THE EFFECT OF COMPOSTING ON ANTIBIOTIC RESISTANCE IN NON-COMMERCIAL CHICKEN LITTER

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IMPACT OF COVID-19 AND AVIAN INFLUENZA

Unfortunately, the timeframe of this study and ability to complete a certain volume of work in a given period of time was impacted by both COVID-19 regulations and an avian influenza outbreak occurring in the autumn and winter of 2020. Heightened biosecurity measures on farms as a result of the outbreak meant that it was difficult to obtain chicken litter. Alternative sources had to be located and liaised with as a result before experimental work could begin, and around five months of time for experimental work was lost. COVID-19 regulations also meant that a limited number of individuals were able to have access to the laboratory at one time (2 people), and so in order to accommodate that everyone had fair access to the laboratory, more experimental time was lost.

ABSTRACT

Extensive antibiotic resistances are found in chicken litter, which can then be transferred to soil when used as fertiliser. This, in turn, leads to elevated levels of enteric bacteria in soil and an altered soil resistome, which may pose risk a to human health through the contamination of crops. Composting of chicken litter has been shown to be a viable option to decrease levels of enteric bacteria, potential pathogens, and antibiotic resistance. This study aimed to explore the effect of composting on chicken litter originated from birds that were not raised commercially or with antibiotics using phenotype-based methods (antimicrobial susceptibility testing), as resistance in these types of chickens is often not examined, and phenotypic methods rarely utilised in this area of research. Chicken litter from a 'back-yard' system was composted outside in a 1000 L volume for 100 days. The study found increased levels of enteric bacteria and most antibiotic resistances during the 100-day composting period, which appeared to be largely attributable to the passage of time (and any other compost changes this would entail) rather than to temperature. Temperature profiles of the compost heap revealed that variable patterns were recorded, and it is hypothesised that these were associated with the lack of aeration available to the heap, leading to poor removal of enteric bacteria and antibiotic resistance.

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LIST OF ACRONYMS

AM	Antimicrobial
AMR	Antimicrobial resistance
AMC	Amoxicillin/clavulanic acid
AMP	Ampicillin
ARG	Antimicrobial resistance gene
AZM	Azithromycin
C	Chloramphenicol
CIP	Ciprofloxacin
CN	Gentamicin
CTX	Cefotaxime
ENR	Enrofloxacin
ESBL	Extended-spectrum β-lactamase
F	Nitrofurantoin
HGT	Horizontal gene transfer
LEV	Levofloxacin
MGE	Mobile genetic element
NA	Nalidixic acid
OT	Oxytetracycline
S	Streptomycin
SXT	Trimethoprim/sulfamethoxazole
TGC	Tigecycline
TRG	Tetracycline resistance gene
ZoI	Zone of inhibition

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1 INTRODUCTION AND LITERATURE REVIEW

1.1 ANTIMICROBIAL RESISTANCE (AMR) IN CHICKENS, THE ENVIRONMENT, AND ANTIBIOTIC USE IN POULTRY FARMING

It was not long after antibiotics became more widely used that they began to be implemented in agriculture, and now, a large proportion of antimicrobials are used in agriculture worldwide, and this may only continue to increase if changes are not made (You & Silbergeld, 2014; Boeckel *et al.*, 2017). Antimicrobial use in agriculture can be utilised in several ways. Firstly, animals can be treated therapeutically, that is, diseased animals receive the antimicrobial treatment. However, this may then be extended to other animals in the group, either for disease prevention or ease of administration purposes (the antimicrobial may be administered through feed or water, due to the number of animals in a group), which is known as metaphylaxis. Antimicrobials may also be given purely for disease prevention, known as prophylaxis, which as Roth *et al.* (2019) notes, is allowed in all counties with significant poultry production. This is particularly relevant in the instances of large-scale and highly intensive farming practices. Finally, antimicrobials may also be used for growth promotion for greater efficiency and, as a likely result, economic gain (McEwen & Fedorka-Cray, 2002; Nguyen *et al.*, 2017).

1.1.1 Antibiotic use in poultry farming in the UK

In the UK in 2019, the most commonly used antibiotic class for treating poultry raised for meat production was the penicillins, followed by tetracyclines. For hens used for egg production however, tetracyclines were the most commonly used antibiotic class. Other antibiotic classes such as aminoglycosides, macrolides, sulfonamides, fluoroquinolones, pleuromutilins and lincomycins were also used (Veterinary Medicines Directorate, 2020). Unfortunately, fluoroquinolones and macrolides are classified as highest priority critically important antimicrobials, and more still that were in use are classed as critically important or highly important antimicrobials (WHO, 2019). For all antimicrobials in meat poultry, usage has decreased compared to 2014, and colistin (another highest priority critically important antimicrobial) was removed from use completely after 2016. Despite this, total antibiotic use in chickens raised for meat has increased since 2017. For egg producers, several antibiotics have increased in use since 2016, and the total antibiotic usage has also increased slightly since this time, but both colistin and fluoroquinolones have seen a cessation in use (Veterinary Medicines Directorate, 2020). It is very important that antimicrobial usage in poultry continues to decrease, and critically important antimicrobials are ideally phased out of use; the reasons why are demonstrated in the numerous studies evaluating the antimicrobial resistance levels in bacteria isolated from poultry and their impacts that follow. This is true not only for the UK, but for all countries applying antimicrobials to agriculture, particularly in those using them for growth promotion.

1.1.2 Antimicrobial resistance in chickens

There is a myriad of papers available, from multiple countries, that detail the antimicrobial resistances of bacteria isolated from poultry and/or their products, particularly in regards to clinically-relevant bacteria present in chickens. Many of these papers also demonstrate that antimicrobial use may aid in and increase the development of antimicrobial resistant bacteria.
Focusing on *E. coli*, Diarrassouba *et al.* (2007) found that in faecal, caecal and litter samples from chickens from numerous farms in a certain area of Canada, more than 20% of isolates were resistant to 12 antibiotics. The highest levels of resistance seen were to amoxicillin, tetracyclines, and streptomycin. 51.4% of isolates were also resistant to ceftiofur, a 3rd-generation cephalosporin (which are also highest priority critically important antimicrobials, albeit ceftiofur is only used in animals; WHO, 2019). This may not be unexpected however, given that another paper notes how ceftiofur is injected into eggs in Canada (Dutil *et al.*, 2010). A further Canadian study (Diarra *et al.*, 2007) also found quite high tetracycline and amoxicillin resistance levels (68.5% and 61.4% of isolates, respectively). The percentage of isolates resistant to ceftiofur was also similar to the previous study's findings (51.3%). Tetracycline-resistant isolates that were further characterised had extremely high resistance levels to several beta-lactam antibiotics, including ceftiofur (80.8%), as well as sulfonamides and aminoglycosides (particularly streptomycin). In another paper from North America (Arkansas, USA), Khan *et al.* (2005) isolated fluoroquinolone-resistant *E. coli* from poultry litter and found them to have resistances to many antibiotics other than fluoroquinolones. One hundred percent of isolates were resistant to erythromycin (which is not largely unexpected as macrolides are a common choice in veterinary medicine (Gibreel *et al.*, 2005)). Similar to the previous studies, there was also high levels of tetracycline resistance (89% of isolates). All isolates were resistant to antibiotics belonging to multiple different classes.

One paper that demonstrates the effects of rearing practices on AMR levels in *E. coli* from poultry is by Hussain *et al.* (2017), wherein the AMR profiles of isolates from the meat and caeca of commercial broiler chickens and free-range chickens were compared. There was almost total resistance amongst the isolates from both groups of meat and commercial caeca samples to tetracycline (95% overall), with only caeca isolates from free-range chickens being significantly different at 54%. After this, however, the significant differences in the resistance levels of isolates from samples of the two groups are seen for many of the antibiotics tested (although not all). For example, against ciprofloxacin, 96% of commercial broiler meat was resistant compared to just 15% for free-range, and for caecal samples it was 73% and 52%, respectively. Furthermore, a higher percentage of isolates from both types of broiler chicken samples possessed extended-spectrum β-lactamase (ESBL) genes in comparison to those of free-range.

A study investigating antimicrobial usage is the one conducted by Ozaki *et al.* (2011), wherein some farms administered antimicrobials (AMs) to the broilers whilst others did not. Whilst it may not have been what was expected, chickens from AM-free farms could harbour *E. coli* isolates resistant to antibiotics at much higher numbers than those from AM-using farms, as is seen with cefazolin and ceftiofur. However, a clear link between the administration of antibiotics in feed and resulting resistance in *E. coli* isolates from the chickens was seen with one particular AM-using farm; this farm administered oxytetracycline (OTC) in feed, and consequently there were consistently more OTC-resistant isolates, even in comparison to the other AM-using farm (as it did not use OTC in feed).

A study by Nguyen *et al.* (2016) also documents the antimicrobials administered in feed, therapeutically and prophylactically. They found that antimicrobials were used much more often for prophylaxis rather than treatment, and involved critically-important antimicrobials such as colistin, macrolides, fluoroquinolones and aminoglycosides, which were always administered through water. *E. coli* isolates from cloacal samples in this study showed extremely high resistance rates to ampicillin, and high rates to ciprofloxacin also, alongside considerable resistance to gentamicin and colistin. Forty-three percent of isolates were classed as multiple drug resistant. The study could not conclude an association between antimicrobial usage and levels of AMR; however, it did not include any AMR-free farms in the study for comparison.

From European studies (Germany and the Netherlands) that focus especially on ESBL-producing *E. coli*, it can be concluded that chicken carcasses and meat products were greatly contaminated with this type of *E. coli* (Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Reich et al., 2013), and thus be resistant to many forms of penicillins and cephalosporins (Scientific Advisory Group on Antimicrobials of the Committee for Medicinal Products for Veterinary Use, 2009). Across the three studies, 88.6%, 94.0%, and 76.8% of carcasses/meat contained this *E. coli*. Given that one of these studies (Reich et al., 2013) found ESBL-producing *E. coli* from 72.5% of caeca samples, it is likely that the meat becomes contaminated from faeces/gut contents, and therefore that the chickens carry resistant *E. coli*, which the study references with the addition that contamination occurs at slaughter. The study also found AmpC-producers in just over half of the carcasses assessed.

Price *et al.* (2005) investigated *Campylobacter* and fluoroquinolone resistance using samples of chicken meat (which were likely contaminated with *Campylobacter* during slaughter). A much stronger and defined correlation is presented in this study than the aforementioned studies; there were significant differences between the antibiotic-free brands' isolates and the 'conventional' brands' isolates in terms of their carriage of fluoroquinolone resistance, with the antibiotic-free brands having much lower rates. Interestingly, the two ‘conventional’ brands had ceased use of fluoroquinolones for around a year before the study, highlighting the important role a flock’s environment has to play in the persistence of resistance even when antibiotic usage has ended.

The role that metals have to play when administered to chickens either within or alongside feed for uses as antimicrobials or growth promoters also requires consideration. This is due to the existence of cross-resistance and co-resistance. In the case of the former, a resistance mechanism to both an antibiotic and metal is shared, and for the latter an antibiotic resistance gene is located alongside a metal resistance gene on a mobile genetic element (Baker-Austin *et al.*, Seiler & Berendonk, 2012; Gillieatt & Coleman, 2024). Both lead to the possibility for co-selection; an antibiotic resistance mechanism/gene is maintained even in the absence of the selective pressure of the respective antibiotic if the metal involved is present. This has been demonstrated experimentally and analytically through several means. In the study by Zhao *et al.* (2019), the presence of several antimicrobial resistance genes was assessed in metal-contaminated soils; a multitude of significant positive correlations were seen, with no negative correlations found, indicating the potential for metals to enrich antibiotic resistance. Evidence of plasmids conferring resistance to both antibiotics and metals is seen in the study conducted by Ghosh *et al.* (2000), where it appeared that a single plasmid provided resistance to ampicillin as well as several heavy metals following plasmid curing and transformation experiments. The broad and wide-spreading nature of metal and antibiotic co-resistance is demonstrated by Pal *et al.* (2015), where a large-scale assessment of bacterial genomes showed the co-occurrence of antibiotic resistance genes and metal resistance genes, both chromosomally and on plasmids. As such, the detrimental impact on antibiotic resistance that the usage of metals as antimicrobial agents and growth promoters can be clearly seen and understood.

1.1.3 Potential transfer of antimicrobial resistance from poultry to humans

From the previous papers it is clear to see that commensal, and potentially pathogenic, bacteria of chickens can harbour high resistance levels to several antibiotic classes, as well as the effects antibiotic administration may have on these rates. However, the impact that these resistant bacteria can have in the other sectors of life – that is, putting it into a One Health perspective – must also be considered in order to fully understand the dangers of the extensive antimicrobial resistance existing in agriculture. In particular, there is a large concern on the danger it poses to human health. There are different routes through which AMR bacteria can reach humans, as outlined by Economou & Gousia (2015). Individuals which work within agriculture are understandably exposed to such bacteria and as such may suffer infections, as implied by Gong *et al.*’s (2016) study, but consumers are also at risk due to transmission via poultry products, as has already been implied with the previous studies on

AMR bacteria isolated from poultry meat. Furthermore, there are different ways these bacteria may pose risks to humans; as foodborne pathogens they can cause infections that may be difficult to treat due to antibiotic resistance, but less obviously they may colonise humans and potentially spread resistance genes to other pathogenic bacteria, as mentioned by van den Bogaard & Stobberingh (2000).

A paper which demonstrates the relationship between antibiotic use and resulting resistance in chickens and humans, indicating transmission, exceptionally well and clearly is that by Dutil *et al.* (2010). By monitoring the levels of ceftiofur resistance over time in chicken meat and clinical human *Salmonella enterica* serovar. Heidelberg isolates, they were able to show a correlation between the usage of ceftiofur in the poultry industry and ceftiofur resistance rates in chicken and human isolates. The correlation is made especially clear thanks to the withdrawal of ceftiofur use in poultry during a certain period of time during the study, followed by its partial return. From the results it can be seen how rates of resistance in both chicken and human *Salmonella* Heidelberg isolates drastically decrease following the cessation of ceftiofur use. In Quebec, the trend was clearly defined, with the partial return of ceftiofur use also accompanying a slow increase in resistance of these two groups of isolates.

1.1.4 Antimicrobial resistance in soil

Before considering the impacts of chicken manure on the soil antibiotic resistome and the part that manuring with chicken litter has to play in altering environmental AMR, it has to be understood that microorganisms in the soil itself are in possession of a multitude of antibiotic resistances. This is not surprising given that several antibiotics originated from environmental microorganisms, and so these antibiotic-producing microbes would need to be in possession of respective resistance genes, as well as other environmental bacteria developing resistances in defence. Antimicrobial resistance genes (ARGs) may also serve in roles outside of antibiotic resistance in the environment. As such, there does not need to be any interference for ARGs to exist in the environment, and it must be considered that there will always be basal levels in addition to that which is introduced through manure. ARGs can also be selected for due to the presence of heavy metals through co-selection, and so faecal contamination is not the only element that can alter environmental resistance levels (Alonso *et al.*, 2001; Berkner *et al.*, 2014).

An excellent study for illustrating this was conducted by D’Costa *et al.* (2006). Twenty-one antibiotics of varying origins (e.g., natural or synthetic) and bacterial targets were used to test the resistances of 480 strains of environmental bacteria isolated from different soils. All isolates were resistant to at least one antibiotic, and 99.6% and 99.8% to a further two. Almost 25% of isolates were resistant to 7 antibiotics, and over 20% resistant to eight. Two isolates were resistant to 15 of the antibiotics tested, and the only antibiotic to which no resistant isolates were seen was streptomycin.

This study therefore demonstrates the extensive resistances and resistance profiles that can exist within environmental bacteria, but as the study itself states, their results are a potential understatement, as they only isolated spore-formers.

1.2 FERTILISATION USING CHICKEN MANURE AND THE EFFECT ON SOIL AMR, ITS PERSISTENCE, AND HOW BACTERIAL COMPOSITIONS MAY CHANGE

As has been described from the aforementioned studies, chicken litter, caecal, and cloacal samples can contain antibiotic resistance bacteria capable of resistance to large amounts and wide varieties of antibiotics, with some of these bacteria being pathogenic. As such, the application of chicken litter as a fertiliser to soils poses a risk to the environment by the introduction and spread of antibiotic resistance and potential pathogens, beyond the normal levels. Beyond soils, it can also be hazardous to the environment from spread of these bacteria to water, due to runoff (Chen & Jiang, 2014). There are a wealth of papers dealing with the effects of chicken litter application on soil, both simulated and in the environment, including how the levels, compositions and resistance rates of bacteria change, how numbers and levels of ARGs are altered, and how long these changes remain after application.

1.2.1 The effect of chicken manure as fertiliser on soil AMR

Studies have used both culture-based and molecular methods to assess how antimicrobial resistance is affected through litter application. Zhao *et al.* (2017) uses both methods to determine the difference in AMR between soil samples from multiple farmland sites which had undergone long-term application of chicken manure, except for one site which received “fertiliser” instead, regarding resistance to two antibiotics. It could be seen that bacteria isolated from the fertiliser-only site had significantly lower resistance rates to oxytetracycline compared to the other, chicken manure-receiving sites. To illustrate, when tested against 10μg ml^{-1} oxytetracycline, the resistance rates for this fertiliser-only site were under 10%, compared to the second site where resistance rates were around 70%. For sulfamethazine, significantly lower resistance rates compared to the other sites were seen also, except at the highest antibiotic concentration where it was not significant against one other site only. The fertiliser-only site consistently had lower relative abundances of resistance genes (except potentially for *tetO*), although this was not always significantly so. This paper alone strongly suggests how poultry manure can introduce and increase the levels of antibiotic resistant bacteria in soils in comparison to other fertilisers, and offers unique and beneficial data in doing so in a completely ‘natural’ and organic setting (that is, analysing existing farmland soil used for crops), although this comes with the issue of not knowing for certain how much manure was applied, the source, and other such variables.

A study which instead used soil microcosms (using agricultural soil that had not had manure applied to it) and only utilises molecular methods is that by Han *et al.* (2018). It also looked at other manures in addition to poultry (swine and cattle), in comparison to a control soil. All soils treated with any manure had significantly higher numbers of ARGs in comparison to untreated soil. Furthermore, 86 ARGs were found in poultry manure-treated soils that were not present in the untreated soil, demonstrating how the addition of poultry manure also came with the addition of new resistance genes. For abundances, both relative and absolute were higher in manure-treated than untreated soils. The paper also looked at numbers and abundances of ARGs in the raw manure itself, and poultry manure possessed both the highest number of ARGs and a significantly higher relative abundance; as such, the importance of decreasing AMR in poultry manure/litter prior to application to soil may be particularly important.

Also looking at numbers and the abundance of ARGs as well as including another manure besides poultry in the study (cattle) is that by Zhang *et al.* (2019). For both parameters, soil with poultry manure was significantly higher compared to both cattle manure fertilised- and untreated soils. For absolute abundance, there was around a fourfold increase compared to the control soil, and in particular sulfonamide resistance genes were much more abundant in poultry manure-amended soil. An earlier study by these authors includes swine manure also (Zhang *et al.*, 2017). As before, the number of ARGs in the poultry-manure treated soils were higher than both cattle manure-treated and untreated soils, significantly so with the latter, however not with swine-manure treated. For absolute and relative abundances of ARGs, poultry-manure treated soils exhibited significantly higher levels than all other soil types. This is despite the fact that raw swine manure contained much higher abundances of ARGs than poultry, signifying again that poultry manure can be an especially hazardous manure option if not previously treated. As with Han *et al.* (2018), ARGs were found in poultry manure-treated soil that were not present in untreated (36 ARGs).

These studies highlight how, regardless of methodology, addition of poultry manure to soil can greatly increase the numbers and abundances of resistance genes and bacterial resistance rates. Furthermore, higher numbers of ARGs and abundances could sometimes be seen with poultry manure in comparison to other animal manures.

1.2.2 Potential risk to human health.

Beyond the altered soil resistome and potential for antimicrobial resistance to spread to water via aforementioned runoff, if crops for human consumption are grown in these manured soils there can also be a risk posed to human health through this route, showing how use of poultry manure as fertiliser is a risk in terms of One Health as it is not only hazardous to environments but also human health. Returning to the study by Zhang *et al.* (2019), they examined how the numbers and absolute abundances of ARGs changed in multiple locations on lettuce plants following application. For both number and abundance of ARGs, poultry manure application resulted in a significant increase amongst the root endophyte compared to the control. The same can be said for the absolute abundance of ARGs in the phyllosphere, but little difference was seen for the leaf endophyte. This paper therefore gives an indication that manuring with untreated poultry litter can indeed alter the levels of ARGs, and by probable extension resistant bacteria, present in certain areas of crops, and so may remain following harvest and lead to human consumers being exposed.

1.2.3 The effect of chicken manure as fertiliser on soil bacterial composition.

As stated previously, papers explore more than just how litter application affects AMR levels, as other factors such as persistence of AMR following application and bacterial composition changes are also important to consider. For the latter, there are studies that suggest that the composition of a bacterial community (such as the soil community) has a role in sculpting AMR profiles. As such, it is useful to examine the changes to a bacterial community following litter application as, according to the implications of studies, it can impact the nature of AMR present. Before examining the effects, papers demonstrating the potential link between bacterial composition and AMR should be reviewed, in order to illustrate the importance of the bacterial community for AMR. All of these papers are unfortunately not regarding chicken manure/litter, but rather swine manure, wastewaters, and water supplies, however it is not necessarily required to be specific to see the links between microbial communities and AMR.

In the study by Qin *et al.* (2021) swine manure, compost, compost-amended soil and control soil were assessed for links between the bacterial composition and ARGs. When considering all samples, there were significant correlations between resistome richness and richness of bacterial Operational Taxonomic Units (OTUs). When samples were considered individually, α-diversity correlations were not significant, but β-diversity correlations were, except for the control soils. Certain bacterial genera were significantly positively correlated with particular ARGs in manure, compost, compost-amended soils and control soils, but the genera-ARG correlations in manure and compost were separate from those in the two different soils. Furthermore, bacterial taxonomic composition explained 36% of the resistome, which was higher than all the other factors considered. Considering samples individually again, bacterial taxonomic composition was still the highest for explaining the resistome in nearly all non-control samples, whereas with the control soil it was negligible (0.03%). Just from this paper, it can already be seen how the bacterial composition of a sample can have strong links to certain ARGs, and therefore the possibility of it affecting the resistome.

Another study that dealt with swine manure application to soil was that by Leclercq *et al.* (2016). The study found that within the manure-amended soil, two of the tetracycline resistance genes that were able to persist throughout the experiment were correlated to two certain OTUs that also persisted throughout the experiment's timeframe, thus indicating that if particular bacteria entering the soil microbial community from the addition of manure are able to survive sufficiently, they also bring with them specific ARGs. Continuing with this idea of certain taxa introducing specific ARGs to the community, Huerta *et al.* (2013) found that the percentage of *Actinobacteria* and *Firmicutes* significantly correlated with the ARGs *ermB* and *sulI*, respectively.

Tao *et al.* (2016) discovered that in their samples, seven genera were significantly correlated with the ARGs that the study assessed. Looking into it more deeply, they found that particular genera of this group were correlated significantly to certain resistance gene types (the types being the antibiotic they confer resistance to). Zhou *et al.* (2017) state that in their study the profiles of the ARGs were significantly correlated to bacterial composition and structure, and were also able to determine specific genera (14) that correlated with "persistent ARG distribution", as well as determining the particular ARGs to which there were correlations as with the previous study. Guan *et al.* (2018) showed also that the bacterial community, at both phylum and class level, were significantly related to certain ARGs, including some of those that were most common in the samples.

Whilst these papers deal with correlations (to specific taxa, diversity, and composition) and explanatory analyses to determine effects of the microbial community to the resistome, which cannot be taken as absolute and only implies an effect, the fact that several studies have found such potential links places the soil microbial community composition as a strong likely contributor to the compositions of resistomes. Knowing this, it is worthwhile observing the impacts that chicken litter application can have on microbial composition in soil, even in studies where the composition-resistome link is not considered or explored, as it is now understood that this impact can indeed affect the results that are seen regarding antimicrobial resistance. It also means that focus must not be reserved for only potential pathogens in studies, as a variety of bacteria may be involved in antimicrobial resistance.

The papers by Zhang *et al.* (2019), Han *et al.* (2018) and Wang *et al.* (2018) all detail how chicken manure/litter application to soil led to changes in the microbial community, with the first two examining the role of the community in the antimicrobial resistance observed also. Zhang *et al.* (2019) found variations in the microbial communities of lettuce plants depending on the location of the plant that was sampled. Nevertheless, for some of these locations, differences between the control and poultry manure-treated samples could still be seen. For all sample locations there was at least a slight increase in the relative abundance of Proteobacteria due to the addition of poultry manure, being most pronounced in the phyllosphere, and at least slight decreases in Actinobacteria could be seen across most locations also. There was a slight increase in Acidobacteria in the root endophyte with poultry manure-treated, but a decrease in the phyllosphere, compared to control. Proteobacteria and Acidobacteria are mentioned specifically here as the author goes on to describe how these phyla were correlated with certain ARGs and explained some ARG variance, and therefore any poultry manure-induced changes to such phyla may go on to affect the resistome. Overall, the ARG profiles were significantly correlated with bacterial community, and the combination of four phyla in addition to mobile genetic elements (MGEs) explained 58% of the ARG variance according to their analysis, demonstrating the heavy impact of the bacterial community on the ARG profile.

