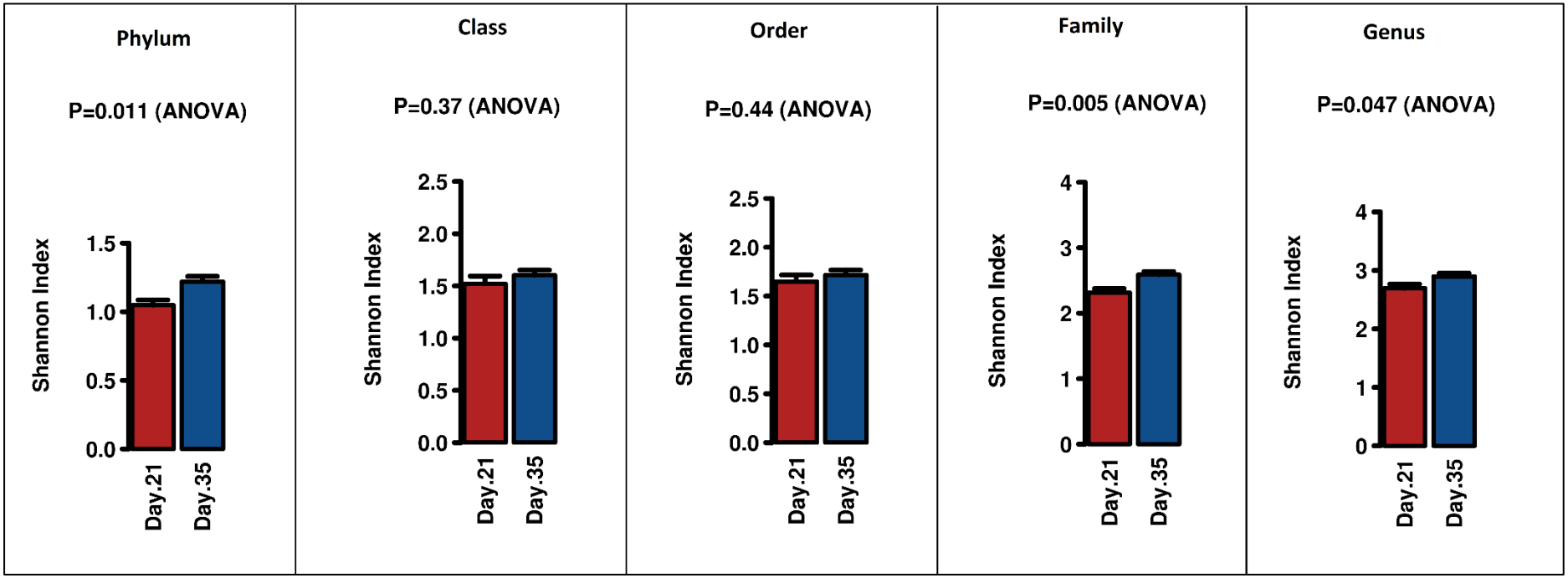

Fig 6. Microbial community alpha diversity assessed using Shannon index.

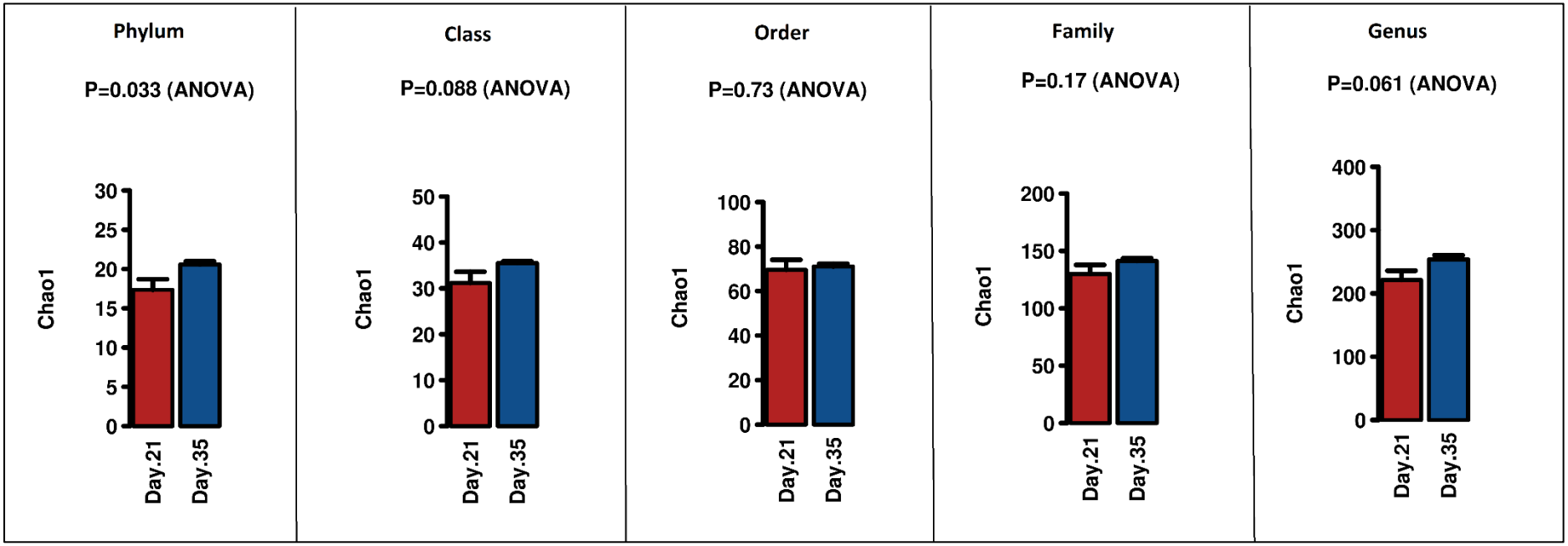

Fig 7. Microbial community richness assessed by Chao1.

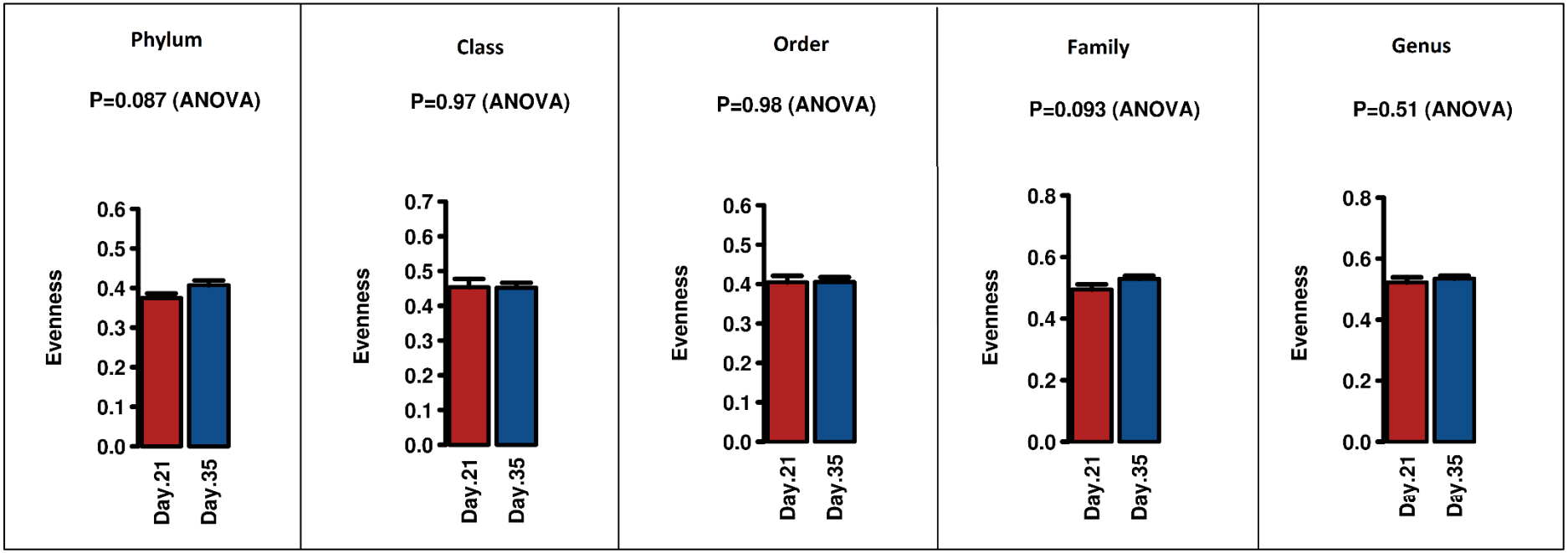

Fig 8. Microbial community evenness at all taxonomic ranks.

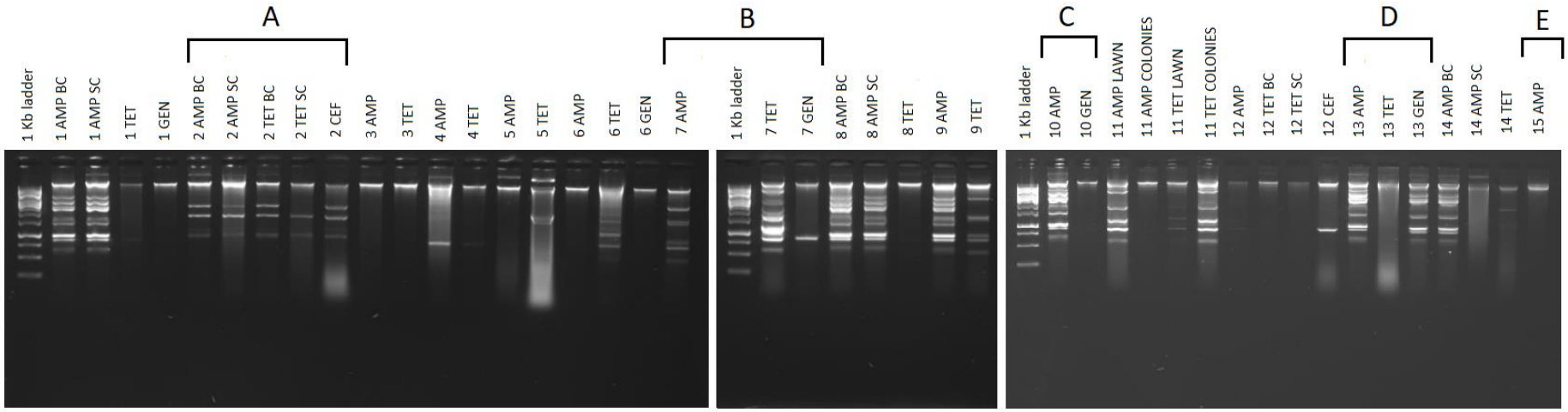

Fig 9. Plasmids isolated from the broiler caecal samples on day 21 visualised on an agarose gel. Samples are named after the bird the caecal sample was taken from and the antibiotic which the transconjugant was selected on, *e.g.* 6 TET= bird 6 selected on tetracycline. Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony) or had both a lawn of growth (LAWN) with some distinct colonies (COLONIES). Samples are labelled with the letter of their corresponding microbiome sample.

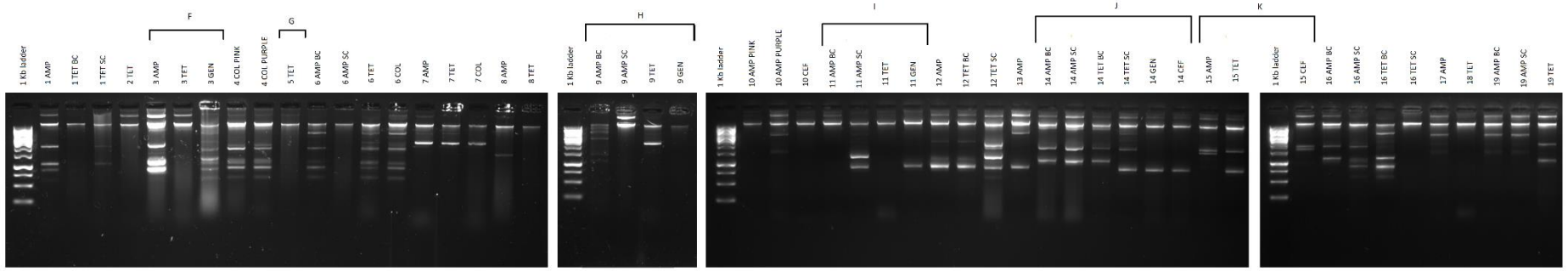

Fig 10. Plasmids isolated from the broiler caecal samples on day 35 and visualised on an agarose gel. Samples are named after the bird the caecal sample was taken from and the antibiotic which the transconjugant was selected on, e.g. 6 TET= bird 6 selected on tetracycline. Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony) or different colours on EMB agar (pink/purple). Samples are labelled with the letter of their corresponding microbiome sample.

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Chapter 5

An investigation into the effect of mannan rich fraction supplementation on the metagenome of broiler chickens

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5.1 ABSTRACT

Antibiotic resistance is regarded as one of the most serious threats to human health worldwide. The rapid increase in resistance rates has been attributed to the extensive use of antibiotics since they became commercially available. The use of antibiotics as growth promotors has been banned in numerous regions due to this. Mannan rich fraction (MRF) has been reported to show similar growth promoting effects. We investigated the effect of MRF on the microbial community present within the caecum of commercial broilers at two different time points within the growth of the broiler, day 27 and day 35. At phylum level, *Firmicutes* and *Bacteroides* were the most abundant, while *Clostridia* and *Bacteroidia* were most dominant at class level. Mannan rich fraction did not appear to affect the most abundant taxa. Food-producing animals are known reservoirs of antibiotic resistance genes (ARGs) within their gut microbiomes. The resistome was comprised of 171 ARGs; 69 core and 102 accessory ARGs. The genes present at the highest abundance in all samples were *tetW, lnuC* and *aadE*. Differences were observed in the MRF supplemented group at day 27 compared with the untreated control. This highlights the potential of MRF to have an effect on ARG abundance. However, significant variability was seen from sample-to-sample. This study also demonstrated the presence of ARGs in the gut of food-producing animals even in the absence of antibiotic selective pressures. These genes could produce detrimental effects for both animal and human health.

5.2 INTRODUCTION

Over 1,000 species of bacteria inhabit the gastrointestinal tracts of poultry and livestock. These bacteria enter the human food chain through the consumption of meat products, which are regarded as a major source of protein for humans[1]. Poultry is the fastest growing agricultural sub-sector, with continued growth expected as the global population increases[2]. This places enormous pressure on poultry producers, with production often being large-scale and highly intensive. Within such systems, large densities of birds are housed in close proximity to each other[3], and are in constant contact with effluent and secretions from other birds[4]. This creates an ideal environment for bacteria, commensal or pathogenic, to spread throughout the flock. For example, bird-to-bird transmission of the enteric pathogen *Campylobacter* occurs rapidly within a flock, with almost the entire flock becoming colonised within a few days of when the first bird was colonised[5]. In the same manner, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are also disseminated throughout poultry flocks.

The threat of antibiotic resistance to global health is ever-increasing. The continued overuse and misuse of antibiotics in both humans and animals has drastically accelerated the development and spread of antibiotic resistance[6]. The link between the use of antibiotics in agriculture, whether for treatment or prevention of disease, or to promote the growth of animals, to increased resistance rates has been documented[7,8,9]. Antibiotic use creates a selective pressure that allows for the proliferation of ARB[10]. The gut microbiome of food-producing animals is a known reservoir of ARGs, with bacteria having the ability to harbour these genes even in the absence of selective

pressure from antibiotic use[11]. These ARGs can transfer to human and animal pathogens[12].

The caecum is the most densely populated region of the chicken gastrointestinal tract, and is known to harbour an assortment of microorganisms which are involved in processes such as the recycling of nitrogen, digestion of resistant carbohydrates, absorption of additional nutrients, prevention of colonisation with pathogens and detoxification of harmful substances[13]. However, it can also harbour pathogens such as *Salmonella enterica* and *Campylobacter jejuni*, which cause disease in humans.

Prebiotics are described as non-digestible feed additives that benefit the host by selectively stimulating the growth or metabolic activity of a small number of intestinal microorganisms[14]. Supplementation of the diet of broilers with the prebiotic mannan-oligosaccharide (MOS) has been reported to improve bird weight and feed efficiency[15]. Because of this, the use of MOS has been suggested as a viable alternative to antibiotic growth promotors[16], which have been banned in the European Union since 2006 and more recently in America in 2017[17]. A study by Sims *et al.*, found that MOS produced an equivalent result to zinc-bacitracin in terms of performance improvement[18]. Ao & Choct reported improved growth performance and flock uniformity in broilers that received MOS[19]. Mannan rich fraction (MRF) is a second-generation MOS product, with increased activities in intestinal health and immune modulation[20].

Our study aimed to investigate the effect of MRF supplementation to the diet of commercial broiler chickens on the microbiome and resistome at two different days

within the growth of the broiler. A metagenomics based approach was employed to examine any MRF-induced changes in the structure and diversity of the microbial community. The resistome was also investigated to assess if MRF had an effect on ARG profiles within the broiler caecum.

5.3 MATERIALS & METHODS

5.3.1 Samples

Broiler caecal samples were obtained from a commercial poultry production unit in the European Union. Broilers received either a standard commercial diet or a standard diet plus MRF at the manufacturer's recommended inclusion rates. The samples were collected at two time-points, on days 27 and 34 post-hatch. Samples were lyophilised and stored at -80°C before analysis.

5.3.2 Total DNA Extraction

Total DNA was extracted from 0.05 g of each caecal sample (*n*=16) using the Qiagen DNeasy PowerSoil kit according to the manufacturer's guidelines. The concentration and purity of the extracted DNA was measured using an Invitrogen Qubit Fluorometer (dsDNA high-sensitivity assay kit) and a DeNovix DS-11 spectrophotometer.

5.3.3 Metagenomic Sequencing

The sequencing was performed at the Centre for Genomics Research, University of Liverpool. Illumina unamplified fragment libraries were prepared using the TruSeq PCR-free kit (350 bp inserts). The samples were paired end sequenced (2×150 bp) using an Illumina HiSeq 4000. Between 60 and 80 million raw reads were obtained per sample. The raw Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1. The 3′ end of any reads which matched the

adapter sequence for 3 bp or more were trimmed. Sickle version 1.200 was used to further trim reads, with a minimum window quality score of 20. Reads which were shorter than 20 bp after trimming were removed.

5.3.4 Bioinformatic Analysis

The trimmed reads were uploaded to the European Nucleotide Archive (ENA). The files were then transferred to MGnify[21] for analysis. InterProScan was used to generate matches against predicted CDS using Pfam, TIGRFAM, PRINTS, PROSITE patterns and Gene3d to provide gene ontology (GO) terms. MAPseq was used for SSU and LSU rRNA annotation, utilising SILVA SSU/LSU version 1.32 reference database to assign taxonomy and OTU classifications.

Antimicrobial resistance annotation was performed using DeepARG. The machine learning solution which utilises CARD, ARDB and UNIPROT databases first removes low quality reads using TRIMMOMATIC. Reads are then merged into one file (VSEARCH) and submitted for classification to the DeepARG algorithm[22]. The relative abundance of ARGs was normalised to the 16S rRNA content of each sample using the following parameters: identity: 80%, e-value: 1e-10, coverage: 50% and probability: 0.8.

5.3.5 Data Analysis

Statistical analysis of the microbiome data was performed using Calypso (http://cgenome.net/calypso/)[23]. The data were normalized for statistical analysis and

samples with less than 1000 sequence reads and rare taxa, with less than 0.001% relative abundance were removed. Rarefaction analyses and Principal Component Analysis (PCA) of the microbiome were carried out using default settings. The microbial community composition was quantitatively visualized by bar charts. ANOVA was used to compare the relative abundances of phylum, class and genus taxonomic levels between treatment groups. Bacterial alpha diversity was estimated using the Shannon index and richness estimated using Chao1.

ARGs were assigned to the core resistome if they were present in all samples. ARGs detected in at least one sample, but less than the total number of samples, were assigned to the accessory resistome. The statistical analysis and correlation analysis of the ARGs was performed using the PAleontological STatistics (PAST) version 3.2[24]. Samples were compared using ANOVA Mann–Whitney pairwise tests with Bonferroni correction for multiple comparisons. PCA were performed in PAST using default settings. Heat-maps were generated using Morpheus (https://software.broadinstitute.org/morpheus/).

The sequences are deposited in the European Nucleotide Archive (ENA) under the primary accession PRJEB29033 and secondary accession ERP111299.

5.4 RESULTS & DISCUSSION

The total reads per sample analysed after quality control and trimming ranged from 57,465,201 reads to 82,809,780 reads.

5.4.1 Microbiome Analysis

Rarefaction analysis showed a sufficient sequencing depth was reached (Fig 1). Samples were compared based on treatment group (control *vs.* MRF) and time-point (day 27 *vs.* day 34). The microbiome was found to be dominated by *Firmicutes* across all samples, with up to 89.45% of all classified reads from day 27 control, up to 80.22% for day 27 treated, 81.46% from day 34 control and 91.49% from day 34 treated classified within the phylum *Firmicutes* (Fig 2A). This was followed by *Bacteroidetes,* with up to 9.55% of all classified reads from day 27 control, up to 7.7% for day 27 treated, 9.31% from day 34 control and 12.38% from the day 34 treated group. We then identified a number of unclassified reads in all samples ranging from 2.45% of all classified reads up to 11.07%. Previous studies have also found unclassified reads within their samples[25]. This was followed by *Tenericutes*, *Actinobacteria* and *Proteobacteria*. Some sample variation was noted here, with 16.45% of reads for *Actinobacteria* in sample A (day 34 treated), compared with the other samples which ranged from 0.5% to 3.86%. Similarly, sample G (day 34 control) contained 10.25% of reads for *Proteobacteria,* in comparison to the 5.23% to 0.21% range of the other samples. These findings are in keeping with other studies investigating the broiler microbiome[26,27]. Variation within samples has also been

observed previously, and has been attributed to factors such as farm workers, housing conditions, biosecurity level, litter, and feed access[28].

The microbiome was largely dominated by *Clostridia* at class level, with up to 70.45% of all classified reads from day 27 control, up to 73.23% for day 27 treated, 74.03% from day 34 control and 85.28% from day 34 treated (Fig 2B). We again identified high percentages of unclassified reads, ranging from 5.33% up to 18.56%. This was followed by *Bacteroidia*, with up to 7.63% of all classified reads from day 27 control, up to 9.41% for day 27 treated, 9.15% from day 34 control and 12.25% from day 34 treated. *Bacilli* comprised up to 15.2% of all classified reads from day 27 control, up to 9.89% for day 27 treated, 7.03% from day 34 control and 7.19% from day 34 treated. Within group variations were seen in the next predominant class, *Mollicutes*, particularly at the day 34 time-point, with percentages ranging from 6.12% to 0.09% in the treated group; and 7.26% to 0.63% in the control group.

Over half of all reads at genera level in all samples were unclassified (between 50.52% and 73.99%) (Fig 2C). *Faecalibacterium* was the next most dominant, with up to 20.23% of all classified reads from day 27 control, up to 14.34% for day 27 treated, 16.21% from day 34 control and 20.87% from day 34 treated. This was followed by *Lactobacillus*, with up to 13.64% from day 27 control, 7.09% for day 27 treated, 6.03% from day 34 control and 5.93% from day 34 treated. *Bacteroides* was the next most dominant genera, with up to 2.37% from day 27 control, 6.81% for day 27 treated, 6.71% from day 34 control and 3.42% from day 34 treated.

Taxa abundance at phylum to genera levels were compared using ANOVA (Fig 3). The difference in relative abundance was considered significant when $P < 0.05$. Pair-wise comparisons were performed by t-test and annotated as *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A significant variance between treatment groups was seen in 2 phyla (*Verrucomicrobia, Candidatus_Melainabacteria*), 2 classes (*Verrucomicrobiae, Betaproteobacteria*), 2 orders (*Verrucomicrobiales, Burkholderiales*), 3 families (*Sutterellaceae, Burkholderiaceae, Akkermansiaceae*) and 3 genera (*Parasutterella, Burkholderia, Akkermansia*). However, these taxa were all present in relatively low abundance, comprising less than 1% of classified reads in any of the samples within the broiler microbiome.

Shannon index was used to assess the microbial alpha diversity (Fig 4A). No significant differences were observed. The bacterial community richness was assessed using Chao1, where a significant difference (where $P < 0.05$) was seen at order level only (Fig 4B), indicating a greater number and therefore richer community at this taxonomic rank in the control group. The evenness of the microbial community is displayed in Fig 4C, where no significant differences were observed. Thus, there were no major changes in the diversity or evenness in the microbiome between treated or control groups.

Principal Component Analysis (PCA) was used to plot the relative abundance of OTUs at phylum, class and genus levels (Fig 5). The observed patterns between treatment groups and time-points were similar. The day 34 time-point and treated group had large intra-cluster distances along both the PC1 axis and the PC2 axis at all taxonomic

levels. The day 27 time-point and control group had a small intra-cluster distance at phylum level, which increased slightly at class level. A much larger intra-cluster distance was observed along the PC2 axis at genus level. Both treatment groups (Fig 5A) and time-points (Fig 5B) clustered together with the exception of a few samples at phylum, class and genera level. This shows that while there was some bird-to-bird variation present, the microbiome diversity and composition was overall consistent between the treatment and control groups.

A previous study into the effect of MRF on the broiler microbiome found a shift from *Firmicutes* to *Bacteroides* at phylum level[29]. We did not observe this same change in microbiota, with ours remaining dominated by *Firmicutes* at phylum level. The authors also note a change to a *Bacteroidia* dominant microbiota at class level from *Clostridia*. We saw a slight increase in the relative abundance of *Bacteroidia* in the treatment group but this was not found to be significant. MOS, the predecessor to MRF, has been described to increase the abundance of *Lactobacillus* spp. in the caecum[30]. However, *Lactobacillus* was already dominant within our samples, and we did not observe notable changes between the control group and the group that received MRF. Numerous studies have shown that pathogenic bacteria which possess mannose-specific fimbriae can bind to mannose which reduces the risk of pathogens including *Salmonella* and *Escherichia coli* in the gastrointestinal tract[31]. Interestingly, we did not detect *Salmonella* spp., *E. coli* or *Campylobacter* spp., which commonly colonise the poultry gut, in any of our samples. Thus, this may be why we did not observe any major changes within the treatment group compared to the control. This suggests that MRF would be beneficial for use in farms or production facilities with pathogen-

challenged chickens, but would not have deleterious effects on the microbiomes of non-pathogen-challenged chickens.

5.4.2 Resistome Analysis

A total of 171 ARGs were identified. These were isolated from healthy broiler chickens which had not been administered antibiotics. From this, 69 ARGs were assigned to the core resistome as they were present in all 16 samples (Table S1). The remaining ARGs that were present in at least one, but not all samples, were assigned to the accessory resistome, totalling 102 ARGs (Table S2). The trends of resistance across all samples were investigated by summing the relative abundance of ARGs per sample by antibiotic class (Fig 6). All of the samples harboured resistance genes conferring resistance to the same classes of antibiotics. The greatest proportions of ARGs present in all samples included tetracycline, aminoglycoside, multi-drug, glycopeptide and macrolide-lincosamide-streptogramin B (MLS_B), nucleoside and peptide resistance genes. Resistance to the remaining classes of antibiotics was relatively low. Variation was seen in the relative abundances of the identified classes both between the groups and between the samples. Overall, the day 27 samples had higher numbers of ARGs than the day 34 samples. Sample D from the day 34 treated group had a higher abundance of multi-drug resistance genes than the other samples within that group. Sample J from the day 27 treated group was shown to have a higher abundance of MLS_B resistance genes than the remaining samples in the group.

The core resistome was composed of 69 ARGs that included a large number of efflux pumps (*n*=21), as well as porins (*n*=3), tetracycline (*n*=9), glycopeptide (*n*=10), beta-lactam (*n*=2), aminoglycoside (*n*=5), peptide (*n*=4), MLS_B (*n*=1), lincosamide (*n*=2), streptogramin (*n*=1), macrolide (*n*=1), unclassified (*n*=6), nucleoside (*n*=2), fluoroquinolone (*n*=1) and diaminopyrimidine (*n*=1) antibiotic resistance genes. The distribution of genes was reasonably consistent across all samples within the core resistome, with *tetW*, *lnuC* and *aadE* being the most abundant. It is represented in Fig 7A, where the most abundant genes clustered together using the Bray–Curtis similarity matrix. Samples were also clustered based on the relative abundance of their core resistance genes by the Bray-Curtis similarity matrix (Fig 8A). None of the clustered groups contained all four samples from the day and treatment group of broilers. This demonstrates the variation in the abundances of ARGs even between samples from the same treatment or age group. For example, from the day 27 control group, samples M and N clustered together; and samples O and P, which had a higher number of ARGs clustered together; but neither of these clusters overlapped and they had a large distance between (labelled in green, Fig 8A). Samples with a higher abundance of ARGs clustered together towards the right of the chart, while samples with a lower abundance clustered together on the left of the chart. A review of the faecal resistome of pigs and broilers from nine European countries also found less consistency and far more variability in the relative proportions of resistance in the broiler samples. The highest abundance of resistance identified was to tetracycline, macrolide, beta-lactam and aminoglycoside antibiotics[32].

The accessory resistome was comprised of 102 ARGs. The *ermF* gene and *rpoB2* were the most abundant accessory genes, which confer resistance to MLS_B antibiotics *via*

efflux pump and rifamycin, respectively. Clustering analysis of the relative abundances of ARGs in the accessory resistome using the Bray-Curtis similarity matrix can be seen in Fig 7B, where the most abundant ARGs clustered together. Analysis of samples was performed also using the Bray-Curtis similarity matrix (Fig 8B). The samples from the day 27 control group clustered into two groups beside each other (labelled in green). This indicates that similar abundances of ARGs were present in each of these samples. Three samples from the day 27 treated group clustered into two groups closely related but separate to sample L (labelled in pink). Overall, the abundance of ARGs present in the accessory resistome at day 27 were consistent. This was not seen at day 34, particularly within the treated group, where none of the samples clustered together (labelled in purple). The day 34 birds had a more varied abundance of ARGs within the accessory resistome.