Due to manure application in the study by Han *et al.* (2018), the absolute abundance of bacteria increased, and alongside this α-diversity decreased (which was determined by several indexes/measurements). The increase of absolute abundance was drastically so with the "high poultry manure" (HPM) group, and this group also experienced very little increase in α-diversity during the period of 120 days following the manure application compared to the other manure groups, whose α-diversities were much higher after 120 days than they were at the start. Before the microbial community composition (in terms of taxa) is even considered for this paper, then, it can already be seen how alteration of the diversity of the microbial community can occur due to manure application, particularly with HPM where this effect may be more persistent than with other manure types. This itself may have implications for antimicrobial resistance, as demonstrated in the paper by Chen *et al.* (2019), wherein the author describes that low microbial diversity may enable AMR to remain in soil more readily. Regression models generated from their study showed that as both OTU richness and diversity decreased, the abundance of ARGs increased. Returning to Han *et al.* (2018), then, the lower diversity maintained with the HPM treatment group may have had a role in the ARG numbers and abundances seen. On the other hand, the sustaining of the low diversity may not be the most important aspect for this, as the "high cattle manure" (HCM) treatment group possessed the highest relative and absolute abundances of ARGs despite the considerable increase in α-diversity as the experiment progressed compared to what it was initially. Instead, HCM began the experiment (day 0) with the lowest α-diversity of all the groups, exceptionally so, and as such this extremely low beginning α-diversity may be more important in respect to the potential AMR impact. Looking at the bacterial composition and specific taxa that may be linked to ARGs, the paper identified that genera belonging Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were positively and significantly correlated with ARGs. All of these phyla, with the exception of Actinobacteria, were of a higher percentage of the bacterial community in poultry manure-treated soils than in the control soil. As such, the increase of these taxa in the bacterial community due to manure addition, as they are positively correlated to ARGs, indicates again how manure can alter AMR in soil potentially through this modification of the bacterial community. Data from this study also enables the changes in absolute abundance of genera potentially carrying ARGs over time to be seen. As such, it is possible to see how long-lasting the impact to the soil microbiome may be, and thus how ARGs may also persist if these bacterial genera do indeed carry ARGs. Looking at HPM, whilst abundances of genera likely to be introduced by poultry manure (given that they are found in the poultry manure sample but not, or at far lower abundances in, the control soil) did degrade over the course of the course of the experiment, many genera still remained at higher abundances at the end of the experiment compared to the control soil. This indicates that microbiota changes induced by poultry manure application can remain for at least 120 days (the length of the experiment), although abundances did tend to decrease for some genera noticeably. Interestingly, environmental bacteria that were not present in the poultry manure, but were in the control soil (and therefore likely to be members of the unaffected soil microbiota), were found in much higher abundances in HPM than even in the control soil itself. Some of these genera also did not drastically decrease in abundance over time, and seem to have persisted more successfully than the genera likely to be introduced from poultry manure. This enrichment of potential ARG-carrying genera of the natural soil microbiota shows that even if poultry manure-introduced bacteria are unable to persist long-term, poultry manure can have surprising effects on pre-existing members of the soil microbiota and still alter bacterial composition and therefore potentially the resistome also. Given that little abundance decrease was seen for many of these enriched original soil members, it also implies that these effects could have the potential to last long-term, especially as these genera are native to the soil regardless. Finally, this paper also did an analysis to determine the impact the bacterial community may have exerted on the resistome, and found that it explained 27.6% of ARG profile variations seen, the highest of the factors that were considered (53.7% was "unexplained").

Unfortunately, the paper by Wang *et al.* (2018) does not make any correlations between bacterial taxa or community composition and ARGs or ARG profiles. Nevertheless, with the previous knowledge that bacterial community composition may have a considerable impact on the ARG profiles seen it is still useful to consider the bacterial community changes, and pay consideration to taxa that have been found to be correlated to ARGs in other studies. Before examining composition changes, Wang *et al.* (2018) offers information on diversity and richness of the communities of control soil and soil with poultry manure. Again, soil with poultry manure had a greatly and significantly lower diversity than control soil, which itself may lend to increasing AMR as explained before with Chen *et al.* (2019). Over the 90 days of the experiment, all richness and diversity indexes for the soil with poultry manure increased, indicating the return of some richness/diversity, but still did not reach the levels of the control soil even by the end of the time period. As such, decreases to richness and diversity among the soil bacterial community from poultry manure addition, and the possible association increase in AMR, takes time to alleviate. Due to the fact that this study does not make any correlations of their own for the relationships between bacterial taxa and ARGs, phyla that have been identified in the other two papers by Zhang *et al.* (2019) and Han *et al.* (2018) will be especially examined, although it must be recognised that this may not be completely accurate for Wang *et al.* (2018)'s study as the ARGs found in the three studies may vary. For Proteobacteria, the relative abundance was significantly higher than control during the middle time points of the experiment (days 10 and 30), but decreased to having no significant difference by the end. Acidobacteria and Firmicutes were found to be significantly lower than control at the ending 90 days. In contrast, Chloroflexi, Actinobacteria, and Bacteroidetes were all significantly higher than the control at 90 days, with Bacteroidetes being consistently so throughout the experiment. Outside of implications for AMR, these results show how the changes to the bacterial community can be quite different between studies, despite all involving poultry manure addition (although all studies used different soil types, and Wang *et al.* (2018) had a different manuring rate to the other two studies). The impacts of poultry application to the soil microbiota can therefore not be generalised, and must be considered for each individual incidence.

1.2.4 The persistence of AMR in soil after the application of chicken manure.

The longer that antimicrobial resistant bacteria remain in the environment, the more opportunities there are for ARGs to be spread through horizontal gene transfer (HGT), for bacteria to contaminate crops and enter waters through runoff, and for exposure to occur to workers and wildlife (which could potentially lead to further spread). It is particularly beneficial, then, when papers also examine the longevity of the effects they record, so it can be gauged how harmful these effects may be towards environmental, and by extension, One Health.

Beginning with papers that look at other manure sources alongside poultry, the incubation period in the study by Han *et al.* (2018) was 120 days, a considerable amount of time and one which enables ARG changes to be easily seen. Examining the data more generally through number of and absolute abundances of ARGs, the HPM treatment group began the incubation with the highest number of ARGs, and second highest abundance in comparison to the other manure treatment types. In regards to the control, HPM had approximately more than double the number of ARGs, and ten times the absolute abundance. Both measurements experienced a decrease during the incubation, absolute abundance more drastically so than number. Despite this decrease, the HPM group still possessed around 90 ARGs, now approximately triple that of the control. The HPM treatment sustaining such a higher number of and more diverse ARGs in comparison to the control, even after 120 days, demonstrates the harm poultry litter can pose to environmental health, potentially more than just short-term. In comparison to the impressive persistence of number of ARGs, absolute abundance underwent a drastic decrease, however even after this the abundance still had not returned to the control levels with HPM. A heat map enables more specific data to be seen (with ARGs grouped by their corresponding antibiotic class). From this, it can overall be observed that HPM was in possession of a more diverse and abundant collection of ARGs compared to the control throughout the experiment. Furthermore, ARGs that were likely introduced from poultry manure (as they were not found in control soil, but in high relative abundances in the manure) were able to persist over the 120 days, meaning that either antimicrobial resistant bacteria carrying these genes were able to survive adequately, or that these ARGs had succeeded in being transferred horizontally. A smaller number of ARGs included in the heat map are present at the lowest relative abundance measurement in poultry manure whilst being present in much higher numbers in the control soil at the start of the incubation, indicating that these ARGs may be those that are native to the soil resistome. For some of these genes in the control soil, they decreased to the lowest measurement by the end of the incubation, whilst in the HPM group they were higher, despite the poultry manure not containing a large relative abundance of these genes. Therefore, some aspect of the poultry manure may have enabled these originally highly-present genes to be enriched and persist for a longer amount of time than they may have normally. As a result, it can be concluded that poultry litter application can lead to the persistence of poultry-derived ARGs in soil for the period of at least 120 days, and possibly extend the persistence of soil-derived ARGs also.

The paper by Zhang *et al.* (2017) follows very similar format to Han *et al.* (2018), wherein changes in numbers and relative abundances of ARGs are given over the period of the experiment, and more specific data are provided through a heatmap. For this study, the incubation period is even longer, at 130 days, and treatments included the addition of tylosin. For the average numbers of ARGs, both poultry manure treatments (with and without tylosin) remained significantly higher than the control soil by the end of the incubation, despite the loss of approximately 42 ARGs from the beginning of the incubation. Tylosin did not seem to have any effect on the numbers of ARGs whatsoever throughout the experiment, with the results presenting more or less the same as the treatment without it. The same cannot be said for the relative abundances, where the poultry manure with tylosin treatment reached the highest relative abundance seen out of all the treatments (including cattle and swine manures). Whilst all of the swine and cattle manure treatments almost reached the relative abundance levels of the control soil by the end of the incubation, poultry manure without tylosin still possessed a higher relative abundance (even to the swine and cattle manure with tylosin), and poultry manure with tylosin was of the highest abundance. This demonstrates that out of all the manure treatments, poultry poses the most hazard in terms of ARG levels, and if soils happen to contain antibiotics due to previous manure exposure, this hazard may be increased even more, as shown with the tylosin treatment. Observing the heatmap, the fates of all the ARGs assessed can be seen (although sadly not all are labelled). Some, such as the *qacH, rarD* and *tolC* genes, were very short-lived in the incubation, decreasing in relative abundance after only a week. Some which decreased in abundance after a period of time increased again at the end of the incubation, such as *vanRA-02* and *vanRB*; however, this effect was only seen in the treatment that had received tylosin, indicating how pre-existing antibiotics in the environment may be important in persistence of certain ARGs that may decline otherwise. The effects of tylosin for persistence can also be seen with *oprD*, where the no-antibiotic treatment had a decrease in abundance of this gene by the end of the incubation (even lower than the control soil, in fact), but with the antibiotic-including treatment the higher abundance remained. Many ARGs remained at a relatively very high abundance throughout the incubation regardless of antibiotic treatment, including those that were already present in soil but became highly enriched (such as the *aadA* genes) due to manure addition, and those which were likely not part of the original soil resistome and introduced through manure (due to the low relative abundance seen in control soil), many of which were multi-drug ARGs. Interestingly, some of the genes that were part of the natural soil resistome (those conferring multi-drug, sulfonamide, and tetracycline resistance, for example) that were lowly abundant in poultry manure itself, were found in very low abundances in the poultry manure treatments, potentially indicating the death of the original soil members that carried these genes upon the introduction of manure.

The study by Wang *et al.* (2018) deals only with poultry manure and a much smaller collection of identified ARGs, as well as having a shorter incubation period of 90 days. Despite this, it can still be seen after a 90-day incubation which genes were beginning to return to control soil levels, and which continued to persist. In addition, this paper calculated dissipation rates for the ARGs, offering an extra perspective on the persistence of these genes. Totalling all ARGs (and one integrase gene) together, the relative abundance of the poultry manure group begins the incubation experiment exceptionally higher than the control soil, followed by it decreasing considerably at each time point. Nevertheless, at 90 days the gene total relative abundance for the poultry manure group is around 30 times that of the control soil. Therefore, some ARGs from poultry manure can persist and remain at higher relative abundances in the soil for at least 90 days, and given that the dissipation rates of the ARGs only decreased as the incubation continued, they are likely to persist for quite some time longer. Examining the individual ARGs reveals which of the genes began to reach the control soil levels by 90 days, and which were persisting more successfully. Genes such as *fexA* and *tetB* appeared to be reaching the control abundances by the end of the incubation, although these genes did not receive as much of an increase in abundance at the start of the experiment in comparison to others. Genes such as *ermF, cfr* and *tetM* did not reach background levels by 90 days, but experienced a stronger and more rapid decrease in abundance during the incubation, with the former two genes having some of the highest dissipation rates. Other genes, such as *sul1* (which was aided by one of the slowest dissipation rates) began the experiment with one of the highest abundances and despite some dissipation, persisted fairly well in comparison to the other genes. Even with this paper not including many ARGs for review or a longer incubation time, it can be seen how different ARGs can have rather drastically different dissipation rates, and as such some are more suited and more of a risk for persistence than others. It also identifies several reasons for this, most of them relating to co-occurrence, locations on plasmids, and a broad range of recipients for the gene.

The final paper for this focus on persistence, by Cook *et al.* (2014), differs from those previous in that it monitors the persistence of a faecal indicator, enterococci. This study also possesses the longest monitoring period of 148 days, and is conducted as a field experiment as opposed to a soil microcosm like the other three papers. This provides the benefit of being able to observe how ARGs and poultry litter-introduced bacteria would behave and persist in the environment more authentically and accurately (although disappointingly the study does not look at many ARGs or bacterial composition as other studies have). The main comparison evaluated in the study was drawn between a conventional till (CT) and no till (NT) regimen of the soil, allowing it to be seen how an aspect of farming practice may alter the outcomes of poultry litter application and its persistence in soil. Unfortunately, and strangely, control data is not provided in the graphs, although a measurement prior to poultry litter application is at least given. Before examining the results seen with the chosen faecal indicator, it is important to remember that this of course does not represent the persistence of other bacteria from poultry litter. The other studies have already provided ample information on how variable the fates of such bacteria can be, with some persisting well whilst others quickly decline. Nevertheless, the data shows how tilling practices of poultry manure-fertilised soils may change persistence, and also implies that other variable features of farming could impact it also. Looking specifically at culturable enterococci, for both groups in both years original levels were radically lower that what was seen following poultry litter addition. After this event all groups received a large spike in culturable enterococci, followed by the expected decline. In the second year of the study, the culturable enterococci levels in the CT soil appear to have reached the original levels again by the 148 days, with the NT soil almost reaching this level (however, this year much less litter was used on the soil). In the first year, which received more litter, it can be seen that by the 148 days, culturable enterococci in the NT soil were considerably higher than the original soil levels, and the same can be said for CT soil, although the levels were somewhat lower compared to NT. It can be deduced, then, that the tilling practice in farm soil can at least affect the persistence of enterococci from poultry litter, and potentially other bacteria. Even with the practice that seemed to decrease the final levels of bacteria (CT), after 148 days in year one enterococci still lingered in the soil, to the extent that they were culturable. For ARGs, data for three genes is provided. Beginning with *strB*, there was a large increase in copies following litter application in year one (the copies in year 2 did not experience such a spike, perhaps due to the lower amount of litter). Copy levels then appeared to fluctuate but generally decrease, for both CT and NT. By 148 days, year one CT copies were not far higher than the beginning levels, with those for year two ending lower than year one. For NT, both the year one and year two possessed more copies at the end of the experiment compared to their CT counterparts, indicating that this practice may facilitate increased persistence of certain ARGs such as *strB* alongside enterococci. This consequently also meant there was a larger difference than for CT between the ending copy numbers and the beginning, original numbers. Interestingly, despite the lower litter application in year 2, the year 2 NT sample at day 148 was higher than year 1, albeit not considerably so. The same pattern of a sharp, large increase then decline is seen for *sul1* also. This time however, year 2 finishing with more copies than year 1 was seen with CT rather than NT. CT copies at 148 days had not reached original levels for this ARG also, although once again this was more pronounced with NT samples. This was especially seen with the year 1 sample, where copies for NT were much higher than for CT, and NT copies being far from reaching its beginning levels. The final gene investigated, *tetW*, was found at lower copy numbers prior to application than the other two genes, being below detection limits in CT soil. As before the large increase was achieved before decreasing and generally levelling off. For year 1 CT soil, the copy numbers returned to below detection limits just after 80 days of the experiment, whereas surprisingly year 2 remained much higher than original levels until the end of the experiment. It even remained at higher levels than its NT counterpart, contrary to what would be expected considering the results for the other two genes. Nevertheless, for NT year 1, copies of the gene were detected well above original levels until the end of the experiment, as opposed to the disappearance in detection for CT.

Persistence of both poultry litter-associated bacteria and ARGs can be easily seen from these papers, lasting up to at least 148 days. To truly understand just how long-term the changes to bacterial composition and the resistome may be due to litter application, studies would need to be conducted for a much longer period of time. Nevertheless, the longer the period of time these ARGs linger in the soil, the more opportunities exist for transfer and spread, and so it seems quite possible that new and/or higher levels of ARGs become integrated into the environment.

1.3 THE EFFECT OF COMPOSTING ON ANTIMICROBIAL RESISTANCE GENES, MOBILE GENETIC ELEMENTS, AMR BACTERIA, AND BACTERIAL COMPOSITION IN CHICKEN MANURE.

Composting has the ability to reduce AMR and even residual antibiotics that have been excreted through the recipient animal (thus removing their selective pressures), as will be detailed with several studies, and as noted by Xie *et al.* (2019). Similar to the aspects examined in the studies conducted on manure application to soil, four elements of composting chicken litter will be focused on: the effect on AMR bacteria, detectable ARGs, bacterial composition, and detectable MGEs. Studies vary in methods, with some manures being composted for as long as ten weeks (Esperón *et al.* 2020), and others as short as 26 days (Li *et al.*, 2017). Some studies were conducted at a laboratory-scale with controlled conditions, whilst others were field-scale and relied on environmental conditions. The source of the chicken manure also varies, as does the diets fed to the chickens. All of this means that studies are not always directly comparable, and some results seen may indeed be impacted by methodology, but it also allows for trends to be seen regardless of method. Field-scale studies, whilst not as controllable in terms of variables, are more realistic, as they will more closely represent the practical usage of chicken litter composting by farmers prior to usage as fertiliser.

1.3.1 The effect of composting on AMR bacteria in chicken manure.

Out of all the studies assessed and chosen, only two adopted the method of enumerating resistant bacteria, with other studies instead focusing on quantification of ARGs, although both studies here also included the identification and enumeration of a small number of ARGs. Both Wang *et al.* (2019) and Subirats *et al.* (2020) demonstrated the antimicrobial resistance of their bacteria through the use of a small number of antibiotics, with the former author using varying concentrations. After this however their methods differ, with Wang *et al.* (2019) choosing to assess total bacteria isolated from the litter/compost, and Subirats *et al.* (2020) instead selectively isolating certain genera and species, although unfortunately only two of these were actually challenged against antibiotics (*Staphylococcus* and *Enterococcus*). With *Enterococcus*, total counts and counts of bacteria resistant to the five chosen antibiotics (tetracycline, vancomycin, erythromycin, penicillin and ciprofloxacin) decreased significantly after composting, with most of the replicates indicating that the bacteria were undetectable. *Staphylococcus* species also displayed significant decreases in counts for each antibiotic and without, however they were still quantifiable for some replicates with the exception of those challenged with tetracycline, ciprofloxacin, and vancomycin (although they were also undetectable before composting for this antibiotic). Composting can therefore significantly decrease the levels of both of these genera, including those resistant to certain antibiotics, however it is more effective for the decrease of enterococci than staphylococci. These are only two genera out of the plethora that are present in chicken litter however, so it would be useful if more bacterial genera were examined, or as is the case with Wang *et al.* (2019), total bacteria were included.

Moving on to Wang *et al.* (2019), different antibiotics were chosen in this study (tylosin, doxycycline, enrofloxacin, and sulfadimidine), and this study also included manure from cows and pigs. Chicken manure consistently possessed more bacteria resistant to each antibiotic at each concentration, including more total bacteria, than the other manures, once again indicating the exacerbated risk that chicken manure poses if it is to be introduced to the environment. The results following composting were variable, and certainly not as drastic as those seen in the previous study by Subirats *et al.* (2020), even when considering that that study recorded considerably lower numbers of bacteria. Composting had virtually no impact on the total number of bacteria, although this does not mean that individual taxa within this amount did not shift and change in levels, as will be seen in further studies when considering bacterial composition. With bacteria resistant to enrofloxacin, decreases were seen between raw and composted samples until the highest concentration was used, wherein all of the resistant bacteria to this concentration were removed after composting. The significance of these decreases can be understood when observing the graphs provided of the resistance rates of bacteria; for enrofloxacin, the decrease of bacteria resistant to the lowest concentration was not significant, but for those resistant to the intermediate concentration it was. For tylosin, decreases were once again seen at every concentration following composting, and whilst all of these decreases were significant, there was no total removal as for the previous antibiotic. The same can be said for the results with doxycycline, with the levels of resistant bacteria to each concentration all significantly decreasing after composting. However, it is with sulfadimidine where results begin to diverge from those seen with the other antibiotics. Composting was unable to significantly decrease the levels of resistant bacteria at any concentration, with bacteria resistant to the highest concentration even increasing slightly (but this was not significant). Such a result was also not seen with the other two manure types. The paper cites multiple explanations for this, such as dosages and the metabolism of sulfonamides, largely leading to the conclusion that exposure of the bacteria to such high sulfonamide concentrations long-term may have led to the high rates of resistance seen. Whilst it can be seen that sulfadimidine was present in fresh chicken manure, it was present at roughly the same amount in swine manure also, which did not suffer the same poor removal of resistant bacteria; it would have been beneficial if the dosages of sulfonamides administered to the chickens that produced the manure for this study were provided, if at all possible, to confirm the explanations provided by the author. According to the analysis conducted that aimed to find links between antibiotic residues and certain antibiotic resistance genes, sulfadimidine was not positively correlated with any resistance genes, whereas doxycycline was correlated with the sulfonamide resistance genes *sul1* and *sul2*. Whilst composted swine manure had comparatively high levels of doxycycline, as composted chicken manure also did, only fresh chicken manure had high levels of this antibiotic prior to composting. It was the most abundant antibiotic residue out of those analysed, and found at much higher levels than in fresh cattle manure, and very little was found in fresh swine manure. As such, perhaps it is the abundance of this antibiotic residue in fresh manure that influenced the lack of decrease of sulfadimidine-resistant bacteria. Whilst not relevant to this section as a whole, the high levels and proportions of the sulfonamide resistance genes in composted chicken manure can be seen in the ARG data. Of course, this is only a correlation, but given that fresh chicken and swine manures had comparable levels of sulfadimidine, it may be that doxycycline played a more important role in the poor removal of sulfadimidine-resistant bacteria for chicken manure. It is entirely possible that the removal of *sul1* and *sul2* is not well-achieved in the composting of chicken manure (and as such not related at all to the amount of doxycycline), and that chicken manure contains higher levels of these ARGs than other manures, which will be further explored with other studies focusing on ARGs.

1.3.2 The effect of composting on ARGs in chicken manure.

As there is such an abundance of studies monitoring the changes of ARGs during the composting process, genes will be considered on the basis of the antibiotic they confer resistances to. Beginning with tetracycline resistance genes (TRGs), it seems that overall composting has a good ability to remove these genes from chicken manure. Across all papers that assessed the following genes, *tetA, tetB, tetC, tetD, tetE, tetK, tetM, tetO, tetQ, tetR, tetS,* and *tetW* all decreased due to composting chicken manure (whether they were measured in relative abundance, absolute abundance, percentage of bacteria, or indicated through removal rate) (Cui *et al.,* 2016; Xie *et al.*, 2016; Li *et al.,* 2017; Peng *et al.*, 2018; Qian *et al.*, 2018; Zhou & Yao, 2020; Esperón *et al.,* 2020; Zhou *et al.,* 2021). Some were not as efficiently removed as others, as was seen for *tetC* in Zhou *et al.* (2021)'s study, as it had the lowest removal rate of 66.8% out of the other three TRGs (the highest, belonging to *tetW,* was 96.1% for comparison). Similarly, *tetQ* in the study by Cui *et al*. (2016) did not appear to undergo as drastic a decrease in relative abundance as the several other TRGs. Some of the TRGs are typically more reported on than others, and when genes that were featured across multiple studies such as *tetM, tetO, tetQ* and *tetW* (with the former in six and latter three in five) were all recorded to decrease in levels due to composting it is particularly impressive and reinforces the idea that composting is an effective method for their removal, especially when considering the dissimilar data that can be seen for other genes. *tetG, tetL* and *tetX* are examples of TRGs that receive such turbulent results. With *tetG,* decreases were seen in the papers by Li *et al.* (2017), Zhou *et al.* (2021) and Qian *et al.* (2018). A removal rate of 80% was achieved in the study by Zhou *et al.* (2021), and composted manure from three out of four farms experienced *tetG* decrease as reported by Qian *et al.* (2018). A noticeable decrease in relative abundance appeared to be seen at the end of the study by Li *et al.* (2017), although the abundances actually increased from previous time points (perhaps due to higher temperatures being reached earlier in the composting timeframe). Peng *et al.* (2018) also noted a change in relative abundance of 42.6%, however this was far lower than what was recorded for other TRGs in the study, and virtually no absolute abundance change was seen, with 0.4%. Despite the findings of these studies, Xie *et al.* (2016) classified *tetG* as a gene where "high persistence in mature composts" was seen, with its absolute abundance increasing during the thermophilic stage of composting and at the end stage. Zhou & Yao (2020) also show that *tetG-01* and *-02* either experienced no change in abundances upon composting or slightly increased. *tetL* is similar, with Peng *et al.* (2018) showing it received changes of 75.9% and 85.3% for absolute and relative abundance respectively, and whilst this was an improvement from *tetG*, they were still lower than those for *tetM, tetO* and *tetB.* Qian *et al.* (2018) showed that only manure from one farm out of four experienced a decrease of *tetL*, the rest remaining the same. Cui *et al.* (2016) on the other hand showed a considerable decrease in *tetL.* As with *tetG*, it was Xie *et al.* (2016) and Zhou & Yao (2020) reporting less favourable results. *tetL* too was classed as persistent, and increased due to composting, in the former study. In the latter, one gene was already low in abundance (*tetL-01*), but the other (*tetL-02*), which was higher, remained high with no change from composting. Lastly, decreases in *tetX* was reported in four studies (Cui *et al.,* 2016; Li *et al.,* 2017; Qian *et al.*, 2018; Zhou & Yao, 2020), but it increased in another (Xie *et al.*, 2016).

Two classes of ARGs that seem to be prone to receiving inconsistent results and poor removal from composting are those providing resistance to chloramphenicol (and its related and derived compounds) and sulfonamides. One chloramphenicol resistance gene, *cfr*, received agreement that composting decreased its levels (Cui *et al.*, 2016; Zhou & Yao, 2020), and *cmx(A)* was the same in terms of it persisting (Xie *et al.,* 2016; Zhou & Yao, 2020). *cmlA* and *floR* are where results begin to become polarised, with two studies finding that they increase (or decrease very little) (Cui *et al.*, 2016; Xie *et al.,* 2016), whereas Zhou & Yao (2020) found that they decreased. Admittedly the decrease of *cmlA* was small, but for *floR* the decrease was markedly larger. Evaluating methodology and sources of the manure for each paper to attempt to find a link for why such different results have been obtained is not straightforward; all studies had thermophilic phases at different temperatures and for different lengths of time, the composting time for Xie *et al.* (2016) was closer to that described by Zhou & Yang (2020), and Cui *et al.* (2016) performed a laboratory-scale compost, whereas manure was composted commercially by Xie *et al.* (2016).

sul2 and *sul1* are the two sulfonamide genes that are often included in ARG-focused papers, the latter of which is not always well-removed, or receives varying results. Out of seven studies, *sul2* is said to have decreased following composting in six, although for some of these papers the reduction was not as successful as was seen for other genes, and increased in only one (Cui *et al.*, 2016; Li *et al.,* 2017; Peng *et al.*, 2018; Wang *et al.,* 2019; Zhou & Yao, 2020; Esperón *et al.*, 2020; Zhou *et al.,* 2021). It can be fairly confidently concluded, then, that composting is successful at removing at least some proportion of *sul2*. The same cannot be said for *sul1*, as it is frequently found to persist, and even increase, throughout composting (Li *et al.*, 2017; Esperón *et al.*, 2020; Subirats *et al.,* 2020; Subirats *et al.,* 2021). There was a 2.2 $\log_{10}$ increase and 7.5 times increase in the gene in the studies by Esperón *et al.* (2020) and Li *et al.* (2017) respectively following composting. Even when it has been found to not persist or increase, the removal of *sul1* has not always been excellent. In terms of absolute abundance change in Peng *et al.* (2018)'s study, only 16% was reached, and it was the most abundant ARG before and after composting. When considering the abundance relative to 16S rRNA the change increased to 52.6%, but was still the second lowest out of the seven ARGs (*tetG* being the lowest). It was also the most abundant ARG in Wang *et al.* (2019)'s study before and after composting, with *sul2* being the second most abundant, and far higher than those in swine and cow manure. It was however diminished considerably from composting compared to the aforementioned study, with a relative abundance change of what appears to be around 77.4%. It appeared to be more or less removed for swine manure however, whilst strangely increasing for cow manure, once again demonstrating the unpredictability of *sul1*'s response to composting. Cui *et al.* (2016) also shows that it had a good reduction (it was the most abundant ARG for this study too), as does Zhou *et al.* (2021), with a removal rate of 88.8%. These inconclusive results mean that it is difficult to determine if composting is truly successful at reducing *sul1* levels in chicken manure particularly, but it can at least be said that it is not as effective as it is for the removal of many other ARGs. Whilst utilising swine rather than chicken manure, Lin *et al.* (2021) offers a more focused investigation into *sul1* and *sul2* genes during composting. It also examines horizontal gene transfer (HGT) for these two ARGs and how this is altered due to composting, therefore evaluating a potential explanation for why *sul1* can have such a propensity to survive composting. Fitting with what most other studies found, *sul2* gradually decreased during the composting before experiencing a large drop from day 35 to 42. *sul1,* on the other hand, demonstrated a stable relative abundance that hardly shifted during the 42-day compost, and the absolute abundance dipped only at one time point before sharply increasing again and finishing the experiment slightly higher than it began. When reviewing their HGT data to see if this persistence may be due to the conjugation of the *sul1* gene between bacteria in the compost, the transfer of *sul1* was actually calculated to have decreased due to composting (*sul2's* ability was completely removed). Therefore, as this reduction in HGT did not impact the endurance of the gene throughout composting, there must be another, more heavily contributing factor for sustaining the gene. Data on the composition of total bacteria shows that Firmicutes remained the most dominant phyla regardless of composting, and for sulfonamide-resistant bacteria specifically the most dominant phyla was Proteobacteria. Around a quarter of the resistant bacteria in manure belonged to Firmicutes also, however, showing that this phylum contained bacteria that were very likely to be carrying *sul1*. After composting, the phyla-level composition of sulfonamide-resistant bacteria shifted to where Firmicutes became the dominant phylum, as it was for total bacteria. Therefore, as the proportion of Firmicutes was not largely affected by composting and consequently comprised 68.6% of total bacteria in the composted manure, and Firmicutes comprised over half of sulfonamide-resistant bacteria, it can be inferred that bacteria belonging to this phylum that presumptively possess *sul1* were also able to survive composting. This could potentially be reflected in the HGT data also; the rate of detection of transconjugants positive for only *sul1* actually increased from 63.3% in manure to 96.7% in compost, despite the decrease in conjugative frequency. It may be, then, that an increase in Firmicutes, particularly if it was bacteria in this phylum that possessed *sul1* rather than *sul2,* was enough to outweigh the lower conjugation rate and lead to more transconjugants possessing *sul1* after composting. Furthermore, looking at genus-level data, it can be seen that although some sulfonamide-resistant genera decreased in relative abundance, they were replaced with the increase and enrichment of other resistant genera like *Bacillus* and *Staphylococcus* (belonging to Firmicutes). Drawing together this data, it may be the case that *sul1* is able to persist so successfully in many of the studies previously seen thanks to the survival and/or enrichment of potential carriers of the gene from the composting process, perhaps more so than it is due to HGT between bacteria within the compost.

Macrolide resistance genes are generally well-removed from manure thanks to the composting progress, with the exception of some genes. *ermQ*, *ereB*, *ereA*, *ermC*, and *ermA* are consistently found to decrease, the latter three consistently so across two or more studies (Li *et al.*, 2017; Qian *et al.*, 2018; Zhou & Yao, 2020; Zhou *et al.*, 2021). On the other hand, the genes *ermX* and *mphA* cannot confidently be commented on about whether composting has a negative or positive effect on their levels, as out of the few papers that included them in their studies, some found increases or persistence whilst others found decreases (Xie *et al.*, 2016; Qian *et al.*, 2018; Zhou & Yao, 2020). *ermY*, however, was found to increase in two studies, rather highly so in one (Qian *et al.*, 2018; Zhou & Yao, 2020). Two genes are particularly well-studies in comparison to the others – *ermB* and *ermF* – and are largely found to decrease from composting, although a smaller number of papers do report otherwise. Two papers (Subirats *et al.*, 2020; Subirats *et al.*, 2021) show that *ermB* significantly decreases after composting. As the 2020 paper includes, chicken raised with antibiotics did have higher abundances of *ermB* than those without, but even so composting was able to significantly decrease the abundance, albeit not to the same lower abundance of composted non-antibiotic fed chicken manure. These same papers show that composting has little effect on *ermF* though, even increasing the abundance, although not significantly. In contrast, Zhou & Yao (2020) report that *ermF* copies decreased, and it is in fact *ermB* that increased, dramatically so. Xie *et al.* (2016) states both genes persist, but all other papers involving these two genes record reductions thanks to composting (Li *et al.*, 2017; Qian *et al.*, 2018; Wang *et al.*, 2019; Esperón *et al.*, 2020; Zhou *et al.*, 2021). It may be worth noting, however, that in the studies where these genes feature together, *ermB* often benefits from a stronger decrease than *ermF*, which perhaps could indicate that *ermB* responds more favourably to composting.

Resistance genes to aminoglycosides appear to be important to monitor and include in studies according to the data presented by Zhou & Yao (2020); out of all the ARG classes, aminoglycoside resistance genes contain the most genes with high log copy numbers, both prior to and following composting. Some level of reduction is achieved for most genes neverless, as is also reflected by Qian *et al.* (2018) who shows that all the aminoglycoside-resistance genes in manure from multiple farms either undergo reductions or have no change. *aadA* genes in Zhou & Yao (2020)'s data that increased after composting exhibited inconsistent results in other studies. They were found to decrease and/or at least remain the same in one (Qian *et al.*, 2018), decrease, even significantly, in a further two (Subirats *et al.*, 2020; Subirats *et al.*, 2021), but persist and increase in others (Xie *et al.*, 2016; Esperón *et al.*, 2020). *aacA/aphD*, while increasing in Zhou & Yao (2020)'s study, actually decreases in Qian *et al.* (2018)'s, and had a high reduction rate in the study by Xie *et al.* (2016). The combination of inconsistencies, and especially the apparent high level of resistance genes before and following composting, means that this class of ARGs are important to examine further and may pose quite significant environmental risks.

Finishing with beta-lactam resistance, this ARG class is the only one reviewed that is consistently well-removed from manure due to composting treatment, including those belonging to the extended spectrum beta-lactamase group. As is displayed by Zhou & Yao (2020), the wide variety of beta-lactam resistance genes that the study encompassed were all reduced to the lowest log copy numbers recorded. In Xie *et al.* (2016)'s list of genes that were highly reduced, five genes were featured and some were declared undetectable after composting completion. Similarly, *bla*$_{TEM}$ in Esperón *et al.* (2020)'s study experienced the largest reduction out of all genes. The successful removal of beta-lactam resistance genes is very likely to be from the removal of the bacteria which commonly carry them, such as *E. coli*. Studies do show that *E. coli* that is inoculated into poultry manure is well-removed in composting experiments. In one paper, when manure was composted under temperatures that would typically and naturally be reached during composting (the experiment used a controlled, lab-based method), *E. coli* underwent rapid and drastic reductions. At 55°C, an average of roughly 78% of *E. coli* were destroyed after just one hour, with the quantity finishing around the same after 24 hours. Results like this were also seen at 65°C, with an average decrease of around 75% after an hour and similarly low levels after 25 hours (Wilkinson *et al.*, 2011). Another study used an ESBL-producing strain to inoculate the manure, and all nine treatments no longer contained *E. coli* that could be directly detected (with a detection limit of less than 100 CFU/g) in a time frame that composting typically exceeds. For illustration, one treatment no longer contained directly detectable *E. coli* after just one day, with the longest lasting 22.5 days. Furthermore, *E. coli* could not even be detected after enrichment for all but two treatments, with the longest amount of time for this to be achieved being just 14 days (Thomas *et al.*, 2020). Studies such as these strongly suggest that *E. coli* is not likely to survive the composting process and numbers quickly deteriorate once higher temperatures are reached. As such, this is likely reflected in the successful removal of beta-lactam resistance genes during composting that is seen in the previous studies. The need for composting chicken litter is further supported by knowledge like this, given that *E. coli* is a well-known pathogen and has a propensity to cause foodborne illness (which is highly relevant if fresh manure is to be used as fertiliser for crops).

Overall, it can generally be said that composting chicken litter has a good capacity to decrease or remove a wide variety of ARGs, although it is also true that a number of genes across the classes instead respond to composting by persisting or increasing, given that so many studies have reported such an occurrence rather than the opposite outcome. Nevertheless, the reduction of such an extensive number of ARGs, and consequently the bacteria harbouring them, means that composting is indeed a viable and successful treatment option to diminish the likelihood that ARGs will be introduced to the environment and pose risks, both to the environment and human and animal health.

1.3.3 The effect of composting on bacterial composition in chicken manure.

Beyond antimicrobial resistant bacteria and ARGs, it is also important to consider the effects composting can exert on the bacterial community of chicken litter. Calling back to the papers dealing with the impacts of chicken manure on soil communities, it is understood that the bacterial community structure has a role in shaping the resistome, that the level of diversity can hinder or encourage antimicrobial resistance, and that ARG-linked taxa present in soils naturally can become enriched from manure addition. Whether sufficient changes are enacted on bacterial composition through composting so that these alterations may be avoided, then, must receive attention.

Whilst variations can be seen study-to-study, two statements can be said to almost always be true; composting certainly alters the bacterial composition of chicken manure quite extensively, and it is successful in increasing the diversity of the community. Only one paper was found to the contrary for one of these statements, when the bacterial diversity actually strangely decreased in composted poultry manure (Wan *et al.*, 2021). It can also be determined with some confidence that composting has a favourable effect for Actinobacteria, with many studies documenting increases in the relative abundance of this phyla, and in others levels at least remain stable (Cui *et al.*, 2016; Peng *et al.*, 2018; Zhou & Yao, 2020; Esperón *et al.*, 2020; Subirats *et al.*, 2020; Wan *et al.*, 2021; Bao *et al.*, 2021). The other phylum that received results mostly in agreement was Firmicutes. It seems that, generally, Firmicutes tend to decrease in response to composting conditions, sometimes rather drastically (Cui *et al.*, 2016; Peng *et al.*, 2018; Subirats *et al.*, 2020). However, two studies record extreme increases in the phylum, from around 30% relative abundance to roughly 70%, and from around 12% to comprising over three quarters of all bacteria in Esperón *et al.* (2020) and Wan *et al.* (2021), respectively. Examining the methods/litter sources/bulking agents used in all papers providing data on Firmicutes identifies one potential cause for such an extreme contradiction; both papers featuring these harsh increases used far longer composting times of approximately 70 days (Esperón *et al.*, 2020) and 90 days (Wan *et al.*, 2021), whereas the length of composting for other papers was on average 41 days. It may very well be that this is why the latter study was also the only one to find decreases in bacterial diversity. Viewing the data for how the orders and genera of Firmicutes change in these two studies, the large increase in Bacillales, *Bacillus* and *Novibacillus* are probably accountable for the inflation of Firmicutes. Therefore, extension of composting time may allow these bacteria to thrive in the composting environment to such a level that the Firmicutes phyla increases overall rather than decreasing. The other well-studied phyla, Bacteroidetes and Proteobacteria, received overall varied results, although they were often found to decrease in abundance (Cui *et al.*, 2016; Peng *et al.*, 2018; Esperón *et al.*, 2020; Subirats *et al.*, 2020; Wan *et al.*, 2021; Bao *et al.*, 2021). Unsurprisingly given the high temperatures reached during composting, thermophilic taxa like

Deinococcus-Thermus and Thermomicrobia began to appear from virtually unnoticeable relative abundances (with Wan *et al.,* 2021 being an exception, no doubt from the dominating percentage of Firmicutes) (Zhou & Yao, 2020; Subirats *et al.,* 2020).

It is worth giving some attention to the fate of pathogenic bacteria also, given that the environment and other organisms will be exposed to these bacteria, a situation that only worsens if surviving pathogens also possess any resistances. Only three papers devoted focus to these bacteria, but these alone demonstrate that composting has an excellent capacity to remove most pathogenic bacteria, with the removal rate for one study near the end of the composting period reaching 86.83%. In particular, Corynebacteriaceae (and its *Corynebacterium* genus), *Clostridium*, and of course *E. coli* were well-removed. Some genera and species that were capable surviving, or even rather proliferating, during composting were *Bacillus* (including *Bacillus anthracis*), *Legionella*, and *Staphylococcus equorum* (Cui *et al.,* 2016; Peng *et al.,* 2018; Esperón *et al.,* 2020). The development of *Bacillus* during composting is not surprising, due to the capacity of species of this genus to produce heat-resistant spores that would easily withstand the thermophilic phase (Setlow, 2006). As for *Legionella*, it likely grew in abundance following composting because the thermophilic temperature reached in the study that recorded it, which was around 45°C, was likely not sufficient to remove members of this genera, or potentially even stop their growth (Ohno *et al.,* 2003; Cui *et al.,* 2016; Lesnik *et al.,* 2016). Therefore, it can be said that so long as the pathogenic species are not thermophilic or able to form heat-resistant spores, and the temperature during the thermophilic phase ideally reaches at least around 60°C, they generally are very well-removed.

The central importance of analysing these community changes in relation to the topic of AMR is to decide if these changes brought about by composting are actually beneficial in terms of decreasing resistances, considering the affect that bacterial community can have on resistance and the links between certain members and ARGs. When dealing with positive correlations between microbial taxa and AMR/ARGs, difficulties are unfortunately posed if whole phyla are considered rather than lower taxonomic ranks, as many studies do; it may be, of course, that many members of a phylum show positive correlations to ARGs, whilst many others have the entirely opposite. Nonetheless, it does seem that, overall, the decreasing of the phyla Firmicutes and Proteobacteria would have favourable effects for reducing a wide variety of resistance types, given the evidence of the network analyses by Han *et al.* (2018) and Peng *et al.* (2018). To illustrate, in the latter, the majority of the families of Firmicutes were discovered to have several significantly positive correlations to select tetracycline and sulfonamide resistance genes, and in the former multiple genera of the aforementioned phyla possessed positive correlations to multiple ARGs of a variety of resistance types. Becoming more specific, lower ranking taxa that are repeatedly highlighted in studies and/or found to possess links to many ARGs are the families of *Clostridiales, Clostridiaceae, Lactobacillaceae, Enterococcaceae,* and *Bacteroidaceae,* and genera include *Bacteroides, Lactobacillus, Clostridium, Corynebacterium,* *Flavobacterium, Pseudomonas,* and *Pedobacter.* Members of the bacterial community can also be found to instead have negative correlations with ARGs; the family *Bacillaceae* is an example of this, including several of its genera such as *Sinibacillus, Oceanobacillus¸ Pseudogracilibacillus,* and *Bacillus,* indicating any increases of these bacteria may in fact be highly helpful in terms of AMR, as for one reason or another their presence coincides with lower levels of particular ARGs (Tao *et al.,* 2016; Zhou *et al.,* 2017; Peng *et al.,* 2018; Han *et al.,* 2018; Zhou *et al.,* 2021).

With this understanding of which taxa may be important for shaping the resistances seen in manure and compost, it can now be determined if composting is a useful process from this perspective. Looking at changes from a shallow, phylum-level, the already-mentioned decreases in Firmicutes that are often seen can be viewed as advantageous. Similarly, repeated diminishment of the other reported ARG carrier-linked phylum, Proteobacteria, also supports composting for the purpose of reduction of AMR. With Actinobacteria, which has already been stated to be found to increase or sustain its levels, it cannot be easily said whether the changes (or lack thereof) induced by composting are detrimental or actually beneficial. This further emphasises the need for studies to investigate community changes beyond purely phylum levels; many families of Actinobacteria do indeed show negative ARG correlations, but many also show positive, such as *Corynebacteriaceae* (Peng *et al.,* 2018). The importance of deeper levels of classification is also seen with Esperón *et al.* (2020) and Wan *et al.* (2021), where at first glance the exceptionally large increases in Firmicutes could be perceived as problematic, until discovering that this increase was very likely due to the growth of Bacillales (which features several negatively-correlated families) and *Bacillus, Oceanobacillus,* and *Sinibacillus,* respectively, rather than more problematic members. Moving on from phyla, families and genera that were previously mentioned as potential carriers (due to their correlations) were never found to increase as a result of composting and were instead decreased or even seemingly removed (Cui *et al.,* 2016; Peng *et al.,* 2018; Zhou *et al.,* 2021; Wan *et al.,* 2021). Furthermore, taxa showing negative correlations to ARGs often appeared and expanded as a result of composting, such as *Bacillus, Oceanobacillus, Sinibacillus, Bacillaceae, Paenibacillaceae, Pseudonocardiaceae, Brevibacteriaceae* (amongst other families), and potentially Bacillales (as it contains members such as *Bacillaceae* and *Paenibacillaceae*) (Cui *et al.,* 2016; Peng *et al.,* 2018; Zhang *et al.,* 2018; Esperón *et al.,* 2020; Wan *et al.,* 2021).

It can most certainly be determined from the existing studies that composting is capable of exerting rather powerful effects on the composition of the bacterial community. It has the capacity to diminish pathogens and as such may prevent contamination of soil and crops when it is applied, and generally can greatly reduce the levels of bacteria that are potential hosts for a wide array of ARGs whilst supporting the proliferation of those that possess negative links to such genes, creating a fertilisation option that is far safer for the environment in respect to AMR.

1.3.4 The effect of composting on mobile genetic elements in chicken manure.

Mobile genetic elements (MGEs) pose dangers to the environmental and human health as they enable the perpetuation and spread of antimicrobial resistance genes. It may be possible for this to occur between manure-borne bacteria in compost and also between compost-derived bacteria and native soil bacteria, resulting in both persistence of ARGs and dissemination amongst the soil community. The MGE types that are featured in studies regarding composting are plasmids, transposons, insertion sequences and, most commonly, integrons, particularly the integrase genes *intI1* and *intI2*.

Few papers explore the fate of plasmids during composting, but from what could be found it appears that the ability for composting to succeed at removing ARG-carrying plasmids is highly dependent on the bacteria in possession of the plasmid. Examining *E. coli,* as Guan *et al.* (2007), it is apparent that composting is easily able to quickly and completely remove *E. coli* carrying two different plasmids with resistance genes. Strains with these plasmids were removed by day 3 of the incubation, when simulated composting temperatures reached around 55°C. Furthermore, no *E. coli* transconjugants were detected, indicating transfer of the plasmids to these recipients was unable to occur and the chosen plasmids could not be detected exogenously either, suggesting high composting temperatures would be able to destroy these plasmids. Even in the incubation at 23°C, the monitored *E. coli* were eventually seemingly eliminated, transfer frequencies were slightly lower than what was recorded in chicken manure as opposed to compost, resulting transconjugants were also removed, and one of the plasmids could not be detected exogenously. Therefore, the composting environment may be unsuitable for *E. coli* even without high temperatures. However, the detection of one of the plasmids after *E. coli* were removed does indicate that there was sufficient opportunity for transfer to other members of the compost community, emphasising the importance that the thermophilic phase is achieved in composting. Lower conjugation frequencies, or complete elimination of conjugation, were also recorded for genes that were likely to be plasmid-borne in another study (Lin *et al.*, 2021). Studies involving *Bacillus subtilis*, on the other hand, are not as positive. This is somewhat to be expected given that it is already established that *E. coli* is readily removed from composting, whereas *B. subtilis* is able to sporulate and survive composting temperatures. Unfortunately, these studies do not involve the composting of chicken manure, but mushroom compost. Nonetheless it can at least be observed what effects composting temperatures may have on this bacterium the and plasmids they contain. In one study, chloramphenicol resistant-*B. subtilis* (which was the antibiotic the plasmid chosen conferred resistance to) vegetative cells were only totally removed at the highest incubation temperature (65°C), with *B. subtilis* recovered on non-selective agar still remaining. This did not indicate that vegetative cells possessing the plasmid were removed however, as the paper goes on to demonstrate, when recovered *B. subtilis* colonies all possessed the plasmid. As such, the radically different efficiencies of removal of plasmids and consequently plasmid-mediated resistance caused by which bacteria possess the plasmids can begin to be seen from this paper alone. Nonetheless, although the vegetative cells did remain for at least 28 days of the 65°C incubation, the amount of these cells decreased, with spore counts also being removed, and there was no evidence found for spread of the plasmid to other bacteria (McDonald *et al.*, 1998). Vegetative cells and spores of the same *B. subtilis* strain also incubated at 65°C in another study decreased even less. Tetracycline resistant (the conferred resistance type from the plasmid in this study) vegetative cells and spores were not removed by 28 days incubation, and total *B. subtilis* vegetative cells and spores, whilst initially reducing by quite some way, appeared to eventually stabilise towards the end of the incubation and were more or less at the same levels of indigenous compost bacteria. Similar to the previous study, the plasmid used also did not seem to be transferred to any other bacteria throughout the experiment (Amner *et al.*, 1991).

Transposons and insertion sequences are also not hugely investigated in the relevant literature, but from what exists it seems that composting has an excellent capacity to reduce these MGEs. The number of insertion sequences significantly decreased in Subirats *et al.* (2021) and the richness was also reduced, with composting having the effect of decreasing the relative abundance of certain ISs whilst increasing the abundance of others, namely IS6 and IS52. A particular insertion sequence, IS613, was shown to be well-removed in multiple studies (Xie *et al.*, 2016; Qian *et al.*, 2018; Zhou & Yao, 2020). Transposon genes in these three studies were also generally diminished as a result of composting, with several genes decreasing or becoming undetectable and therefore, presumably, totally removed. Transposon genes were also shown to co-occur with several resistance genes and multiple resistance gene types in two of these studies, meaning removal of these MGEs is very important for decreasing spread of groups of genes. In Xie *et al.* (2016) however, *tnpA-02* and *tnpA-05* were shown to persist, contrary to what was shown for *tnpA-02* by Qian *et al.* (2018). In the remaining third study, these genes were also shown to decrease, although admittedly they were two of the three transposon genes to possess a positive log copy number after composting. One transposon gene that is prominent for its seeming persistence despite composting is *tnp-04*, which remained at extremely high log copy numbers with no apparent changes from composting in one study, and increased in another (Xie *et al.*, 2016; Zhou & Yao, 2020). Correlation data from the former study shows *tnpA-04* was the only transposon gene to be highly significantly correlate with chloramphenicol resistance genes; since FCA genes were shown to not decrease during composting in this same study, it could be that this co-occurrence is what enabled this transposon gene and the chloramphenicol resistance genes to persist.