There were significant differences ($P < 0.05$) observed between the relative abundances of ARGs among samples in the core resistome (Table 1). Samples in the day 27 control group showed the most differences to all other samples. In particular, samples P (day 27 control), D (day 34 treated) and O (day 27 control) were significantly different to all other samples, even within the same group. Again, the high levels of sample-to-sample variance was detected, with sample D being significantly different to all other samples within the day 34 treated group. Significant differences were also observed between samples within the accessory resistome (Table 2). In particular, samples B, A, C (day 34 treated) and H (day 34 control) had significant differences to all other samples. Sample D was significantly different to all other samples within the same group (day 34 treated), again highlighting the variability

that can be seen between samples of the same group that was also observed in the core resistome.

Principal component analysis was performed on the core (Fig 9A) and accessory (Fig 9B) resistomes. An overlap in the core resistomes of all sampled groups was observed. The core resistomes of samples from the day 27 treated group had a large intra-cluster distance along the PC1 axis, while the core resistomes of other sample groups had a greater intra-cluster distance along the PC2 axis. The core resistomes of samples from the day 34 clustered together. Those from the day 27 groups had a defined inter-cluster distance, indicating the differences in the abundance of core ARGs between the treated and control groups. This was also observed within the accessory resistome, where the day 27 groups also had a large inter-cluster distance, with a clear separation between the control and treated groups. The day 34 treated group (purple) had a larger intra-cluster distance along the PC1 axis but still clustered with the day 34 control samples (blue). It is therefore possible that MRF had an effect on ARG numbers at day 27. However, the variations seen in the samples from each group make this harder to definitively conclude. These ARGs may have been harboured by taxa present in lower abundances in the microbiome, and are therefore less likely to be detected, as no significant changes were observed within the most dominant taxa. It is also possible that some of these ARGs were present on broad-host range plasmids, and therefore changes in the plasmid population may be independent of changes within the microbiome.

5.4.3 Functional Analysis

The functional profile of the broiler microbiome was derived from the GO (Gene Ontology) assignments from the metagenomics analysis. A total of 2706 genes were assigned GO terms in at least one sample. The GO terms were divided into three categories: biological processes (*n*=1111 GO terms), cellular components (*n*=327 GO terms) and molecular functions (*n*=1268 GO terms) (Fig 10).

The cellular components with the highest presence were basic cellular components including membrane, ribosome, intracellular and cytoplasm. The presence of the fungal-type cell wall and the viral capsid and envelope indicates the presence and function of fungi and viruses within the broiler microbiome. Host cell components were also present. The molecular functions with the highest abundance in all samples were ATP binding, DNA binding, catalytic activity and oxidoreductase activity. Sample E had a higher presence of nucleotide binding, nucleic acid binding, DNA-directed DNA polymerase activity and 3'-5' exonuclease activity genes than all other samples, but has a slightly lower abundance of ATP binding and catalytic activity genes than the other samples.

The biological process most prominent in all samples was oxidation-reduction processes, metabolic processes, carbohydrate metabolic processes and regulation of transcription DNA-templated. Again, sample E was found to have a significantly higher abundance of DNA replication genes than all other samples. A number of viral components were also identified including viral capsid assembly, viral genome

replication and viral life cycle. Antibiotic catabolic process genes, which result in the breakdown of an antibiotic, were present in all samples. Also, antibiotic metabolic process genes were present in 7 samples, while antibiotic biosynthetic process genes, which result in the formation of an antibiotic, were present in all samples except samples I and P.

5.5 CONCLUSION

The advances in molecular technologies has allowed for more sensitive detection of the components of the metagenomes of numerous environments. We examined the metagenome of sixteen broiler chickens from a commercial production facility at two different time points. Half of the broilers received a MRF supplementation to their diets. We noted that significant variabilities were found between birds, even within the same group living in identical conditions. We did not observe any notable changes in the most dominant taxa in MRF supplemented groups, but suggest this may be due to the lack of pathogen-targets for the MRF in our samples. A large number of ARGs were identified (n=171) across all samples, displaying the presence of ARGs, even in the absence of selective pressures from antibiotics. A significant difference was detected in the relative abundance of ARGs between the MRF and control groups at day 27. As a similar difference was not observed in the most abundant taxa, these ARGs may be harboured by less dominant taxa within the microbiome or located on broad-host range plasmids, where changes may be independent of the microbiome. However, the samples were highly variable, even within the same group. The presence of high numbers of ARGs in food-producing animals could adversely affect both animal and human health.

5.6 TABLES

Table 1. Mann-Whitney pairwise test, Bonferroni corrected *p* values, core resistome.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A		0.006741	0.02182	0.01235	0.9945	0.08937	0.09803	0.004942	0.7321	0.2356	1	0.2289	1	0.7809	0.116	0.008518
B	0.006741		1	0.000148	0.0827	0.3854	0.2459	1	0.2161	0.5337	0.003813	0.2604	0.001358	0.001441	7.884E-05	3.227E-05
C	0.02182	1		8.059E-05	0.1589	1	1	1	1	1	0.005619	1	0.005222	0.003162	4.734E-05	2.091E-05
D	0.01235	0.000148	8.059E-05		0.004507	0.001498	0.0002219	0.0001071	0.005829	0.0002739	0.02903	0.0009713	0.04309	0.1952	1	1
E	0.9945	0.0827	0.1589	0.004507		1	1	0.06435	1	1	0.1841	1	0.1952	0.08664	0.02626	0.001854
F	0.08937	0.3854	1	0.001498	1		1	1	1	1	0.01018	1	0.02808	0.01904	0.001358	0.0002038
G	0.09803	0.2459	1	0.0002219	1	1		0.563	1	1	0.01131	1	0.006156	0.003045	0.0005201	3.955E-05
H	0.004942	1	1	0.0001071	0.06435	1	0.563		0.9698	1	0.001231	0.7513	0.001716	0.001184	5.293E-05	3.697E-05
I	0.7321	0.2161	1	0.005829	1	1	1	0.9698		1	0.1453	1	0.3042	0.1868	0.005936	0.001332
J	0.2356	0.5337	1	0.0002739	1	1	1	1	1		0.02762	1	0.036	0.02038	0.0005759	8.42E-05
K	1	0.003813	0.005619	0.02903	0.1841	0.01018	0.01131	0.001231	0.1453	0.02762		0.02903	1	1	0.4359	0.02497
L	0.2289	0.2604	1	0.0009713	1	1	1	0.7513	1	1	0.02903		0.03316	0.0166	0.001094	0.0001301
M	1	0.001358	0.005222	0.04309	0.1952	0.02808	0.006156	0.001716	0.3042	0.036	1	0.03316		1	0.2129	0.009822
N	0.7809	0.001441	0.003162	0.1952	0.08664	0.01904	0.003045	0.001184	0.1868	0.02038	1	0.0166	1		0.6683	0.04905
O	0.116	7.884E-05	4.734E-05	1	0.02626	0.001358	0.0005201	5.293E-05	0.005936	0.0005759	0.4359	0.001094	0.2129	0.6683		1
P	0.008518	3.227E-05	2.091E-05	1	0.001854	0.0002038	3.955E-05	3.697E-05	0.001332	8.42E-05	0.02497	0.0001301	0.009822	0.04905	1	

Table 2. Mann-Whitney pairwise test, Bonferroni corrected *p* values, accessory resistome.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A		0.06312	1	0.008164	1	1	1	1	1	1	1	1	1	0.9799	0.456	0.03637
B	0.06312		1	8.543E-11	0.001158	0.02117	0.009528	0.05846	0.000184	0.001301	0.0001235	0.00755	5.118E-07	1.949E-07	7.191E-07	6.569E-09
C	1	1		5.377E-08	0.167	1	1	1	0.04358	0.21	0.01674	0.8709	0.0001	0.0001297	0.0001728	1.029E-05
D	0.008164	8.543E-11	5.377E-08		0.02999	0.00198	0.001398	1.997E-05	0.02169	0.008006	0.7082	0.006054	1	1	1	1
E	1	0.001158	0.167	0.02999		1	1	1	1	1	1	1	0.8327	1	0.4126	0.1606
F	1	0.02117	1	0.00198	1		1	1	1	1	1	1	0.1758	0.2699	0.1026	0.01942
G	1	0.009528	1	0.001398	1	1		1	1	1	1	1	0.1092	0.1826	0.06517	0.01605
H	1	0.05846	1	1.997E-05	1	1	1		1	1	1	1	0.01205	0.02244	0.01343	0.001733
I	1	0.000184	0.04358	0.02169	1	1	1	1		1	1	1	1	1	0.7225	0.2408
J	1	0.001301	0.21	0.008006	1	1	1	1	1		1	1	0.6281	1	0.3158	0.08379
K	1	0.0001235	0.01674	0.7082	1	1	1	1	1	1		1	1	1	1	1
L	1	0.00755	0.8709	0.006054	1	1	1	1	1	1	1		0.313	0.3992	0.1543	0.04529
M	1	5.118E-07	0.0001	1	0.8327	0.1758	0.1092	0.01205	1	0.6281	1	0.313		1	1	1
N	0.9799	1.949E-07	0.0001297	1	1	0.2699	0.1826	0.02244	1	1	1	0.3992	1		1	1
O	0.456	7.191E-07	0.0001728	1	0.4126	0.1026	0.06517	0.01343	0.7225	0.3158	1	0.1543	1	1		1
P	0.03637	6.569E-09	1.029E-05	1	0.1606	0.01942	0.01605	0.001733	0.2408	0.08379	1	0.04529	1	1	1	

5.7 FIGURES

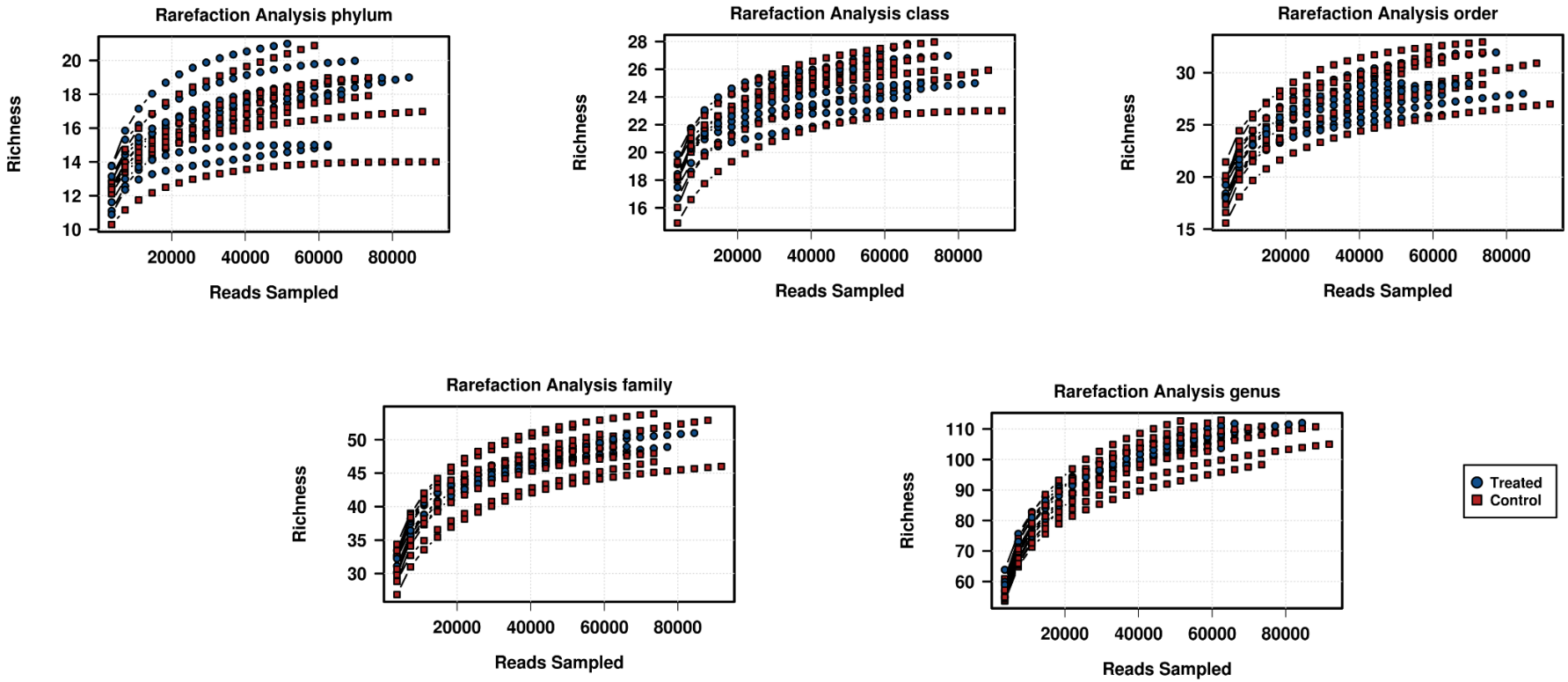

Fig 1. Rarefaction analysis at all taxonomic ranks.

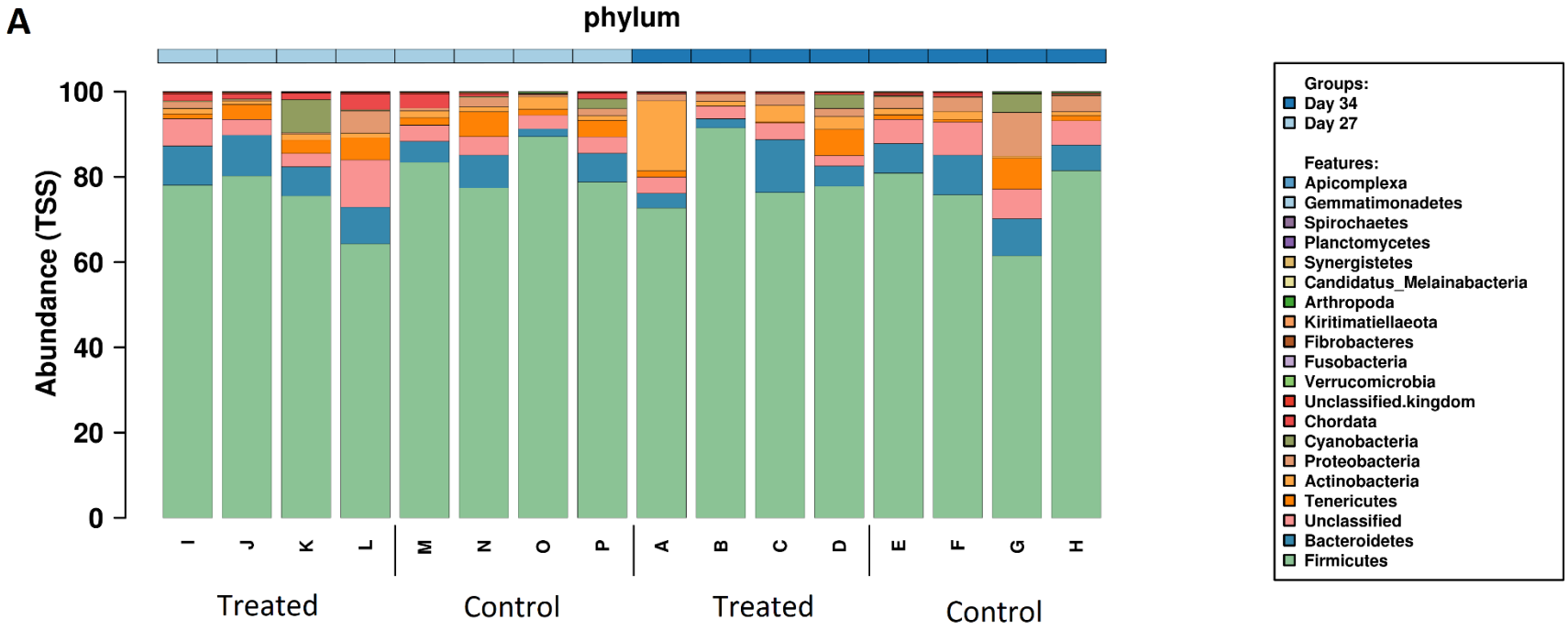

Fig 2. Microbial community composition displaying the top 20 most dominant taxa at (A) phylum, (B) class and (C) genus taxonomic levels.

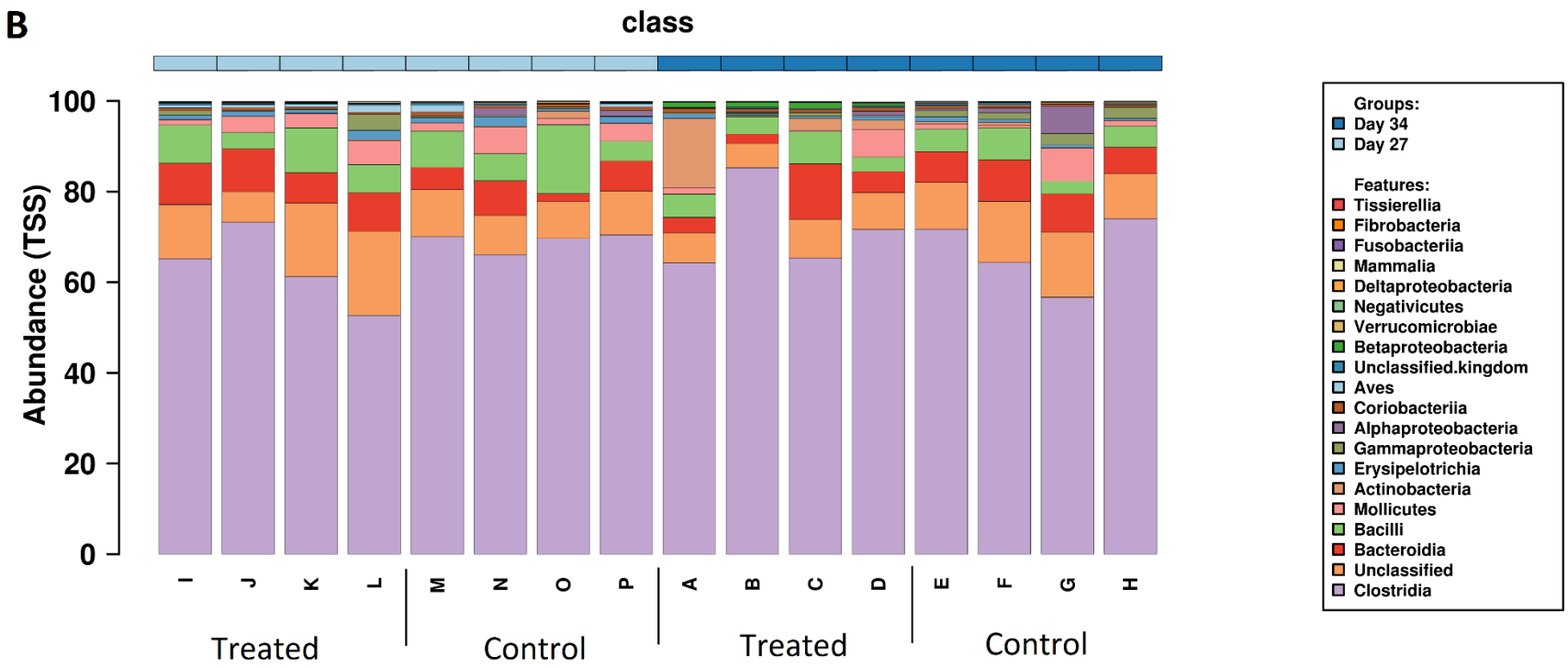

Fig 2. Microbial community composition displaying the top 20 most dominant taxa at (A) phylum, (B) class and (C) genus taxonomic levels.

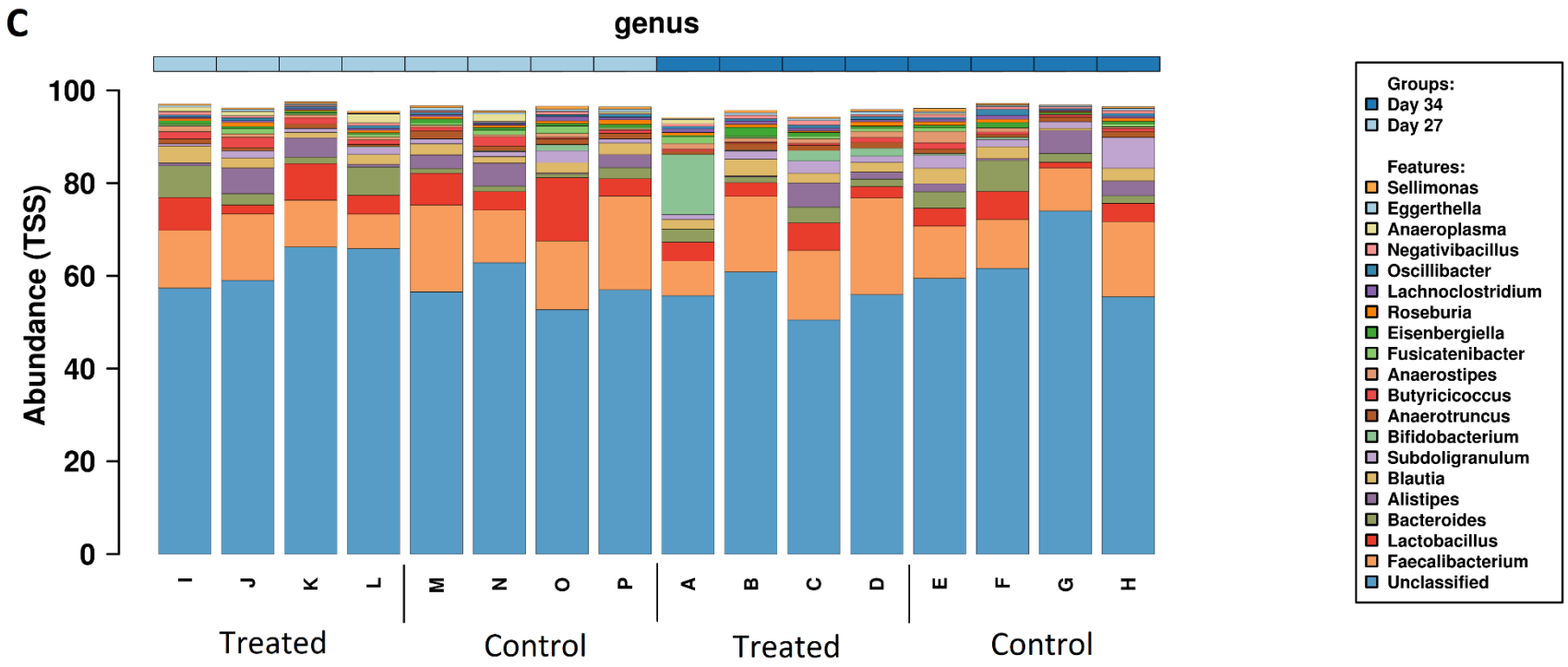

Fig 2. Microbial community composition displaying the top 20 most dominant taxa at (A) phylum, (B) class and (C) genus taxonomic levels.

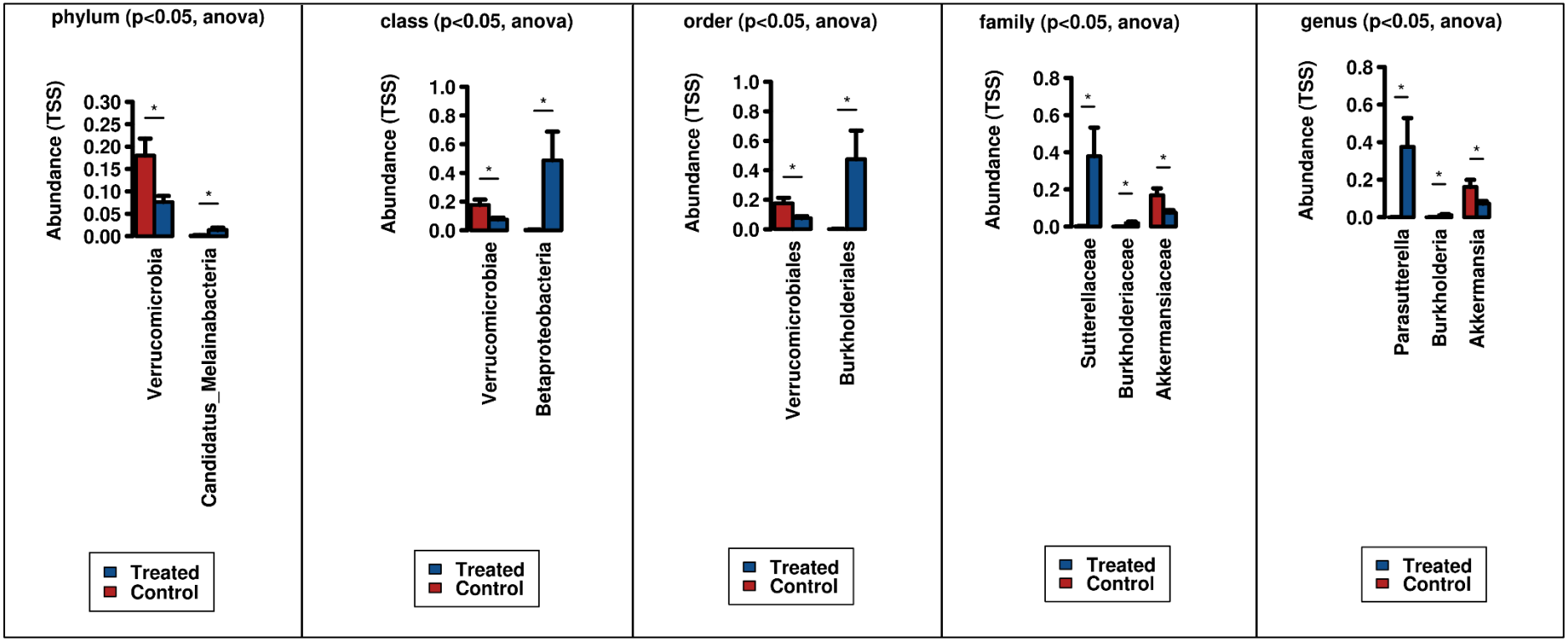

Fig 3. Comparison of taxa abundance across sample groups using ANOVA.

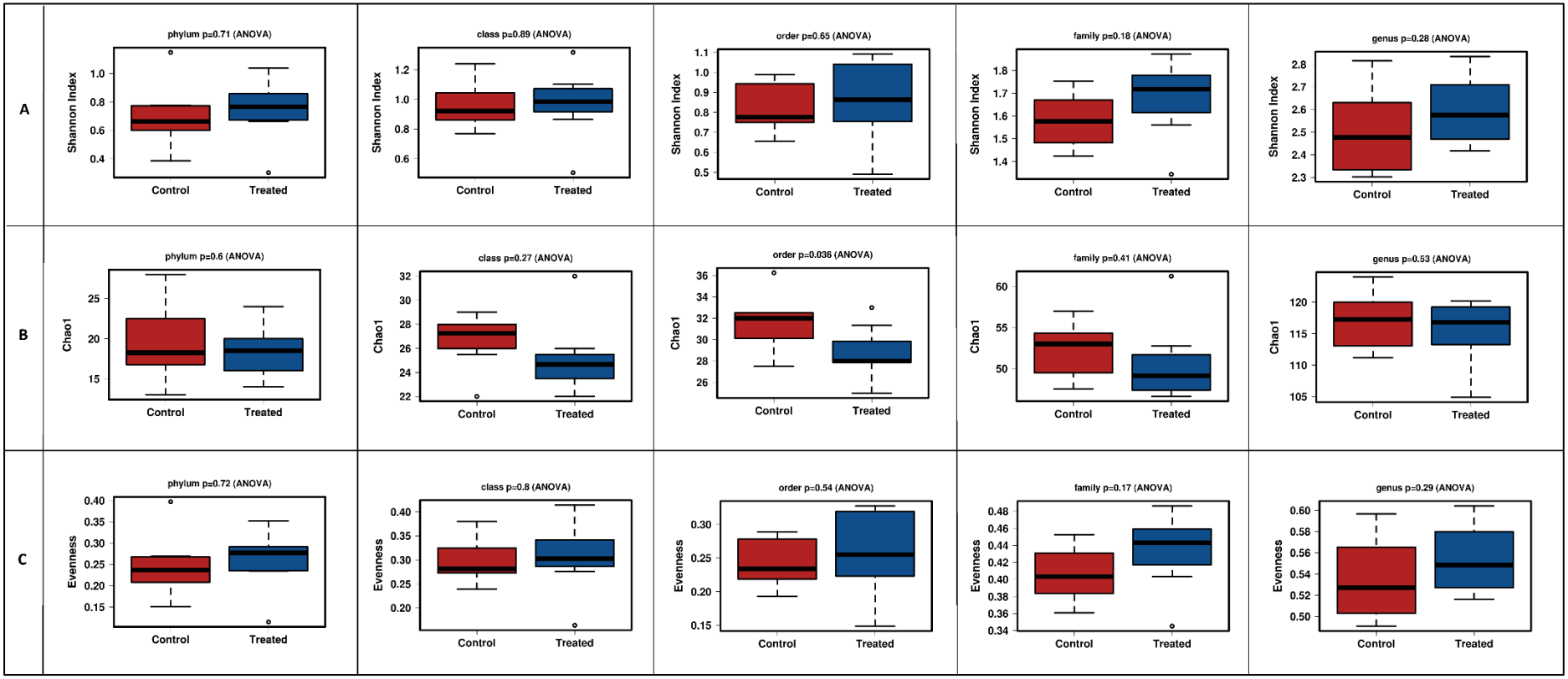

Fig 4. (A) Microbial community alpha diversity assessed using Shannon index, (B) microbial community richness assessed by Chao1 and (C) microbial community evenness at all taxonomic ranks.