Integrons are the final MGEs to be explored, whose related genes are the most well-studied in papers, particularly the integrase genes *intI1* and *intI2*. The latter gene generally seems to respond favourably to composting, often heavily decreased or approaching total removal, with high removal rates/efficiencies (Peng *et al.*, 2018; Zhu *et al.*, 2019; Subirats *et al.*, 2021). *intI1* on the other hand receives mixed results. A handful of papers report *intI1* decreasing in abundance following composting, with a surprisingly excellent removal rate in one paper, although in others it takes quite a long incubation time for a considerable decrease to occur (Li *et al.*, 2017; Qian *et al.*, 2018; Zhu *et al.*, 2019; Zhou *et al.*, 2021). A slightly higher number of studies discover the opposite, with *intI1* either persisting, increasing, or suffering poor removal rates (Xie *et al.*, 2016; Peng *et al.*, 2018; Wang *et al.*, 2019; Zhou & Yao, 2020; Subirats *et al.*, 2020; Subirats *et al.*, 2021). It seems unlikely that methods chosen for composting may have affected the results, given the varieties between each study, although it can be seen that the majority of studies finding decreases for *intI1* used a laboratory-scale approach as opposed to a field-scale/commercial approach for those finding persistence and increases. Looking at correlation data between *intI1* and bacterial taxa, it is still difficult to determine why *intI1* manages to be decreased more successfully in some studies, and may very well be due to which particular bacteria are carrying this gene in a certain sample. To illustrate, *intI1* is described as being positively correlated to Firmicutes and Proteobacteria, and especially *Lactobacillus*, by several studies (Xie *et al.*, 2016; Zhu *et al.*, 2019; Zhou *et al.*, 2021); the excellent removal rate of *intI1* seen in one of these studies may be linked to the decrease of *Lactobacillus* that composting achieved, for example. However, some studies which found persistence and increases of *intI1* achieved reductions in Firmicutes and Proteobacteria, the estimated hosts of *intI1*, possibly indicating that which bacteria are harbouring *intI1* can vary from sample-to-sample, and data from other studies cannot be carried over to explain results seen in another (therefore, it is necessary for papers to generate their own complete data on *intI1*, bacterial composition, and their correlations for explanations to be made)(Peng *et al.*, 2018; Subirats *et al.*, 2020). It is highly important for it to be defined what can ensure *intI1* removal during composting, as this MGE is repeatedly found to be highly correlated with a myriad of resistance genes across multiple classes. This includes ARG types that were previously described as being hard to remove or that experience variable results, such as chloramphenicol, sulfonamide aminoglycoside resistance genes, and as such the problematic resistance genes of these classes may be able to survive due to their co-occurrence and spread with *intI1* (Xie *et al.*, 2016; Li *et al.*, 2017; Peng *et al.*, 2018; Wang *et al.*, 2019). It may be beneficial for certain additives to be applied to the compost to aid the removal of *intI1*, such as biochar and zeolite, as two papers found these materials were able to increase the deterioration of *intI1* (Li *et al.*, 2017; Zhou *et al.*, 2021). These studies were amongst those that found decreases in *intI1* even without such materials however, so it is unknown what effect they may have in the instances where composting alone is unable to remove *intI1* at any capacity.

1.4 RESEARCH OBJECTIVES.

The purpose of this study was to investigate the effects of an outdoor, medium-scale composting system on the antibiotic resistance of chicken litter sourced from non-commercial UK laying chickens that had been raised without antibiotics. The literature available on antibiotic resistance in UK chicken faeces is not as extensive as that available for many other countries, and composting studies also tend to focus exclusively on commercial chickens, leaving this very specific area of research underrepresented and overlooked. With this considered, the objectives of this study were:

1. To measure the temperatures of different locations/depths in a compost heap.
2. To characterise the antibiotic resistance that exists in non-commercial, antibiotic-free UK chicken litter and compost, and how it differs within the compost heap, depending on location/depth and lactose-fermenting ability of bacteria.
3. To determine the effects of composting on the levels of enteric bacteria from chicken litter.
4. To determine the effects of composting chicken litter on the levels of resistance against various antibiotics.

2 GENERAL MATERIALS AND METHODS.

2.1 COMPOSTING PROCESS AND SAMPLING.

Chicken litter was collected in March 2021 from a chicken-owner in East Midlands, United Kingdom. The hens were layers, and were kept in three sheds containing approximately 50 hens per shed. They were kept under cover due to the avian influenza regulations at the time of litter collection. Information on the feed and any supplements the chickens received was provided. Chickens were fed GoldnLay performance pellets (GLW Feeds Limited) and given the supplement Poultry Spice (Battles Poultry). Metals included in the feed appear to be for satisfying nutritional requirements or for growth promotion rather than for any antimicrobial purpose. Metal compounds within the feed comprised of cobalt(II) carbonate, manganese(II) oxide, copper(II) sulfate pentahydrate, iron(II) sulfate, zinc oxide, and sodium selenate, at concentrations of 0.082mg/kg, 126.271mg/kg, 16.525mg/kg, 93.182mg/kg, 81.218mg/kg and 0.259mg/kg, respectively. Iron(II) sulfate was also present in the supplement as a nutritional additive. Alongside faeces, the litter contained chopped maize straw.

A 1 metre × 1 metre × 1 metre composting bin constructed from wooden pallets and reinforced with fresh hay was filled with the chicken litter. Three temperature probes with Tinytag View 2 data loggers (Tinytag) were inserted at different depths: one at approximately 40cm depth in the centre of the pile, one at approximately 20cm depth, and one at 10cm depth (referred to as “centre”, “intermediate” and “outer”, respectively). These probes recorded the temperatures at 9.00am each day, as well as the minimum and maximum temperatures achieved over the 24-hour period. The intermediate probe was not inserted until day 31, when additional chicken litter was added. The heap was left to complete composting for 100 days. The probes were removed on day 100, and recorded data were transferred to Microsoft Excel 2019. Information on the maximum and minimum ambient temperatures was obtained from an online database (Time and Date AS, 2022). Beginning on day 81, the heap was turned once a week. During rainier months the heap was covered with a tarpaulin sheet, and during drier months water was added to the heap during weekly turning, in order to try and keep the moisture of the compost relatively stable.

Sampling of the compost heap was conducted once a week by hand. Samples were taken from three random locations in the heap for each depth (~40cm, ~20cm and 10cm) to ensure more representative results. The samples were collected in 50ml centrifuge tubes, and stored at 4°C if not immediately processed. The sample tubes were vortexed to facilitate homogenisation of the litter from the different heap locations before 500mg was measured and transferred to Bijou containers. A 4.5ml aliquot of maximum recovery diluent (MRD) was added to the litter to create a 10^{-1} dilution, which was vortexed to disrupt any larger pieces of litter and homogenise the sample. Further 10^{-3} and 10^{-4} dilutions were made with MRD. A 100µl volume of each dilution was spread-plated onto MacConkey Agar No. 2 (Oxoid) plates, in duplicate. Plates were incubated at 37°C for 24 hours. Resulting colony morphologies and the proportions of lactose-negative versus lactose-positive colonies were observed and noted as a broad indicator of changing bacterial dynamics in this specific regard. Lactose-positive and lactose-negative colonies were counted, and CFU g^{-1} F Wt. calculated.

3 THE EFFECT OF LOCATION AND DEPTH IN THE COMPOST HEAP ON COMPOSTING TEMPERATURES ACHIEVED

3.1 INTRODUCTION

Composting is the process of organic matter becoming degraded through the action of microbes. This results in an increase in temperature of the compost heap from the metabolic activity of the microbes, and subsequently the removal of potentially pathogenic organisms, as well as the maximisation of nutrient availability (which is why it is particularly attractive for agriculture). Many factors are important for successful composting, such as the nature of the organic matter, pH, temperature, moisture content, and oxygen availability through aeration. There is also interplay of these factors, as the temperatures reached during composting depend on moisture content and aeration, for example (Finstein & Morris, 1975). Composting of chicken manure has been completed successfully in many studies using a variety of techniques, from laboratory-scale, to large-scale, outdoor compost heaps, as would occur in agricultural practice. During the thermophilic phase, composting temperatures of chicken manure can reach anywhere from 40°C to above 75°C (Cui *et al.*, 2016; Li *et al.*, 2017; Peng *et al.*, 2018; Zhang *et al.*, 2018; Esperón *et al.*, 2020; Bao *et al.*, 2021). However, these authors did not specify the depth at which temperatures were taken, or only measured the temperature at the centre of the pile, or just measured temperatures at the same depth. It is being assumed that, at different positions and depths in a composting heap, there would be varying densities of material (and so, also, pressure, heat insulation, and oxygen availability), and subsequently different temperatures and microbial populations, especially if a pile was aerated (i.e., through an aeration pipe), but not turned (which would allow for some level of homogenisation). Therefore, it may be very important to consider the different depths of a composting heap, and monitor the separate temperatures achieved. As such, the objectives of the study were:

1. To monitor temperatures at three distinct locations and depths of the compost heap over a period of 100 days.
2. To determine how these different locations/depths relate to each other and the ambient temperature.

3.2 MATERIALS AND METHODS

3.2.1 Placement of temperature probes and measurement of temperature

Please refer to section 2.1

3.2.2 Statistical analyses

Student’s t-tests were executed in Microsoft Excel 2019 to analyse the temperature data of the different compost locations/depths, and ambient temperature, at specific time points (i.e., temperature peaks). Using Genstat 21.1 (VSN International, Ltd.), simple linear regression analyses were used to produce regressions and identify potential relationships with temperature data.

3.3 RESULTS

3.3.1 Maximum temperatures across the composting period

The maximum temperatures recorded for each position in the compost heap (centre, intermediate, and outer) were plotted and analysed (Figure 3.1). The maximum ambient temperature was also plotted to serve as a comparison with the composting temperatures. Rather than reaching a singular, long thermophilic phase before entering the cooling and maturation phases for the remainder of the composting period, several short peaks followed by dips in temperature were recorded. The first noticeable increase in maximum temperature occurred on day 11, with temperatures at the intermediate position reaching 40.7°C and centre position temperatures reaching 38.8°C. The temperatures of the outermost position remained significantly lower at 29.5°C ($p<0.01$). These temperatures were maintained for two days only, with a sharp decline occurring on day 14. A pattern of temperature spikes and troughs repeatedly occurred throughout the composting period. The highest temperatures recorded for the centre and intermediate positions were on day 16 (50.8°C and 51.5°C, respectively), whilst the highest temperature of the outer position was 41.6°C on day 84. Beginning on day 18, temperatures remained under 20°C for around two weeks after a large temperature spike that began on day 16. After the passage of the spike beginning on day 44, any increases in temperature did not surpass 40°C as before, with the exception of day 84 where the outer position achieved 41.6°C as previously mentioned.

Observing the temperature profiles (Figure 3.1), the maximum composting temperatures and maximum ambient temperatures often shared similar patterns, particularly with some temperature increases and decreases. However, peak maximum compost heap temperatures were significantly different ($p<0.01$) from the corresponding ambient temperatures.
Nevertheless, when comparing the maximum ambient and compost temperatures across the composting period rather than at specific points (i.e., at the peaks), there were positive relationships

between the ambient temperatures and the temperatures for each of the compost positions (r= 0.17, $F_{1,96}$=19.42, p<0.001, r=0.28, $F_{1,90}$=34.40, p=<0.001, and r=0.23, $F_{1,96}$=29.05, p<0.001 for centre, intermediate, and outer, respectively).

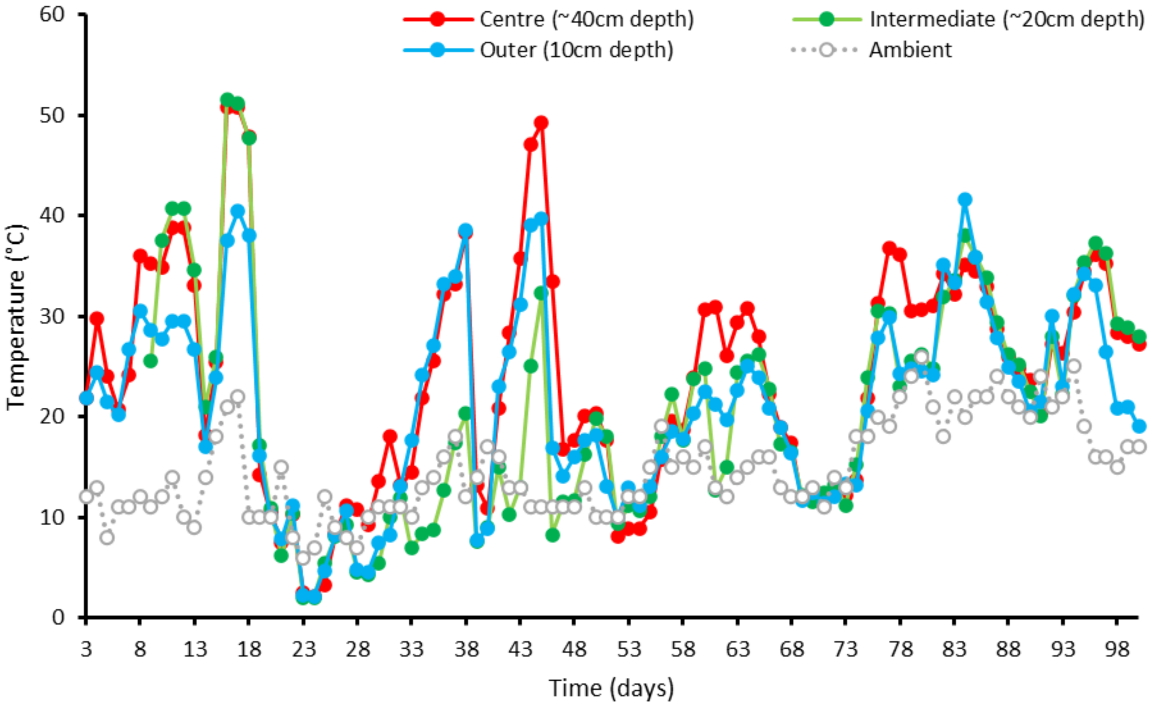

Figure 3.1: *Maximum temperatures of the different positions and maximum ambient temperatures across the composting period.*

The maximum temperatures of the different compost heap positions also had positive relationships with each other (r=0.68, $F_{1,90}$=194.52, p<0.001; r=0.86, $F_{1,96}$=581.10, p<0.001; and r=0.65, $F_{1,90}$=164.85, p<0.001 for between centre and intermediate, centre and outer, and intermediate and outer, respectively).

3.3.2 Minimum temperatures across the composting period.

Minimum temperatures were also examined with the maximum temperatures (Figure 3.2, Figure 3.3 and Figure 3.4) to determine the degree of fluctuation within a 24-hour period, as the maximum temperatures of the different composting positions were likely to have occurred during the day, whereas the minimum temperatures were most likely to have been recorded during the night. From this, it can be seen that each compost position has distinct results. For the centre position, the minimum temperatures closely resemble that of the maximum; whilst they may not reach the same height in temperature during the ‘peaks’ or sustain them as long, the pattern of the plots are almost identical and the lower temperatures are similar (Figure 3.2). The outer position’s temperatures are not dissimilar to this result, except for minimum temperatures during the ‘dips’ being considerably lower than the maximum (Figure 3.4). The intermediate results differ by way that the small ‘peaks’ seen with the maximum temperature on days 38 and 45 are not reflected in the minimum recorded temperature, instead remaining low and stable during this period. This is not seen with later peaks however, where the minimum temperature increases just as the maximum does, nor with the major peak occurring on days 16-18 and the smaller peak preceding it (Figure 3.3). With every position, the averages of the maximum and minimum temperatures were significantly different (*p*<0.001).

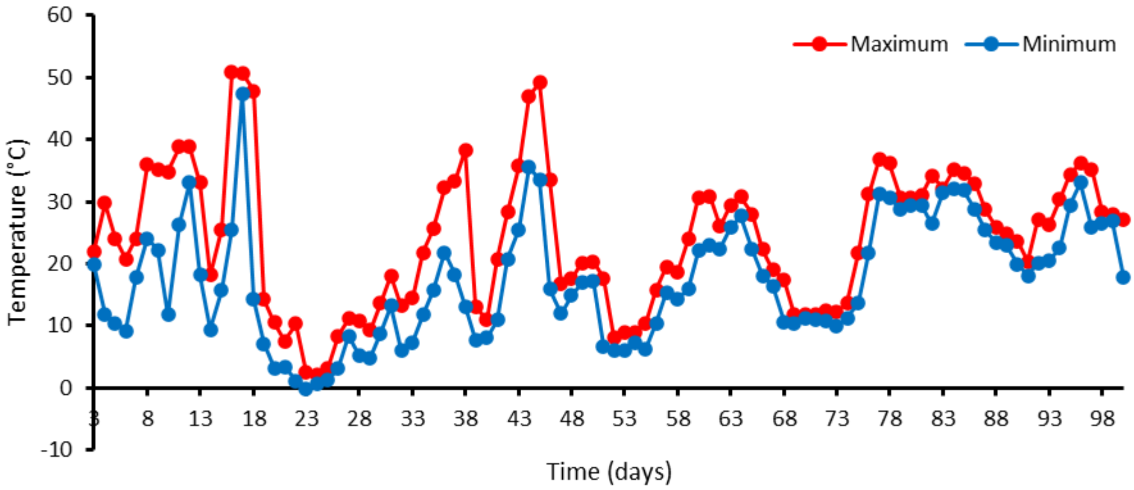

Figure 3.2: *Maximum and minimum temperatures of the centre position (~40cm depth) across the composting period.*

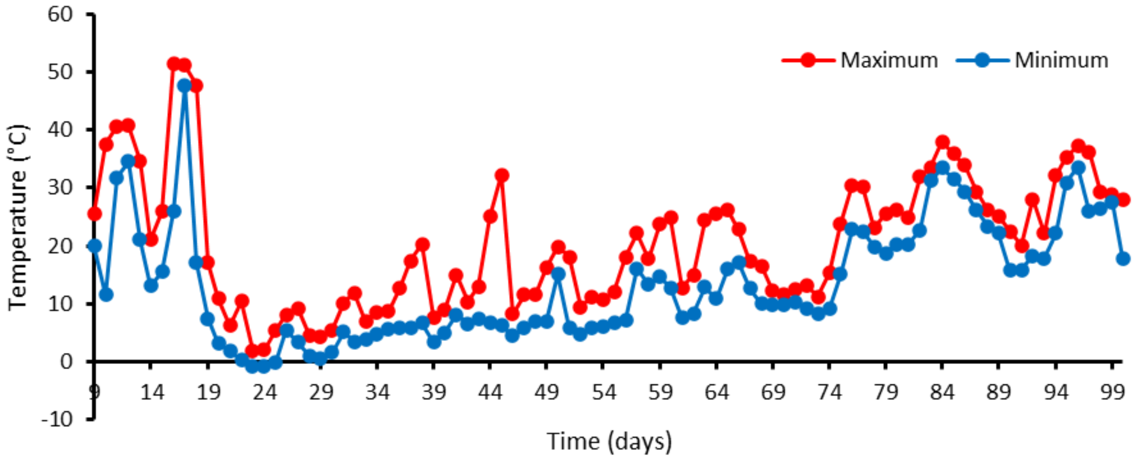

Figure 3.3: *Maximum and minimum temperatures of the intermediate position (~20cm depth) across the composting period.*

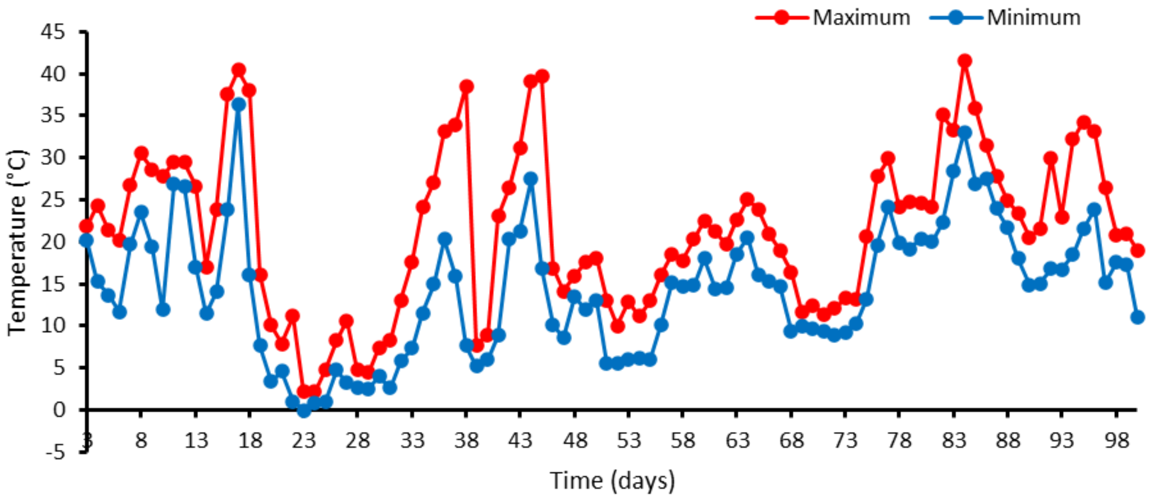

Figure 3.4: *Maximum and minimum temperatures of the outer position (10cm depth) across the composting period.*

Minimum temperatures of the positions and the minimum ambient temperature were also plotted (Figure 3.5). The minimum temperatures of all positions within the compost heap were still significantly higher (p<0.05) than the minimum ambient temperatures. The temperature increase for the centre and outer positions on day 36 occurred whilst ambient temperatures remained below 5°C, and the much larger increase on day 44 for these positions was accompanied by a significantly far lower temperature peak for the ambient temperature, at 6°C (p<0.01). The ambient temperature remained stable over the period of days 58-67, whilst the centre and outer positions rose in temperature. Considering the temperatures across the composting period, the averages of the minimum temperatures of each position and those of the minimum ambient temperatures were all significantly different (p<0.001). As with the maximum temperatures, there were positive relationships between the minimum temperatures of each compost heap position and the minimum ambient temperatures (r =0.34, $F_{1,96}$=49.36, p<0.001; r =0.50, $F_{1,90}$=91.65, p<0.001; and r =0.33, $F_{1,96}$=46.73, p<0.001 for centre, intermediate and outer, respectively).

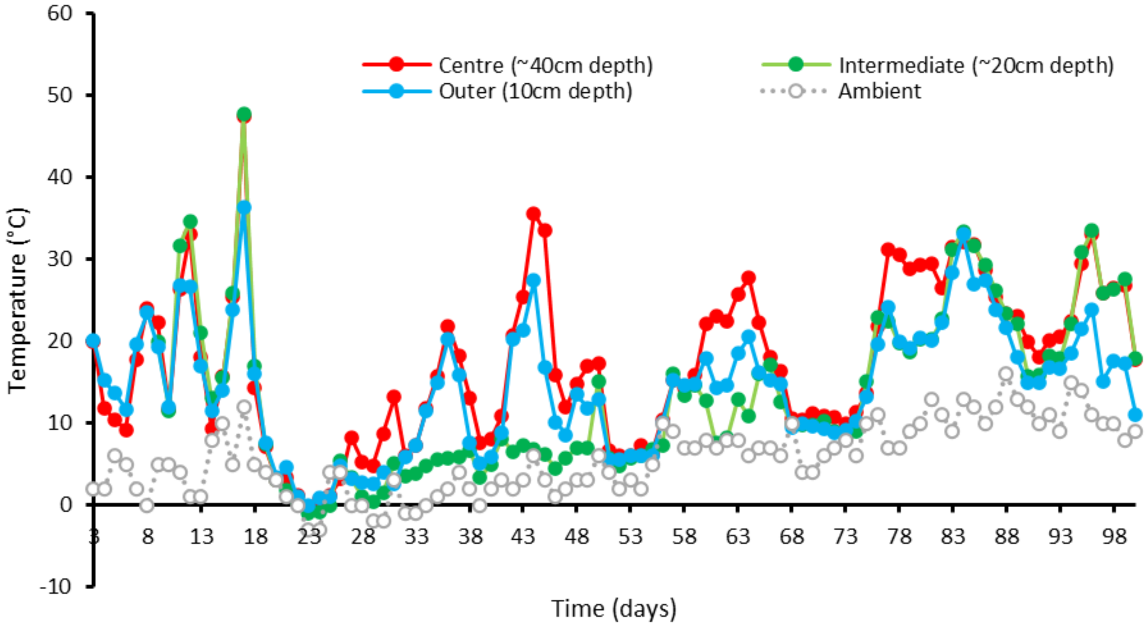

Figure 3.5: *Minimum temperatures of the different positions and minimum ambient temperatures across the composting period.*

3.4 DISCUSSION

3.4.1 The effect of ambient temperature on composting temperatures

It is clear from the data that there are obvious shared patterns between the maximum ambient temperatures and the temperatures of the different composting positions, particularly as there is a delay (often of 24 hours) between the pattern being seen in ambient and the pattern being replicated in composting temperatures (Figure 3.1). Furthermore, it was determined that these shared patterns are actually significant for each position, and so it can be assumed that the ambient temperature was having some level of effect on the composting temperatures seen in this study. Nevertheless, the potential influence is not likely to have been too extensive based on the regressions seen, and there will have been many other factors contributing to the temperatures of the compost, of which metabolic activity of the bacterial community would have very likely been a large contributor. This is reflected in the data also, with the patterns for the temperatures of the different composting positions being found much higher than that for the ambient temperature (Figure 3.1). This contribution of metabolic activity rather than environmental factors is indicated with the outer position in the first few weeks of the composting period especially; ambient temperatures were low and the weather was overcast, which would have more readily affected the outer position, yet higher temperatures were still achieved. The probable contribution is also indicated when comparing maximum and minimum temperatures of both the centre and outer positions (Figures 3.2 and 3.4). The patterns of these two temperature measurements are identical, and often without a large difference between them, whereas no real pattern is discernible overall for minimum ambient temperatures besides natural fluctuations. Conversely, higher temperatures achieved by the outer position towards the end of the composting period were probably more affected by ambient temperatures and the heat of the Sun on the compost heap, given the position's tendency to reach higher or comparable temperatures to the centre position despite its shallowness. It is not unusual, however, for the composting temperatures to have been impacted by the ambient temperatures; it was reported by Huang *et al.* (2005) that such an influence occurs in sewage sludge composting also, and likewise accompanied by a "lag" between the ambient and composting temperatures, as seen in the current study (although, the delay in their study could be measured in hours, which the probes would not have been able to detect in the setting used in this study). A conclusion can be drawn, then, that composting would benefit greatly from being carried out during warmer months in terms of promoting higher composting temperatures. Whilst the ambient temperature seemingly affected all positions, regressions indicate that it may have done so more for the intermediate and outer positions' temperatures than the centre, which is very probable as these depths were in closer proximity to the external environment.