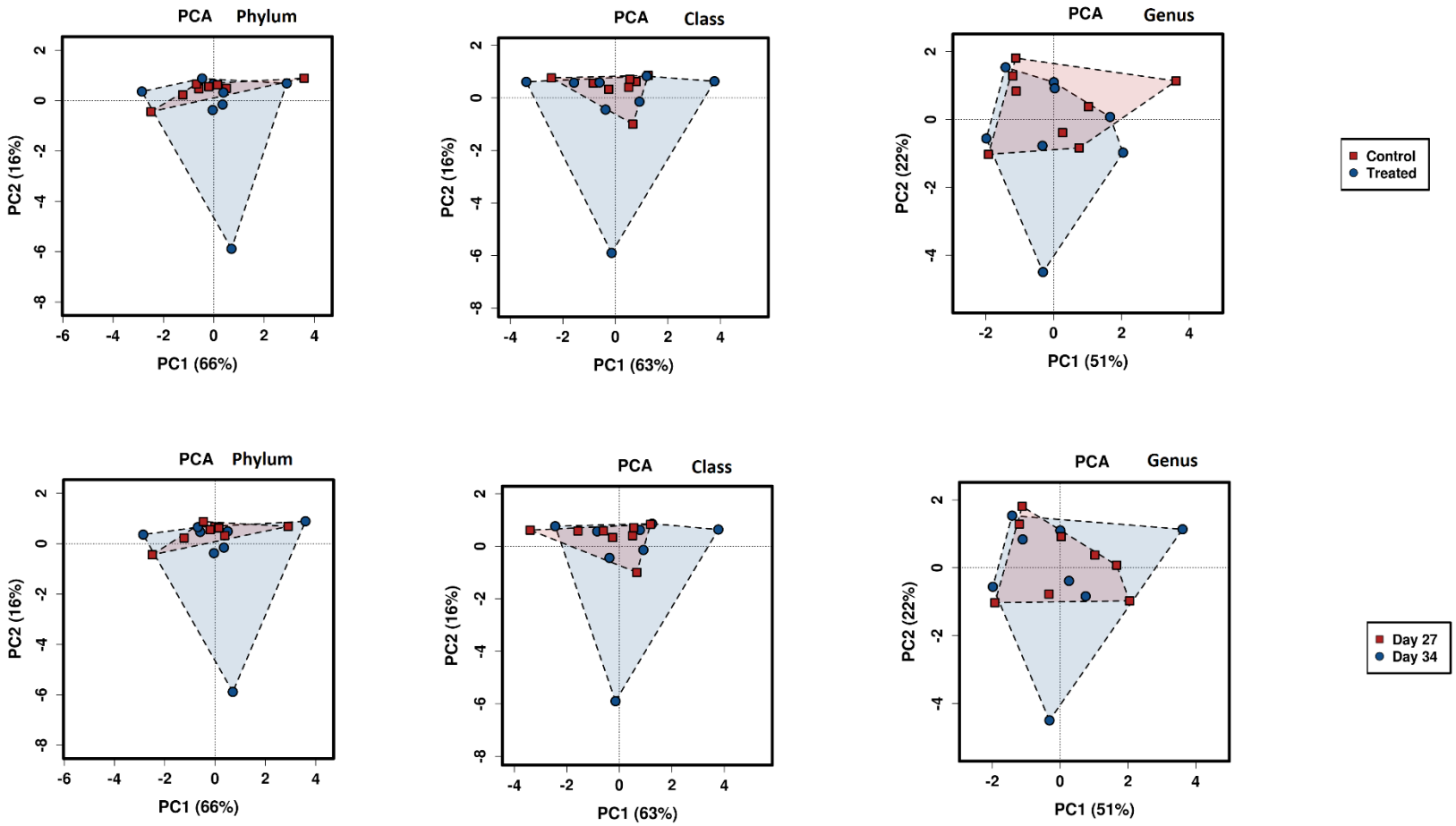

Fig 5. Principal component analysis of sample groups at phylum, class and genus taxonomic levels comparing treatment and time-points.

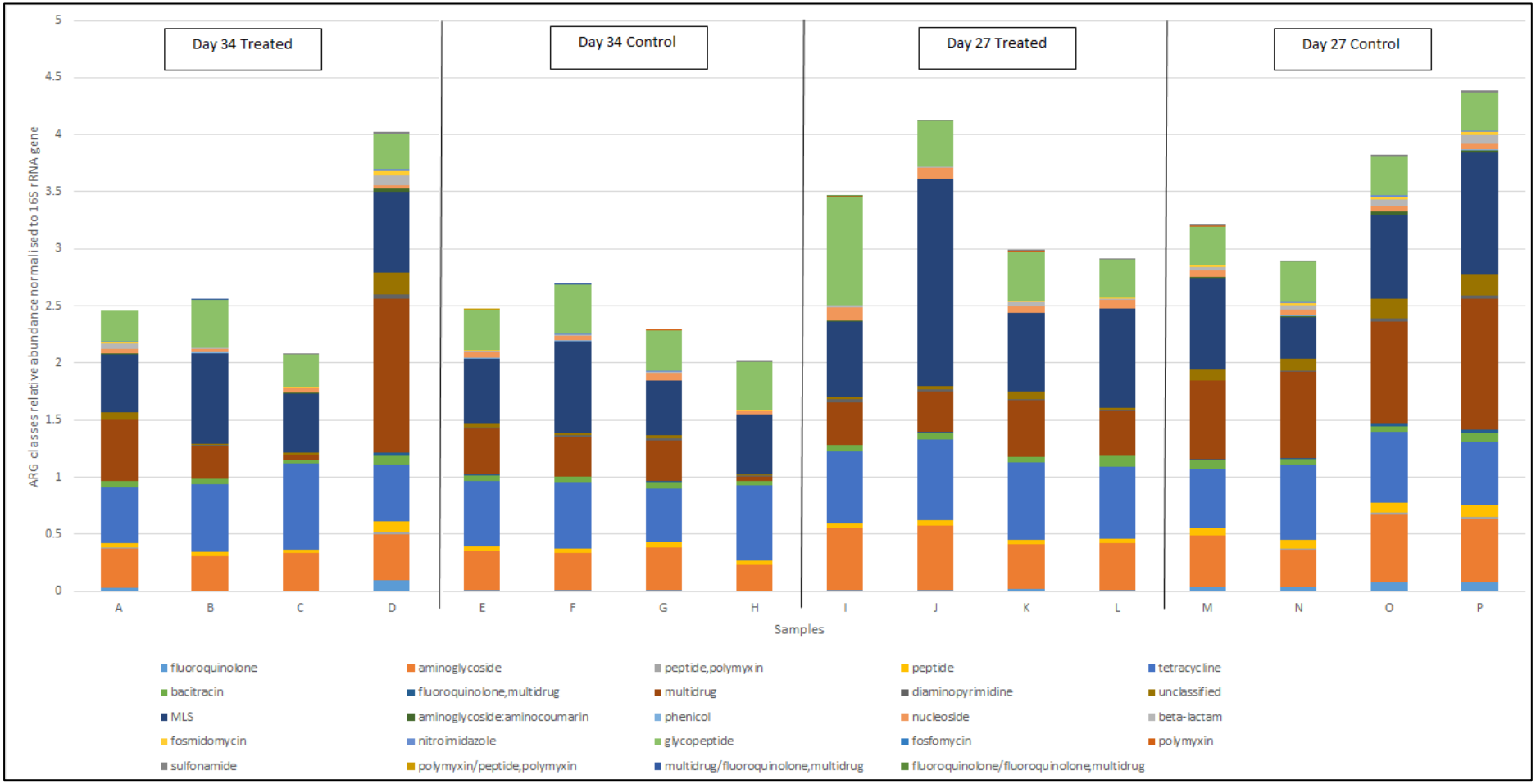

Fig 6. Relative abundance of ARGs present by antibiotic class.

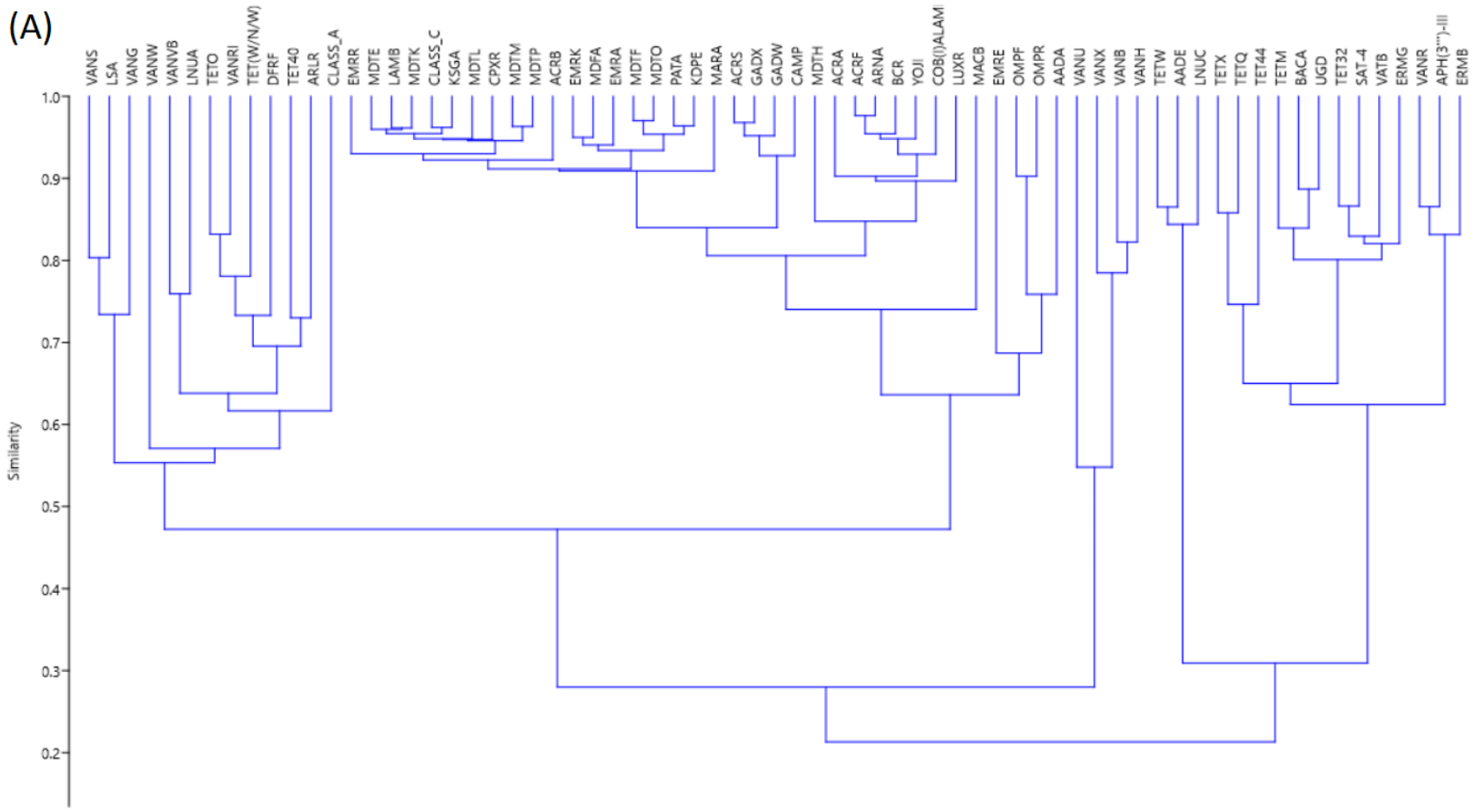

Fig 7. Cluster analysis of ARGs present in the (A) core resistome and (B) accessory resistome using the Bray-Curtis similarity matrix.

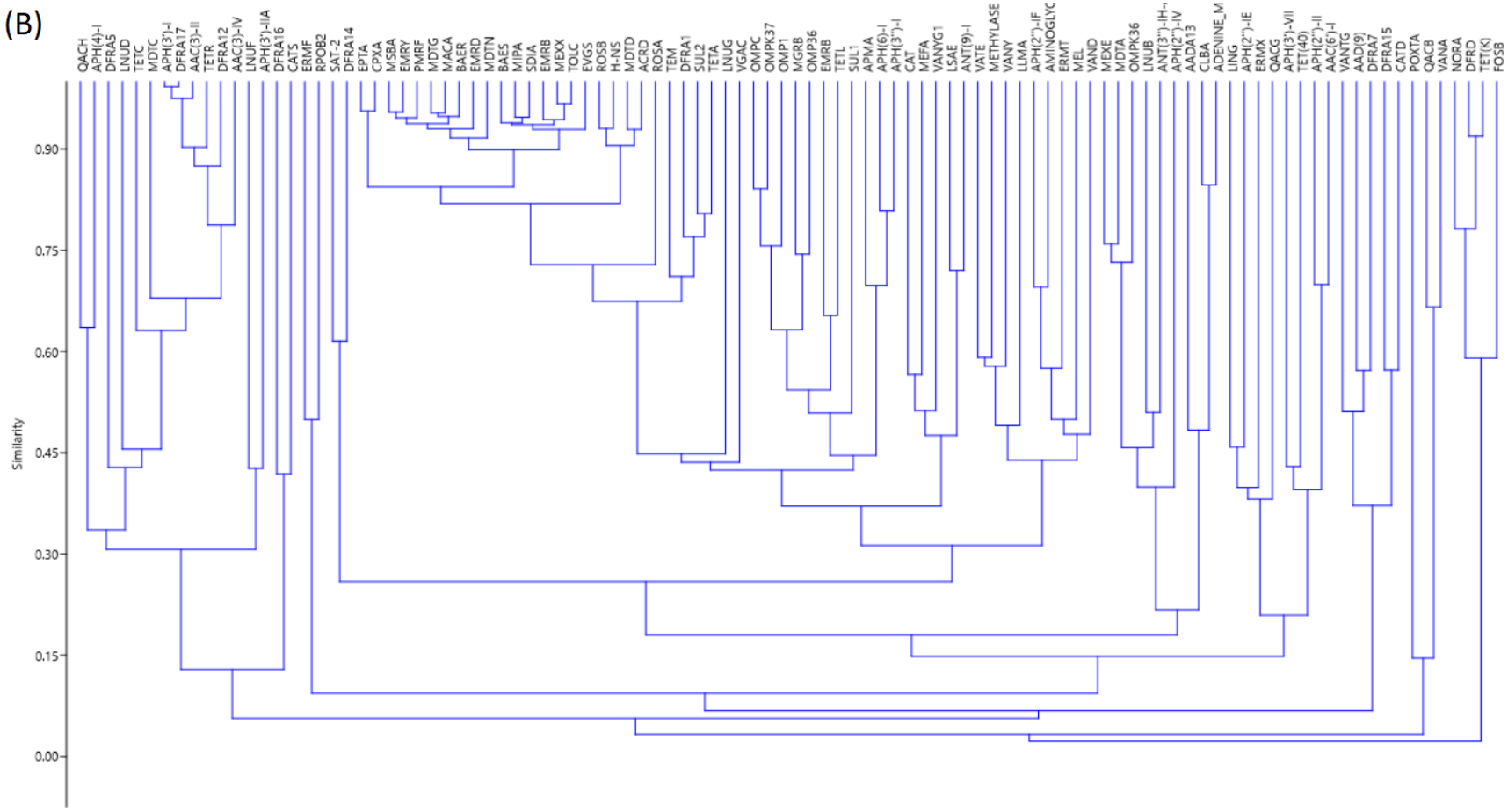

Fig 7. Cluster analysis of ARGs present in the (A) core resistome and (B) accessory resistome using the Bray-Curtis similarity matrix.

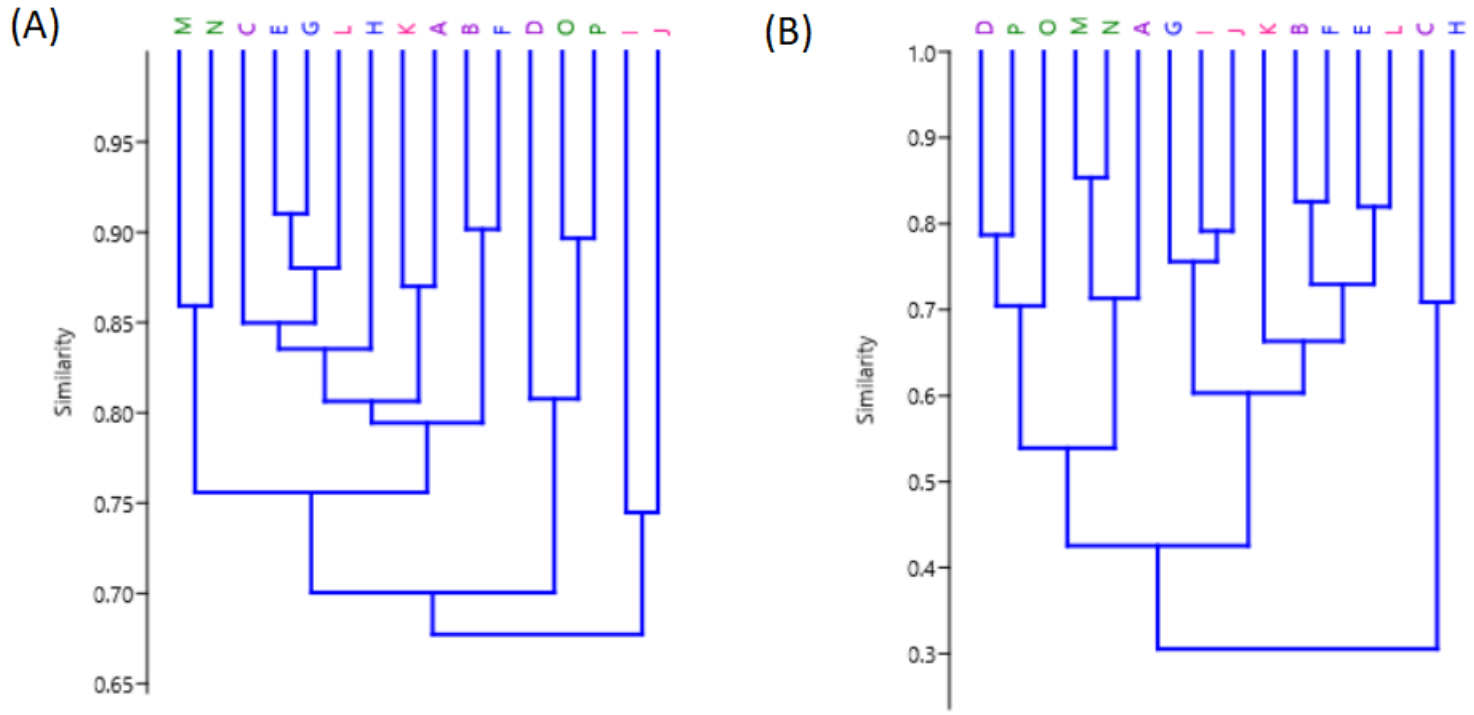

Fig 8. Cluster analysis of samples based on the relative abundance of ARGs in the (A) core resistome and (B) accessory resistome using the Bray-Curtis similarity matrix.

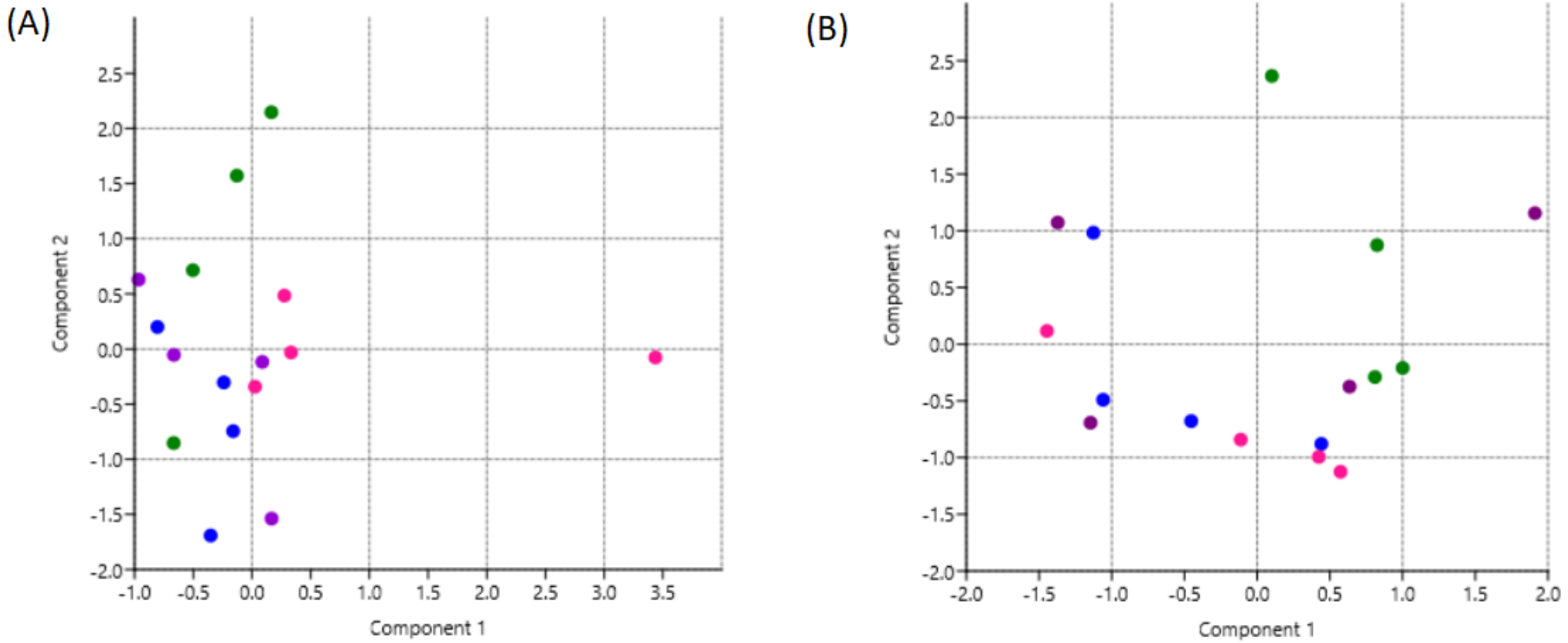

Fig 9. Principal Component Analysis of samples based on the relative abundance of ARGs in the (A) core resistome and (B) accessory resistome.

Purple: day 34 treated, blue: day 34 control, pink: day 27 treated green: day 27 control.

Fig 10. Gene ontology content from GO slim.

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5.9 SUPPLEMENTARY DATA

Table S1. List of antibiotic resistance genes present in the core resistome.

CORE RESISTOME
TET44
TET40
TETO
TET(W/N/W)
TETX
TETQ
TETW
TETM
TET32
VANS
VANR
VANU
VANX
VANB
VANG
VANH
VANW
VANVB
VANRI
EMRR
EMRK
EMRA
EMRE
ERMB
ERMG
MDTF

MDTE
MDTH
MDTM
MDTL
MDFA
MDTP
ACRB
ACRA
ACRF
ACRS
MARA
GADW
PATA
BICYCLOMYCIN-MULTIDRUG_EFFLUX_PROTEIN_BCR
CLASS_C
CLASS_A
MACB
LNUA
LNUC
VATB
LSA
OMPF
OMPR
ESCHERICHIA_COLI_LAMB
BACA
YOJI
UGD
ARNA
SAT-4

MDTO
AADA
AADE
APH(3'')-III
KDPE
KASUGAMYCIN_RESISTANCE_PROTEIN_KSGA
MDTK
DFRF
COB(I)ALAMIN_ADENOLSYLTRANSFERASE
CAMP-REGULATORY_PROTEIN
BACTERIAL_REGULATORY_PROTEIN_LUXR
TRUNCATED_PUTATIVE_RESPONSE_REGULATOR_ARLR
DNA-BINDING_TRANSCRIPTIONAL_REGULATOR_GADX
TRANSCRIPTIONAL_REGULATORY_PROTEIN_CPXR_CPXR

Table S2. List of antibiotic resistance genes present in the accessory resistome.

ACCESSORY RESISTOME
LNUG
VATE
EPTA
MSBA
PORIN_OMPC
ROSA
ROSB
APH(2")-IF
MEXE
MDTG
MDTD
LLMA_23S_RIBOSOMAL_RNA_METHYLTRANSFERASE
CPXA
MDTN
VANTG
VANY
VAND
EMRY
BAER
BAES
PMRF
BIFUNCTIONAL_AMINOGLYCOSIDE_N-ACETYLTRANSFERASE_AND_AMINOGLYCOSIDE_ PHOSPHOTRANSFERASE
EMRB
EMRD
ERMF

ESCHERICHIA_COLI_MIPA
ERMT
TEM
SERRATIA_MARCESCENS_OMP1
SDIA
CAT_CHLORAMPHENICOL_ACETYLTRANSFERASE
MEXX
16S_RRNA_METHYLASE
VGAC
EVGS
ACRD
MACA
AADA13
RPOB2
DNA-BINDING_PROTEIN_H-NS
KLEBSIELLA_PNEUMONIAE_OMPK37
TOLC
SAT-2
LING
APMA
LNUB
APH(6)-I
MEFA
NORA
APH(2")-IE
DFRD
LSAE
ANT(9)-I
DFRA14

EMRB-QACA_FAMILY_MAJOR_FACILITATOR_TRANSPORTER
APH(3")-I
FOSB
ERMX
DFRA1
SUL1
SUL2
QACH
TETA
TETL
QACG
TET(K)
VANYG1
APH(3')-VII
APH(2")-II
MGRB
MEL
TET(40)
AAC(6')-I
APH(2")-IV
POXTA
QACB
CLBA
ANTIBIOTIC_RESISTANCE_RRNA_ADENINE_METHYLTRANSFERASE
OMP36
KLEBSIELLA_PNEUMONIAE_OMPK36
LNUF
AAD(9)
DFRA7

DFRA15
CATD
MDTA
DFRA5
ANT(3'')-IH-AAC(6')-IID
LNUD
DFRA16
CATS
APH(3')-IIA
TETC
MDTC
APH(3')-I
DFRA17
DFRA12
TETR
APH(4)-I
AAC(3)-IV
AAC(3)-II
VANA

Chapter 6

The effect of mannan rich fraction supplementation on the diet of broiler chickens using metagenomic and plasmid based approaches

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6.1 ABSTRACT

Antibiotic resistance has been declared by the WHO as one of the biggest threats to health worldwide. The ban on the use of antibiotics as growth promotors has seen the introduction of prebiotics as alternative products. Mannan rich fraction (MRF) has been described to improve bird weight and feed efficiency. The metagenomes of broilers were compared. The groups included those that received either a standard commercial diet (control) or a standard diet plus MRF (treated), and a group that were receiving MRF were also administered amoxicillin on days 22-24 (treated + antibiotic). The microbiome of all birds investigated was dominated by *Firmicutes* and *Bacteroides* at phylum level, while *Clostridia* and *Bacteroidia* were the most abundant classes across all samples. A total of 164 antibiotic resistance genes (ARGs) were identified, 19 of which were core and 145 were accessory genes. The most abundant genes across all samples were *tetW, lnuC* and *aadE.* In addition, the mobile resistome was specifically characterised using exogenous plasmid capture. The MRF treated group at both time-points had a lower and less varied resistance profile than the control or antibiotic treated group. We suggest that MRF may reduce the effects of antibiotic administration on the selection of antibiotic resistance *via* the plasmid populations present within the broiler caecum and may also reduce the effects of antibiotic administration microbiome change. In addition, the administration of MRF did not appear to have deleterious effects on the metagenomes of the broilers.

6.2 INTRODUCTION

Antibiotic resistance leads to treatment failure and increased mortality[1]. Antibiotic resistance in food-producing animals can be spread to the human population through the food chain. This is transferred to humans through consumption of contaminated food products. Antibiotic resistance can be disseminated by both pathogenic and non-pathogenic bacteria, with the resistances genes from the latter being transferred to a human pathogen after consumption of the food product[2]. The global population is expected to reach 9.6 billion by 2050[3]. Correspondingly, the production of food-producing animals will increase to meet this demand. Intensive farming practices would traditionally have relied heavily on antibiotics in the production process[4]. The routine addition of antibiotics into the diets of poultry has become less prevalent[5], with many countries banning the practice due to increasing concerns over the increase of antibiotic resistance. Aarestrup *et al*., found that the use of avilamycin as a growth promotor in broilers led to avilamycin resistant *Enterococcus faecium*[6,7]. However, the absence of growth promotors from poultry feed may increase bird disease rates. Thus, effective alternatives that improve chicken health while maintaining efficiency of production are required[8].

Mannan oligosaccharides (MOS) are prebiotics derived from the outer cell wall of *Saccharomyces cerevisiae* yeast cells[9]. MOS has been found to improve growth rate and feed conversion ratios[10] and has been supplemented to the diet of broilers in recent years[11]. MOS has been shown to have a negative effect on pathogenic bacteria by stimulating beneficial bacteria in the gut microbiome[12]. Mannan Rich Fraction (MRF)

is the next generation of MOS technology, which can be included in diets at lower inclusion rates than MOS while still delivering all of the benefits to the animal[13].

Metagenomics is a technique described to have the ability to overcome the limitations of culture dependent studies[14]. These traditional culture-based methods are dependent on the growth of viable and culturable microbes in a laboratory environment, and will most often require further testing to confirm microbe identification[15]. Metagenomic sequencing techniques have resulted in the generation of large sequence datasets from various environments and given great insight into the enormous taxonomic and functional diversity of the microbial communities within these environments[16]. Plasmid detection and assembly from metagenomic samples is highly challenging. Sequences from plasmids are comprised of a large number of short fragments hindering their identification. Due to this many plasmids remain undetected in studies of such datasets[17,18]. We therefore employed a separate plasmid-based study alongside the metagenomic analysis. We targeted conjugative plasmids, thought to be the main drivers of antibiotic resistance due to their ability to transfer to different bacterial species[19].