3.4.2 The effects of the temperatures of the different composting positions on one another.

Interactions between the temperatures of the different positions in the compost heap can be difficult to ascertain, even if significant regressions were found; whilst it is true that the temperatures recorded displayed patterns that were more or less analogous overall (Figure 3.1), this is now known to have likely been dictated by maximum ambient temperature. Nevertheless, it is also unlikely for there to have been absolutely no impact of one position's temperature on another. Following this, as significant regressions were found between each position, the most likely occurrence is that the metabolically-originated heat from the centre position spread outwards to affect the temperatures of the intermediate and outer positions. However, the potential relationship between the intermediate and outer positions may work two ways; any metabolic heat managed to be generated at the intermediate position may have impacted the outer position, or the ambient temperatures from the environment may have reached the intermediate position via the outer. The only study that can be found that also features temperatures at different depths of a compost heap is that by Avnimelech *et al.* (2004). The highest temperatures were reached by depths of 30cm and 50cm, comparable to the highest temperatures being reached by our centre (40cm) and intermediate (20cm) positions, although their 10cm depth measurements (corresponding to the outer position in this study) was found at a far lower temperature than the other depths, unlike in this study.

3.4.3 Abnormality of the temperatures during the composting period.

The temperatures recorded for all positions do not resemble those that would expect to be found during a composting process. There was no extended, single thermophilic phase, and increases in temperature were found periodically throughout the composting period. It appears that temperature began to build up shortly after the assembly of the compost heap (albeit with disturbances), before a true thermophilic phase arises around at around two weeks, before ending prematurely and entering what could be considered as cooling and then maturation phases (Figure 3.1). A remarkably similar pattern was found within the first two weeks in the study by Cui *et al.* (2016), albeit this short thermophilic phase was not discussed in the paper (although the low temperatures achieved in this study were attributed to composting heap size, and so that may have potentially had some impact). This is in comparison to the patterns and thermophilic phases exhibited in other studies featuring the composting of chicken manure; temperatures of at least 55°C were frequently achieved and maintained for a period of, at minimum, 2 weeks, with temperatures exceeding this (>60°C) in many cases (Xie *et al.,* 2016; Peng *et al.,* 2018; Zhang *et al.,* 2018; Esperón *et al.,* 2020). Even in the instance of a thermophilic phase not being sustained beyond two days, as in Li et al. (2017)'s study, far greater (>60°C) temperatures were still recorded than those in the present study.

It is possible that the reason for the abnormalities seen in the temperature data in this study was lack of sufficient aeration of the composting pile, and in turn the effect this had on temperature and microbial populations. Both oxygen availability and temperature are noted to be self-limiting factors if not mitigated against successfully. Firstly, if temperatures are enabled to reach a high enough level due to lack of aeration, death of the active microbes generating the heat can occur, then followed by a temperature decline from lack of activity. Secondly, oxygen is greatly required and its consumption sharply increases following the onset of the composting process; insufficient aeration, again, and resulting lack of repletion of oxygen can cause the anaerobic conditions to develop and deplete the levels of active aerobic microorganisms, leading to a decline in temperature. Indeed, this is shown in studies where temperatures at deeper levels of compost actually possess lower temperatures than 'intermediate' levels, which was reasoned as being due to insufficient oxygen levels, and where microaerophilic composts experience sharper temperature declines than those with higher oxygen (MacGregor *et al.,* 1981; Tiquia *et al.,* 1996; Avnimelech *et al.,* 2004; Wang *et al.*, 2007; Qasim *et al.,* 2018). Furthermore, in another chicken litter composting study that was also likely to have experienced the development of anaerobic conditions (as there was no mention of aeration techniques and piles were covered with a "non-breathable" material), it can be observed that temperatures above 50°C were held for only 32 hours before beginning to decrease (also mirroring the short thermophilic phase seen in this study) (Macklin *et al.,* 2006). As it is highly unlikely that the sharp temperature decline (Figure 3.1) that occurred within the first three weeks of composting was due to excessive temperature (thermophiles would have thrived and been active at this point), it can be determined that it was potentially due to oxygen levels having been largely depleted from lack of aeration, and the subsequent death of aerobic thermophiles. The heat generated after this point would likely have been produced by anaerobic bacteria. It may also be presumed that these were mesophiles, and that no anaerobic thermophiles were found in the pile, as the sharp temperature decrease (Figure 3.1) occurring towards the middle of the composting period may have been due to the death of these mesophiles as temperatures became unfavourable (if anaerobic thermophiles were present, the temperature would have been able to rise further). The fact that the temperatures of the intermediate and outer positions drop at this point (Figure 3.1) regardless of whether the peak temperature preceding was suitable for mesophiles or not (such as with the intermediate temperature, which only reached 32°C) may very well be attributed to the fact that the temperatures seen at these positions were heavily provided by, and dependent on, the temperature of the centre position.

3.5 CONCLUSION.

From this study, it can be determined that the external, ambient temperature has a role to some level in the temperatures reached and temperature patterns experienced by a chicken litter compost pile. To further elucidate, visualise and analyse this impact, it would be useful to perform a time lag correlation analysis on the temperature data gathered. It can also be determined that whilst patterns may be shared, the temperatures reached by the different positions and depths of a chicken litter compost pile at a given time can be different, and that the temperatures of intermediate and outer layers of a compost heap are likely to be derived at some level from the temperature generated at the centre. Insight was potentially provided into the importance of sufficient aeration during the composting process and what temperature measurements may ensue if the aeration needs are not met, as well as why these measurements may be occurring, if it is assumed that the results of this study were seen due to lack of sufficient oxygen levels.

4 ANTIBIOTIC RESISTANCE IN CHICKEN LITTER AND CHICKEN LITTER COMPOST

4.1 INTRODUCTION

Antibiotic resistance in poultry can be estimated as being virtually ubiquitous, and is found in farms across the globe. Furthermore, whilst it is well-understood that highly intensive and large-scale poultry-rearing operations can lead to the emergence and growth of antibiotic resistance due to the proximity of birds, ease of spread of disease (and thus also presumably of resistant bacteria), and widespread administration of antibiotics, instances of antibiotic resistance can be found in a variety of farms with very different rearing practices. This includes farms described as commercial and "semi-industrial", to those of integrated production systems, and even those that are free-range, small-scale and household-based. It can also be found regardless of antibiotic use (Miles *et al.,* 2006; Padungtod *et al.,* 2006; Diarrassouba *et al.,* 2007; Lu *et al.,* 2010; Furtula *et al.,* 2010; Lei *et al.,* 2010; Ozaki *et al.,* 2011; Olonitola *et al.,* 2015; Nguyen *et al.,* 2016; Roth *et al.*, 2019).

The increasing understanding of the importance of antimicrobial resistance in an agricultural and veterinary environment has led to decreased overall antibiotic usage in the UK for chickens reared for meat, with a -69% change in total antibiotic usage between 2014 and 2019, although sulfonamides have had little decrease in use, experiencing just a -1% difference in usage in the same period. This decrease in general use is not extended to laying hens, with a total increase of 3% between 2016 and 2019. Antibiotic classes accounting for the increase were aminoglycosides, macrolides and tetracyclines, at rates of increase of 100%, 40% and 57%, respectively (Veterinary Medicines Directorate, 2020). Nevertheless, it cannot be denied that steps are being taken to slow the progression of antibiotic resistance by the generally reduced use of antibiotics, and indeed the only feed additives authorised for use in the UK are coccidiostats and non-antibiotic growth promoters (Veterinary Medicines Directorate, n.d.).

However, this attempt to sway antibiotic resistance is not reflected in the data (Veterinary Medicines Directorate, 2020). Resistance to all of the antibiotics included in the surveillance exhibited an increase between 2017 and 2019, with the exception of one, neomycin. High rates of resistance were seen to ampicillin, doxycycline and tetracycline in particular, with over 40% of isolates resistant to these three antibiotics in 2019. This, in conjunction with the fact that antibiotic resistance can still be found at high levels in an antibiotic-free environment (Diarra *et al.,* 2007; Ozaki *et al.,* 2011), indicates that the ways to halt the progression of antibiotic resistance in agriculture may still not be fully understood, or that other factors may need to be modified, such as rearing practices. It also indicates that attention should be paid to operations outside of just commercial/industrial settings, as there may be many categories of poultry-rearing operations being overlooked (particularly in the UK) that could potentially harbour extreme resistance levels. Furthermore, by focusing solely on *E. coli* and other pathogenic bacteria (e.g., *Salmonella* and *Campylobacter* species), the true extent of resistance of commensal enteric bacteria from chickens may not be fully realised.

As such, the objectives of this study were:

1. To characterise the percentage resistance, resistance profiles, and number of resistances of chicken litter and chicken litter compost enteric isolates derived from chickens in a small-scale, non-commercial, antibiotic-free operation.
2. To determine how the depth within a compost heap from which the enteric bacteria are isolated may change the resistances observed.
3. To determine how the lactose-fermenting ability of the enteric isolates may change the resistances observed.

4.2 MATERIALS AND METHODS

4.2.1 Antimicrobial susceptibility testing

Six colonies were selected from across the aforementioned (section 2.1) MacConkey No. 2 agar plates for each of the three depths. Half of the colonies chosen were lactose-positive, and half lactose-negative, where possible in an attempt to broaden the representation of phenotypic resistances present. Selected colonies were streaked onto LB Broth with agar (Lennox; Sigma-Aldrich) plates, and incubated for 24 hours at 37°C (for a total of 18 isolates at each sampling point). Any isolates that had not grown sufficiently were incubated for a further 24 hours at the same temperature. Three to four colonies of each isolate were suspended in 2ml of MRD, to reach a turbidity of 0.5 McFarland. Using a sterile cotton swab, the suspension was then spread onto 150mm and 90mm Mueller-Hinton agar (Oxoid) plates (a larger and smaller plate for each isolate). All antibiotic discs used were Oxoid, and the following were applied to the 150mm plates with a disc dispenser (Thermo Fisher Scientific): ampicillin 10µg (AMP), ciprofloxacin 5µg (CIP), amoxicillin/clavulanic acid 30µg (AMC), trimethoprim/sulfamethoxazole 25µg (SXT), cefotaxime 5µg (CTX), tigecycline 15µg (TGC), azithromycin 15µg (AZM), gentamicin 10µg (CN), enrofloxacin 5µg (ENR), nitrofurantoin 300µg (F), levofloxacin 5µg (LEV), and nalidixic acid 30µg (NA). These antibiotics were chosen as they cover several antibiotic classes (β-lactams, macrolides, fluoroquinolones and quinolones, aminoglycosides, nitrofurans, tetracyclines, and sulfonamides), and antibiotics of varying potential potencies. Three further antibiotic discs were applied to the 90mm plates by hand: oxytetracycline 30µg (OT), streptomycin 10µg (S), and chloramphenicol 30µg (C). All plates were incubated at 37°C for 24 hours. After incubation, the zones of inhibition were measured and recorded.

4.2.2 Data processing

The zone of inhibition measurements from the antimicrobial susceptibility tests were entered into Microsoft Excel 2019. EUCAST breakpoints for Enterobacterales were used to determine whether isolates possessed susceptibility, intermediate resistance, or resistance to a given antibiotic, as enteric bacteria were selected regardless of potential identity, and not all isolates were identified. The three phenotypes were given a numerical value to enable calculations and statistical analyses: 1 for susceptibility, 2 for intermediate resistance, and 3 for resistance. Resistance 'scores' were calculated using these values, wherein a result of 15 indicated susceptibility to all antibiotics (as 15 antibiotics were used in the ASTs) and a result of 45 indicated full resistance to all antibiotics. Resistance profiles were identified and enumerated. Averages, the numbers of resistances possessed by collections of isolates, the percentage of antibiotics to which isolates were resistant, and percentage resistances of isolates were also calculated in Microsoft Excel 2019.

4.2.3 Statistical analyses

Student's t-tests were executed in Microsoft Excel 2019 to analyse the various parameters of the AST data, to determine the presence of significant differences between data of different positions and lactose-fermenting ability. Pearson's correlation was carried out in Genstat 21.1 (VSN International, Ltd.) to analyse the resistance score data, including every antibiotic, and a two-sided test of the correlations against 0 was used to determine significance. Data from day 44 was excluded from this analysis due to the uncharacteristically low resistances seen at this sampling point.

4.3 RESULTS

4.3.1 Number of antibiotic resistances of the isolates

Data from the chicken litter and its compost from across the composting period were analysed to explore the level and nature of resistances present. Data were utilised from both fresh litter and litter from the compost heap as it was rationalised that any resistances developing throughout the composting process would have already been present in the fresh litter. For all the isolates combined, the number of resistances detected spanned from 0 (susceptible) to 11 of the 15 antibiotics tested. The most common number of resistances possessed was 6, representing 21% of the isolates. Less than 2% of the isolates tested were susceptible to all of the antibiotics, whilst 1.4% (3 isolates of 221) were resistant to 11 of the 15 antibiotics (Figure 4.1).

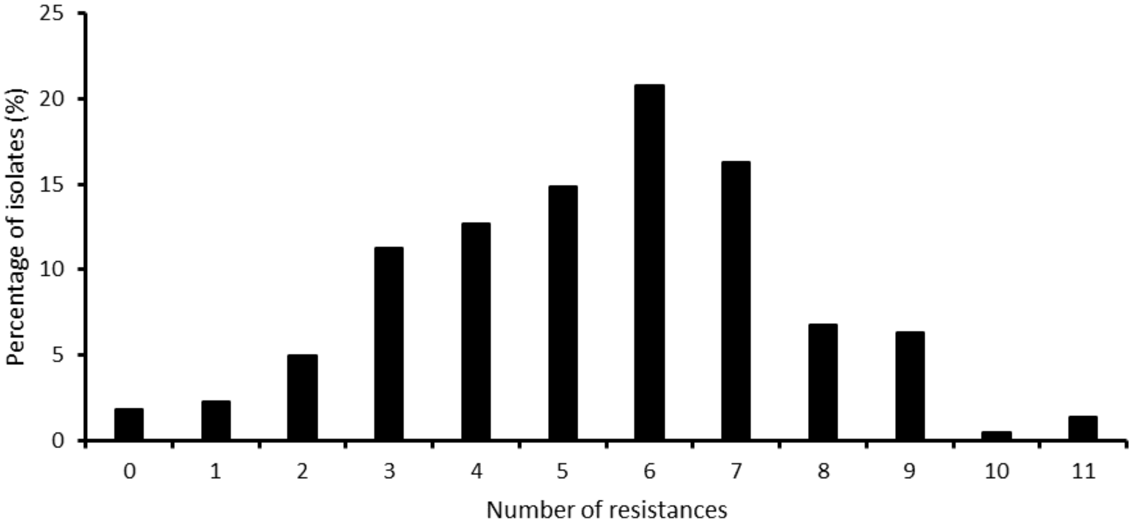

Figure 4.1: *The number of antibiotic resistances of total chicken litter and compost isolates.*

The most common number of resistances recorded for isolates from the centre, intermediate and outer positions of the compost heap were 7 (18.9%), 6 (22.1%) and 6 (25.4%), respectively. The single isolate resistant to 10 antibiotics was isolated from the centre of the heap, and those resistant to 11 antibiotics were isolated from the centre (2 isolates) and intermediate (1 isolate) positions (Figure 4.2).

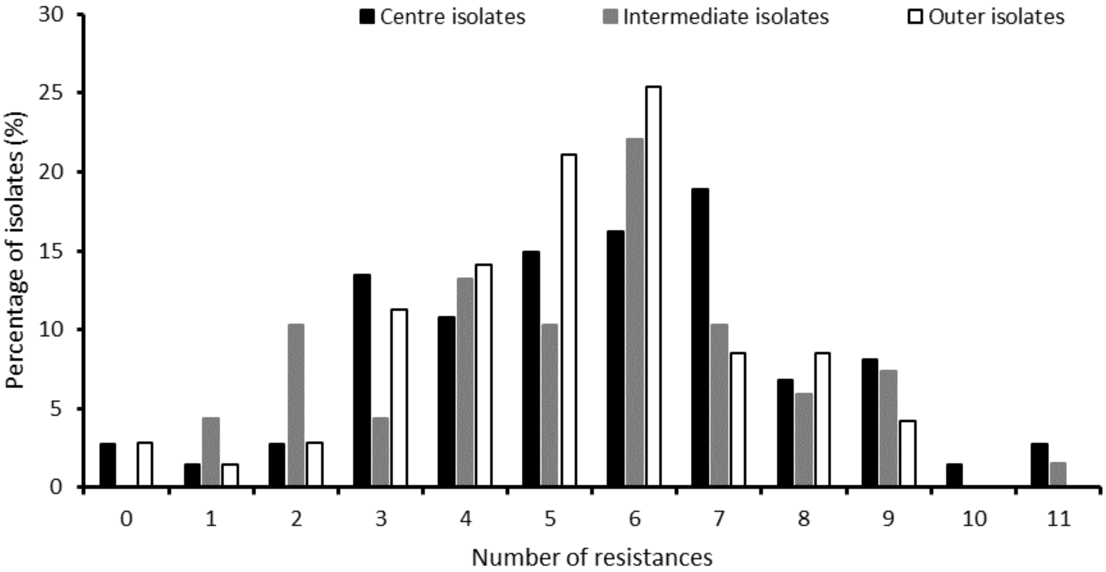

Figure 4.2: *The number of antibiotic resistances of chicken litter and compost isolates separated by position (~40cm centre, ~20cm intermediate, and 10cm outer).*

The most common number of resistances for lactose-positive isolates was 3 (18.8%), and 6 (26.2%) for lactose-negative isolates. In lactose-positive isolates, one isolate was resistant to 11 antibiotics (0.9%). In lactose-negative isolates, one isolate was resistant to 10 (1%) and two isolates resistant to 11 (1.9%) antibiotics (Figure 4.3). The isolates resistant to 11 antibiotics were isolated from samples from days 51 and 65, and the isolate resistant to 10 from day 81. Susceptible isolates were found on days 5, 36, and 44.

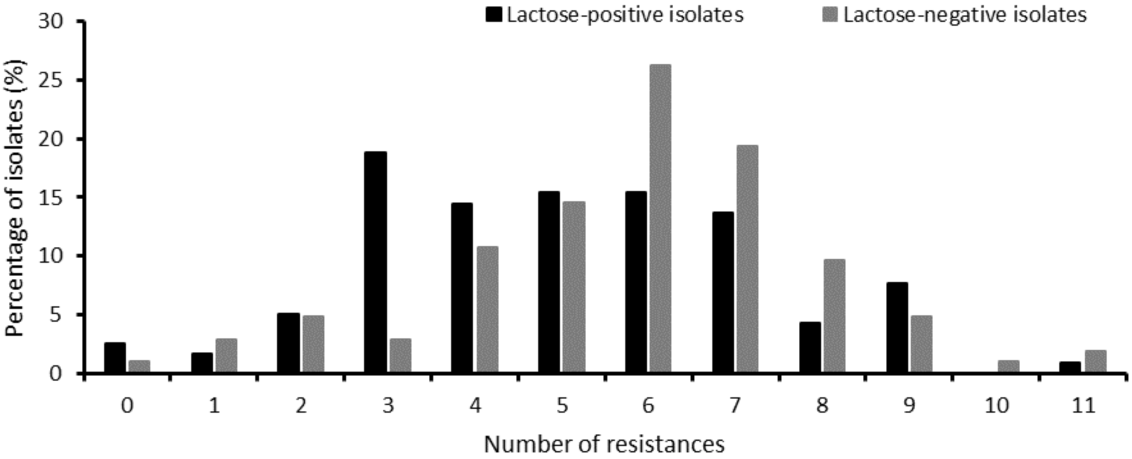

Figure 4.3: *The number of antibiotic resistances of chicken litter and compost isolates separated by their ability to ferment lactose.*

4.3.2 Percentage antibiotic resistances of the isolates

The percentage of total isolates (n=221) resistant to ampicillin, gentamicin, azithromycin, and nitrofurantoin were above 50%, with gentamicin resistance being the highest (70.6% of isolates). For some resistances, namely those to the fluoroquinolones (ciprofloxacin, enrofloxacin, and levofloxacin), intermediate resistance was more often, or exclusively, seen rather than full resistance (Figure 4.4).

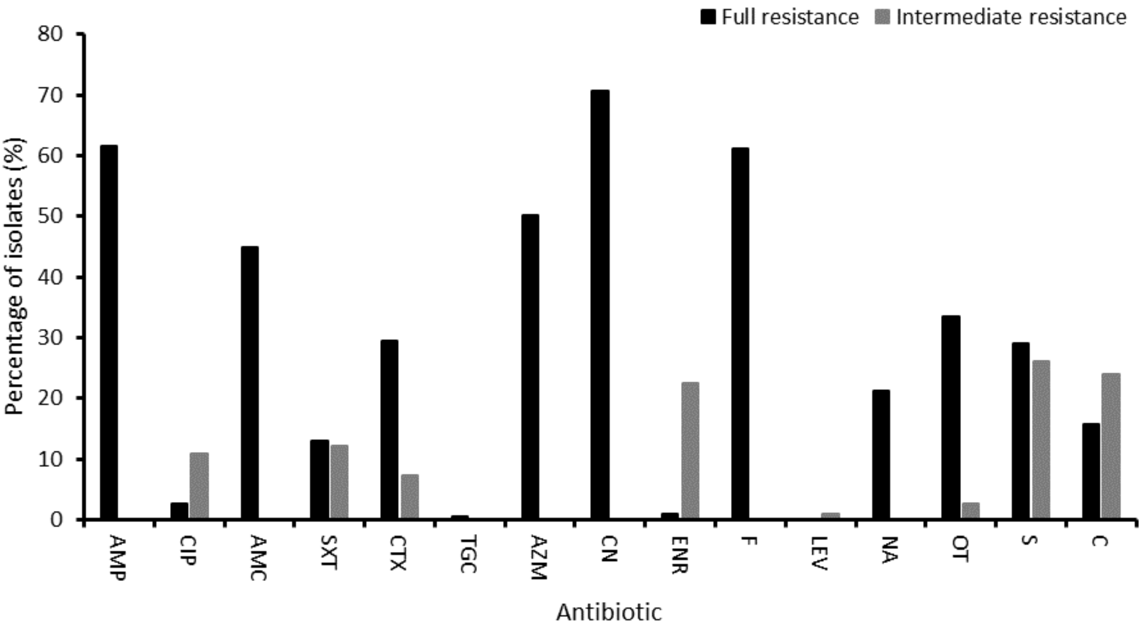

Figure 4.4: *The percentage full and intermediate antibiotic resistances of total chicken litter and compost isolates.*

There were no drastic differences between the different positions of the compost heap in terms of which position's isolates contributed most to full resistance. Where there appeared to be some difference, such as between the centre and outer positions for streptomycin and oxytetracycline, it was not significant (Figure 4.5). Whilst there appeared to be large differences between the positions for contribution to intermediate resistances for a number of antibiotics, they were also non-significant (Figure 4.6).

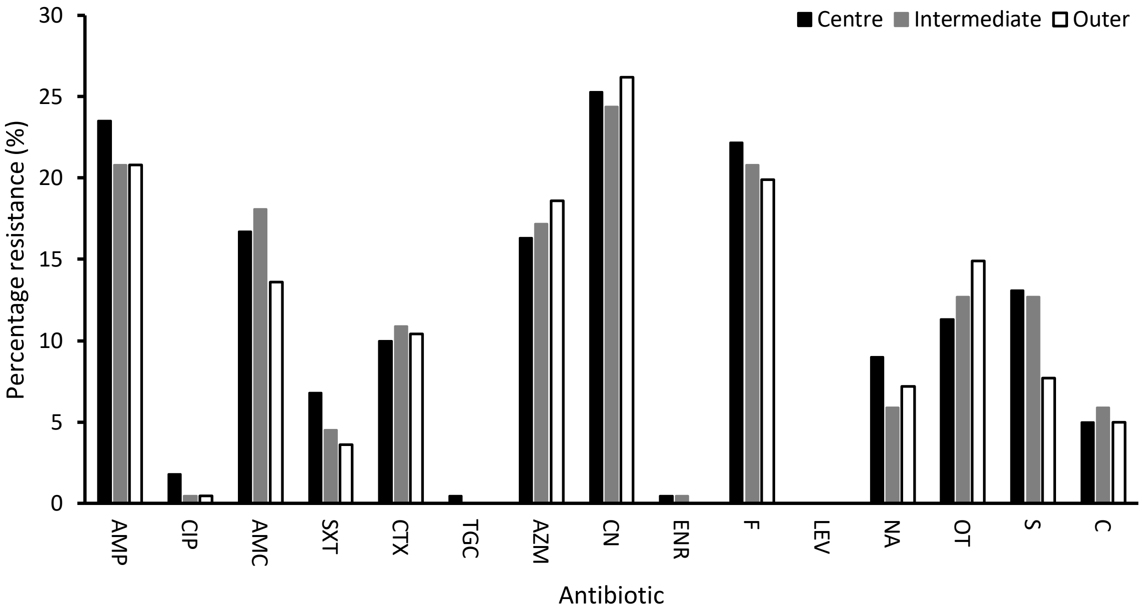

Figure 4.5: *The percentage full antibiotic resistances of chicken litter and compost isolates separated by position (~40cm centre, ~20cm intermediate, and 10cm outer).*

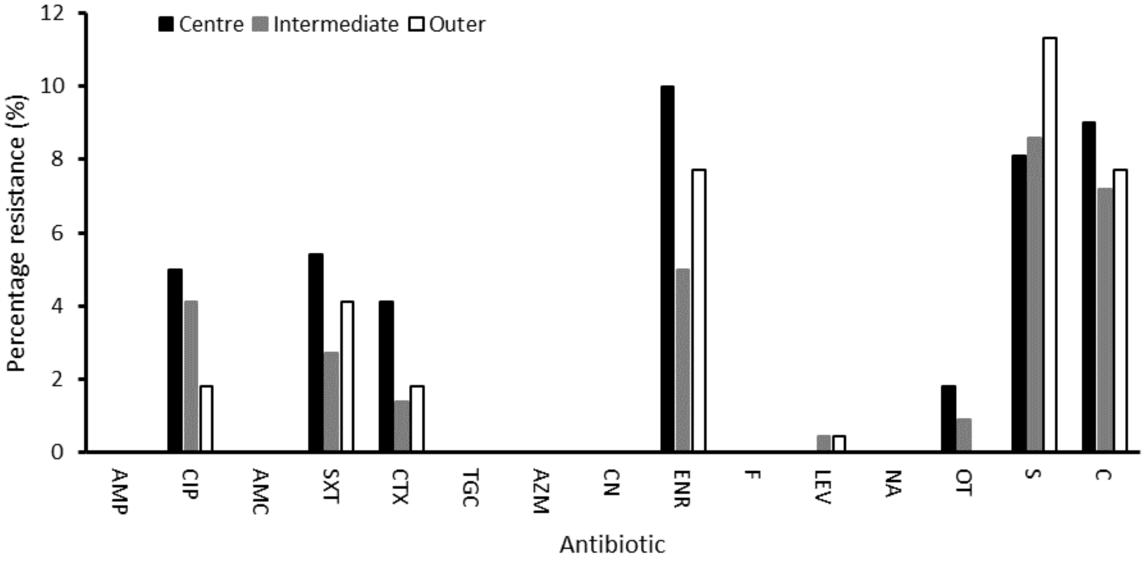

Figure 4.6: *The percentage intermediate antibiotic resistances of chicken litter and compost isolates separated by position (~40cm centre, ~20cm intermediate, and 10cm outer).*

The percentage of lactose-positive isolates resistant to azithromycin, gentamicin, oxytetracycline and streptomycin was significantly higher than the percentage of lactose-negative isolates ($p<0.001$). The percentage of lactose-negative isolates fully resistant to nitrofurantoin and cefotaxime, on the other hand, was significantly greater than lactose-positives ($p<0.001$) (Figure 4.7).