Our work aimed to investigate the effect of MRF on the caecal microbiome of broiler chickens. As a group who received MRF were also administered amoxicillin for 3 days, we also aimed to identify if MRF had an effect on the resistome with and without amoxicillin challenge. We also hypothesise that MRF may possess the ability to reduce the transfer of plasmids in the broiler gastrointestinal tract.

6.3 MATERIALS & METHODS

6.3.1 Samples

Broiler caecal samples were obtained from a commercial poultry production unit in the European Union. Broilers received either a standard commercial diet (control) or a standard diet plus MRF (treated) at the manufacturer's recommended inclusion rates. A group that were receiving MRF were also administered amoxicillin on days 22-24 (treated + antibiotic). The samples were collected at two time-points, days 28 and 35 post-hatch. Samples were lyophilised and stored at -80°C before analysis.

6.3.2 Total DNA Extraction and Metagenomic Sequencing

Total DNA was extracted from 0.05 g of each caecal sample (*n*=36) using the Qiagen DNeasy PowerSoil kit according to the manufacturer's guidelines. The concentration and purity of the extracted DNA was measured using an Invitrogen Qubit Fluorometer (dsDNA high-sensitivity assay kit) and a DeNovix DS-11 spectrophotometer.

Paired-end sequencing libraries were prepared using the Illumina Nextera XT Library Preparation Kit. The samples were paired-end sequenced (2 x 150 bp) on the Illumina NextSeq 500 platform using high-output chemistry (300 cycles). Delivered raw FASTQ sequence files were quality checked with poor quality and duplicate reads removed, and trimming implemented using a combination of SAM and Picard tools.

6.3.3 Bioinformatic Analysis

The quality controlled reads were uploaded to the European Nucleotide Archive (ENA). The files were then transferred to MGnify[20] for host decontamination and assembly of the primary metagenome. Samples were uploaded to MG-RAST[21] using default parameters to perform quality control, protein prediction, clustering and similarity based annotation on nucleic acid sequence datasets. For each sample, the sum of reads per genera were extracted and further ranked taxonomic data was applied using NCBI taxonomy. A series of filters were applied to remove undesired and insignificant taxa; genera not annotated as bacteria were removed, genera with a cumulative $n < 10$ across the dataset were removed; and genera that were annotated as *incertae sedis* and with a cumulative $n < 25$ were removed.

DeepARG[22], was used to annotate antibiotic resistance. The pipeline first removes low quality reads using TRIMMOMATIC. Reads are then merged into one file (VSEARCH) and submitted for classification to the deepARG algorithm which applies CARD, ARDB and UNIPROT databases. The following parameters: identity: 80%, e-value: 1e-10, coverage: 50% and probability: 0.8, were used to normalise the relative abundance of ARGs to the 16S rRNA content of each sample.

6.3.4 Data Analysis

Calypso (http://cgenome.net/calypso/)[23] was used to statistically analyse the microbiome. The data were normalized for statistical analysis and rare taxa, with less than 0.001% relative abundance and samples with less than 1000 sequence reads were

removed. Rarefaction analyses and Principal Component Analysis (PCA) were performed using default settings. The microbial community composition was quantitatively visualized by bar charts. The relative abundances of taxonomic levels between treatment groups were compared by ANOVA between treatment groups. Shannon index was used to estimate bacterial alpha diversity and Chao1 was used to estimate richness.

Antibiotic resistance genes (ARGs) detected in across samples were assigned to the core resistome. The accessory resistome comprised ARGs detected in at least one sample, but less than the total number of samples. The statistical analysis and correlation analysis of the ARGs was performed using the PAleontological STatistics (PAST) version 3.2[24]. ANOVA Mann–Whitney pairwise tests with Bonferroni correction for multiple comparisons to compare samples were performed. PCA were performed using default settings. Heat-maps were generated using Morpheus (https://software.broadinstitute.org/morpheus/).

The sequences are deposited in the European Nucleotide Archive (ENA) under primary accession PRJEB33644 and secondary accession ERP116454.

6.3.5 Exogenous Plasmid Isolation

Plasmids were isolated from the caecal samples (*n*=46) using the exogenous plasmid isolation method as previously described with the following modifications. Samples were resuspended in 5 ml Tryptic Soy Broth (TSB) (Sigma). We included both a

'rinse' step, where the supernatant was immediately used as the donor culture (denoted with an asterisk (*)); and an 'enriched' step, where the resuspended caecal sample was left rocking overnight before being used as the donor. 100 µL of both donor and recipient *Escherichia coli* DH5α Rif^R were combined, centrifuged and resuspended in 100 µL of TSB before being applied to a 0.2 µm filter. Cells were resuspended from the filters in 10 ml 0.85% NaCl. Exogenous transconjugants were selected on Luria-Bertani (LB) agar (Duchefa-Biochemie) with rifampicin (100 mg/L) and ampicillin, tetracycline, kanamycin, colistin, cefotaxime, ciprofloxacin or imipenem at breakpoint concentrations according to CLSI guidelines (2018)[25]. A transconjugant from each antibiotic selective plate with growth was selected at random. If bacteria from the same plate appeared to have different features (*e.g.* morphology), both were selected for further testing.

6.3.6 Plasmid Analysis

Plasmids were extracted from the putative recipient *E. coli* strains using the Macherey-Nagel NucleoSpin Plasmid kit according to the manufacturer's guidelines and following the low-copy number protocol. The extracted plasmid DNA was visualised on a 1% agarose gel run at 70 volts for 60 minutes and stained with 1X GelRed (Biotium). Antibiotic susceptibility testing was performed in duplicate *via* the disk diffusion method according to CLSI guidelines (2018)[25] for 11 antibiotics (ampicillin, tetracycline, kanamycin, cefotaxime, ciprofloxacin, gentamicin, ceftazidime, meropenem, imipenem, trimethoprim and chloramphenicol) from 8 different classes.

6.4 RESULTS & DISCUSSION

The total reads per sample analysed after quality control and trimming ranged from 5,194,020 reads to 16,466,390 reads.

6.4.1 Microbiome Analysis

A total number of 28 phyla, 55 classes, 126 orders, 256 families and 600 genera passed the quality control. Rarefaction analysis was performed at each level to confirm that a sufficient depth of sequencing was reached (Fig 1). Samples were compared based on treatment group (control *vs.* treated *vs*. treated + antibiotic) and time-point (day 28 *vs.* day 35).

Firmicutes was the predominant phyla, the relative abundance ranged from 61.96% to 83.33% across all samples (Fig 2A). This was followed by *Bacteroidetes*, ranging between 31.34% and 10.01% of all classified reads. *Proteobacteria* ranged from 7.34% to 1.78% and *Actinobacteria* had between 5.99% and 1.57% of all classified reads. Our findings are in line with previous studies of the most predominant phyla in the chicken microbiome, with *Firmicutes* known to represent 50–90% of all taxa in the caecum[26].

The most dominant class was *Clostridia*, with a range of 66.67% of all classified reads to 46.15% (Fig 2B). *Bacteroidia* was the next most dominant (31.14% to 8.89%), followed by *Bacilli* (17.88% to 4.01%) and *Erysipelotrichia* (4.65% to 1.83%). This

distribution is similar to a study by Ma *et al.*, where broilers diets were supplemented with *Bacillus subtilis*[27]. *Bacillus* species are known to be beneficial to the host by balancing the properties of the native microbiota resulting in better growth performance[28]. *Erysipelotrichia* are less commonly detected with in the top 5 most dominant classes. Specific species within the *Erysipelotrichia* class have been linked to being of benefit to host energy metabolism[29].

Clostridium was the most dominant genus across all samples, with a range of 20.01% to 13.2% of classified reads (Fig 2C). Certain *Clostridium* species such as *Clostridium islandicum* have been associated with host benefits such as cellulytic activity and feed conversion[30]. This was followed by *Bacteroides* (30.52% to 8.32%), *Ruminococcus* (12.6% to 6.43%) and *Lactobacillus* (13.39% to 0.29%). MOS supplementation has previously been associated with increased in *Lactobacillus* community diversity[31]. While the overall relative abundance of taxa remained constant, variations within sample groups were evident. For example, *Bacteroides* in the day 28 treated group ranges from 9.97% of all classified reads to up 30.52%. Meanwhile, *Lactobacillus* at day 35 in the control group ranged from 0.29% up to 12.75% of all classified reads.

Taxa abundance across treatments were compared using ANOVA at phylum through to genera levels (Fig 3). When $P < 0.05$ the difference in relative abundance was considered significant. Pair-wise comparisons were performed by t-test and annotated as *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A significant variance between treatment groups was seen at phylum level (*Chlorobi*), within 2 classes (*Oligoflexia*, *Chlorobia*), 6 orders (*Rhodocyclales, Rhodobacterales, Neisseriales, Desulfovibrionales,*

Chlorobiales, Bdellovibrionales), 11 families (*Rikenellaceae, Rhodobacteraceae, Psychromonadaceae, Neisseriaceae, Halobacteroidaceae, Desulfovibrionaceae, Comamonadacae, Chromatiaceae, Chlorobiaceae, Beutenbergiaceae, Bdellovibrionaceae*) and 13 genera (*Alistipes, Bilophila, Pelotomaculum, Neisseria, Dethiobacter, Moorella, Chlorobium, Acetohalobium, Beutenbergia, Thermus, Catonella, Flavobacterium, Micromonospora*). These variations all occurred within the less predominant taxa in the caecal microbiome. *Chlorobi* was only the 17th most dominant phyla, while the significant variances seen at class level were in classes outside the top 20 most dominant. *Alistipes* was the 19th most dominant genera, with all others showing variance between treatment groups falling outside the top 20 most abundant genera.

Microbial alpha diversity was assessed with Shannon index (Fig 4A) and bacterial community richness was assessed with Chao1 (Fig 4B). Microbial community evenness is represented in Fig 4C. No significant differences (where $P < 0.05$) were observed for diversity, richness or evenness at any taxonomic rank. This indicates a highly consistent microbiota present among all the sampled birds, even between treatment groups.

These results indicate that the addition of MRF may counteract the deleterious effects of the antibiotic amoxicillin on the caecal microbiome, while not significantly changing the normal microbiome of the caeca relative to the control. This suggests that MRF maintains the alpha-diversity and evenesss of the microbial community in the presence of antibiotic amoxicillin. Schokker *et al*., observed an increased diversity

in 5 day old chickens that had received amoxicillin at 1 day of age. This increase in diversity is more chaotic than a stable microbiota, and therefore less resilient[32]. The authors also found that the abundance of *Lactobacillaceae* was decreased in the antibiotic group. As *Lactobacilli* are involved in the competitive exclusion of pathogens, the antibiotic appears to have had a negative effect on gut health[32].

The relative abundance of OTUs at phylum, class and genus levels were plotted using Principal Component Analysis (PCA) (Fig 5). The patterns observed were similar between treatment groups and time-points. The control group had a small intra-cluster distance at phylum level. The treated group had a slightly larger intra-cluster distance along the PC2 axis, while the treated + antibiotic group had a similar distance along the PC1 axis. At class level, there was an increase in the intra-cluster distance along the PC1 axis at phylum level in the control group. The intra-cluster distance increased for the treated groups also. A more significant increase in intra-cluster distance is seen at genus level for all groups, highlighting a greater diversity at this taxonomic level. At all taxonomic levels, the groups cluster together, with no clearly defined inter-cluster distances between them. This shows that while there is some variation present within each sample group, overall the microbiome is consistent across all samples.

Both the MRF supplementation and the administration of antibiotics had the potential to significantly alter the caecal microbiome. Antibiotics reduce the gut microflora, however, the microbial community in the groups that received antibiotics and MRF were highly similar to those in the groups that did not. It is possible that the microbiome recovered after the antibiotic treatment which occurred before sampling.

This recovery may have been aided by the MRF supplementation, providing a substrate for selective beneficial commensal bacteria to recover and proliferate throughout the caecal microbiome. Previous studies summarised by Teng and Kim noted that *Lactobacillus* were the main species influenced by MOS, whereby their prevalence in the microbiome increased[11]. The benefits of this have been recognised, *Lactobacillus salivarius* was shown to limit *Salmonella* colonization, and *Lactobacillus crispatus* has been documented as having anti-*E. coli* and anti-*Salmonella* properties[33]. We observed similar levels of *Lactobacillus* in the control group as the treated groups. The presence of *Lactobacillus* within the birds may have meant that the effect of MOS was as high as previously observed as it would have been in birds where *Lactobacillus* was present in much lower levels or absent. A study by Corrigan *et al.,* investigating the effect of MOS on the broiler microbiome found an increase in *Bacteroidetes* from a *Firmicutes* dominated microbiota, while correspondingly observing a shift from a predominantly *Clostridia* populated microbiome to *Bacteroidia* at class level[34]. While our microbial community structure was similar at phylum and class levels, we did not observe these same changes in the microbiome with MRF supplementation. This suggests that the effect of MRF may be specific to certain genera or species which were not present in our samples. MOS are also recognised in their ability to bind enteric pathogens with type-1 fimbriae such as *Salmonella* and *Campylobacter* species[35], neither of which were identified in our study. It is therefore likely that the effect of MRF and major changes in the microbiome is seen to a much greater extent in pathogen-challenged birds. From our results, we determine that MRF may play a role in maintaining a healthy microbiome within the broiler caecum. We also suggest it may assist with the recovery of the

microbiome after antibiotic administration and promote the development of a stable microbial community.

6.4.2 Resistome Analysis

A total of 164 antibiotic resistance genes (ARGs) were identified across all samples. The core resistome contained 19 ARGs, which were present in all 36 samples (Table S2). The remaining 145 ARGs that were present in at least one but not all samples were assigned to the accessory resistome (Table S3). The relative abundance of ARGs per sample by antibiotic class were summed to identify if the trends in ARGs were consistent across all samples (Fig 6). All samples contained resistance genes from the same classes of antibiotics. The greatest proportions of ARGs present in all samples comprised tetracycline, aminoglycoside, macrolide-lincosamide-streptogramin B (MLS_B), glycopeptide and multi-drug resistance genes. The identified resistance to the remaining classes of antibiotics was relatively low. The relative abundances of the identified classes varied across the groups and between the samples. For example, sample 31 had a larger proportion of multi-drug resistance compared to the other samples (32-36) within the same group (Fig 6). Sample 7 from the day 28 treated + antibiotic group had the largest variety of ARGs with 124 different ARGs. Interestingly, sample 26 from the day 35 treated + antibiotic group had the lowest variety of ARGs overall (n=59).

The core resistome was composed of 19 ARGs: tetracycline (n=6), glycopeptide (n=4) aminoglycoside (n=2), MLS_B (n=2), beta-lactam (n=1), bacitracin (n=1), lincosamide

(*n*=1), streptogramin (*n*=1) and nucleoside (*n*=1) resistance genes. The distribution of genes within the core resistome was consistent across all samples, with *tetW, lnuC* and *aadE* being the most abundant. Both *tetW* and *aadE* were found to be the most abundant core ARGs in the pig faecal metagenome[36]. A study into the faecal resistome on nine European broiler farms found bla_{TEM}, *tetW*, *dfrA1*, *ermB* and *aadA* to be the most abundant genes[37]. In Fig 7A, it can be seen where the most abundant genes in our study clustered together using the Bray–Curtis similarity matrix. Samples were also clustered based on the relative abundance of their core resistance genes by the Bray-Curtis similarity matrix (Fig 8A). Interestingly, there were variations where samples from the same group (control, treated or treated + antibiotic) did not cluster together. Most notably was the day 28 treated + antibiotic group, (samples 7-12, labelled pink in Fig 8A), where the samples were distributed throughout the cluster. Similarly, within the day 28 treated group, half of the samples clustered together while half did not (samples 1-6, labelled blue in Fig 8A). This highlights the differences between samples of the same group based on core ARG abundance. Meanwhile, the five of the six samples in the day 35 treated + antibiotic group (samples 25-30, labelled red in Fig 8A) clustered quite closely together and closely with three control samples from D28 (*n*= 2) and D35 (*n*= 1), indicating a more consistent population of core ARGs within this group.

The accessory resistome comprised 145 different ARGs. The most abundant accessory genes were *rpoB2*, *tetX*, *ermX*, *ugd* and *ermF*. The *ugd* gene is also known as *pmrE*. Mutations in the *pmrE* gene are associated with colistin resistance, but the gene itself is part of several bacterial genomes. Clustering analysis of the relative abundances of ARGs in the accessory resistome using the Bray-Curtis similarity matrix can be seen

in (Fig 7B) A similar pattern to the core resistome was observed, but with more variation between samples within each group (Fig 8B). For example, within the day 35 group, samples 32 and 36 clustered closely together, and samples 34 and 35 clustered together, but the two clusters were far away from each other (labelled orange in Fig 8B). This pattern is evident among all groups, where two or three samples cluster together, but away from the other samples within that group. This highlights the variations in ARGs present in bird samples of the same group.

There were no significant differences ($P < 0.05$) between the relative abundances of ARGs between samples in the core resistome. However, within the accessory resistome significant differences were observed between samples (Table S1). The contrast between time-points was the most striking here, with significant differences observed between the day 28 and day 35 samples, indicating a very different distribution of ARGs in the accessory resistome as the broilers age. As we did not observe any significant changes in the most dominant taxa within the microbiome, it is possible that the ARGs from the accessory resistome may be harboured by taxa present in lower abundances. PCA analysis of the core (Fig 9A) and accessory (Fig 9B) resistomes was performed. There was a general overlap of all sampling groups in the analysis of the core resistome, with a greater intra-cluster distance observed for the day 35 treated + antibiotic group (red) and the day 28 treated + antibiotic (pink) group. Within the analysis of the accessory resistome, larger intra-cluster distances are seen for all sampling groups. The inter-cluster distances are also more clearly defined between groups, particularly between the day 35 treated + antibiotic group (red), day 28 treated + antibiotic (pink) and day 28 treated group (blue). This displays both the variation between samples but also between groups.

Our most notable results were within the groups of birds that received both MRF and the antibiotic. The broilers were administered amoxicillin on days 22-24. This did not appear to increase the abundance of beta-lactamases within those birds. A class A beta-lactamase gene was identified but it was not the most abundant in the core resistome. Sample 7 had much higher numbers of ARGs present, which could be attributed to the administered antibiotics creating a selective pressure and encouraging proliferation of ARGs throughout the caecum. However, as this was not seen in the other birds in this group, it seems that other factors are involved in determining ARG numbers in this complex environment. While high abundances of ARGs were present in the day 28 treated + antibiotic group, the lowest observed ARGs were within the day 35 treated + antibiotic group. It could be that the withdrawal from antibiotic over time removed the selective pressure which had created the ideal environment ARGs would have favoured, thus resulting in lower abundances.

6.4.3 Functional Analysis

The functional profile of the broiler microbiome was derived from the KO (KEGG Orthology) assignments in MG-RAST. Genes were assigned to six functions: Cellular Processes, Human Diseases (which contain infectious diseases and pathogen interaction), Genetic Information Processing, Environmental Information Processing, Organismal Systems or Metabolism (Fig 10). Samples within the day 28 control group had the highest abundance of genes assigned to all functions. To again highlight the sample-to-sample and within-group variations, samples 2 and 3 from the day 28 treated group had high abundances of genes corresponding to all functions, while

sample 1 had the lowest abundance of assigned functional genes overall. Similarly, samples 32 and 35 from the day 35 control group had higher abundances of assigned function genes than the others from the same group. However, these two samples were not identical, with sample 35 having a lower abundance of organisational systems genes and higher abundance of environmental information processing and metabolism genes.

6.4.4 Mobile Resistomes

A total of 139 transconjugants from 24 birds at day 28 (control *n*=8, treated *n*=8, treated + antibiotic *n*=8) and 105 transconjugants from 22 birds at day 35 (control *n*=6, treated *n*=7, treated + antibiotic *n*=8) were analysed (Fig S1-S6).

Within the day 28 samples, the control group displayed resistance to the greatest number of antibiotics with 58.69% of all transconjugants resistant to ampicillin, 52.17% to tetracycline, 13.04% to trimethoprim, 8.88% to gentamicin, 4.34% to kanamycin and 2.17% to cefotaxime (Table 1). Overall, 10.86% of transconjugants within this group were multi-drug resistant, conferring resistance to three or more classes of antibiotics. From the day 28 treated + antibiotic group, 78.86% were resistant to ampicillin, 53.84% to tetracycline, 28.84% to trimethoprim and 7.69% to kanamycin and chloramphenicol respectively (Table 2). No resistance was observed to gentamicin or cefotaxime antibiotics, which was present in the control group. 26.92% of transconjugants in this group were multi-drug resistant. Resistance to a lower number of antibiotics was observed in the day 28 treated group, with 46.15% of

transconjugants resistant to ampicillin and 12.82% resistant to tetracycline, kanamycin and trimethoprim, respectively (Table 3). Only 2.56% displayed a multi-drug resistant phenotype. Overall, the highest proportion of plasmid mediated resistance was observed towards ampicillin and tetracycline antibiotics followed by trimethoprim.

Within the day 35 control group, 65.51% of transconjugants were resistant to ampicillin, 51.72% to tetracycline, 34.48% to kanamycin, 31.03% to trimethoprim, 24.13% to gentamicin, 10.34% to ceftazidime and 3.44% to cefotaxime, chloramphenicol and imipenem respectively (Table 4). This group had the greatest variation in resistance profiles of all the sampled groups, indicating that this group harboured the greatest variety of plasmids. 34.48% of transconjugants were multi-drug resistant. From the day 35 treated + antibiotic group, 72.72% of transconjugants were resistant to ampicillin, 50% to tetracycline, 40.90% to trimethoprim and 2.27% to kanamycin (Table 5). 34.09% of transconjugants were multi-drug resistant. In the day 35 treated group, 59.37% of transconjugants were resistant to ampicillin, 21.87% to tetracycline, 9.37% to trimethoprim and 3.12% were resistant to kanamycin (Table 6). Only 3.12% were found to exhibit a multi-drug resistant phenotype.

The highest proportion of resistance overall was observed towards ampicillin and tetracycline antibiotics, as was seen in the day 28 groups. Only the plasmids from the control group at day 28 (cefotaxime only) and day 35 conferred resistance to chloramphenicol, ceftazidime, cefotaxime or imipenem. Thus, the inclusion of MRF either with or without amoxicillin did not result in the detection of plasmids conferring resistance to these antibiotics of importance for human medicine. Plasmid mediated

resistance mechanisms to the third generation cephalosporins (cefotaxime or ceftazidime), and imipenem also confer resistance to the antibiotic amoxicillin. However, in the presence of MRF these plasmids were not detected at day 28 or day 35 even when amoxicillin was administered to the birds.

Overall, the highest levels and greatest variety in resistance profiles was seen in the control groups in the plasmid study. This was more evident at day 35, with resistance to 9 of the 11 tested antibiotics identified, indicating that the birds obtained and harboured a greater variety of plasmids as they aged. Some of the greatest number of transconjugants that were resistant to trimethoprim was seen in the treated + antibiotic groups, which were administered amoxicillin on days 22 to 24. A class A beta-lactamase was identified in the core resistome after sequencing, and *dfr* genes were detected in the accessory resistome. The link between ampicillin-based antibiotic use and trimethoprim resistance has been documented since the 1980s[38]. Amoxicillin and trimethoprim resistance genes may be linked on the same mobile genetic element[39]. Pouwels *et al.*, found that co-selection by use of amoxicillin or ampicillin antibiotics is a more important driver of trimethoprim resistance levels than trimethoprim use itself[40]. The lowest range of resistance was seen in the treated groups from both time-points. They displayed resistance to fewer classes than the control group or the groups that received the antibiotic. Only one transconjugant from each of the day 28 and the day 34 treated groups were found to display a multi-drug resistant phenotype.

This may be attributed to the addition of MRF to the diet of broilers. The effect may be specific to plasmid populations. MOS, the predecessor to MRF, is known for its

capability to bind pathogens via mannose specific type-I fimbriae[41]. Type-3 fimbriae have been linked to an increased frequency of conjugation[42] but also to mannan-binding[43]. There is the possibility therefore that MRF reduces the conjugative ability of plasmids, and thus lower resistance is detected. Plasmids are not easily detected within complex samples as they are present in lower abundances than chromosomal DNA, meaning they can often be missed in total DNA extractions. Likewise, they can also be easily lost in the metagenomic sequencing process, as short-read sequencing is not ideal for the assembly of the small pieces of plasmid DNA that can possess numerous repeat regions. This may explain why the same differences were not observed between the transconjugant data and the data gathered from the core and accessory resistomes. Broad host range plasmids are capable of being transferred and maintained in a wide range of bacteria[44]. Therefore, if the plasmids present in our samples were mainly broad-host range, changes in the plasmid population may not necessarily be seen alongside changes in the microbial community. Further investigations to characterise the isolated plasmids will provide a better understanding of the observed changes in the mobile resistome.

6.5 CONCLUSION

The effect of MRF on broilers from a commercial production unit was assessed. A sub-set of treated birds were also administered antibiotic. We did not detect any major changes in the dominant taxa between the sampled groups, and identified a highly consistent caecal microbiome. We characterised the core and accessory resistome, where we saw notable variation between samples of the same group. We also examined the mobile resistome, where a lower and less varied resistance profile was observed in the treated groups at both time-points. We suggest that MRF may have an effect on plasmid populations and encourage further investigation into the possible impact of the use of MRF on the control of mobile antibiotic resistance in the digestive tracts of food animals.

6.6 TABLES

Table 1. Resistance profile of exogenous transconjugant strains from the day 28 control group[a,b,c,d].

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S13	*1 LB	S	S	S	S	S	S	S	S	S	S	S
S13	1 LB	R	R	S	S	S	S	S	S	S	S	S
S13	1 AMP	R	R	S	S	S	S	S	S	S	S	S
S13	*1 TET	S	R	S	S	S	S	S	S	S	S	S
S13	1 TET	R	R	S	S	S	S	S	S	S	S	S
	*2 LB	S	S	S	S	S	S	S	S	S	S	S
	2 LB	S	S	S	S	S	S	S	S	S	S	S
	*2 AMP	R	S	S	S	S	S	S	S	S	S	S
	2 AMP	R	S	S	S	S	S	S	S	S	S	S
	*2 TET BC	R	R	S	S	S	S	R	S	S	S	S
	*2 TET SC	R	R	S	S	S	S	R	S	S	S	S
	2 KAN	S	R	R	S	S	S	S	S	S	S	S
S14	*3 LB	S	S	S	S	S	S	S	S	S	S	S
S14	3 LB	S	S	S	S	S	S	S	S	S	S	S
S14	*3 AMP	R	R	S	S	S	S	S	S	S	S	S
S14	3 AMP	R	S	S	S	S	S	S	S	S	S	S
S14	3 TET	R	R	S	S	S	S	S	S	S	S	S
S14	3 KAN	R	S	I	R	S	R	R	S	S	S	S
	*4 LB	S	S	S	S	S	S	S	S	S	S	S
	4 LB LAWN	S	S	S	S	S	S	S	S	S	S	S
	4 LB COLONIES	R	R	S	S	S	S	S	S	S	S	S
	*4 AMP	R	R	S	S	S	S	S	S	S	S	S
	4 AMP BC	R	R	S	S	S	S	S	S	S	S	S
	4 AMP SC	R	R	S	S	S	S	S	S	S	S	S
	*4 TET LAWN	R	R	S	S	S	S	S	S	S	S	S
	*4 TET COLONIES	R	R	S	S	S	S	S	S	S	S	S
	4 TET	R	R	S	S	S	S	S	S	S	S	S
S15	*5 LB	S	S	S	S	S	S	S	S	S	S	S
S15	5 LB	S	S	S	S	S	S	S	S	S	S	S
S15	*5 AMP	R	R	S	S	S	S	S	S	S	S	S
S15	5 AMP	R	R	S	S	S	S	S	S	S	S	S

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S15	*5 TET	R	R	S	S	S	S	S	S	S	S	S
S15	5 TET	R	R	S	S	S	S	S	S	S	S	S
S16	*6 LB	S	S	S	S	S	S	S	S	S	S	S
S16	*6 AMP	R	S	S	S	S	S	S	S	S	S	S
S16	6 AMP	R	S	S	S	S	S	S	S	S	S	S
S17	*7 LB	S	S	S	S	S	S	S	S	S	S	S
S17	7 LB	S	S	S	S	S	S	S	S	S	S	S
S17	7 TET	S	R	S	S	S	S	R	S	S	S	S
S17	7 KAN	S	S	R	S	S	R	S	S	S	S	S
S18	*8 LB	S	S	S	S	S	S	S	S	S	S	S
S18	8 LB	S	S	S	S	S	S	S	S	S	S	S
S18	*8 AMP	R	S	S	S	S	S	S	S	S	S	S
S18	8 AMP BC	R	S	S	S	S	S	S	S	S	S	S
S18	8 AMP SC	R	R	S	S	S	S	S	S	S	S	S
S18	*8 TET	S	R	S	S	S	R	R	S	S	S	S
S18	8 TET	S	R	S	S	S	R	R	S	S	S	S

[a]M-S= corresponding sequenced sample.

[b]Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment.

[c]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, CAZ=Ceftzidime. MER=Meropenem, IMP=Imipenem, C=Chloramphenicol.

[d]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

[e]Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony) or had both a lawn of growth (LAWN) with some distinct colonies (COLONIES).

Table 2. Resistance profile of exogenous transconjugant strains from the day 28 treated + antibiotic group[a,b,c,d].

	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S7	*1 LB	R	S	S	S	S	S	S	S	S	S	S
S7	1 LB	S	S	S	S	S	S	S	S	S	S	S
S7	1 AMP BC	R	S	S	S	S	S	R	S	S	S	S
S7	1 AMP SC	R	S	S	S	S	S	S	S	S	S	S
S7	*1 TET	R	R	S	S	S	S	R	S	S	S	S
S7	1 TET	R	R	S	S	S	S	S	S	S	S	S
S8	*2 LB	R	S	S	S	S	S	S	S	S	S	S
S8	2 LB	R	S	S	S	S	S	S	S	S	S	S
S8	*2 TET BC	R	R	S	S	S	S	S	S	S	S	S
S8	*2 TET SC	R	R	S	S	S	S	S	S	S	S	S
S8	2 TET	R	R	S	S	S	S	S	S	S	S	S
S9	*3 LB	S	S	S	S	S	S	S	S	S	S	S
S8	3 LB	S	S	S	S	S	S	S	S	S	S	S
S9	*3 AMP	R	R	S	S	S	S	S	S	S	S	S
S9	3 AMP BC	R	R	S	S	S	S	S	S	S	S	S
S9	3 AMP SC	R	R	S	S	S	S	R	S	S	S	S
S9	*3 TET BC	R	R	S	S	S	S	S	S	S	S	S
S9	*3 TET SC	R	R	S	S	S	S	S	S	S	S	S
S9	3 TET BC	R	R	I	S	S	S	R	S	S	S	S
S9	3 TET SC	R	R	S	S	S	S	R	S	S	S	S
S10	*4 LB	S	S	S	S	S	S	S	S	S	S	S
S10	4 LB	R	S	S	S	S	S	S	S	S	S	S
S10	*4 AMP	R	S	S	S	S	S	S	S	S	S	S
S10	4 AMP BC	R	R	S	S	S	S	R	S	S	S	R
S10	4 AMP SC	R	R	S	S	S	S	R	S	S	S	R
S10	4 TET BC	R	R	S	S	S	S	R	S	S	S	R

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S10	4 TET SC	R	R	S	S	S	S	R	S	S	S	R
	*5 LB	S	S	S	S	S	S	S	S	S	S	S
	5 LB	S	S	S	S	S	S	S	S	S	S	S
	*5 AMP	R	S	S	S	S	S	S	S	S	S	S
	5 AMP BC	R	S	S	S	S	S	R	S	S	S	S
	5 AMP SC	R	R	S	S	S	S	R	S	S	S	S
	5 TET	R	R	S	S	S	S	S	S	S	S	S
S11	6 LB	R	R	S	S	S	S	S	S	S	S	S
S11	6 AMP	R	S	S	S	S	S	S	S	S	S	S
S11	6 TET	R	R	R	S	S	S	S	S	S	S	S
S11	6 KAN	R	R	R	S	S	S	S	S	S	I	S
S12	*7 LB	S	S	S	S	S	S	S	S	S	S	S
S12	7 LB	R	R	S	S	S	S	S	S	S	S	S
S12	*7 AMP	R	R	S	S	S	S	S	S	S	S	S
S12	7 AMP	R	R	S	S	S	S	R	S	S	S	S
S12	*7 TET	R	R	S	S	S	S	S	S	S	S	S
S12	7 TET	R	R	S	S	I	S	R	S	S	S	S
S12	7 KAN	S	S	R	S	S	I	S	S	S	S	S
	*8 LB	S	S	S	S	S	S	S	S	S	S	S
	8 LB	S	S	S	S	S	S	S	S	S	S	S
	*8 AMP	R	S	S	S	S	S	S	S	S	S	S
	8 AMP BC	R	S	S	S	S	I	R	S	S	S	S
	8 AMP SC	R	S	S	I	S	S	S	S	S	S	S
	*8 TET	R	R	S	S	S	S	S	S	S	S	S
	8 TET	R	R	S	S	S	S	R	S	S	S	S
	8 KAN	S	S	R	S	S	S	S	S	S	S	S

[a]M-S= corresponding sequenced sample.

[b]Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment.

[c]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, CAZ=Ceftzidime. MER=Meropenem, IMP=Imipenem, C=Chloramphenicol.

[d]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

[e]Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony).

Table 3. Resistance profile of exogenous transconjugant strains from the day 28 treated group[a,b,c,d].

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S1	*1 LB	S	S	S	S	S	S	S	S	S	S	S
S1	1 LB	S	S	S	S	S	S	S	S	S	S	S
S1	*1 AMP	R	S	S	S	S	S	S	S	S	S	S
S1	1 AMP BC	R	S	S	S	S	S	R	S	S	S	S
S1	1 AMP SC	R	S	S	S	S	S	S	S	S	S	S
S1	*1 TET	R	R	S	S	S	S	R	S	S	S	S
S1	1 KAN	S	S	R	S	S	I	S	S	S	S	S
	*2 LB	S	S	S	S	S	S	S	S	S	S	S
	2 LB	S	S	S	S	S	S	S	S	S	S	S
	2 AMP	R	S	S	S	S	S	S	S	S	S	S
	2 KAN	S	S	R	S	S	I	S	S	S	S	S
S2	*3 LB	S	S	S	S	S	S	S	S	S	S	S
S2	3 LB	S	S	S	S	S	S	S	S	S	S	S
S2	*3 AMP	R	S	S	S	S	S	S	S	S	S	S
S2	3 AMP	R	S	S	S	S	S	S	S	S	S	S
S3	*4 LB	S	S	S	S	S	S	S	S	S	S	S
S3	4 LB	S	S	S	S	S	S	S	S	S	S	S
S3	*4 AMP	R	S	S	S	S	S	R	S	S	S	S
S3	4 AMP	R	S	S	S	S	S	R	S	S	S	S
S3	4 KAN BC	S	S	R	S	S	I	S	S	S	S	S
S3	4 KAN SC	S	S	R	S	S	S	S	S	S	S	S
S4	*5 LB	R	R	S	S	S	S	S	S	S	I	S
S4	5 LB	S	S	S	S	S	S	S	S	S	S	S
S4	*5 AMP	R	R	S	S	S	S	S	S	S	I	S
S4	5 AMP	R	R	S	S	S	S	S	S	S	S	S
S4	*5 COL	S	R	S	S	S	S	S	S	S	I	S
	*6 LB	S	S	S	S	S	S	S	S	S	S	S
	6 LB	S	S	S	S	S	S	S	S	S	S	S
	*6 AMP	R	S	S	S	S	S	S	S	S	S	S
	6 AMP	R	S	S	S	S	S	R	S	S	S	S
	6 KAN	S	S	R	S	S	I	S	S	S	S	S
S5	*7 LB	S	S	S	S	S	S	S	S	S	S	S
S5	7 LB	S	S	S	S	S	S	S	S	S	S	S

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S5	*7 AMP	R	S	S	S	S	S	S	S	S	S	S
S5	7 AMP	R	S	S	I	S	S	S	S	S	S	S
S6	*8 LB	S	S	S	S	S	S	S	S	S	S	S
S6	8 LB	S	S	S	S	S	S	S	S	S	S	S
S6	*8 AMP	R	S	S	S	S	S	S	S	S	S	S
S6	8 AMP	R	S	S	S	S	S	S	S	S	S	S

[a]M-S= corresponding sequenced sample.

[b]Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment.

[c]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, CAZ=Ceftzidime. MER=Meropenem, IMP=Imipenem, C=Chloramphenicol.

[d]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

[e]Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony).

Table 4. Resistance profile of exogenous transconjugant strains from the day 35 control group[a,b,c,d].

M-S	Plasmid Sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S31	*1 LB	S	S	S	S	S	S	S	S	S	S	S
S31	1 LB	S	S	S	S	S	S	S	S	S	S	S
S31	*1 AMP	R	R	I	S	S	S	S	S	S	I	S
S31	1 AMP	R	R	S	S	S	S	S	S	S	S	S
S31	1 TET	R	R	R	S	S	R	R	S	S	S	S
S32	*2 LB	S	S	S	S	S	S	S	S	S	S	S
S32	2 AMP	R	S	S	S	S	S	S	S	S	S	S
S33	5 LB	S	S	S	S	S	S	S	S	S	S	S
S33	*5 AMP	R	S	S	S	S	S	S	S	S	S	S
S33	5 AMP	R	R	S	S	S	S	S	S	S	S	S
S34	*6 LB	S	S	S	S	S	S	S	S	S	S	S
S34	6 LB	S	S	S	S	S	S	S	S	S	S	S
S34	6 AMP	R	R	S	S	S	S	S	S	S	S	S
S34	*6 TET	R	R	S	S	S	S	S	S	S	S	S
S34	6 TET	R	R	R	S	S	R	R	S	S	S	S
S34	6 KAN	R	R	R	I	I	R	R	S	S	S	S
S35	*9 LB	S	S	S	S	S	S	S	S	S	S	S
S35	9 LB	S	S	S	S	S	I	S	R	S	R	S
S35	*9 AMP	R	S	R	R	S	I	R	R	I	S	S
S35	9 AMP	R	R	R	S	I	R	R	S	S	S	S
S35	9 TET	R	R	R	S	I	R	R	S	S	S	S
S35	9 KAN	R	R	R	S	I	S	S	S	S	S	S
S36	*12 LB	S	S	S	S	S	I	S	R	S	S	S
S36	12 LB	S	S	S	S	S	S	S	S	S	S	S
S36	12 AMP	R	R	S	S	S	S	S	S	S	S	S
S36	12 TET BC	R	R	S	S	S	S	S	S	S	S	S
S36	12 TET SC	R	R	R	S	I	R	R	S	S	S	S
S36	*12 KAN	R	S	R	S	I	R	R	S	S	S	R
S36	12 KAN	R	R	R	S	I	I	R	S	S	S	S

[a]M-S= corresponding sequenced sample.

[b]Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment.

[c]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, CAZ=Ceftzidime. MER=Meropenem, IMP=Imipenem, C=Chloramphenicol.

[d]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

[e]Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony).

Table 5. Resistance profile of exogenous transconjugant strains from the day 35 treated + antibiotic group[a,b,c,d].

M-S	Plasmid sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S25	*2 LB	R	R	S	S	S	S	R	S	S	S	S
S25	2 LB	R	R	S	S	S	S	R	S	S	S	S
S25	*2 AMP	R	R	S	S	S	S	R	S	S	S	S
S25	2 AMP	R	R	S	S	S	S	R	S	S	S	S
S25	*2 TET	R	R	S	S	S	S	R	S	S	S	S
S25	2 TET	R	R	S	S	S	S	R	S	S	S	I
	3 LB	S	S	S	S	S	S	S	S	S	S	S
	*3 AMP	R	R	S	S	S	S	S	S	S	S	S
	*3 TET	R	R	S	S	S	S	S	S	S	S	S
S26	*4 LB	S	S	S	S	S	S	S	S	S	S	S
S26	4 LB	S	S	S	S	S	S	S	S	S	S	S
S26	*4 AMP	R	S	S	S	S	S	S	S	S	S	S
S26	4 AMP	R	R	S	S	S	S	R	S	S	S	S
S26	*4 TET	R	R	S	S	S	S	S	S	S	S	S
S26	4 TET	R	R	S	S	S	S	R	S	S	S	S
	*5 LB	S	S	S	S	S	S	S	S	S	S	S
	5 LB	R	S	S	S	S	S	S	S	S	S	S
	5 AMP	R	S	S	S	S	S	S	S	S	S	I
	5 TET	R	R	S	S	S	S	S	S	S	S	S
	5 KAN	S	S	S	S	S	S	S	S	S	S	S
S27	*7 LB	S	S	S	S	S	S	S	S	S	S	S
S27	7 LB	R	S	S	S	S	S	S	S	S	S	S
S27	*7 AMP	R	S	S	S	S	S	S	S	S	S	S
S27	7 AMP	R	S	S	S	S	S	S	S	S	S	S
S27	*7 TET	S	R	S	S	S	S	R	S	S	S	S
S27	7 TET BC	R	R	S	S	S	S	R	S	S	S	S
S27	7 TET SC	S	R	S	S	S	S	R	S	S	S	S
S28	*8 LB	S	S	S	S	S	S	S	S	S	S	S
S28	8 LB	S	S	S	S	S	S	S	S	S	S	S
S28	*8 AMP	R	S	S	S	S	S	S	S	S	S	S
S28	8 AMP	R	R	S	S	S	S	S	S	S	S	S
S29	*9 LB	S	S	S	S	S	S	S	S	S	S	S
S29	9 LB	R	S	S	S	S	S	R	S	S	S	S
S29	*9 AMP	R	S	S	S	S	S	S	S	S	S	S
S29	9 AMP	R	S	S	S	S	S	R	S	S	S	S

M-S	Plasmid sample	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S29	*9 TET	R	R	S	S	S	S	R	S	S	S	S
S29	9 TET BC	R	R	S	S	S	S	R	S	S	S	S
S29	9 TET SC	R	R	S	S	S	S	S	S	S	S	S
S30	*10 LB	S	S	S	S	S	S	S	S	S	S	S
S30	10 LB	R	S	S	S	S	S	R	S	S	S	S
S30	*10 AMP	R	R	S	S	S	S	S	S	S	S	S
S30	10 AMP	R	S	S	S	S	S	S	S	S	S	S
S30	*10 TET	R	R	S	S	S	S	R	S	S	S	S
S30	10 TET	R	R	S	S	S	S	R	S	S	S	S

[a]M-S= corresponding sequenced sample.

[b]Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment.

[c]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, CAZ=Ceftzidime. MER=Meropenem, IMP=Imipenem, C=Chloramphenicol.

[d]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

[e]Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony).

Table 6. Resistance profile of exogenous transconjugant strains from the day 35 treated group[a,b,c,d].

M-S	Plasmid Samples	AMP	TET	KAN	CTX	CIP	CN	W	CAZ	MER	IMP	C
S19	*1 LB	S	S	S	S	S	S	S	S	S	S	S
S19	1 LB	S	S	S	S	S	S	S	S	S	S	S
S19	*1 AMP	R	S	S	S	S	S	S	S	S	S	S
S19	1 AMP	R	S	S	S	S	S	S	S	S	S	S
S19	1 KAN	S	S	R	S	S	S	S	S	S	S	S
	*2 LB	S	S	S	S	S	S	S	S	S	S	S
	2 LB	S	S	S	S	S	S	S	S	S	S	S
	*2 AMP	R	S	S	S	S	S	S	S	S	S	S
	2 AMP	R	S	S	S	S	S	S	S	S	S	S
S20	*3 LB	S	S	S	S	S	S	S	S	S	S	S
S20	3 LB	S	S	S	S	S	S	S	S	S	S	S
S20	*3 AMP	R	R	S	S	S	S	S	S	S	S	S
S20	3 AMP	R	R	S	S	S	S	S	S	S	S	S
S20	*3 TET	R	R	S	S	S	S	S	S	S	S	S
S20	3 TET	R	R	S	S	S	S	S	S	S	S	S
S21	*4 LB	S	S	S	S	S	S	S	S	S	S	S
S21	4 LB	S	S	S	S	S	S	S	S	S	S	S
S21	*4 AMP	R	S	S	S	S	S	S	S	S	S	S
S21	4 AMP	R	S	S	S	S	S	S	S	S	S	S
S22	*6 LB	R	S	S	S	S	S	S	S	S	S	S
S22	6 LB	R	R	S	S	S	S	S	S	S	S	S
S22	*6 AMP	R	S	S	S	S	S	S	S	S	S	S
S22	6 AMP	R	S	S	S	S	S	S	S	S	S	S
S22	*6 TET	R	R	S	S	S	S	R	S	S	S	S
S22	6 TET	R	R	S	S	S	S	S	S	S	S	S
S23	*11 LB	S	S	S	S	S	S	S	S	S	S	S
S23	11 LB	S	S	S	S	S	S	S	S	S	S	S
S23	*11 AMP	R	S	S	S	S	S	R	S	S	S	S
S23	11 AMP	R	S	S	S	S	S	R	S	S	S	S
S24	*12 LB	S	S	S	S	S	S	S	S	S	S	S
S24	12 LB	S	S	S	S	S	S	S	S	S	S	S
S24	12 AMP	R	S	S	S	S	S	S	S	S	S	S

[a]M-S= corresponding sequenced sample.

[b]Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment.

[c]AMP=Ampicillin, TET=Tetracycline, KAN=Kanamycin, CTX=Cefotaxime, CIP=Ciprofloxacin, CN=Gentamicin, W=Trimethoprim, CAZ=Ceftzidime. MER=Meropenem, IMP=Imipenem, C=Chloramphenicol.

[d]R=Resistant, I=Intermediate, S=Susceptible; according to CLSI guidelines (2018).

6.7 FIGURES

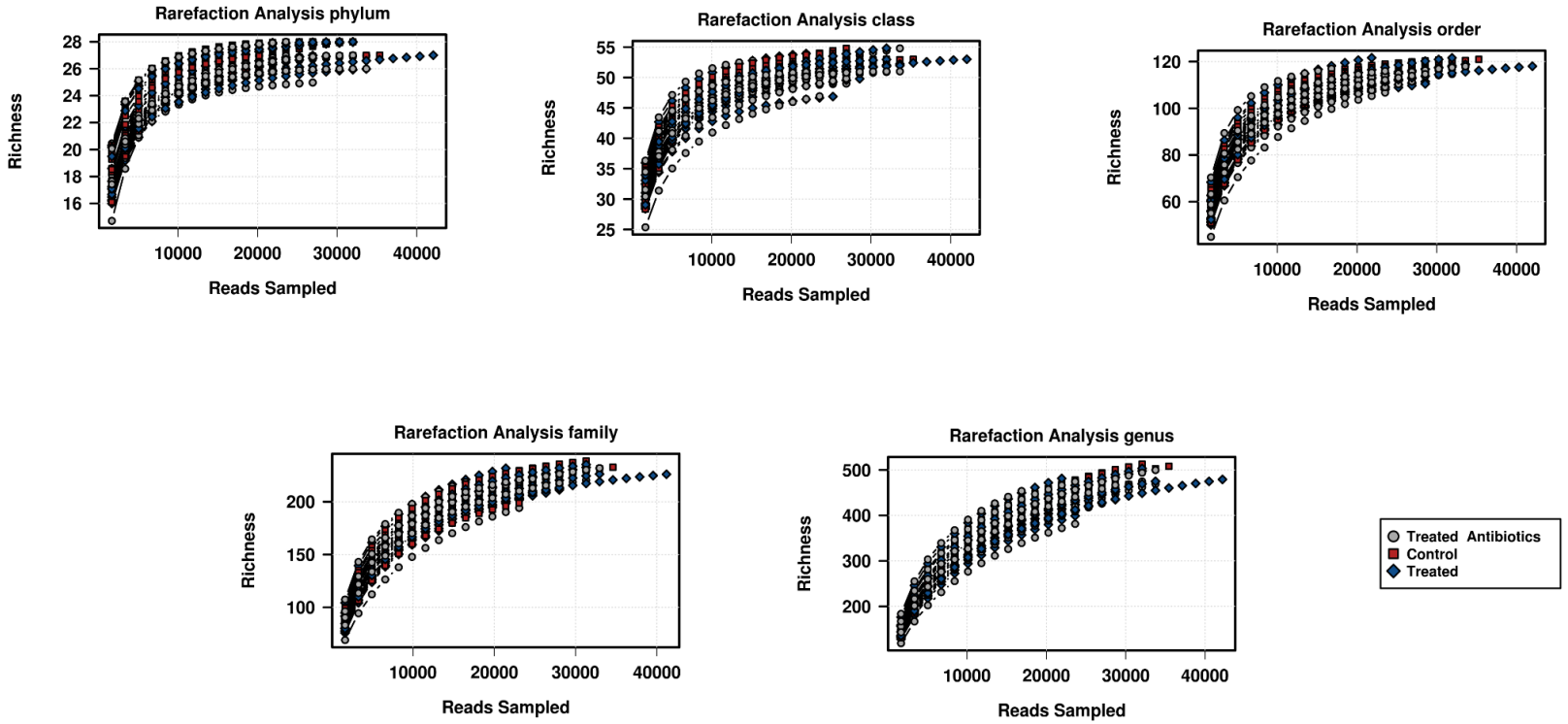

Fig 1. Rarefaction analysis at all taxonomic levels.

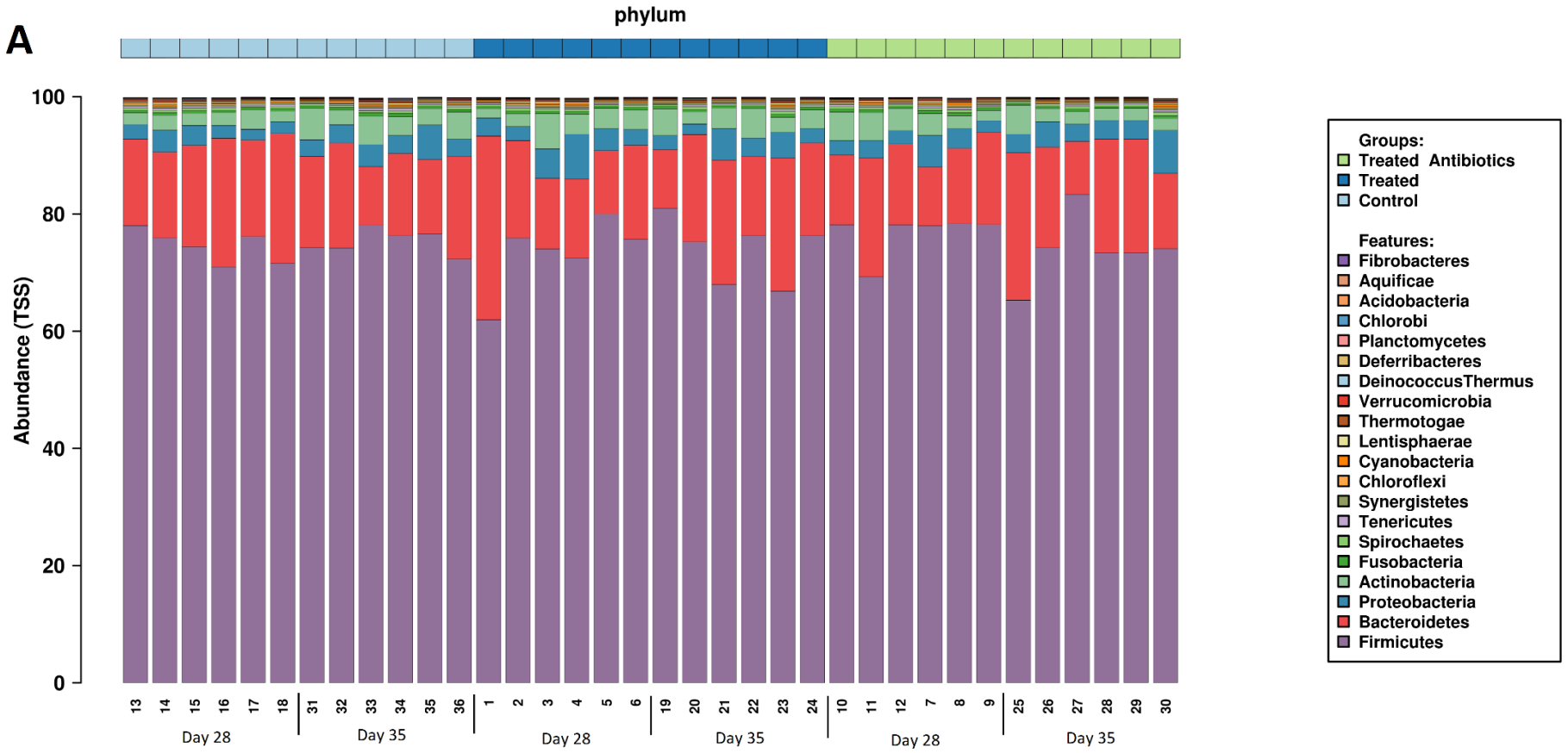

Fig 2. Microbial community composition at (A) phylum, (B) class and (C) genus taxonomic levels.

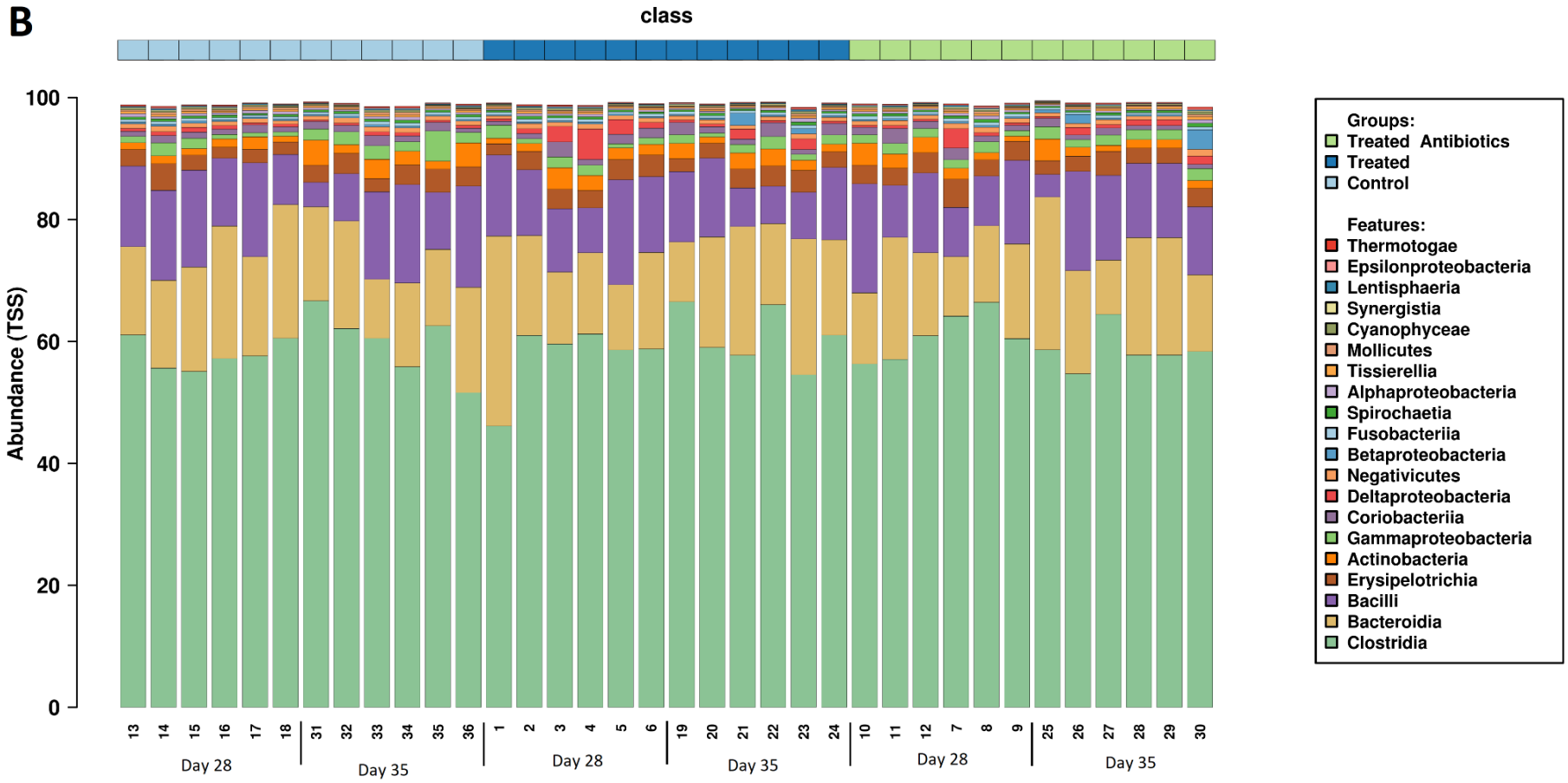

Fig 2. Microbial community composition at (A) phylum, (B) class and (C) genus taxonomic levels.

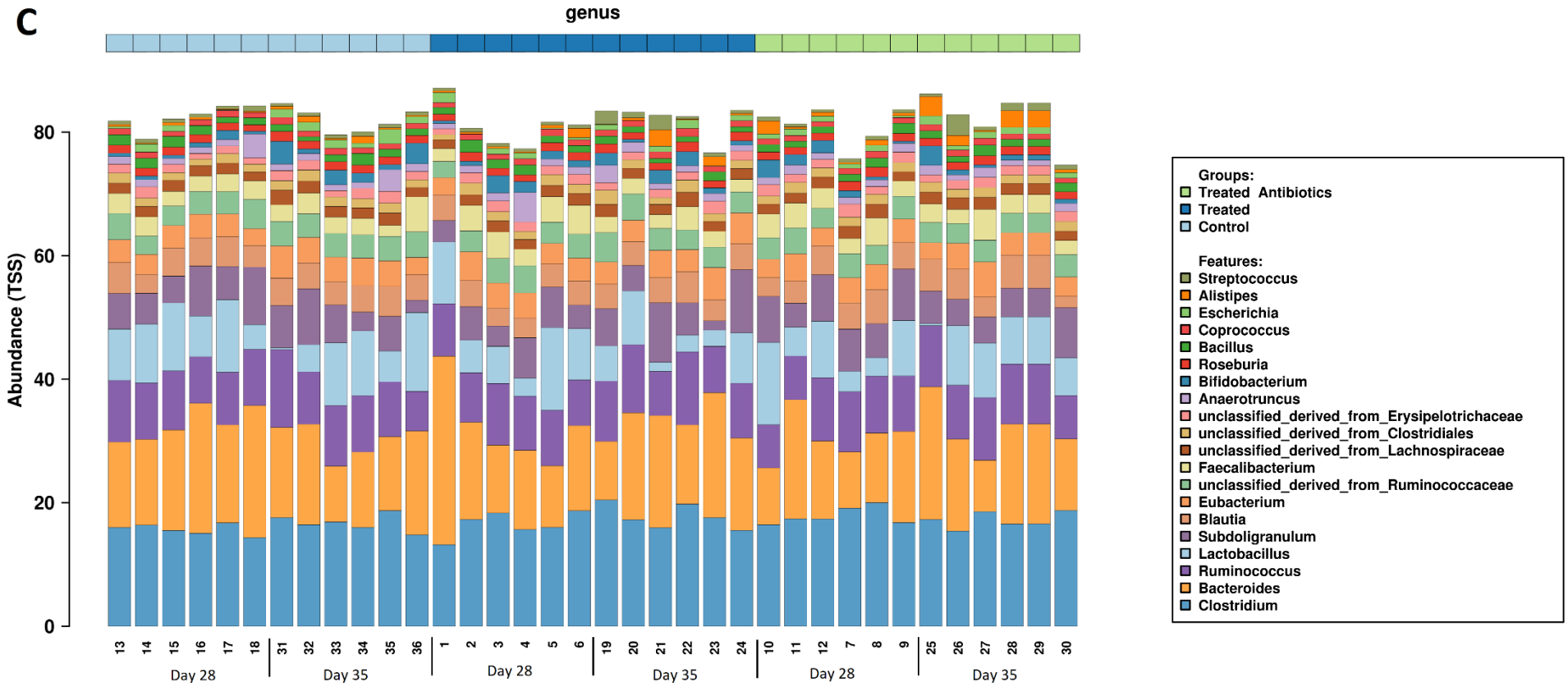

Fig 2. Microbial community composition at (A) phylum, (B) class and (C) genus taxonomic levels.