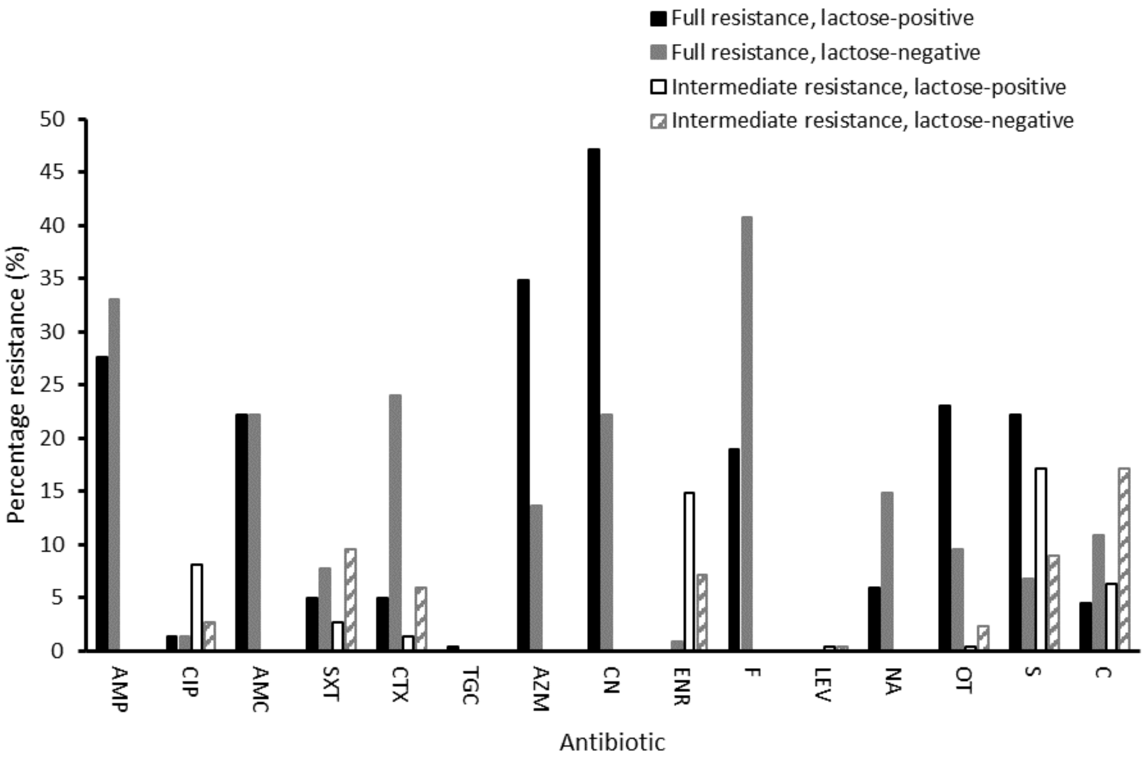

Figure 4.7: *The percentage full and intermediate antibiotic resistances of chicken litter and compost isolates separated by ability to ferment lactose.*

4.3.3 Antibiotic resistance profiles of the isolates

A total of 114 different antibiotic resistance profiles were determined from the data for the total isolates. The majority of the profiles were unique to one isolate (78 isolates, 68.4% of profiles recorded). Just 12 profiles were associated with four or more isolates. The profile representing the most isolates was CN-OT-S (17 isolates), followed by AZM-CN-OT-S (9), AMP-CIP-AMC-AZM-CN-ENR-S (9), AMP-SXT-CTX-CN-F-C (8), and AMP-AMC-CTX-F-NA-C (8). The aforementioned single isolate resistant to 10 antibiotics had the profile AMP-AMC-SXT-CTX-AZM-ENR-F-NA-S-C. The three isolates resistant to 11 antibiotics all had separate profiles (AMP-CIP-CTX-AZM-CN-ENR-F-LEV-NA-OT-S, AMP-AMC-SXT-CTX-AZM-CN-ENR-F-NA-OT-S, and AMP-CIP-AMC-SXT-CTX-CN-ENR-F-NA-S-C).

Isolates with the antibiotic resistance profile CN-OT-S were all lactose-positive, and were isolated largely from the centre position of the compost heap (8 isolates, compared to 4 for the outer and 1 for the intermediate positions). The AZM-CN-OT-S profile largely belonged to lactose-positive isolates (8 compared to one for lactose-negatives), and was found only in the intermediate and outer positions. AMP-CIP-AMC-AZM-CN-ENR-S belonged mostly to centre position isolates (6, with 2 for intermediate and one for outer) and those which were lactose-positive (8, with one being lactose-negative). Conversely, AMP-SXT-CTX-CN-F-C was mainly attributed to lactose-negative isolates (7, compared to one for lactose-positives). This profile was found across all positions, but most commonly from outer (4). Similarly, AMP-AMC-CTX-F-NA-C belongs mainly to lactose-negative isolates (7, with one being lactose- positive), although it was found most commonly in the intermediate position (5, with 2 from outer and one from centre).

4.3.4 Correlations between the antibiotic resistances

Correlations between the resistance scores for each antibiotic were performed. Significant positive correlations of particular note due to the strength of their relationship were amoxycillin/clavulanic acid and ampicillin (r=0.40, p<0.001), ampicillin and cefotaxime (r=0.54, p<0.001), azithromycin and gentamicin (r=0.43, p<0.001), chloramphenicol and nitrofurantoin (r=0.58, p<0.001), ciprofloxacin and enrofloxacin (r=0.62, p<0.001), cefotaxime and nitrofurantoin (r=0.49, p<0.001), cefotaxime and nalidixic acid (r=0.46, p<0.001), and gentamicin and oxytetracycline (r=0.41, p<0.001). Those of significant negative correlations were ampicillin and oxytetracycline (r=-0.61, p<0.001), azithromycin and cefotaxime (r=-0.52, p<0.001), cefotaxime and gentamicin (r=-0.63, p<0.001), cefotaxime and oxytetracycline (r=-0.48, p<0.001), cefotaxime and streptomycin (r=-0.45, p<0.001), gentamicin and nalidixic acid (r=-0.53, p<0.001), and nitrofurantoin and streptomycin (r=-0.49, p<0.001).

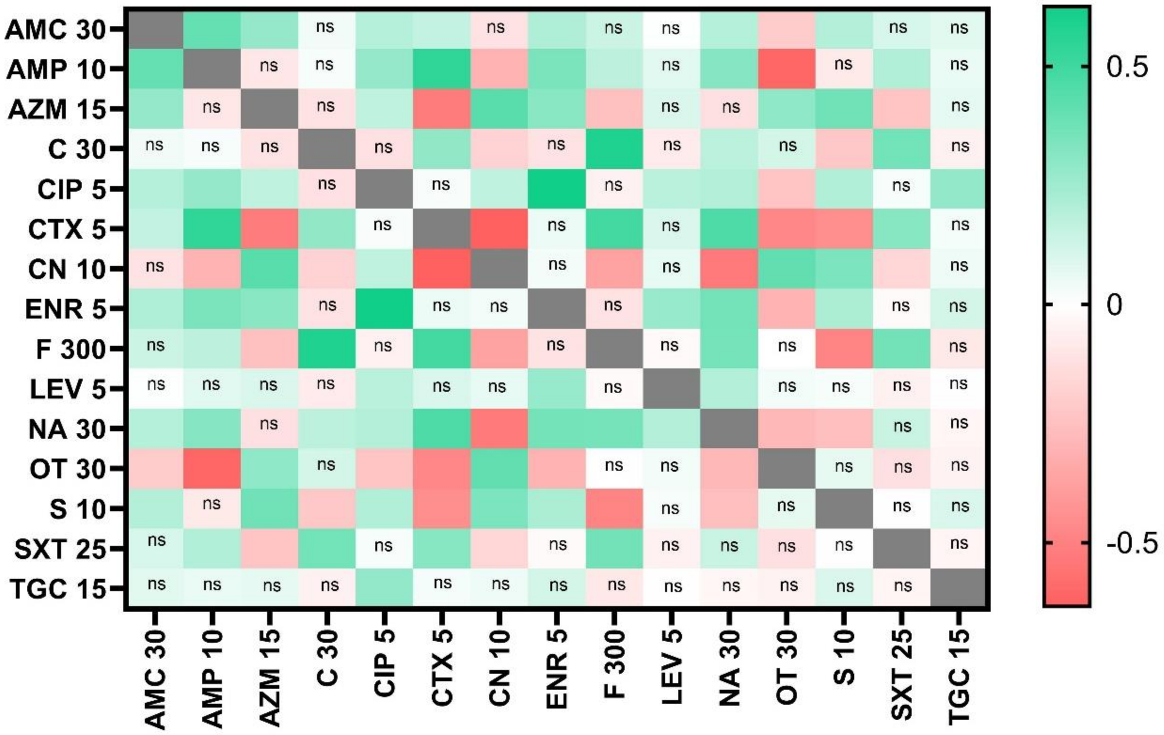

Figure 4.8: *Correlations between the tested antibiotic resistances of the isolates, as determined through Pearson's correlation analysis. Non-significance (p >0.05) is indicated with the text 'ns'.*

4.4 DISCUSSION

4.4.1 The antibiotic resistances of the isolates

High and moderate levels of resistance were found to many of the antibiotics tested in this study (Figure 4.4). Compared to another study with data on UK chickens (Bywater *et al.*, 2004), full ampicillin, cefotaxime, chloramphenicol, and gentamicin resistance were all found at higher levels in the current study, when compared with the *E. coli* isolates in Bywater *et al.*'s (2004) study. For some antibiotic resistances this difference is quite extensive, such as with cefotaxime (where no resistance was found in their study, compared to 29.4% resistance in the current one) and gentamicin (3% compared to 20.6%). This trend remains true when comparing against their *Salmonella* isolates also, but not with the *Campylobacter* isolates; ciprofloxacin resistance was higher in the *Campylobacter* isolates (6%) than it was for the isolates in this study (2.7%). Instances of the isolates in the present study possessing lower resistances also occurred, as trimethoprim/sulfamethoxazole resistance was considerably higher for both their *E. coli* and *Salmonella* isolates (46.5% and 25.6%, respectively, compared to 13.1%), and streptomycin resistance was higher with *E. coli* isolates (41.5% compared to 29%), albeit not with *Salmonella* isolates (14%). Nalidixic acid was included for their *Campylobacter* isolates only, where resistance was much lower than what was reported with the current study's isolates (6.5% compared to 21.3%). When observing these differences in results, however, it must also be considered that these data are now over a decade old, and so it is likely that bacterial resistance to certain antibiotics has increased in general since this time in UK chickens. Indeed, comparing government reports from 2004 and 2020, the majority of the resistances that both reports feature have increased between these years for *E. coli.* (Communicable Disease Surveillance Centre Northern Ireland *et al.,* 2004; Veterinary Medicines Directorate, 2020). Furthermore, it was also not specified by Bywater *et al.* (2004) what the chickens were fed, and so if antimicrobials were present in feed, this may have also impacted their data.

To compare results with studies that also utilised chickens on an antibiotic-free diet, data must be considered from outside of the UK. The ampicillin resistance found in this study's isolates is comparable to that of Diarra *et al.*'s (2007) *E. coli* isolates (61.5% and ~64%, respectively). This is where the similarities end however, as sulfamethoxazole, gentamicin and enrofloxacin resistance were all lower, whilst oxytetracycline, streptomycin and chloramphenicol were higher. Few similarities were found with the *E. coli* studied by Miles *et al.* (2006) either, with only full ciprofloxacin resistance being very loosely comparable (2.7% in the current isolates, 8.8% in their isolates), as well as intermediate enrofloxacin resistance (22.6% and 38.2%). In the study by Rehman *et al.* (2018), the streptomycin resistance found for *Enterococcus faecium* isolates (28.6%) is reflective of that found here (29%). The only study to feature azithromycin was by Padungtod *et al.* (2006), who found that 5.8% of their *Campylobacter* isolates were resistant, which was much lower than observed here (50.2%). No data for levofloxacin were available from chickens confirmed as being raised on an antibiotic-free diet. However, Lei *et al.* (2010) reported 51.3% of their *E. coli* isolates were resistant to levofloxacin, whereas no full resistance was found in isolates here. van den Bogaard *et al.* (2001) did not observe any resistance to nitrofurantoin for either broilers or laying hens, in opposition to the high resistance in this study (61.1%).

Multiple papers utilising antibiotic-free chickens included information on the numbers of resistances that isolates possessed, but only one had data comparable to what was found for this study, as the others had isolates that were much less multidrug-resistant (Miles *et al.,* 2006; Padungtod *et al.,* 2006). This was the study conducted by Ozaki *et al.* (2011), where isolates from chickens (those of the oldest age sampled) from antibiotic-free farms were most resistant to 5, 2, and 6 antibiotics (in our study, it was 6). Another study had information on multi-resistance in their isolates, with results that were somewhat similar to this study's (the most common number of resistances in their broiler isolates was 5 or above), but it is not known if the chickens were antibiotic-free (van den Bogaard *et* *al.,* 2001).

In regards to the role that metals may have played in the resistances seen in these studies involving chickens on antibiotic-free diets through the occurrence of possible co-selection, it sadly cannot be reliably determined due to the lack of complete information on feed that was provided to the animals. Many studies only provided information on the antibiotics used in addition to regular diet in the non-antibiotic-free groups. In the one study that does provide information on feed composition (Diarra *et al*, 2007) however, including the presence of various metals, it appears these were of a required supplemental nature, similar to this study.

The potential reasons for why most of the data in this study does not correspond to data in others are numerous. Not only are the studies separate temporally, geographically, and by using different rearing practices, but admittedly the distribution and levels of resistant bacteria in our study's samples have been modified by composting. However, data on phenotypic antibiotic resistance exists only in studies using fresh faeces, as unfortunately very few composting studies focus on phenotypic resistance, and so only these studies could be used for comparison.

4.4.2 The difference in antibiotic resistance between lactose-positive and lactose-negative isolates

The percentage of isolates resistant to certain antibiotics, namely azithromycin, gentamicin, nitrofurantoin, cefotaxime, streptomycin, and oxytetracycline, was greatly polarised by the ability of the isolate to ferment lactose. As this could be linked to the identities of the lactose-positive and lactose-negative bacteria, literature was evaluated for commonly-seen bacteria (which were capable of growth on MacConkey No. 3 agar) of chicken faeces, compost, and the lower gastrointestinal tract. Remaining were the genera *Psychrobacter, Ignatzschineria, Pseudomonas, Escherichia* (most likely referring to *E. coli*) and *Shigella* (Gong *et al.,* 2007; Oakley *et al.,* 2014; Zhang *et al.*, 2018; Subirats *et al.*, 2020; Wan *et al.,* 2021). Of these, only *E. coli* was lactose-positive.

If the lactose-positive isolates in the current study are presumed to largely consist of *E. coli*, the large difference in resistance for azithromycin between lactose-positive and lactose-negative isolates can potentially be explained. Despite azithromycin being considered a more effective option than other macrolides against *E. coli*, it is still stated as being able to have natural resistance, which can be attributed to inefficiency for azithromycin to pass through porins in the outer membrane and the action of efflux pumps (Retsema *et al.,* 1987; Farmer *et al.,* 1992; Stock & Wiedemann, 1999; Gomes *et al.,* 2017). Indeed, studies that review the effects of increasing membrane permeability or disrupting efflux pumps on azithromycin resistance in *E. coli* provide evidence for the importance of these two factors in *E. coli* having some level of natural resistance (Vaara, 1993; Gomes *et al.,* 2019). Therefore, this may be why there is such an extensive and significant difference in azithromycin resistance between the lactose-positive and lactose-negative isolates (again, this is based on presumption only that lactose-positive isolates are largely *E. coli*).

Continuing with the presumption of lactose-positives largely pertaining to *E. coli*, the higher resistance to gentamicin and streptomycin could potentially be explained by a level of intrinsic resistance afforded by the transport and export proteins *sapC* and *secG*, as knockout mutants of these genes were more susceptible to gentamicin and streptomycin (Liu *et al.*, 2010). Similarly, intrinsic resistance to tetracycline may also be afforded in *E. coli* by several efflux transporter genes, as expression of these genes was shown to be increased in resistant *E. coli* compared to susceptible (Viveiros *et al.*, 2005).

The elevated resistance of lactose-positive isolates to these antibiotics may not necessarily be due to identity (or identity alone), however. It may also be due to the presence of plasmids. Studies have described various plasmids that confer not only the ability to ferment lactose, but also the resistance to various antibiotics, including some of those seen in this study. Some of these plasmids were also demonstrated as being able to be transferred to other bacteria, including *E. coli*, *Shigella flexneri*, and *Salmonella* species (Williams Smith & Parsell, 1976; Carey *et al.*, 1983; Walia *et al.*, 1987).

Nitrofurantoin resistance was instead higher in lactose-negative isolates. This may also be explained by the potential identities of the isolates; lactose-negative enteric bacteria such as *Pseudomonas* and *Proteus* species (which whilst not listed previously can also be isolated from chicken faeces) are stated as having intrinsic resistance to nitrofurantoin (Brumfitt & Hamilton-Miller, 1998; Kobashi *et al.*, 2007; Gautam *et al.*, 2021). Cefotaxime resistance can also be intrinsic to *Pseudomonas aeruginosa*, through the action of chromosomally-encoded β-lactamases like AmpC and efflux pumps, and so this may also be why it was found at a higher level in lactose-negative isolates (Lodge *et al.*, 1990; Li *et al.*, 1995; Masuda *et al.*, 2000).

4.4.3 Correlations between the antibiotic resistances

Several pairs of antibiotics had significantly positive correlations; multiple possible reasons for these correlations, such as a shared resistance mechanism, or location of ARGs encoding resistances on the same mobile genetic element.

Observing a correlation between ampicillin and cefotaxime is not surprising, as both antibiotics are β-lactams, and so may share a resistance mechanism/resistance gene, or certain β-lactam resistance genes may commonly be found together. Evidence can be found for the latter case being potentially true for this experiment; the β-lactamase encoding genes bla_{TEM} and $bla_{CTX\text{-}M}$ are repeatedly found to be possessed together in cefotaxime-resistant isolates, from a variety of sources (including British chickens), with one study even finding all isolates to possess both of them (Randall *et al.*, 2011; Korzeniewska & Harnisz, 2013; Mir *et al.*, 2016; Adegoke *et al.*, 2020). $bla_{\text{CTX-M}}$ genes confer resistance to cefotaxime, whilst bla_{TEM} are able to provide resistance to ampicillin (Livermore *et al.*, 1986; Bradford, 2001). Therefore, with the co-occurrence of these β-lactamase genes in many cefotaxime-resistant isolates from various environments, it may be possible that this is why a correlation between ampicillin and cefotaxime resistance was seen in this study. Whilst correlation data between these two antibiotics in other studies could not be found, it can be said at least that cefotaxime resistance commonly occurs alongside ampicillin resistance in other studies (Amaya *et al.*, 2021; Mir *et al.*, 2016; Lambrecht *et al.*, 2017).

Regarding the positive correlation between ampicillin and amoxycillin/clavulanic acid, it is entirely possible that the mechanisms enabling isolates to be resistant to the action of the latter drug naturally extends to resistance of the former. One such mechanism of resistance to amoxycillin/clavulanic acid in *E. coli* is overproduction of certain β-lactamases, such as bla_{TEM}, as found by Wu *et al.* (1994). Overproduction of this enzyme and others, including AmpC, was indeed found to be the predominant mechanism of amoxycillin-clavulanic acid resistance amongst *E. coli* in the study by Pérez-Moreno *et al.* (2004). In such an instance, isolates would possess resistance to ampicillin also, and may explain a positive correlation.

Ciprofloxacin and enrofloxacin also belong to the same antibiotic class (fluoroquinolones), and so the positive correlation may also be due to a shared resistance mechanism/gene. The two major mechanisms of resistance to fluoroquinolones may have, just by their nature, enabled resistance to both ciprofloxacin and enrofloxacin to occur. Mutations in the genes encoding the target enzymes of both antibiotics (DNA gyrase and topoisomerase IV) could logically lead to resistance to both, and so too could the use of efflux pumps potentially lead to the extrusion of both antibiotics (Hooper, 2001a; Hooper, 2001b; Bai *et al.*, 2012).

It is extremely likely that for the correlation between chloramphenicol and nitrofurantoin resistance, which do not belong to the same antibiotic class, the multidrug efflux pump OqxAB is the cause. This efflux pump is one of the known mechanisms that is shared for both nitrofurantoin and chloramphenicol resistance, as studies have shown how isolates possessing plasmids harbouring this gene are provided resistance to both chloramphenicol and nitrofurantoin (albeit this has been shown separately) (Hansen *et al.*, 2004; Hansen *et al.*, 2007; Ho *et al.*, 2016).

oqxAB genes have also been repeatedly found alongside bla^{CTX-M} on several different plasmids from *E. coli* of avian (of which the majority were from chickens) origin, indicating the potential for chloramphenicol, nitrofurantoin and cefotaxime resistance to co-occur and be co-selected, and potentially providing an explanation for the positive correlations seen between cefotaxime and these two other antibiotics (Liu *et al.*, 2011; Yang *et al.*, 2016; Pan *et al.*, 2016; Wong *et al.*, 2016; Li *et al.*, 2019).

Another positive correlation existing between antibiotics of different classes was that of azithromycin and gentamicin, belonging to the macrolides and aminoglycosides, respectively. This may be unsurprising, given that a significant association between the resistance to these two antibiotic classes was noted in *Campylobacter* species from Lopez-Chavarrias *et al.* (2021)'s study also. Whilst both antibiotics inhibit protein synthesis through targeting components of the bacterial ribosome, their specific drug target differs, and so a shared modification of the drug targets is unlikely. As such, it may be that existence of a multidrug efflux pump capable of expelling both macrolides and aminoglycosides has led to the correlation. An example of this may be the MexXY system in *P. aeruginosa*, which was indicated as providing intrinsic resistance to both macrolides and aminoglycosides in two separate studies (Mine *et al.,* 1999; Aires *et al.*, 1999). Similarly, the notable efflux pump AcrAB-TolC may partially provide the positive correlation seen between gentamicin and oxytetracycline resistance, given the propensity for it to transport gentamicin and several antibiotics of the tetracycline class (Jang, 2023). It also has the capability to export both nalidixic acid and ceftazidime (a third-generation cephalosporin, as cefotaxime is), which also displayed positive correlations.

All of the negatively correlated pairs of antibiotic resistances were between a resistance that increased with time and one that decreased with time (Table 5.1), and so the negative correlation is highly likely to be due to these opposing changes rather than an underlying mechanism. In the event that this is not the case however, there are several possibilities that may have occurred to result in the negative relationships seen. One of which is that the corresponding resistance genes were located on separate plasmids belonging to different incompatibility groups (Thomas, 2014), so that a given isolate could only possess one or the other antibiotic resistance. Indeed, given that one resistance increased over the other, it may be that the plasmid responsible for the increasing resistance became dominant in the bacterial population, decreasing the occurrence of the other resistance. Another possibility is that the negative correlation is rooted in the fitness cost imparted by a resistance gene or the element which carries it (Humphrey *et al.,* 2012; Vogwill & MacLean, 2015; San Millan & MacLean, 2017), which has been shown to be well-documented for plasmids in several studies involving various bacterial species and resistances as reviewed by Björkman & Andersson (2000). This, in conjunction with the assumption that no antibiotic selective pressures were provided by the litter environment (given that the chickens' diet was antibiotic-free), may explain why some resistances decreased with time (if they were particularly costly to the bacterium) whilst others did not (if the impact on fitness was lesser). Pertaining to some of the resistances involved in negative correlations that decreased in relation to time, fitness costs for streptomycin (Westhoff *et al*, 2017) tetracycline (Card *et al.,* 2021) and azithromycin (Maher *et al.,*2012) resistance have been demonstrated experimentally, with the former two studies being in the absence of antibiotic also.