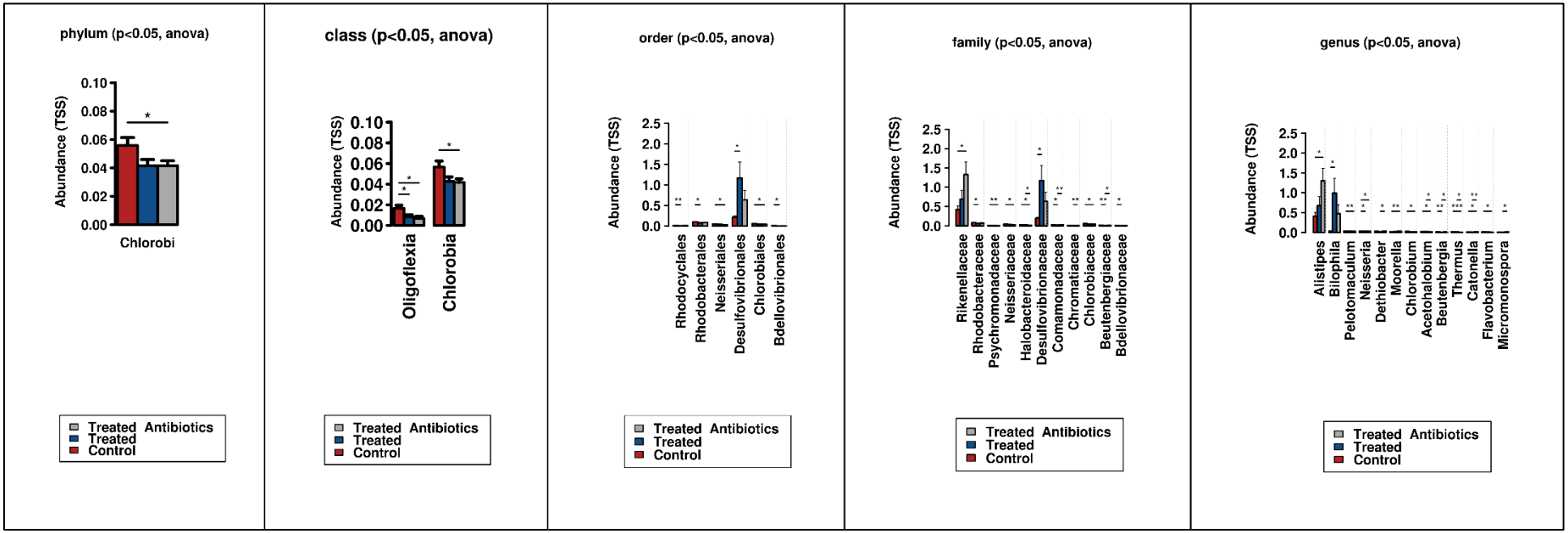

Fig 3. Comparison of taxa abundance across sample groups using ANOVA.

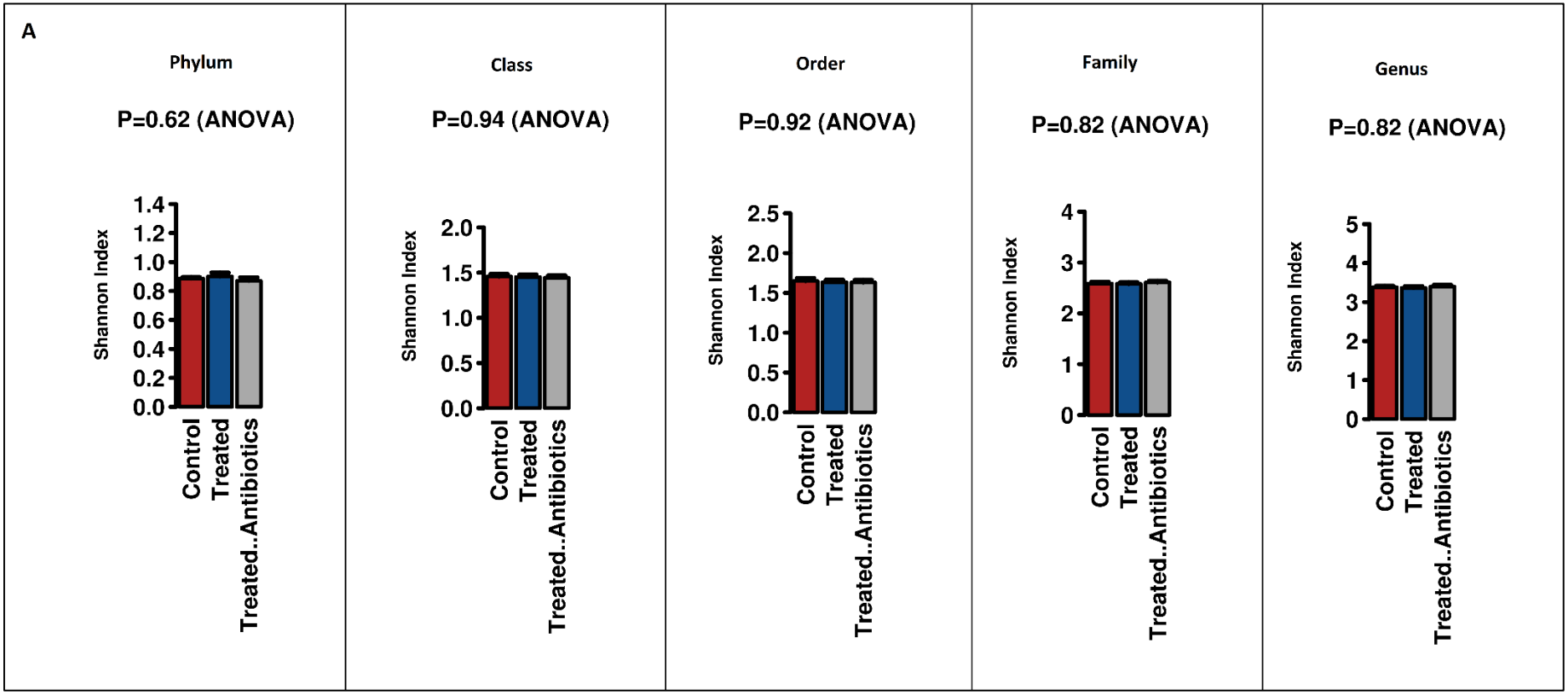

Fig 4. (A) Microbial community alpha diversity assessed using Shannon index, (B) microbial community richness assessed by Chao1 and (C) microbial community evenness at all taxonomic ranks.

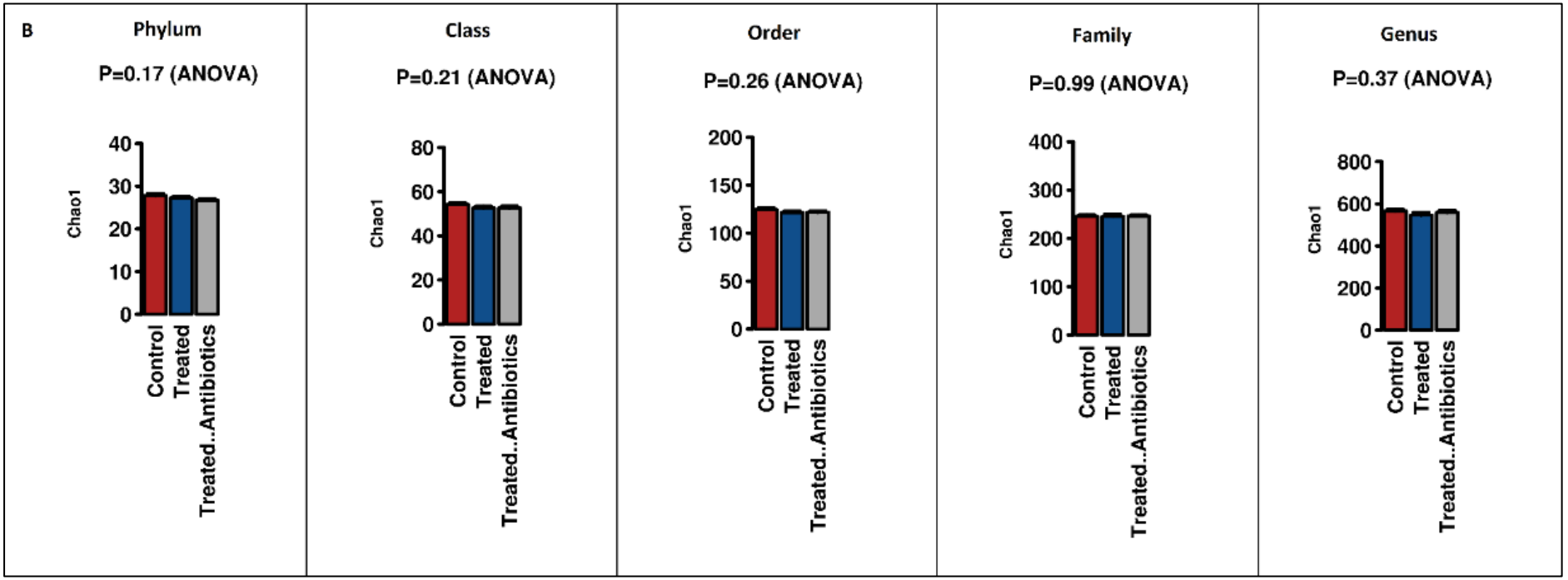

Fig 4. (A) Microbial community alpha diversity assessed using Shannon index, (B) microbial community richness assessed by Chao1 and (C) microbial community evenness at all taxonomic ranks.

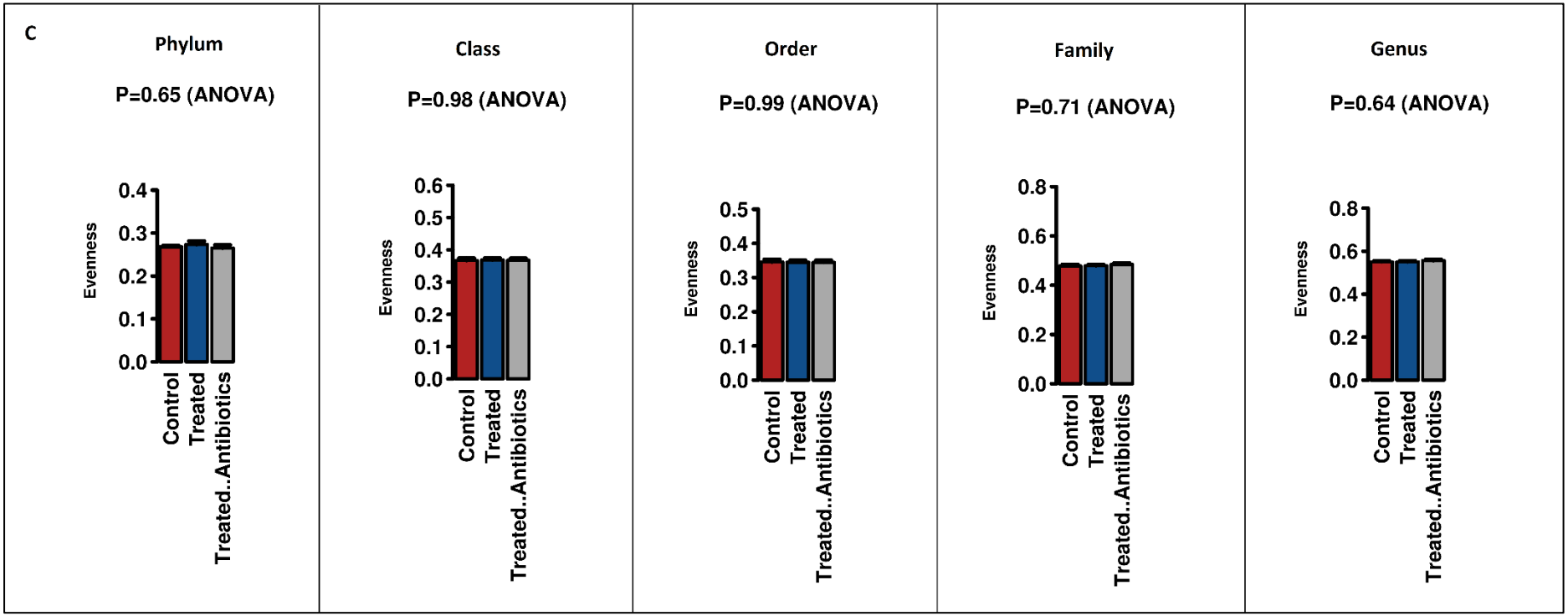

Fig 4. (A) Microbial community alpha diversity assessed using Shannon index, (B) microbial community richness assessed by Chao1 and (C) microbial community evenness at all taxonomic ranks.

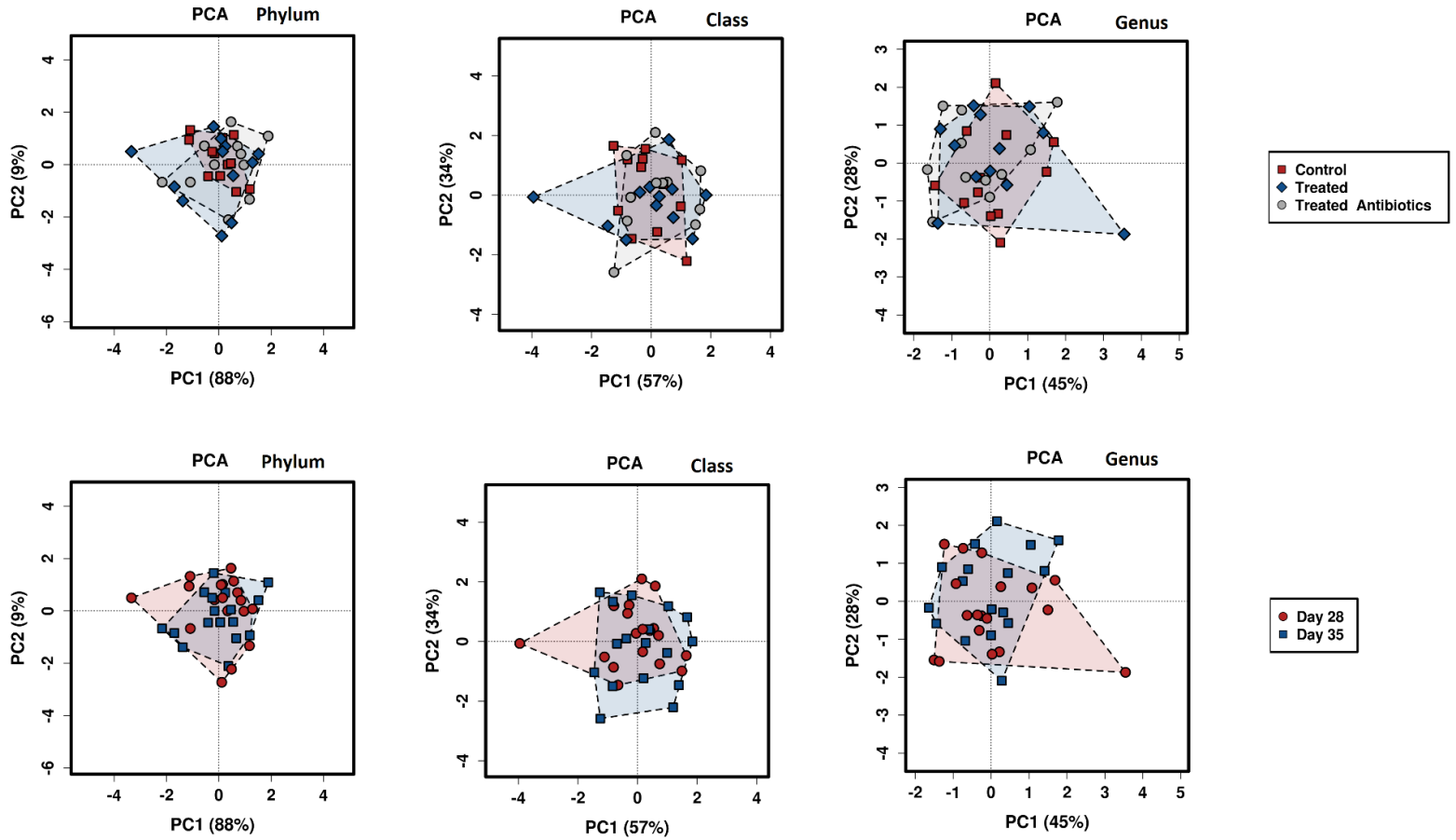

Fig 5. Principal component analysis of sample groups at phylum, class and genus taxonomic levels comparing treatment and time-points.

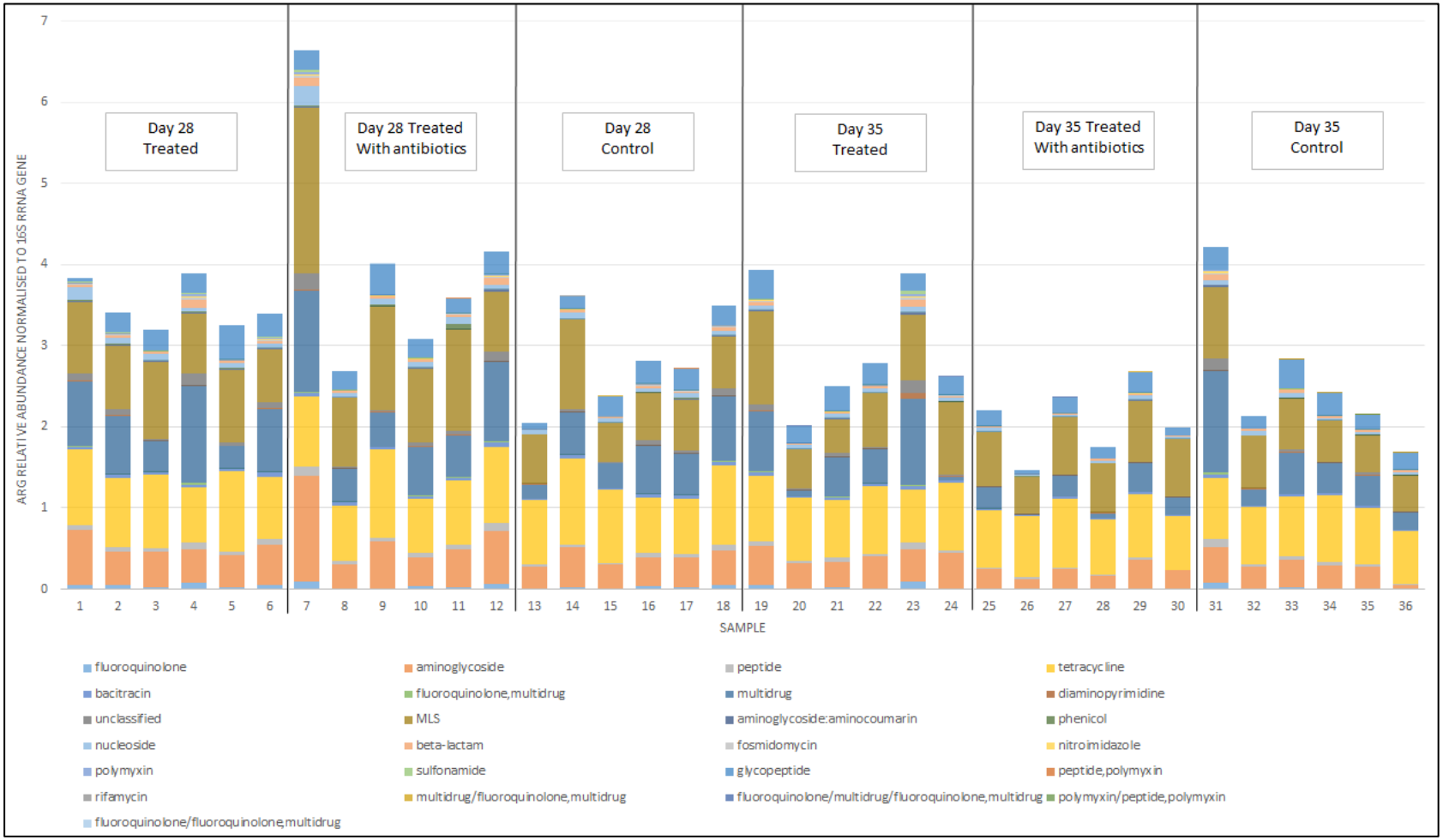

Fig 6. Relative abundance of ARGs present by antibiotic class.

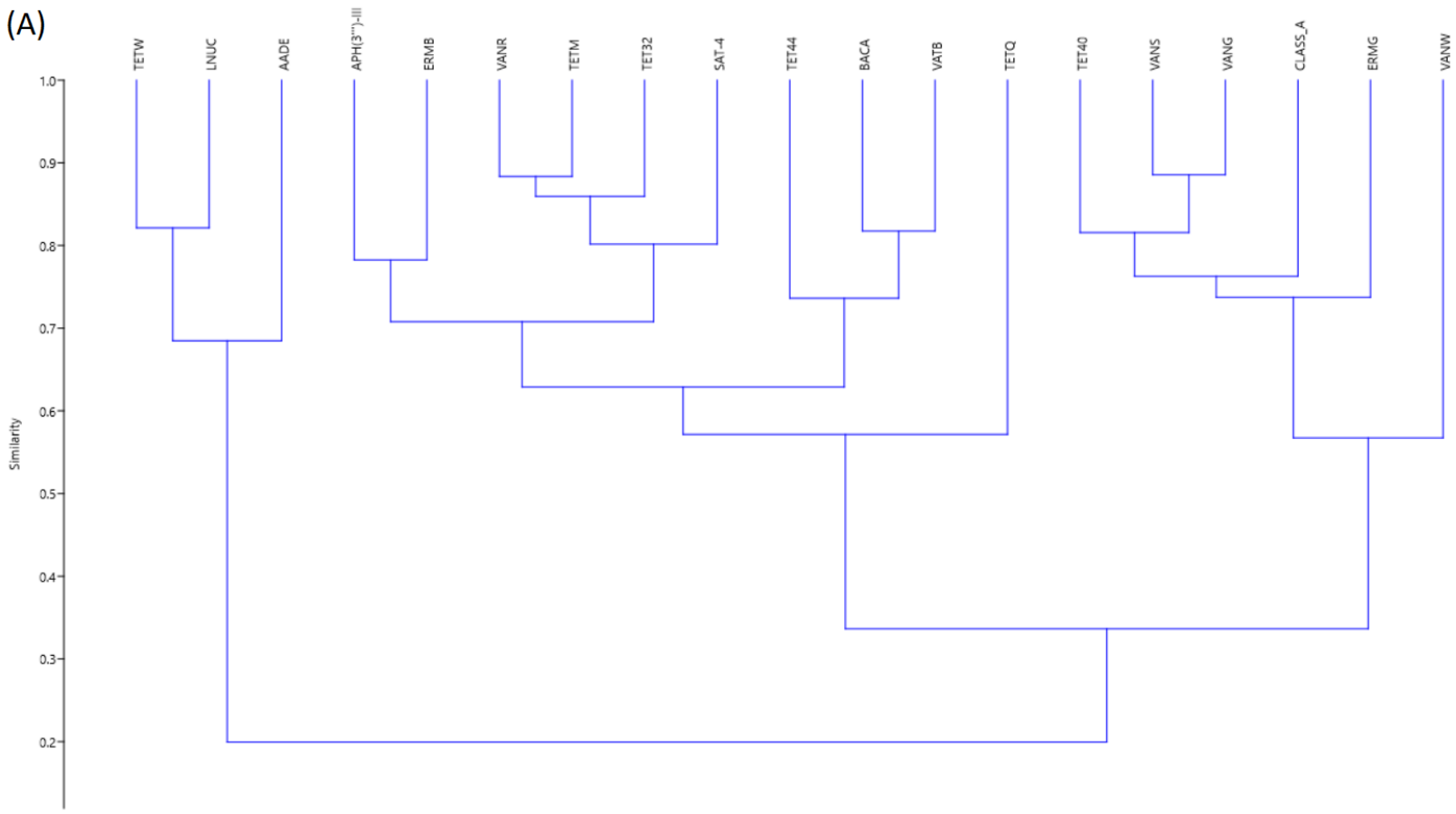

Fig 7. Cluster analysis of ARGs present in the (A) core resistome and (B) accessory resistome using the Bray-Curtis similarity matrix.

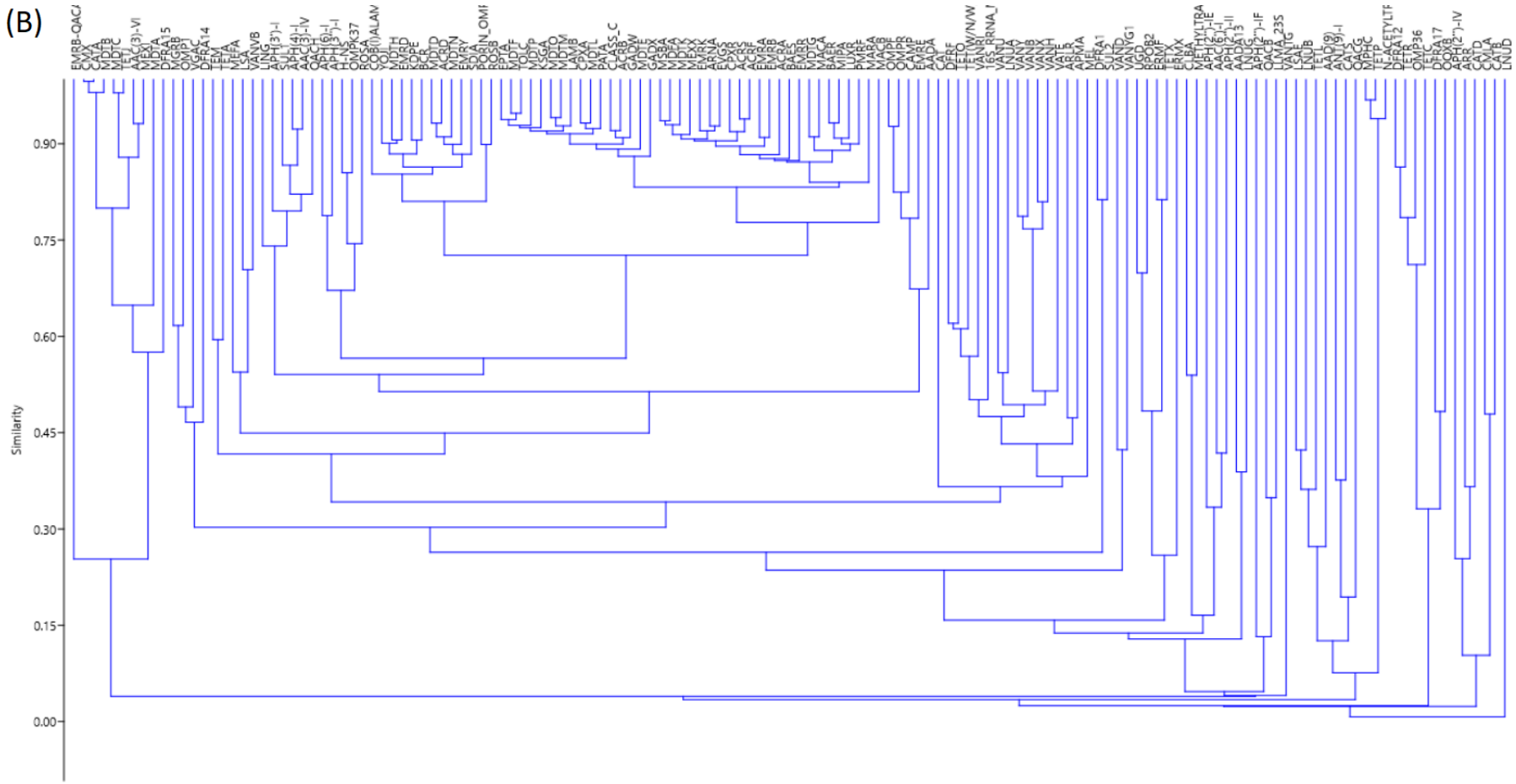

Fig 7. Cluster analysis of ARGs present in the (A) core resistome and (B) accessory resistome using the Bray-Curtis similarity matrix.

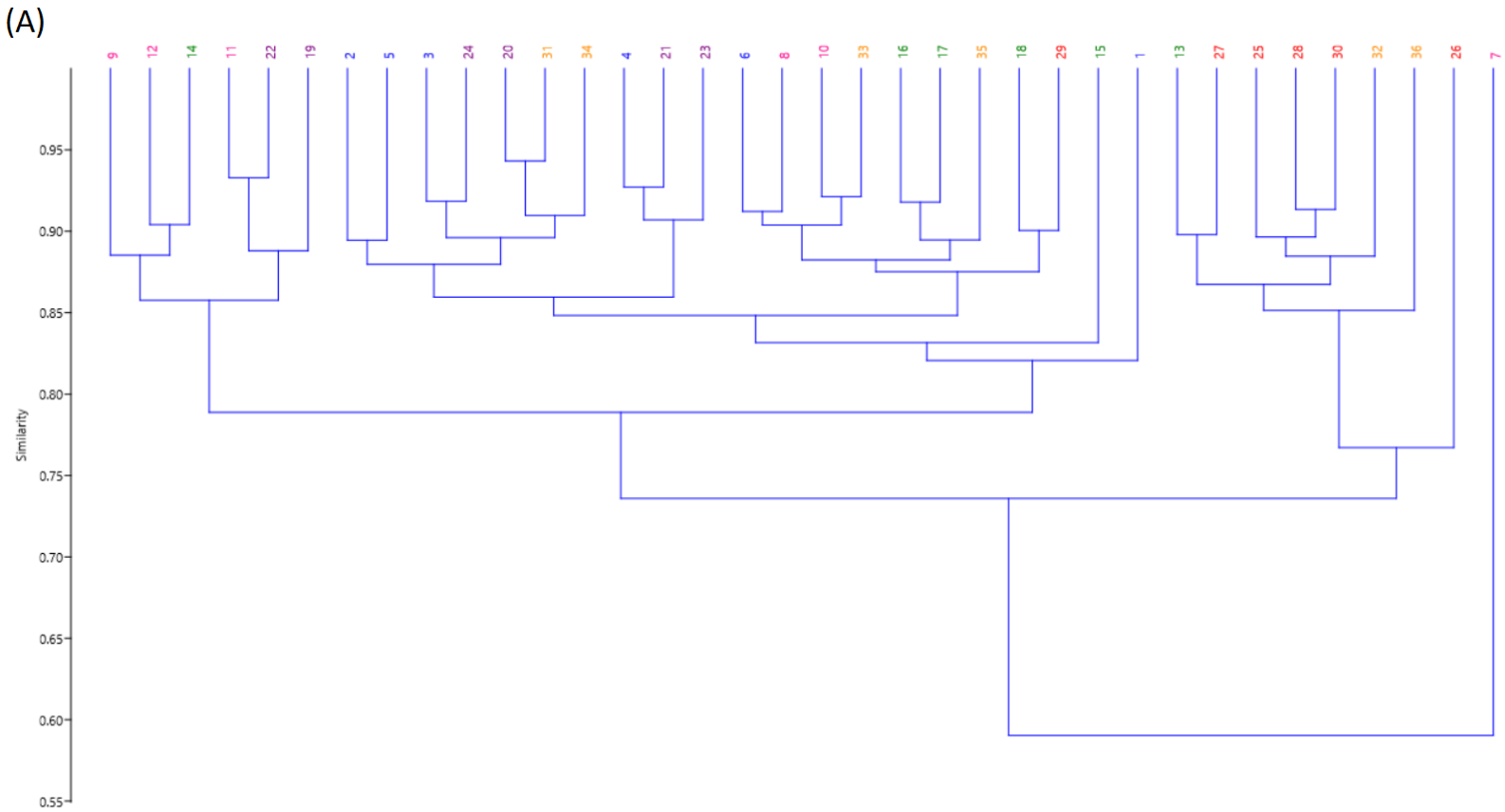

Fig 8. Cluster analysis of samples based on the relative abundance of ARGs in the (A) core resistome and (B) accessory resistome using the Bray-Curtis similarity matrix.

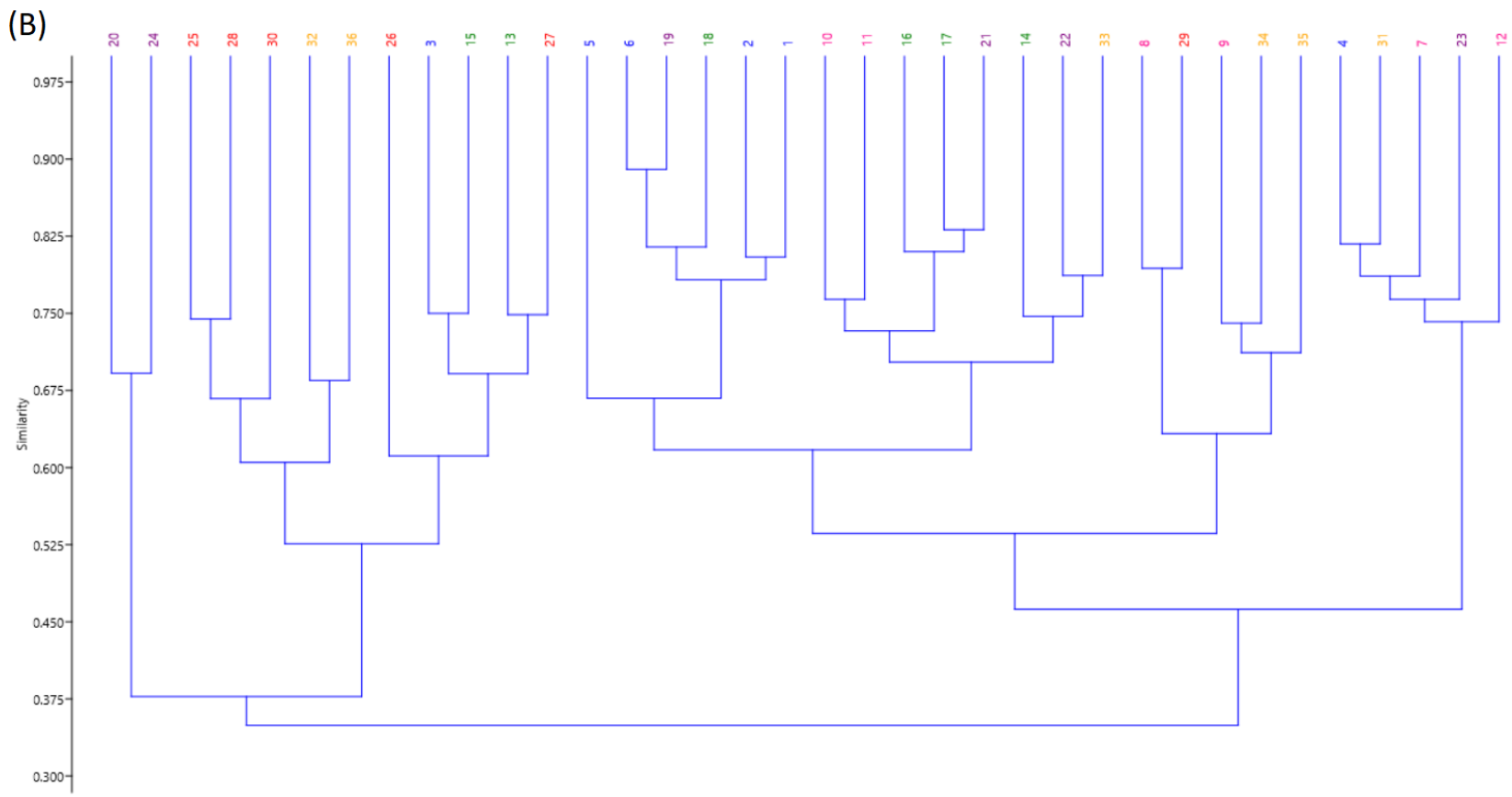

Fig 8. Cluster analysis of samples based on the relative abundance of ARGs in the (A) core resistome and (B) accessory resistome using the Bray-Curtis similarity matrix.

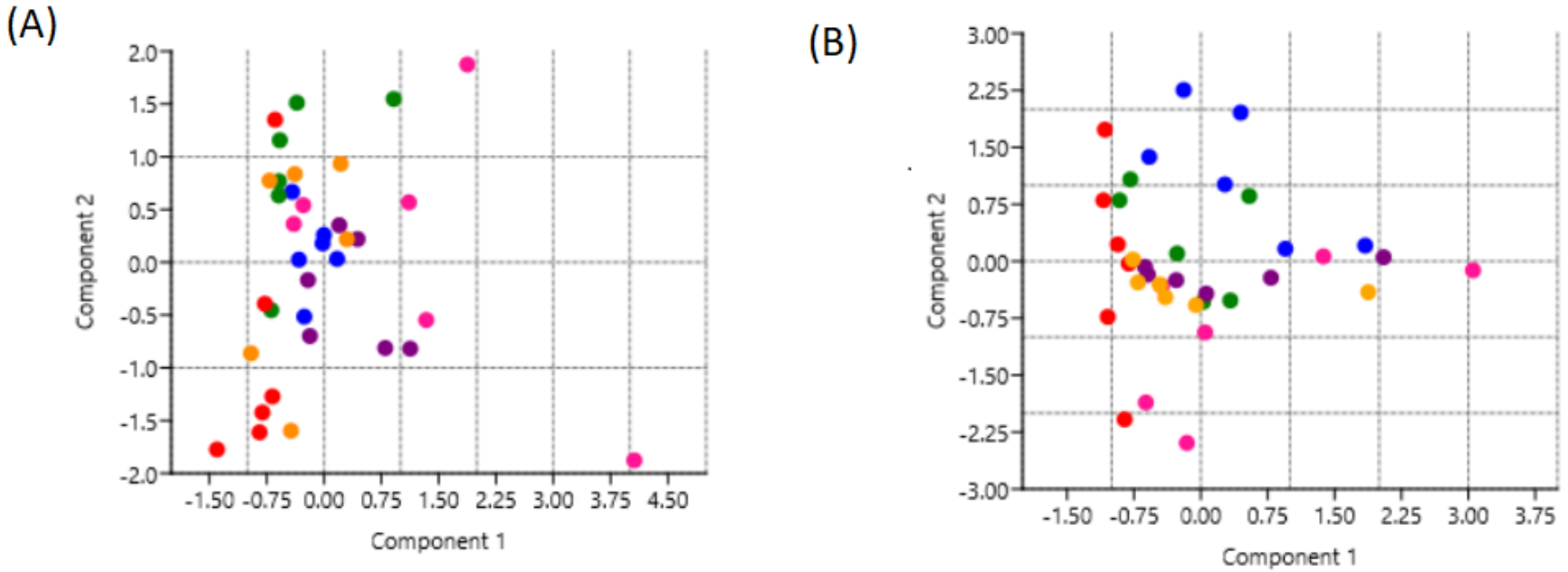

Fig 9. Principal Component Analysis of samples based on the relative abundance of ARGs in the (A) core resistome and (B) accessory resistome. Blue: day 28 treated (S1-6), pink: day 28 treated + antibiotic (S7-12), green: day 28 control (S13-18), purple: day 35 treated (S19-24), red: day 35 treated + antibiotic (S25-30), orange: day 35 control (S30-36).

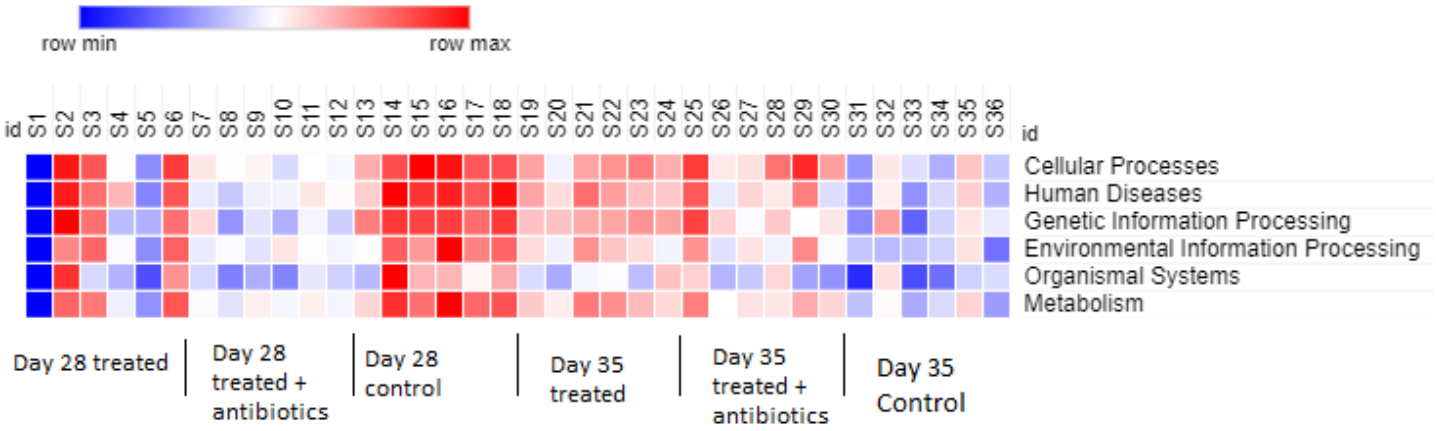

Fig 10. Functional profile based on the KEGG Orthology (KO).

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6.9 SUPPLEMENTARY DATA

Table S1. Mann-Whitney pairwise test, Bonferroni corrected *p* values, accessory resistome.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18
S1		1	0.0228	1	1	1	0.1607	0.02207	0.01295	1	1	1	9.526E-14	1	4.025E-05	1	1	1
S2	1		0.000274	1	1	1	0.1542	0.0003435	0.0002888	1	1	1	4.086E-18	0.1143	5.104E-08	1	1	1
S3	0.0228	0.000274		3.523E-06	1	0.0003811	4.397E-06	1	1	0.367	0.4203	0.004566	1.148E-11	1	1	0.4307	1	0.09187
S4	1	1	3.523E-06		0.0016	1	1	1.783E-06	5.152E-07	0.01504	0.0113	1	1.479E-19	0.0001407	1.229E-09	0.01696	0.002625	1
S5	1	1	1	0.0016		0.3366	0.0003267	1	1	1	1	0.259	3.598E-12	1	0.001398	1	1	1
S6	1	1	0.0003811	1	0.3366		1	0.000335	0.000173	1	1	1	5.77E-17	0.0381	1.951E-07	1	0.3971	1
S7	0.1607	0.1542	4.397E-06	1	0.0003267	1		1.752E-06	2.413E-07	0.002845	0.00282	1	9.256E-20	5.326E-05	1.031E-09	0.001819	0.001007	0.09725
S8	0.02207	0.0003435	1	1.783E-06	1	0.000335	1.752E-06		1	0.5879	1	0.002085	1.426E-10	1	1	0.5257	1	0.06726
S9	0.01295	0.0002888	1	5.152E-07	1	0.000173	2.413E-07	1		0.2254	0.346	0.0004471	8.65E-08	1	1	0.2439	0.4538	0.03564
S10	1	1	0.367	0.01504	1	1	0.002845	0.5879	0.2254		1	1	3.546E-15	1	0.000124	1	1	1
S11	1	1	0.4203	0.0113	1	1	0.00282	1	0.346	1		1	1.398E-14	1	8.377E-05	1	1	1
S12	1	1	0.004566	1	0.259	1	1	0.002085	0.0004471	1	1		1.101E-16	0.06048	1.681E-06	1	0.6626	1
S13	9.526E-14	4.086E-18	1.148E-11	1.479E-19	3.598E-12	5.77E-17	9.256E-20	1.426E-10	8.65E-08	3.546E-15	1.398E-14	1.101E-16		5.873E-12	9.078E-06	1.087E-14	2.944E-16	5.009E-14
S14	1	0.1143	1	0.0001407	1	0.0381	5.326E-05	1	1	1	1	0.06048	5.873E-12		0.02989	1	1	1
S15	4.025E-05	5.104E-08	1	1.229E-09	0.001398	1.951E-07	1.031E-09	1	1	0.000124	8.377E-05	1.681E-06	9.078E-06	0.02989		0.0003556	3.77E-05	0.0001331
S16	1	1	0.4307	0.01696	1	1	0.001819	0.5257	0.2439	1	1	1	1.087E-14	1	0.0003556		1	1
S17	1	1	1	0.002625	1	0.3971	0.001007	1	0.4538	1	1	0.6626	2.944E-16	1	3.77E-05	1		1
S18	1	1	0.09187	1	1	1	0.09725	0.06726	0.03564	1	1	1	5.009E-14	1	0.0001331	1	1	
S19	1	1	0.001378	1	1	1	0.1833	0.001114	0.001245	1	1	1	3.568E-15	0.09187	2.28E-06	1	1	1
S20	3.895E-06	1.377E-09	0.1188	1.721E-11	0.0001729	9.031E-09	1.004E-11	0.3697	1	4.77E-06	6.736E-06	3.587E-08	0.0001013	0.004589	1	1.68E-05	1.765E-06	8.611E-06
S21	1	0.5717	1	0.0002282	1	0.06053	8.259E-05	1	1	1	1	0.07253	6.077E-13	1	0.004961	1	1	1
S22	0.1758	0.006149	1	1.499E-05	1	0.00466	1.251E-05	1	1	1	1	0.01825	2.455E-12	1	0.04101	1	1	0.523
S23	1	1	0.5114	1	1	1	1	0.2422	0.03154	1	1	1	1.996E-13	1	0.0009874	1	1	1
S24	1.318E-05	1.107E-08	0.2422	9.03E-11	0.0009349	3.065E-08	4.962E-11	0.7869	1	4.046E-05	4.154E-05	1.301E-07	4.302E-06	0.0188	1	0.0001537	9.809E-06	3.544E-05
S25	0.0005465	3.27E-07	0.793	1.318E-08	0.007809	2.625E-06	6.736E-09	1	1	0.0004794	0.0003549	9.396E-06	1.062E-07	0.0686	1	0.002304	6.408E-05	0.001139
S26	1.5E-11	4.775E-16	6.185E-09	2.722E-17	6.599E-10	7.501E-15	2.088E-17	5.147E-08	2.469E-05	9.941E-13	3.089E-12	2.507E-14	1	1.394E-09	0.004074	4.031E-12	7.469E-14	9.51E-12
S27	1.668E-08	1.458E-12	8.243E-06	4.461E-14	6.203E-07	1.631E-11	3.22E-14	5.803E-05	0.008159	1.563E-09	5.996E-09	3.265E-11	1	1.79E-06	0.5938	6.527E-09	2.264E-10	1.14E-08
S28	3.673E-14	1.771E-18	4.93E-12	4.696E-20	1.454E-12	2.06E-17	3.055E-20	5.354E-11	2.902E-08	1.472E-15	5.544E-15	3.871E-17	1	1.594E-12	3.312E-06	4.757E-15	1.175E-16	1.809E-14
S29	0.0005879	4.953E-07	1	1.074E-08	0.01412	2.427E-06	7.832E-09	1	1	0.0008008	0.0007887	1.256E-05	2.289E-08	0.1835	1	0.002995	0.0002685	0.001301
S30	3.042E-11	5.013E-15	9.058E-08	7.005E-17	2.423E-09	2.745E-14	3.081E-17	4.828E-07	0.0001176	9.22E-12	2.037E-11	7.778E-14	1	9.152E-09	0.01652	2.255E-11	1.452E-12	2.88E-11
S31	1	1	0.108	1	1	1	1	0.04348	0.009552	1	1	1	1.362E-13	0.5366	0.0002986	1	1	1
S32	6.72E-12	9.238E-16	3.416E-09	8.004E-18	6.765E-10	5.085E-15	3.174E-18	3.164E-08	5.878E-06	9.005E-13	3.54E-12	5.44E-15	1	1.369E-09	0.0003243	1.694E-12	1.977E-13	2.933E-12
S33	1	0.2029	1	0.0002522	1	0.0421	0.0001357	1	1	1	1	0.1193	7.268E-14	1	0.002644	1	1	1
S34	0.001494	6.77E-06	1	4.561E-08	0.1195	1.212E-05	3.556E-08	1	1	0.01703	0.02283	6.614E-05	1.602E-08	1	1	0.02641	0.02043	0.004471
S35	0.01186	0.0001675	1	8.085E-07	0.9977	0.0001585	9.155E-07	1	1	0.3295	0.3775	0.001571	5.086E-11	1	1	0.3408	0.8771	0.04852
S36	3.963E-06	1.021E-09	0.002483	7.03E-12	0.0003063	3.857E-09	3.836E-12	0.02168	1	2.038E-06	4.069E-06	6.115E-09	0.001309	0.00128	1	7.924E-06	2.912E-07	3.211E-06

	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
S1	1	3.895E-06	1	0.1758	1	1.318E-05	0.0005465	1.5E-11	1.668E-08	3.673E-14	0.0005879	3.042E-11	1	6.72E-12	1	0.001494	0.01186	3.963E-06
S2	1	1.377E-09	0.5717	0.006149	1	1.107E-08	3.27E-07	4.775E-16	1.458E-12	1.771E-18	4.953E-07	5.013E-15	1	9.238E-16	0.2029	6.77E-06	0.0001675	1.021E-09
S3	0.001378	0.1188	1	1	0.5114	0.2422	0.793	6.185E-09	8.243E-06	4.93E-12	1	9.058E-08	0.108	3.416E-09	1	1	1	0.002483
S4	1	1.721E-11	0.0002282	1.499E-05	1	9.03E-11	1.318E-08	2.722E-17	4.461E-14	4.696E-20	1.074E-08	7.005E-17	1	8.004E-18	0.0002522	4.561E-08	8.085E-07	7.03E-12
S5	1	0.0001729	1	1	1	0.0009349	0.007809	6.599E-10	6.203E-07	1.454E-12	0.01412	2.423E-09	1	6.765E-10	1	0.1195	0.9977	0.0003063
S6	1	9.031E-09	0.06053	0.00466	1	3.065E-08	2.625E-06	7.501E-15	1.631E-11	2.06E-17	2.427E-06	2.745E-14	1	5.085E-15	0.0421	1.212E-05	0.0001585	3.857E-09
S7	0.1833	1.004E-11	8.259E-05	1.251E-05	1	4.962E-11	6.736E-09	2.088E-17	3.22E-14	3.055E-20	7.832E-09	3.081E-17	1	3.174E-18	0.0001357	3.556E-08	9.155E-07	3.836E-12
S8	0.001114	0.3697	1	1	0.2422	0.7869	1	5.147E-08	5.803E-05	5.354E-11	1	4.828E-07	0.04348	3.164E-08	1	1	1	0.02168
S9	0.001245	1	1	1	0.03154	1	1	2.469E-05	0.008159	2.902E-08	1	0.0001176	0.009552	5.878E-06	1	1	1	1
S10	1	4.77E-06	1	1	1	4.046E-05	0.0004794	9.941E-13	1.563E-09	1.472E-15	0.0008008	9.22E-12	1	9.005E-13	1	0.01703	0.3295	2.038E-06
S11	1	6.736E-06	1	1	1	4.154E-05	0.0003549	3.089E-12	5.996E-09	5.544E-15	0.0007887	2.037E-11	1	3.54E-12	1	0.02283	0.3775	4.069E-06
S12	1	3.587E-08	0.07253	0.01825	1	1.301E-07	9.396E-06	2.507E-14	3.262E-11	3.671E-17	1.256E-05	7.778E-14	1	5.44E-15	0.1193	6.614E-05	0.001571	6.115E-09
S13	3.568E-15	0.0001013	6.077E-13	2.455E-12	1.996E-13	4.302E-06	1.062E-07	1	1	1	2.289E-08	1	1.362E-13	1	7.268E-14	1.602E-08	5.086E-11	0.001309
S14	0.09187	0.004589	1	1	1	0.0188	0.0686	1.394E-09	1.79E-06	1.594E-12	0.1835	9.152E-09	0.5366	1.369E-09	1	1	1	0.00128
S15	2.28E-06	1	0.004961	0.04101	0.0009874	1	1	0.004074	0.5938	3.312E-06	1	0.01652	0.0002986	0.0003243	0.002644	1	1	1
S16	1	1.68E-05	1	1	1	0.0001537	0.002304	4.031E-12	6.527E-09	4.757E-15	0.002995	2.255E-11	1	1.694E-12	1	0.02641	0.3408	7.924E-06
S17	1	1.765E-06	1	1	1	9.809E-06	6.408E-05	7.469E-14	2.264E-10	1.175E-16	0.0002685	1.452E-12	1	1.977E-13	1	0.02043	0.8771	2.912E-07
S18	1	8.611E-06	1	0.523	1	3.544E-05	0.001139	9.51E-12	1.14E-08	1.809E-14	0.001301	2.88E-11	1	2.933E-12	1	0.004471	0.04852	3.211E-06
S19		1.068E-07	0.2096	0.009551	1	5.494E-07	3.181E-05	4.183E-13	5.471E-10	1.557E-15	2.728E-05	1.275E-12	1	2.334E-13	0.1325	8.869E-05	0.0006928	1.514E-07
S20	1.068E-07		0.0006884	0.007891	4.133E-05	1	1	0.03239	1	2.901E-05	1	0.1299	1.365E-05	0.002676	0.0003052	1	0.2074	1
S21	0.2096	0.0006884		1	1	0.005262	0.02472	1.785E-10	2.961E-07	1.638E-13	0.06581	9.95E-10	0.7204	1.601E-10	1	0.5315	1	0.0004031
S22	0.009551	0.007891	1		0.9179	0.0162	0.06415	6.537E-10	1.43E-06	8.896E-13	0.2839	7.364E-09	0.1453	1.036E-09	1	1	1	0.0006482
S23	1	4.133E-05	1	0.9179		0.0001811	0.006724	1.073E-10	7.491E-08	5.393E-14	0.007016	9.263E-11	1	4.182E-12	1	0.01705	0.1778	1.114E-05
S24	5.494E-07	1	0.005262	0.0162	0.0001811		1	0.002106	0.2647	1.273E-06	1	0.01514	7.094E-05	0.0001322	0.001082	1	0.3498	1
S25	3.181E-05	1	0.02472	0.06415	0.006724	1		0.0001242	0.03889	2.211E-08	1	0.0009485	0.002642	4.479E-06	0.005907	1	1	1
S26	4.183E-13	0.03239	1.785E-10	6.537E-10	1.073E-10	0.002106	0.0001242		1	1	3.036E-05	1	5.097E-11	1	2.062E-11	7.147E-06	2.199E-08	0.6432
S27	5.471E-10	1	2.961E-07	1.43E-06	7.491E-08	0.2647	0.03889	1	0.9097	0.01103	1	3.673E-08	1	4.697E-08	0.003608	3.323E-05	1	
S28	1.557E-15	2.901E-05	1.636E-13	8.896E-13	5.393E-14	1.273E-06	2.211E-08	1	0.9097		5.205E-09	1	3.748E-14	1	2.558E-14	4.168E-09	2.33E-11	0.0003372
S29	2.728E-05	1	0.06581	0.2839	0.007016	1	1	3.036E-05	0.01103	5.205E-09		0.0002922	0.002635	1.26E-06	0.02049	1	1	1
S30	1.275E-12	0.1299	9.95E-10	7.364E-09	9.263E-11	0.01514	0.0009485	1	1	1	0.0002922		4.747E-11	1	2.538E-10	5.692E-05	2.339E-07	1
S31	1	1.365E-05	0.7204	0.1453	1	7.094E-05	0.002642	5.097E-11	3.673E-08	3.748E-14	0.002635	4.747E-11		3.645E-12	1	0.003659	0.02984	7.316E-06
S32	2.334E-13	0.0002676	1.601E-10	1.036E-09	4.182E-12	0.0001322	4.479E-06	1	1	1	1.26E-06	1	3.645E-12		2.989E-11	1.324E-06	1.311E-08	0.01752
S33	0.1325	0.0003052	1	1	1	0.001082	0.005907	2.062E-11	4.697E-08	2.558E-14	0.02049	2.538E-10	1	2.989E-11		0.9338	1	3.321E-05
S34	8.869E-05	1	0.5315	1	0.01705	1	1	7.147E-06	0.003608	4.168E-09	1	5.692E-05	0.003659	1.324E-06	0.9338		1	0.9129
S35	0.0006926	0.2074	1	1	0.1778	0.3498	1	2.199E-08	3.323E-05	2.33E-11	1	2.339E-07	0.02984	1.311E-08	1	1		0.007497
S36	1.514E-07	1	0.0004031	0.0006482	1.114E-05	1	1	0.6432	1	0.0003372	1	1	7.316E-06	0.01752	3.321E-05	0.9129	0.007497	

Table S2. List of antibiotic resistance genes present in the core resistome.

CORE RESISTOME
LNUC
BACA
CLASS_A
VATB
VANS
VANR
VANW
VANG
ERMB
ERMG
SAT-4
APH(3'')-III
AADE
TET32
TET44
TET40
TETW
TETQ
TETM

Table S3. List of antibiotic resistance genes present in the accessory resistome.