4.5 CONCLUSION

The results of this study have shown that the enteric bacteria of chicken litter and compost from non-commercial, antibiotic-free chickens can harbour moderate and high levels of resistance to a variety of antibiotics from several different classes, as well as having a high proportion of bacteria that are multi-drug resistant. It was also determined that the position in a compost heap from which the bacteria are derived may not have much of an impact on the resistances seen, but the ability of bacteria to ferment lactose does for a handful of antibiotics, potentially due to genes enabling lactose-fermentation being co-transferred with resistance genes on plasmids. Enteric bacteria of chicken litter and compost may also contain mechanisms enabling cross-resistance, as determined by correlations between the resistances of antibiotics, and so may potentially be resistant to many other antibiotics that were not included in this study. Overall, it can be established that this type of chicken litter (i.e., that from non-commercial chickens raised without antibiotics) is deserving of more attention in future studies, as it may often be overlooked and considered not as hazardous as commercial chicken litter in terms of AMR, which this study has shown to not necessarily be true.

5 THE EFFECT OF COMPOSTING ON THE LEVELS OF ENTERIC BACTERIA AND ANTIBIOTIC RESISTANCE IN NON-COMMERCIAL CHICKEN LITTER

5.1 INTRODUCTION

There are a multitude of reasons why using untreated chicken litter/manure as fertiliser on soil and crops can be hazardous to environmental, and potentially human, health. Potentially pathogenic bacteria, notably *Escherichia coli* and species of *Campylobacter*, *Clostridium*, *Enterococcus*, and *Salmonella*, can be found in chicken litter, oftentimes at high levels, according to data from various countries (Stern & Robach, 2003; Chinivasagam *et al.*, 2010; Cook *et al.*, 2014; Subirats *et al.*, 2020; Subirats *et al.*, 2021). It is also well-established that it often contains bacteria with resistances to a plethora of antibiotics, or contains many different types of resistance genes from the resident bacteria (Furtula *et al.*, 2010; He *et al.*, 2014; Xie *et al.*, 2016; Zhang *et al.*, 2017; Qian *et al.*, 2018; Han *et al.*, 2018; Wang *et al.*, 2019; Zhou & Yao, 2020; Zhou *et al.*, 2021; Subirats *et al.*, 2021), sometimes more so than other animal manures. As displayed in the aforementioned studies, ARGs relating to several major antibiotic classes can be frequently detected in chicken manure, with resistance genes to aminoglycosides, tetracyclines, beta-lactams and those imparting multidrug resistance appearing to be particularly prevalent. These bacteria are then transferred to the soil upon use as fertiliser, increasing the levels of enteric bacteria in soil, and altering the amounts of and which resistance genes are found in the soil (Cook *et al.*, 2014; Zhang *et al.*, 2017; Wang *et al.*, 2018; Han *et al.*, 2018; Zhang *et al.*, 2019; Subirats *et al.*, 2021). These effects can also last for quite some time in some cases, with ARG numbers/levels, and enteric bacteria levels, remaining above those of the natural soil for over 100 days in multiple studies (Cook *et al.*, 2014; Zhang *et al.*, 2017; Han *et al.*, 2018). It may also impact antimicrobial resistance in crops (Zhang *et al.*, 2019). Furthermore, antibiotics themselves can sometimes be found in the litter (excreted from the chickens that were fed them), with tetracyclines seeming prominent, doubtless due to the extensive time necessary for their degradation in chicken manure (Kasumba *et al.*, 2020), which could potentially drive resistance in soil upon fertilisation also (Xie *et al.*, 2016; Wang *et al.*, 2019).

As such, it is important that chicken litter is treated prior to use as a fertiliser in some way that can decrease the levels of enteric and potentially pathogenic bacteria (so that they do not contaminate soils and crops) and ameliorate the antibiotic resistance present to at least some extent (so as not to alter and expand the soil resistome). Composting is a very viable option for this, as has been shown through many studies reporting decreases in antibiotic resistance/ARG levels (Cui *et al.*, 2016; Xie *et* *al.,* 2016; Li *et al.,* 2017; Qian *et al.,* 2018; Peng *et al.,* 2018; Wang *et al.,* 2019; Zhou & Zhou & Yao, 2020; Esperón *et al.*, 2020; Zhou *et al.*, 2021), as well as reduced effects on soil when used as fertiliser in comparison to fresh chicken manure in terms of resistome impact (Zhang *et al.,* 2019). However, the majority of these studies use genetic-based methods, often through the number of and quantification of ARGs present in a sample, in order to determine the effect composting has had on antibiotic resistance. This may not be the best method of ascertaining the resistances seen in compost, with an example of the reason why being displayed in Wang *et al.*'s (2019) study; although the relative abundances of the *sul1* and *sul2* genes decreased due to composting, a culture-based method revealed that there was virtually no decrease in sulfamethazine resistance that could be seen phenotypically. The result may have been due to the fact that alongside *sul1* and *sul2*, sulfonamide resistance can also be achieved through the development of a resistant target enzyme (Wise & Abou-Donia, 1975). Resistances to other antibiotics can also be mediated by mutations in the genes of the target enzymes, by over-expression of target enzymes (presumably to compensate), or by under-expression of porin genes such as *ompW* (as this would reduce entry of the antibiotic into the cell), as is found for fluoroquinolone-resistance (Hooper, 2001; Bai *et al.,* 2011). The upregulation of efflux pumps which have purposes outside of antibiotic expulsion is another mechanism by which bacteria can gain resistance (Rahman *et al.,* 2017). Therefore, it can be seen that mechanisms of antibiotic resistance reach far beyond acquired resistance genes, and so measuring only these genes may not provide a full, detailed idea of what impact composting is having on antibiotic resistance. As it is not feasible for genetic-based studies to try and detect all acquired resistance genes, mutations in the genes of target proteins, and measure the level of expression of numerous key genes, it is important for a phenotype-based element to be included in studies. Indeed, it may even be more important for phenotypes to be observed rather than for a number of ARGs to measured, as there are so many factors at play. As such, this study utilised culture-based methods and focused on phenotypic resistance in order to gain insight into the changes of antibiotic resistance.

The objectives of this study were:

1. To determine the effect of composting for 100 days on antibiotic resistance in non-commercial chicken litter using a phenotypic method.
2. To determine the effect of composting for 100 days on enteric bacteria levels in non-commercial chicken litter.
3. To understand the contribution of time and temperature to the results seen.
4. To understand the impact of the location/depth in the compost heap that the isolates were derived from on the results seen.
5. To understand the impact of the isolates' ability to ferment lactose on the results seen.

5.2 MATERIALS AND METHODS

Please refer to section 2.1 for full details.

5.2.1 Statistical analyses

Student’s t-tests were executed in Microsoft Excel 2019 to analyse AST and CFU count data. All other analyses were conducted using Genstat 21.1 (VSN International, Ltd.). Principal component analysis (PCA) was used to analyse total $\log_{10}$ CFU g^{-1} F Wt. data and total resistance scores, based on a variance-covariance matrix. Simple linear regression analyses were used to produce identify potential relationships with temperature data. Unbalanced ANOVAs were conducted on total $\log_{10}$ CFU g^{-1} F Wt. data, using time, maximum temperature, minimum temperature, heap position, and lactose-fermenting ability as the factors. Log_{10} CFU g^{-1} F Wt. data segregated for each position and lactose-fermenting ability was also used in unbalanced ANOVAs, using the same treatment structures (excluding position for the former set of data, and lactose-fermenting ability for the latter). Unbalanced ANOVAs were also used to analyse total zone of inhibition data for each antibiotic individually, using the same factors as for total $\log_{10}$ CFU g^{-1} F Wt. data. Regressions were plotted for the results of every unbalanced ANOVA for the treatment structures time, maximum temperature, and minimum temperature. Data from day 44 was excluded from ANOVAs, linear regressions, and PCAs due to the uncharacteristically low resistances seen at this sampling point.

5.3 RESULTS.

5.3.1 Changes in CFU g^{-1} F Wt. of enteric bacteria during the composting process.

Data for colony forming units (CFU) were collected from day 31 onwards, although some data are missing because of uncertainty over differentiating between lactose-positive and lactose-negative bacteria due to inconclusive colour change. On day 31, there was no significant effect of position within the compost heap on CFU ($p>0.05$; Figures 5.1 and 5.2). At the next sampling point on day 36 the bacterial counts had decreased; a significant 99% reduction was observed for lactose-positive enteric bacteria from the centre of the heap ($p=0.049$; Figure 5.1), and a significant 92% reduction for lactose-positive intermediate enteric bacteria ($p=0.04$; Figure 5.1). There was a smaller 66.7% reduction in lactose-positive enteric bacteria from the outer position, but it is unknown whether or not this is significant due to incomplete data (Figure 5.1). From day 51 both lactose-positive and lactose-negative bacterial counts fluctuated, although overall, there was a gradual increase, for all groups (Figures 5.1 and 5.2). Over the duration of the composting process (from day 31 to day 100), lactose-negative enteric bacteria from the centre of the heap significantly increased in number ($p=0.027$). Lactose-positive enteric bacteria were more abundant than lactose-negative on day 31 across all positions, but by the end of they were lower, significantly so in the case of the centre position (*p*=0.003).

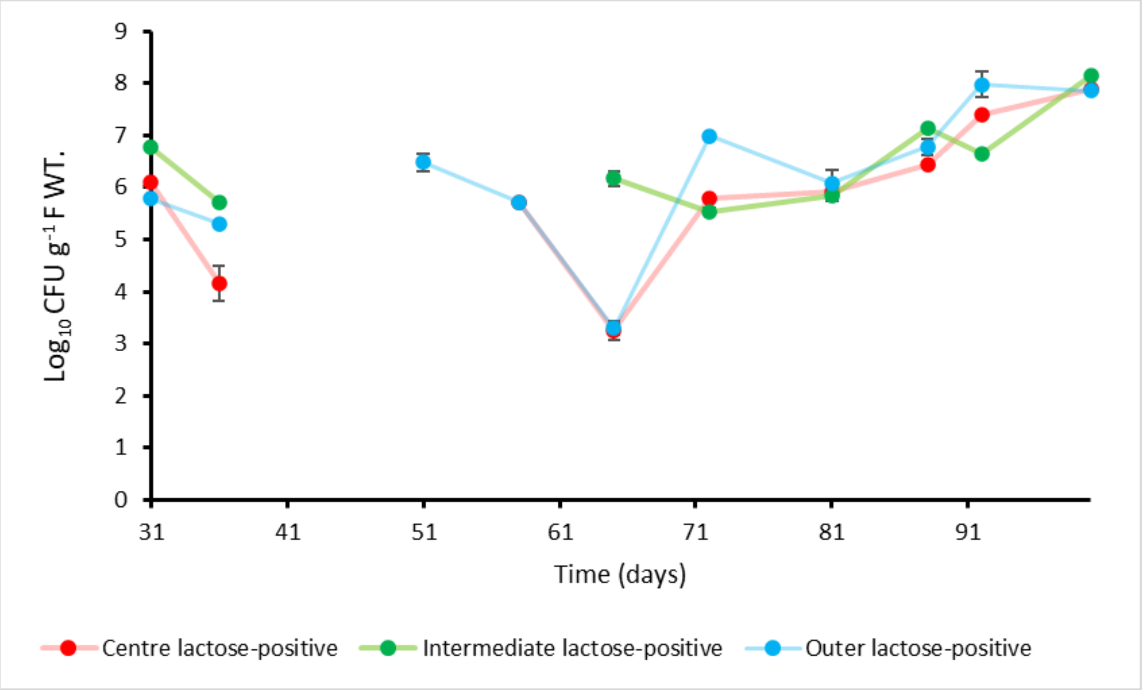

Figure 5.1: *The CFU g-1 F Wt. of lactose-positive enteric bacteria across the sampling period, beginning on day 31.*

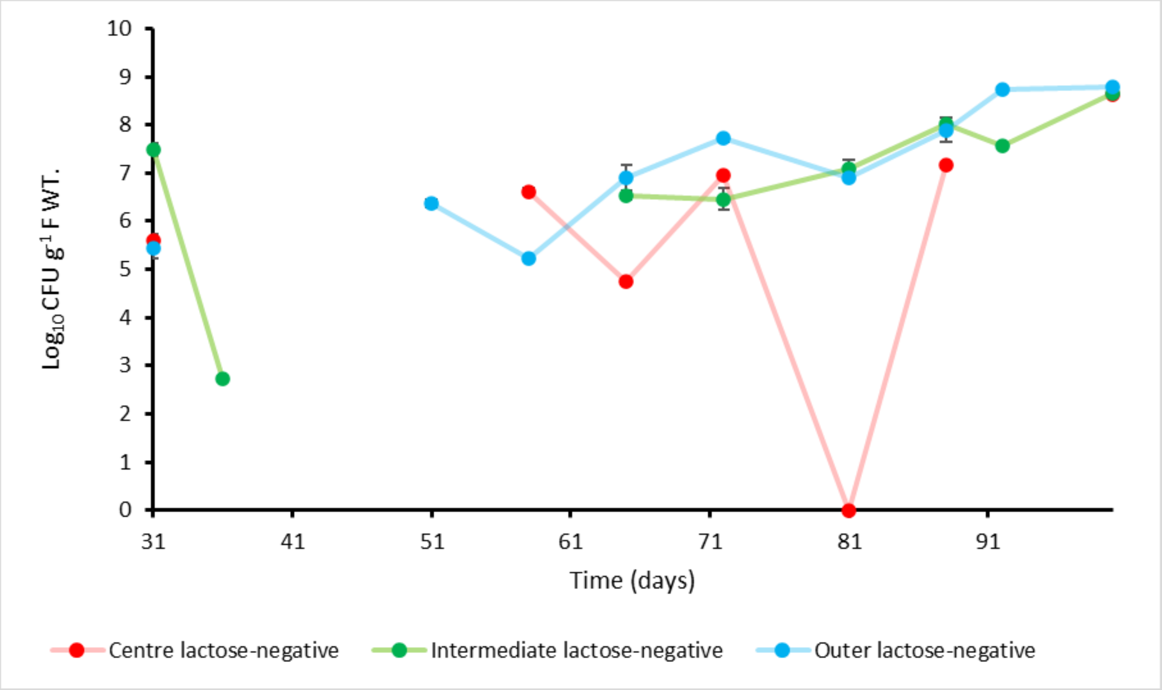

Figure 5.2: *The CFU g-1 F Wt. of lactose-negative enteric bacteria across the sampling period, beginning on day 31.*

5.3.2 Analyses to determine potential factors influencing changes in CFU g^{-1} F Wt. enteric bacteria during composting

Total CFU data, including that for all positions and both lactose-positive and lactose-negative counts, were analysed by principal component analysis (PCA). Three principal components (PCs) accounted for most of the variation in the data. PC1 was responsible for 64.92% of the variation, PC2 for 25.47%, and PC3 for 7.09%. Although PC1 accounted for much of the variation in the data, the most evident visual effects depended somewhat on which PCs were plotted against each other. However, key observations were that the lactose-negative and lactose-positive bacteria from the centre of the heap were split along PC2 and the lactose-negative and lactose-positive bacteria from the outer heap region were separated along PC3. Neither lactose-positive nor lactose-negative bacteria isolated from the intermediate region of the compost heap were separated along any of the principal components. Centre lactose-negatives were the only group with negative values for both PC2 and PC3, and outer lactose-positives the only group with a positive PC2 value and negative PC3 value, resulting in their isolation (Figure 5.5). It was apparent that the results for centre lactose-negatives were positively driven by PC1, but negatively driven by PC2. Conversely outer lactose-positives were positively driven by PC2, and negatively driven by PC3 (Figures 5.3, 5.4 and 5.5).

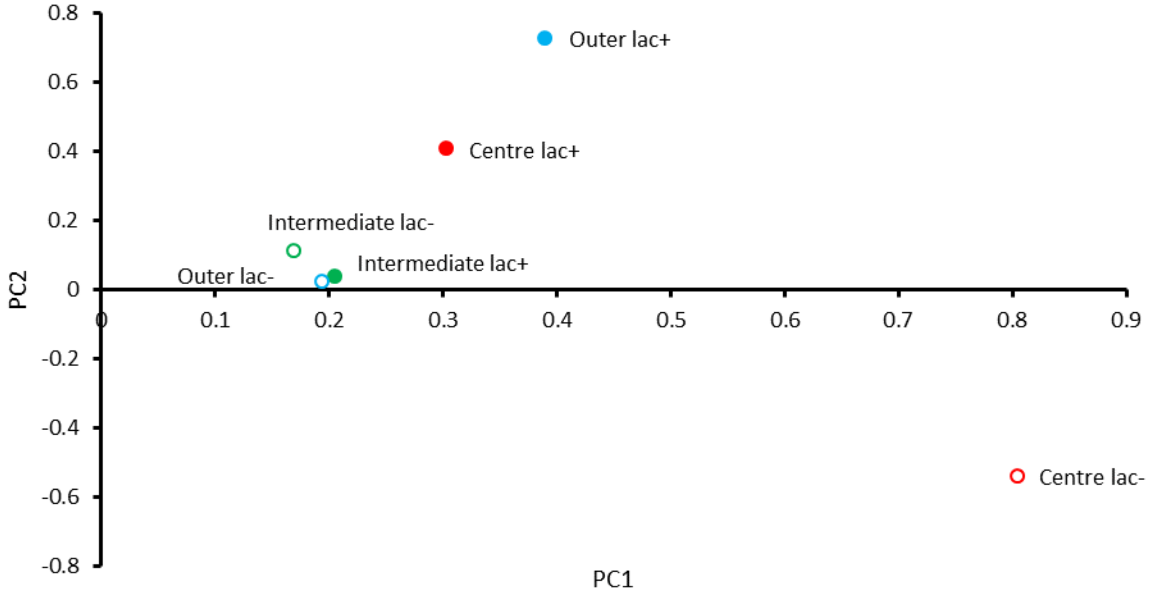

Figure 5.3: *PC1 and PC2 loadings from the principal component analysis of CFU g^{-1} F Wt. over the composting period.*

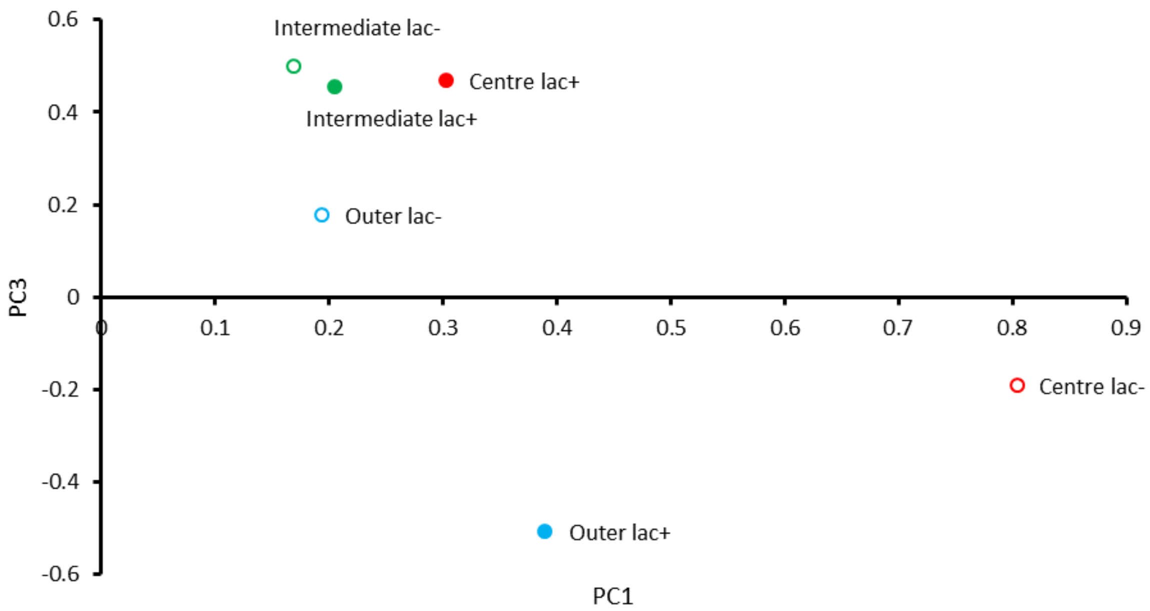

Figure 5.4: *PC1 and PC3 loadings from the principal component analysis of CFU g^{-1} F Wt. over the composting period.*

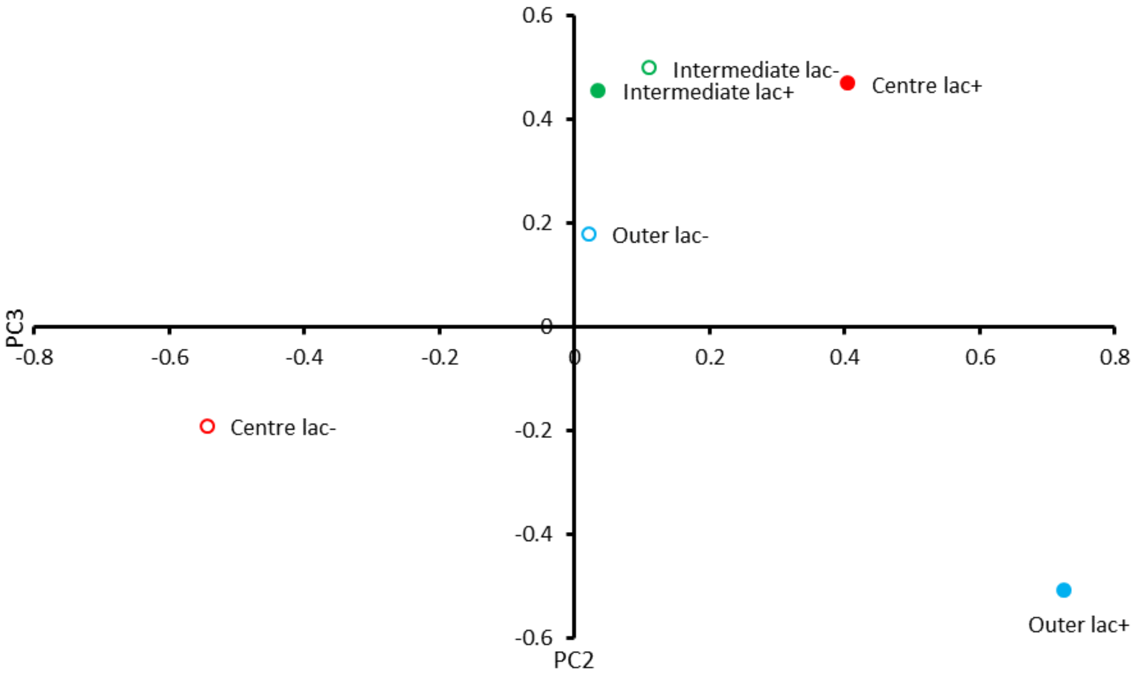

Figure 5.5: *PC2 and PC3 loadings from the principal component analysis of CFU g^{-1} F Wt. over the composting period.*

Alongside PCA, known factors (time, average temperature, position and lactose-fermenting ability) were included in ANOVA analyses to assess what effect they may have had on CFUs throughout composting. Using total CFU data (i.e., from all positions), a significant relationship was found with position ($F_{2,79}$=3.38, p=0.037), with the centre position being associated with the lowest $\log_{10}$ CFU g^{-1} F Wt. measurement. No significant relationship was found for lactose-fermenting ability (p=0.179). The relationship with time was significant and strongly positive for the intermediate and outer positions (r=0.78, $F_{5,18}$=10.88, p<0.001 and r=0.63, $F_{7,24}$=3.53, p=0.01, respectively), but was far weaker for the centre (r=0.27, $F_{6,19}$=6.21, p<0.001). Relationships with average temperature were very different for each position. Virtually no relationship existed for the outer position (r=0.006, $F_{7,24}$=3.53, p=0.01), and a weakly positive relationship existed for the intermediate position (r=0.40, $F_{5,18}$=10.88, p<0.001). However, the centre position actually had a negative relationship with average temperature, albeit weakly (r=-0.19, $F_{6,19}$=6.21, p<0.001).

Two-way ANOVA analyses determined a significant interaction between average temperature and lactose-fermenting ability ($F_{19,41}$=5.09, p<0.001). Lactose-positive enteric bacteria had essentially no relationship with average temperature (r=0.0008), whilst lactose-negative had an extremely weakly negative relationship (r=-0.065).

5.3.3 Changes in antibiotic resistances during the composting process.

Multiple parameters were used to determine and explore the changes in antibiotic resistance throughout the composting process: resistance ‘scores’ (as explained in 4.2.2), the numbers of resistances, and percentage resistances.

Considering time as a single factor and combining data for all isolates and antibiotics as a whole, resistance scores fluctuated throughout the composting process. Nevertheless, the combined resistance scores gradually increased until the experiment’s completion at 100 days. The resistance score at day 100 was not much higher than that of the chicken litter prior to composting (an increase of 2.83), but the small increase was significant (p=0.05; Figure 5.6).