ACCESSORY RESISTOME
LNUA
COB(I)ALAMIN_ADENOLSYLTRANSFERASE
OMPF
LING
EPTA
MSBA
OMPR
PORIN_OMPC
MDFA
APH(6)-I
MDTK
CLASS_C
MEFA
CPXA
ROSB
CAMP-REGULATORY_PROTEIN
YOJI
MDTP
DFRF
LSA
UGD
MDTG
MDTF
MDTE
MDTD
MDTH
MDTO

MDTN
MDTM
MDTL
VANTG
VANU
VANY
VANX
VANB
APH(3')-I
EMRY
BAER
BAES
EMRR
PMRF
EMRK
EMRA
EMRB
EMRD
EMRE
APH(3")-I
ERMF
MGRB
VANH
ESCHERICHIA_COLI_MIPA
ESCHERICHIA_COLI_LAMB
TETX
TEM
SUL1
QACH

TETO
TETA
KDPE
SDIA
AADA
CAT_CHLORAMPHENICOL_ACETYLTRANSFERASE
BICYCLOMYCIN-MULTIDRUG_EFFLUX_PROTEIN_BCR
KASUGAMYCIN_RESISTANCE_PROTEIN_KSGA
TRUNCATED_PUTATIVE_RESPONSE_REGULATOR_ARLR
CLBA
MARA
ARNA
GADW
PATA
DNA-BINDING_TRANSCRIPTIONAL_REGULATOR_GADX
APH(4)-I
MEXX
16S_RRNA_METHYLASE
VGAC
EVGS
TOLC
TRANSCRIPTIONAL_REGULATORY_PROTEIN_CPXR_CPXR
ACRD
AAC(3)-IV
MACB
MACA
ACRS
RPOB2
DNA-BINDING_PROTEIN_H-NS

ACRB
ACRA
ACRF
KLEBSIELLA_PNEUMONIAE_OMPK37
BACTERIAL_REGULATORY_PROTEIN_LUXR
APMA
VANVB
VANRI
ROSA
APH(2")-IE
TET(W/N/W)
VAND
DFRA14
ERMX
DFRA1
SUL2
MEL
AAC(6')-I
AADA13
VATE
ANTIBIOTIC_RESISTANCE_RRNA_ADENINE_METHYLTRANSFERASE
LNUG
SERRATIA_MARCESCENS_OMP1
APH(2")-IF
LSAE
QACB
APH(2")-IV
APH(2")-II
ADP-RIBOSYLATING_TRANSFERASE_ARR

CATD
VANYG1
AAD(9)
LLMA_23S_RIBOSOMAL_RNA_METHYLTRANSFERASE
ANT(9)-I
TETL
LNUD
CATS
LNUB
MPHC
BIFUNCTIONAL_AMINOGLYCOSIDE_N-ACETYLTRANSFERASE_AND_AMINOGLYCOSIDE_ PHOSPHOTRANSFERASE
TETP
EMRB-QACA_FAMILY_MAJOR_FACILITATOR_TRANSPORTER
CMLA
CATB
QACG
DFRA12
CMX
MDTC
MDTB
MDTA
AAC(3)-VI
CATA
DFRA15
TETJ
MEXI
DFRA17
TETR

OQXB
OMP36
TETC

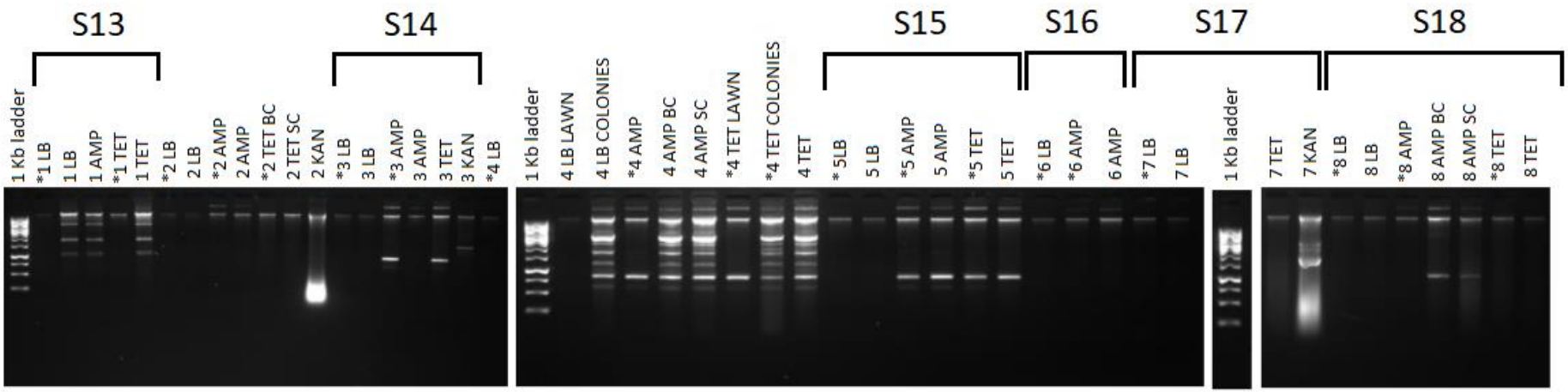

Fig S1. Plasmids from the 'Day 28 Control' group visualised on an agarose gel. Samples are named after the bird the sample was taken from and the antibiotic which the transconjugant was selected on, e.g. 6 TET= bird 6 selected on tetracycline. Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment. Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony) or had both a lawn of growth and distinct colonies on the same plate (LAWN/COLONIES). Samples are labelled with their corresponding sequenced sample.

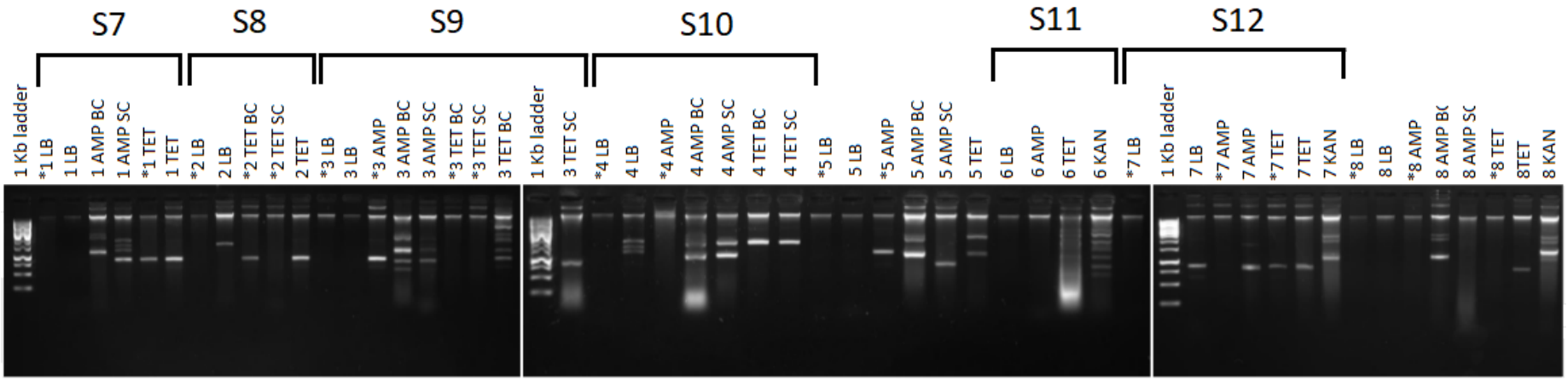

Fig S2. Plasmids from the 'Day 28 Treated +Antibiotics' group visualised on an agarose gel. Samples are named after the bird the sample was taken from and the antibiotic which the transconjugant was selected on, e.g. 6 TET= bird 6 selected on tetracycline. Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment. Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony). Samples are labelled with their corresponding sequenced sample.

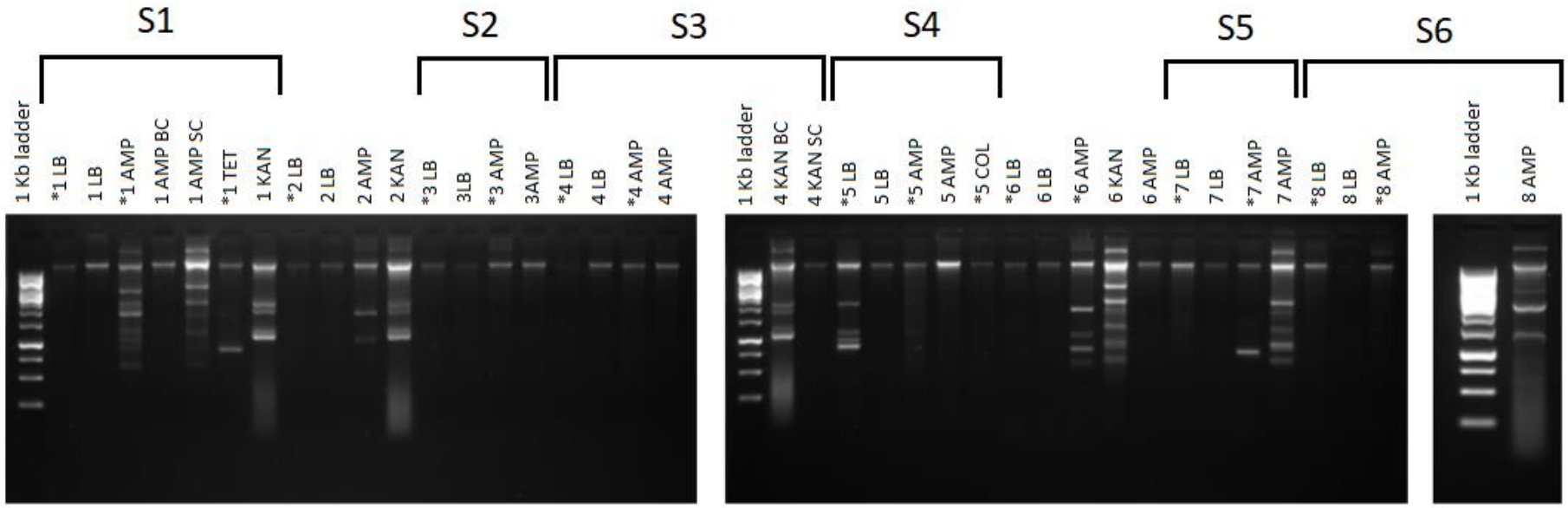

Fig S3. Plasmids from the 'Day 28 Treated' group visualised on an agarose gel. Samples are named after the bird the sample was taken from and the antibiotic which the transconjugant was selected on, e.g. 6 TET= bird 6 selected on tetracycline. Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment. Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony). Samples are labelled with their corresponding sequenced sample.

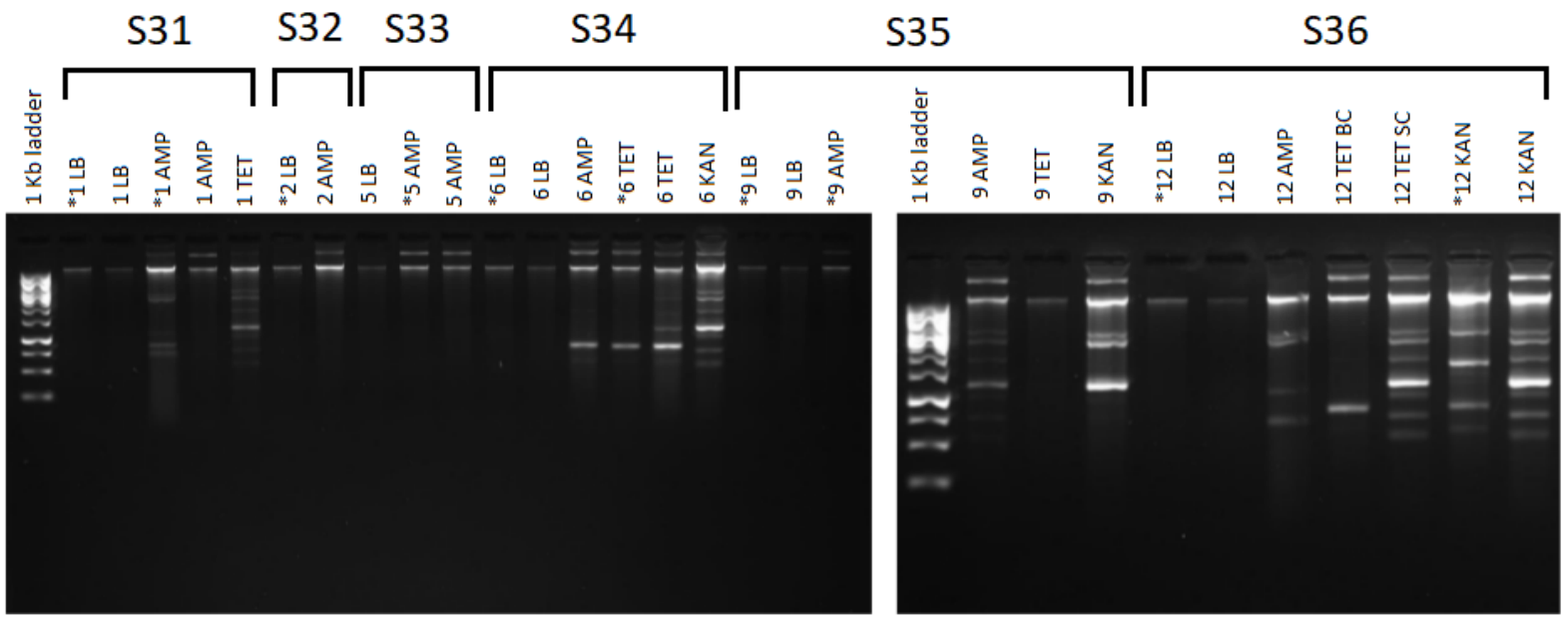

Fig S4. Plasmids from the 'Day 35 Control' group visualised on an agarose gel. Samples are named after the bird the sample was taken from and the antibiotic which the transconjugant was selected on, e.g. 6 TET= bird 6 selected on tetracycline. Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment. Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony). Samples are labelled with their corresponding sequenced sample.

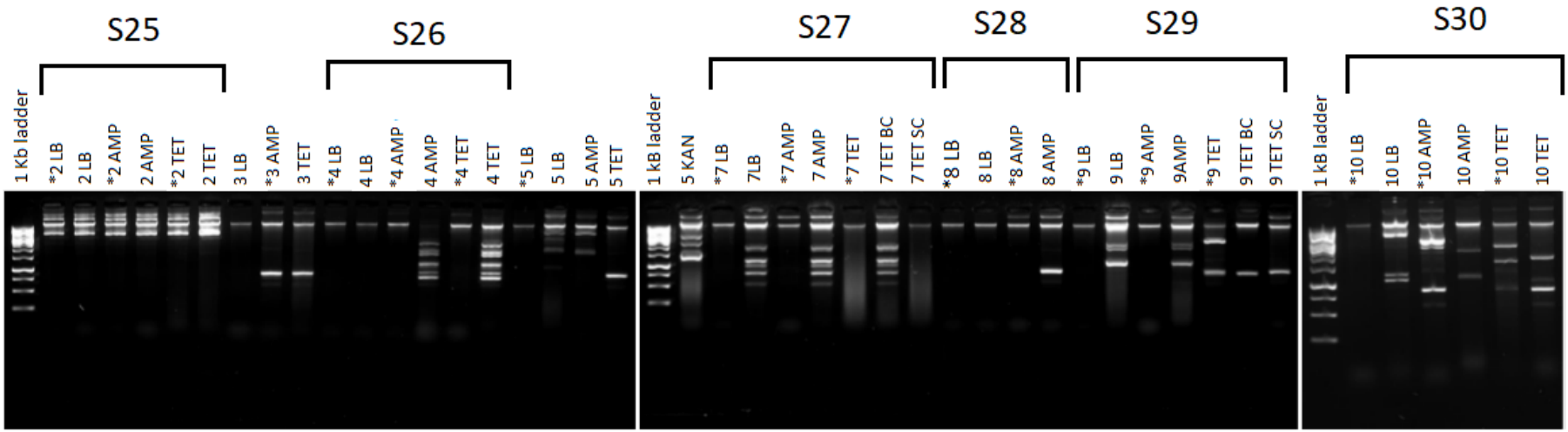

Fig S5. Plasmids from the 'Day 35 Treated +Antibiotics' group visualised on an agarose gel. Samples are named after the bird the sample was taken from and the antibiotic which the transconjugant was selected on, e.g. 6 TET= bird 6 selected on tetracycline. Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment. Some transconjugants appeared to have different colony morphologies on the same antibiotic selective plate (BC=big colony; SC=small colony). Samples are labelled with their corresponding sequenced sample.

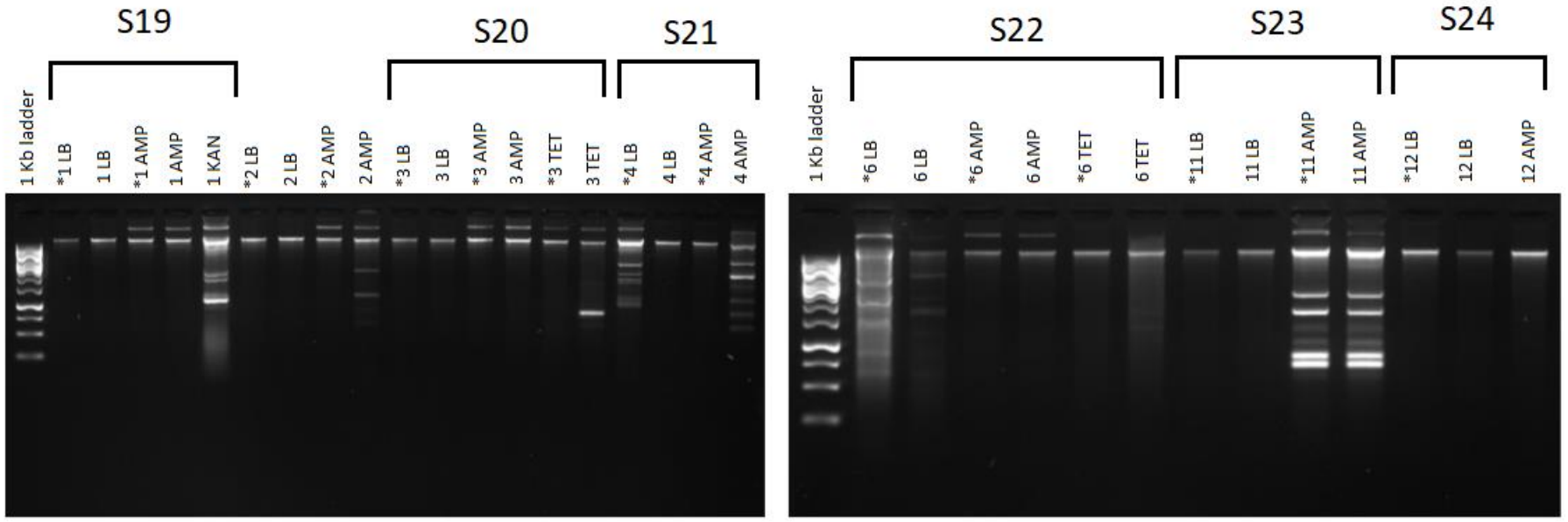

Fig S6. Plasmids from the 'Day 35 Treated' group visualised on an agarose gel. Samples are named after the bird the sample was taken from and the antibiotic which the transconjugant was selected on, e.g. 6 TET= bird 6 selected on tetracycline. Plasmid samples with an asterisk (*) were isolated from the 'rinsed' sample; the remaining plasmids were isolated from the sample after enrichment. Samples are labelled with their corresponding sequenced sample.

Chapter 7

Discussion

7 DISCUSSION

Antibiotic resistance will continue to maintain its position as one of the great threats to human and animal health unless alternatives and interventions are instigated. As it stands, antibiotic resistance is estimated to cause more deaths than cancer by 2050[59]. The use of antibiotics in agriculture has been regarded as a major contributor to the increase in resistance rates that have been observed since the commercial availability of antibiotics.

While there is great academic support towards limiting the use of antibiotics in agriculture, it is not enough to simply cease their use. While the use of antibiotics must be maintained for the therapeutic treatment of infected animals as per animal welfare guidelines, it is their use as prophylactic, metaphylactic and growth promoting agents that is targeted for reduction. Increasing numbers of regions are banning the use of antibiotics as growth promotors daily, but without antibiotics to reduce pathogen load, this may cause an increase in disease to the animals. Therefore, products which produce a similar effect to growth promotors, while also maintaining the health of the animals to reduce infection are required. Mannan-oligosaccharide (MOS) is a prebiotic which has been shown to meet these criteria. Unpublished preliminary data[60] found a reduction in antibiotic gene numbers in broilers whose diet had been supplemented with MOS. With the growing concern of antibiotic resistance transfer to humans through the food chain, a product capable of reducing resistance, while also providing additional benefits to host health, is highly desirable. We hypothesised that this reduction was due to the ability of mannan rich fraction (MRF), the next generation of MOS technology, to reduce the variety or transfer of plasmids. Plasmids

are small, self-replicating, extrachromosomal elements that are ubiquitous in bacteria. They often harbour genes that provide a benefit to the host cell, such as antibiotic resistance. Many plasmids are conjugative and have the ability to transfer to other bacteria, even those of other species. For this reason, plasmids are thought to play a key role in the dissemination of antibiotic resistance.

To test our hypothesis, the isolation of plasmids harbouring antibiotic resistance genes (ARGs) from the caecum of broiler chickens was required. The caecum contains the largest density of bacterial cells than any other section of the chicken gastrointestinal tract. Due to this, it is a highly complex environment, where plasmids only comprise a small proportion of the total DNA present. Therefore, an investigation into a method that could adequately capture the greatest range of resistance plasmids present from this complex sample was required. We examined six methods of plasmid extraction within our study. As we wished to analyse the total plasmid population, we did not want to specifically culture certain strains of bacteria that would strongly *bias* the selection of plasmids from just the cultivable bacteria present in the sample. Current plasmid extraction kits are designed to extract from pure culture and were not well tolerated to the complexity of the sample. A traditional alkaline lysis method also resulted with the same difficulties. We did not obtain any intact plasmid DNA from a kit designed for extraction from complex samples. The transposon-aided capture of plasmids (TRACA) method did permit for the attainment of resistance plasmids, however, they were highly similar to each other. Multiple Displacement Amplification (MDA) provided us with the greatest range of resistance plasmids from our sample. However, this method had numerous time-consuming difficulties, and therefore would be less than ideal for studies involving a large number of samples. We concluded that

the exogenous isolation method would allow for the most efficient and effective acquirement of resistance plasmids. Plasmids isolated by this method are also conjugative, and therefore highly likely to contribute to the spread of antibiotic resistance, and are also capable of being maintained in a human pathogen, highlighting the serious threat to human health that they may possess.

To gain a better understanding of the genetic sequences of the plasmid-mediated antibiotic resistance in the broiler caecum, further analysis into the types of plasmids present was performed. Plasmid DNA from two transformants obtained after the MDA method from a single broiler caecal sample was sequenced using MinION technology. From these transformants, five plasmids were identified. The plasmids varied in size from 151,806 bp to 42,654 bp in length. All of the plasmids carried ARGs, with four of the five plasmids being multi-drug resistant. The plasmids carried genes conferring resistance to tetracycline, aminoglycoside, macrolide, trimethoprim, chloramphenicol and beta-lactam antibiotics. One plasmid contained genes for resistance not only towards antibiotics, but also heavy metals, which function as co-selecting agents in the spread of antibiotic resistance, and quaternary ammonium compounds, which are frequently used as disinfectants. Only three of the plasmids were found to have been previously reported as originating from *Escherichia coli*. The others were reported previously as having originated from *Salmonella enterica* and *Klebsiella pneumoniae*, but were capable of being maintained in our *E. coli* host. Three of the plasmids contained genes for conjugation and may have the potential to transfer to other bacteria. Most notable from our results was the similarity of our plasmids to other plasmids identified from a variety of sources, both animal and human, worldwide. One plasmid matched to a plasmid previously isolated from a chicken meat sample in

America, while another matched to a plasmid from a chicken faecal sample from Switzerland. We also characterised a plasmid that was previously identified in a pig faecal sample in China. One of the isolated plasmids was highly similar to a plasmid found in the faeces of a healthy human from Switzerland, while another was identified in a patient with bacteraemia in South Korea. This raises the concern as to how these plasmids have disseminated worldwide. Our results reveal what is potentially only a small subset of the plasmids that are present in the caecum of a single broiler chicken from within the European Union. We demonstrate the ability of plasmids to survive through the broiler gastrointestinal tract, in chicken faeces and on meat products; while also displaying the ability of plasmids to spread and be maintained in other food-producing animals and human hosts. We highlight the need for interventions to reduce the transmission and spread of plasmid-mediated antibiotic resistance.

We assessed the mobile resistome present in the caecum of broiler chickens over time. The exogenous plasmid isolation method was employed to isolate antibiotic resistance plasmids at days 21 and 35 post-hatch. Ninety five transconjugants displaying a resistance phenotype were identified. Plasmids harbouring resistance to ampicillin, tetracycline, trimethoprim, cefotaxime and chloramphenicol were detected at both time-points. Multi-drug resistance plasmids were identified at both time-points, but a higher level of resistance and more varied resistance profile overall was evident at day 35. The effect of the microbial community present in these caecal samples was also considered using 16S rRNA gene based analysis. We observed significant differences in the most dominant taxa in the microbiome between time-points. For example, a clear shift is seen from a *Faecalibacterium* dominated microbiota at day 21 to a *Bacteroides* dominant microbiome at day 35 at genus level. In contrast to the mobile

resistome results, higher levels of variability were observed in the microbiome at day 21. The relative abundance of certain bacteria could differ greatly from bird-to-bird. *Epsilonproteobacteria* ranged from 19.97% of all classified reads in one bird to just 0.02% in another at the same time-point, for example. We determined that a more stabilised and less variable microbiota is established as the birds age. We noted this stabilisation to occur later than previous reports. We concluded that the highly variable microbiota present at day 21 is less favourable for the capture and maintenance of resistance plasmids. The stabilisation of the microbiome as the bird ages allows the better established microbial community to harbour plasmids which would give them a survival advantage, and to deal with any fitness cost associated with this. We also noted that a sample with a high abundance of *Bifidobacterium* did not harbour any multi-drug resistance plasmids. We suggest that a larger population of *Bifidobacterium* is associated with reduced antibiotic resistance in the broiler caecum. MRF increases the proliferation of *Bifidobacteria*, and therefore has the potential to assist in reducing the spread of resistance.

Continual advances are being made with molecular techniques to study complex environments. We employed a metagenomics-based approach to assess the effect of MRF as an additive to the diet of broiler chickens. This allowed for characterisation of both the microbiome and resistome of the broiler caecum at day 27 and 34 post-hatch. We identified a stable microbiome at both time-points, with *Firmicutes* and *Bacteroides* comprising the most abundant phyla while *Clostridia* and *Bacteroidia* were the most dominant classes in all samples. We did not observe any significant changes in the most dominant taxa in MRF supplemented groups compared to the control. This may be due to the lack of bacterial targets for MRF, such as those which

possess mannose-specific fimbriae upon which MRF is known to have an effect. These include the enteric pathogens *Salmonella* or *Campylobacter*, which were absent in our samples. This suggests that the effects of MRF would be more evident in pathogen-challenged chickens and may be beneficial for use in farms and production facilities dealing with such issues. A total of 171 ARGs were identified, with 69 of these present in all samples. The genes present at the highest abundance were *tetW*, *lnuC* and *aadE*, which confer resistance to tetracycline, lincosamide and aminoglycoside antibiotics. A difference was detected between the MRF supplemented and control groups at day 27. However, there were immense variabilities between samples, even those within the same group, making it difficult to definitively attribute any observed effect to MRF. We draw attention to the presence of ARGs in the caecum of broilers even without antibiotic selective pressures, highlighting the scale of the resistance crisis. While the progression in sequencing technology and bioinformatic analysis has allowed for insights into these complex environments that has been previously unachievable, this area still requires further development. A large number of reads within our samples were unclassified, with other studies having reported this too. Also, it is difficult to ascertain if the entire plasmid population is detected using these methods, as they are present in small quantities compared to the total DNA, and their assembly is difficult with short-read technology. These gaps hinder the attainment of a full understanding of the interactions within these complex environments.

To this end, we embarked on a study that would utilise both a metagenomics-based and a plasmid-based approach. We investigated the effect of MRF as a dietary additive in broiler chickens on the microbiome, resistome and mobile resistome at days 28 and 35. A group that were receiving MRF were also administered amoxicillin on days 22-

24, and were also included in the study. A highly consistent microbial community was identified across all sampled groups. We did not identify any significant changes in the most dominant taxa; or in diversity, richness or evenness at any taxonomic level. As in the previous study, *Salmonella* or *Campylobacter* were not detected, and therefore our samples may lack the specific bacterial target for MRF and may be a reason why more significant changes are not observed. *Lactobacillus* is noted as being the main species on which MOS and MRF have an effect, by increasing their abundance in the microbiome. As *Lactobacillus* was already present in our samples, the effect of MRF was not observed as it would have been in birds where *Lactobacillus* is in lower abundance or absent. Interestingly, no major changes were observed in the group that received antibiotics. We suggest that MRF may have assisted in the recovery of the microbiome by providing a substrate for selective beneficial commensal bacteria to proliferate within the microbiome. In the resistome, 164 ARGs were identified, 19 of which were present in all samples. Similar to the previous study, *tetW, lnuC* and *aadE* were the ARGs present in the highest abundance. No significant changes were seen in the abundance of genes in the core resistome, however within the accessory resistome, significant differences were observed, particularly between time-points. The difference in the abundance of ARGs as the birds age could possibly be attributed to changes in the less dominant taxa in the microbiome. Resistance genes might also be located on broad-host range plasmids, and therefore changes in the resistome may be difficult to trace back to specific changes in the microbiome.

A total of 349 plasmid-harbouring transconjugants were isolated in the plasmid-based study. The highest levels and greatest variation in resistance profiles were seen in the control groups. This was more evident at day 35, which corresponds to our previous

finding that higher and more varied levels of resistance are present as the birds increase in age. The lowest range of resistance was identified in the treated groups at both time-points. The treated group also had the lowest percentage of multi-drug resistance strains from all groups. We conclude that MRF may have a specific effect on the plasmid populations present in the caecum. We suggest that this may possibly be linked to the ability of MRF to bind to mannose-specific fimbriae. While MRF is well-characterised in its ability to bind to type-1 fimbriae, it may also be able to bind type-3 fimbriae which have been linked to an increase in frequency of conjugation. By binding to the fimbriae, MRF may therefore decrease the conjugative ability of plasmids, and thus reduce the spread of antibiotic resistance within the broiler caecum. Further plasmid based studies are required to provide a better understanding of this potential mechanism.

Without action, antibiotic resistance will continue to endanger the efficacy of antibiotics, and lead to increased mortality. The widespread use of antibiotics in agriculture has been scrutinised for contributing to the spread of resistance. Products which are capable of reducing the risk of resistance transfer are required but remain elusive. The potential of MRF in reducing resistance when added to the diets of broilers was examined. A reduction in plasmid-mediated resistance was identified in MRF treated groups. This may be due to the ability of MRF to decrease the conjugative capability of the plasmids. Future work is required to establish the mechanisms by which this occurs.

Chapter 8

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