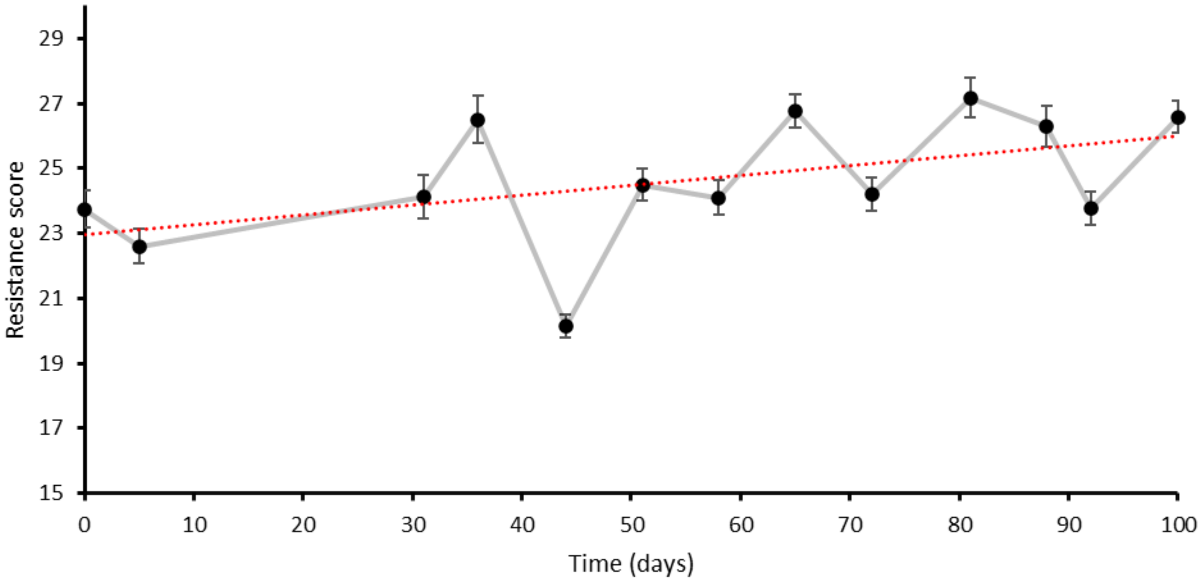

Figure 5.6: *The total resistance score (sum of the average resistance scores of all antibiotic resistances at a sampling point) for total isolates across the sampling period.*

Focusing on individual antibiotics, but still combining data for all isolates, the variable effects of composting on bacterial resistance to different antibiotics shows that from day 0 to day 100, the resistance to 10 antibiotics increased, 4 decreased, and one remained the same (Figure 5.7). The largest increase in resistance was to chloramphenicol (0.8), closely followed by ampicillin (0.78). Before composting, no chloramphenicol resistance was observed in the isolates derived from chicken litter (Figure 5.10), but by day 100, 50% of isolates were resistant; a significant increase (p<0.001). Enhanced ampicillin resistance, from 50% of the isolates from chicken litter before composting to 88.9% at the end of composting was only weakly significant (p=0.06). Significant increases in resistance from before composting to composting completion were observed for ciprofloxacin, enrofloxacin, and nalidixic acid (all p<0.02; Figure 5.7). The apparent increases in amoxycillin/clavulanic acid, cefotaxime, tigecycline, azithromycin, and nitrofurantoin resistance over the composting period were not significant. For tigecycline, only one resistant isolate was found over the entire sampling period, on day 100 (Figure 5.9). Resistance to ciprofloxacin, enrofloxacin, and nalidixic acid increased from 0 to 50%, 0 to 50%, and 12.5 to 44.4% of isolates, respectively. Resistance to oxytetracycline and streptomycin was significantly reduced over the composting period (p<0.02; Figure 5.7). Oxytetracycline resistance also experienced the largest decrease in resistance score (1.4). Apparent decreases in resistance to the antibiotics streptomycin, gentamicin, and trimethoprim/sulfamethoxazole were not significant. Resistance to levofloxacin was unaffected by composting (Figure 5.7), and only two isolates expressing intermediate resistance against this antibiotic were found throughout the entire sampling period (Figure 5.10).

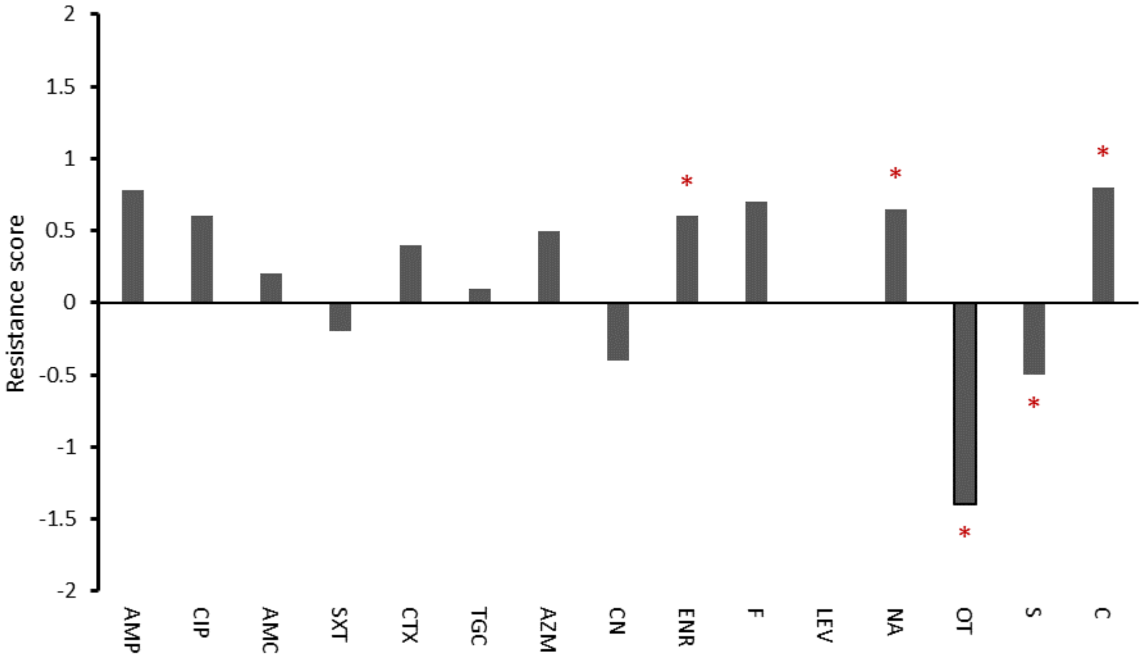

Figure 5.7: *The changes in resistance scores of total isolates (all positions, both lactose-positive and lactose-negative) between day 0 and day 100 for each antibiotic. Red asterisks indicate significant changes.*

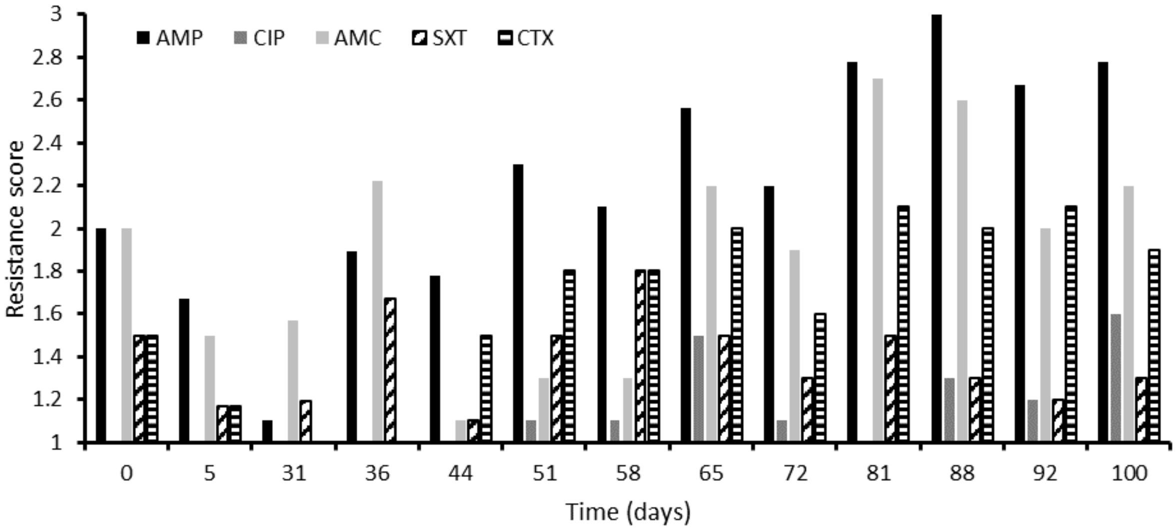

Figure 5.8: *The average resistance scores of total isolates (all positions, both lactose-positive and lactose-negative) of ampicillin (AMP), ciprofloxacin (CIP), amoxycillin/clavulanic acid (AMC) trimethoprim/sulfamethoxazole (SXT), and cefotaxime (CTX) across the sampling period.*

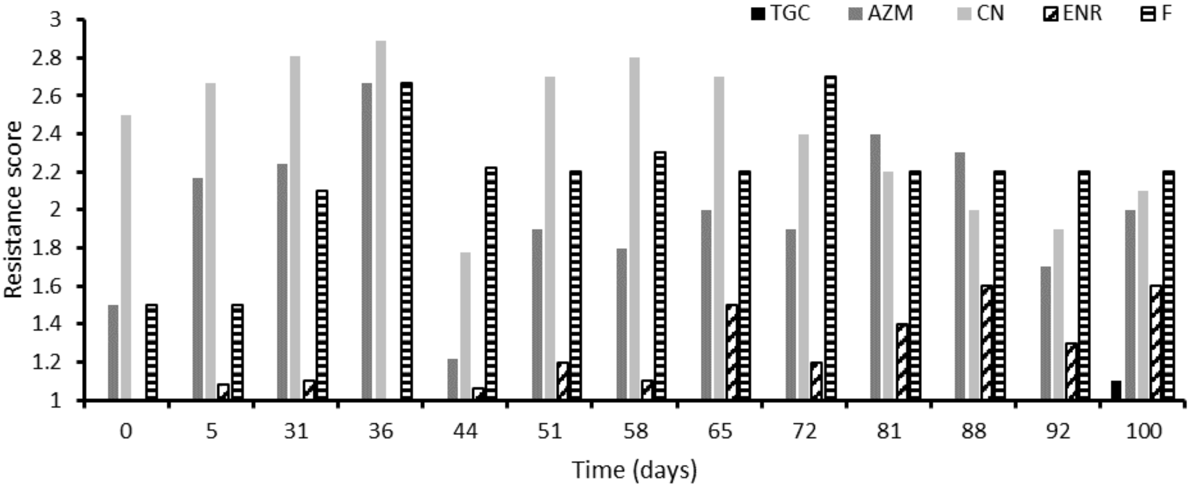

Figure 5.9: *The average resistance scores of total isolates (all positions, both lactose-positive and lactose-negative) of tigecycline (TGC), azithromycin (AZM), gentamicin (CN), enrofloxacin (ENR), and nitrofurantoin (F) across the sampling period.*

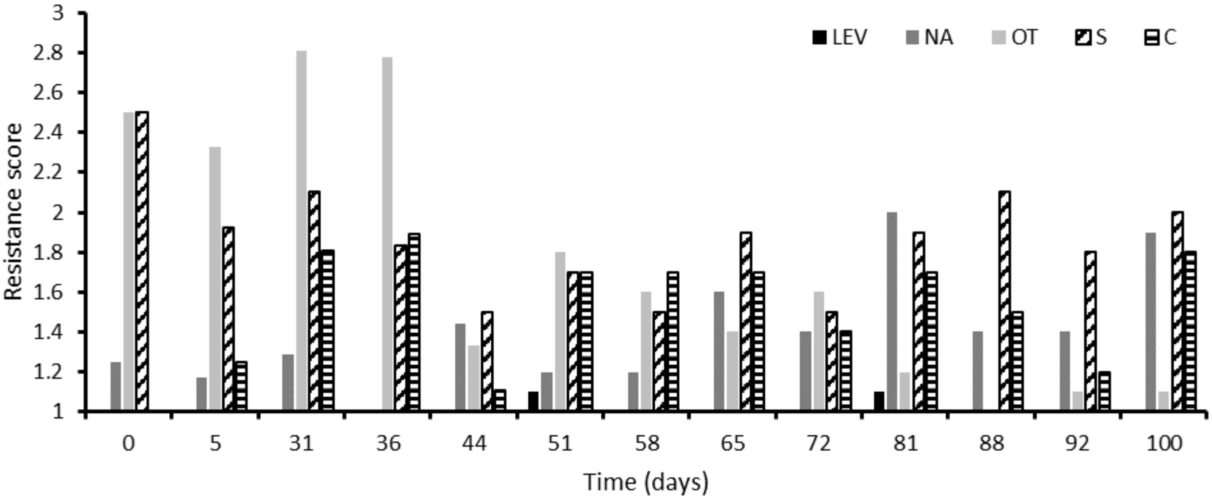

Figure 5.10: *The average resistance scores of total isolates (all positions, both lactose-positive and lactose-negative) of levofloxacin (LEV), nalidixic acid (NA), oxytetracycline (OT), streptomycin (S), and chloramphenicol (C) across the sampling period.*

The most commonly observed number of resistances possessed by isolates at day 0 was three, representing 50% of isolates, whilst at day 100 it was nine, representing 22.2% of isolates. Resistance to nine (or more) antibiotics was not seen in isolates prior to composting, and at day 100, resistance to three antibiotics only represented 5.6% of isolates, with resistance to one or two antibiotics not observed (Figure 5.11). Therefore, there was an increase in the proportion of isolates expressing resistance to a greater number of antibiotics.

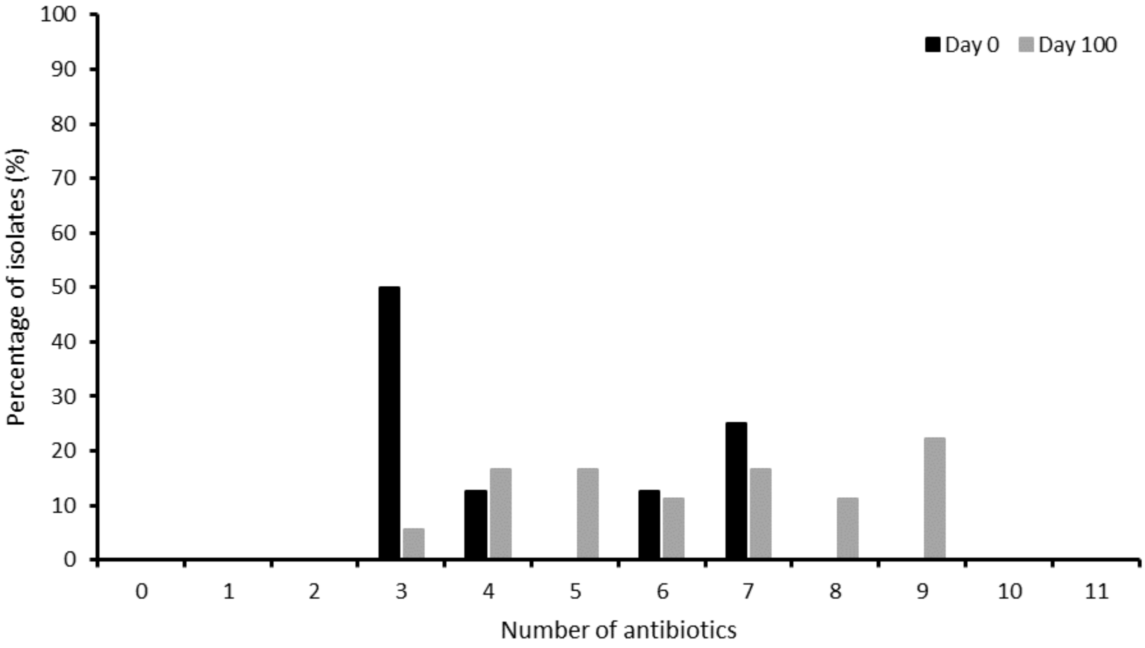

Figure 5.11: *The number of resistances of the total isolates at day 0 and day 100.*

By investigating the effect of composting on enteric bacteria separated by their position/depth in the compost heap and/or their lactose-fermenting ability however, different results to those for time as a single factor (combined position and lactose-fermenting ability data) were observed, and these results also distinguish one group of isolates from another.

The 'total resistance scores' for each position in the compost heap (that is, the collective resistance scores of each antibiotic for the centre, intermediate and outer positions) showed that although the centre possessed the highest resistance score at day 100 (28), followed by the intermediate (27), and then the outer positions (24.8), the differences were not significant ($p>0.2$; Figure 5.12).

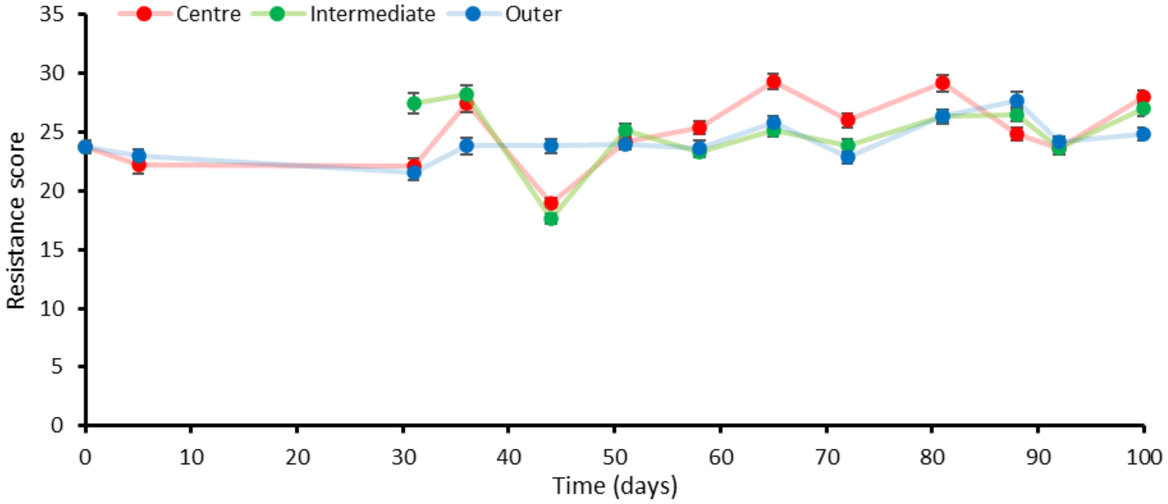

Figure 5.12: *The total resistance scores (sum of the average resistance scores of all antibiotic resistances at a sampling point) for isolates across the sampling period separated by position.*

Inspecting the resistance scores for each antibiotic, for each position, any differences seen between the positions and variation from results seen for the total isolate resistance scores are identifiable. The increases or decreases refer to those between day 0 and 100. Differences from the total isolates and between each position were seen with amoxycillin/clavulanic acid and tigecycline resistance, as the isolates of the centre of the heap were the only isolates exhibiting an increase in resistance score for these antibiotics, whilst no change or decreases were seen for the other positions. With trimethoprim/sulfamethoxazole resistance, decreases were seen for isolates of the intermediate and outer positions (a decrease was seen with total isolates also), but an increase was reported for the centre (Figure 5.13).

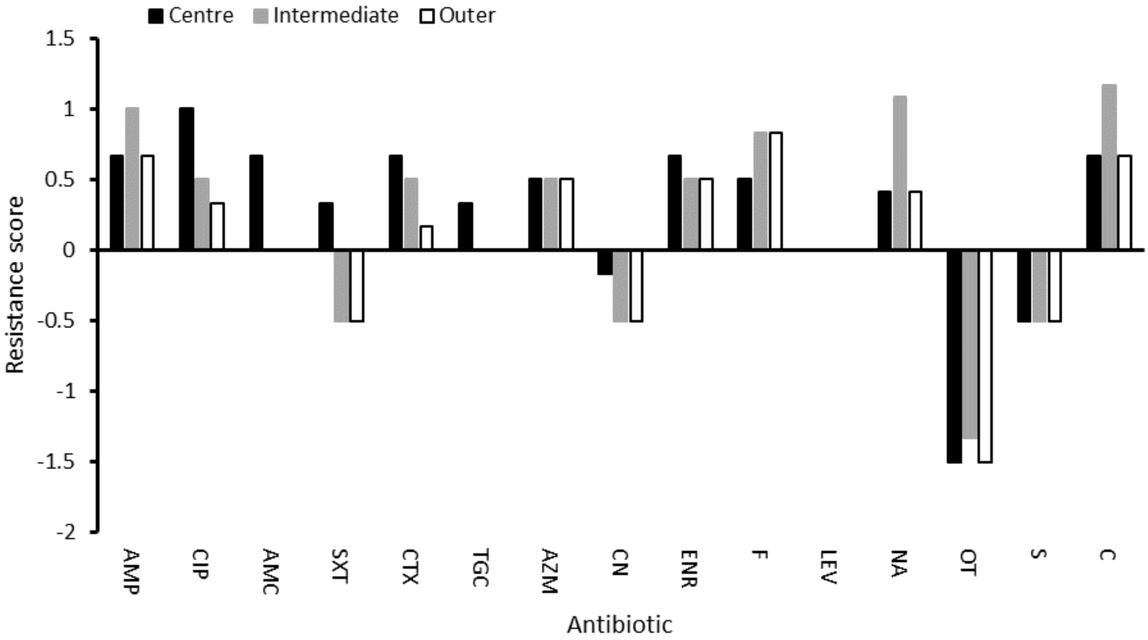

Figure 5.13: *The changes in resistance scores of isolates between day 0 and day 100 separated by position for each antibiotic.*

For most of the sampling period, lactose-negative isolates appeared to possess higher resistance scores than the lactose-positive isolates, but towards the end of the composting period, a shift was apparent with lactose-positive isolates having higher resistance scores. Nevertheless, these differences were not significant. For changes of the antibiotic resistance score for each antibiotic, the difference between day 0 and day 100 was not used, because only one lactose-negative bacterium was isolated on day 0. Instead, day 5 was used, as it was the next sampling point and no significant difference was found between day 0 and day 5 in lactose-positive isolates (p>0.1). As when separating by position, differences from the total isolates and between lactose-positive and lactose-negative isolates could be determined. For tigecycline, the increase in resistance score existed solely in lactose-positive isolates. With azithromycin resistance (which increased in total isolates [Figure 5.7]), there was an increase in lactose-positive isolates, but a decrease in lactose-negative isolates. Bizarrely, with streptomycin (for which resistance decreased in total isolates [Figure 5.7]), resistance actually increased in lactose-positive isolates, with no change in lactose-negative isolates. This was also the case for trimethoprim/sulfamethoxazole resistance, except it was lactose-negative isolates that increased in resistance. With gentamicin resistance (which decreased with total isolates [Figure 5.7]), a change in resistance score occurred only for lactose-negative isolates, where it decreased (Figure 5.15).

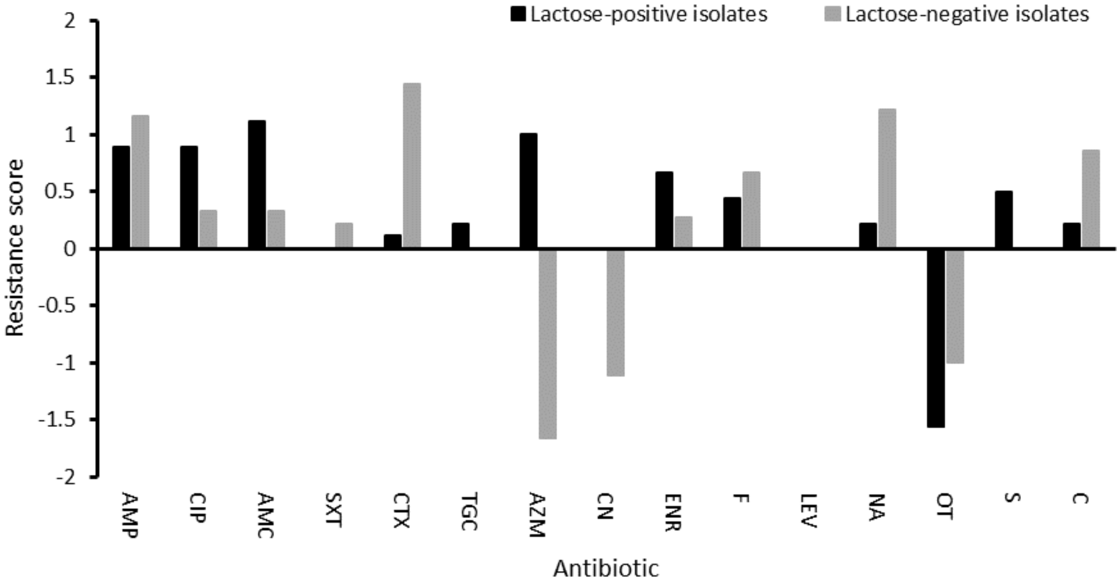

Figure 5.15: *The changes in resistance scores of isolates between day 5 and day 100 separated by their ability to ferment lactose for each antibiotic.*

Specifying further, isolates can be separated by their lactose-fermenting ability in conjunction with their position. The difference in resistance score between day 5 and day 100 was used for the centre and outer positions, but not for intermediate as sampling for this position was not conducted until day 31. As such, day 0 was used for the intermediate position. The results for the centre position majorly followed the patterns seen for the lactose-positive and lactose-negative total isolates (i.e., inclusive of all positions) (Figure 5.15), with four antibiotics differing; nitrofurantoin (the resistance scores did not change for lactose-positives nor lactose-negatives), nalidixic acid (there was a resistance score increase in lactose-negatives only), chloramphenicol (increase for lactose-negatives only), and streptomycin (no change in resistance score for lactose-positives) (Figures 5.16 and 5.17). On the other hand, only 5 antibiotics followed the pattern for lactose-positive and lactose-negative total isolates (Figure 5.15) when reviewing the outer position (nitrofurantoin, chloramphenicol, streptomycin, gentamicin, and levofloxacin). The divergence from the pattern is due to many of the changes in the resistance scores for the outer position being lower than what was seen for the total isolates, there being no change, or the changes being negative (i.e., decreases occurred) rather than positive (Figure 5.16 and 5.17).

For the intermediate position, the results were more often different to those of the total lactose-positive and lactose-negative isolates than they were similar. This appears to be largely due to intermediate isolates having lower changes in resistance score, except in the case for azithromycin, gentamicin and oxytetracycline, where it was actually due to them having higher changes (Figures 5.16 and 5.17).

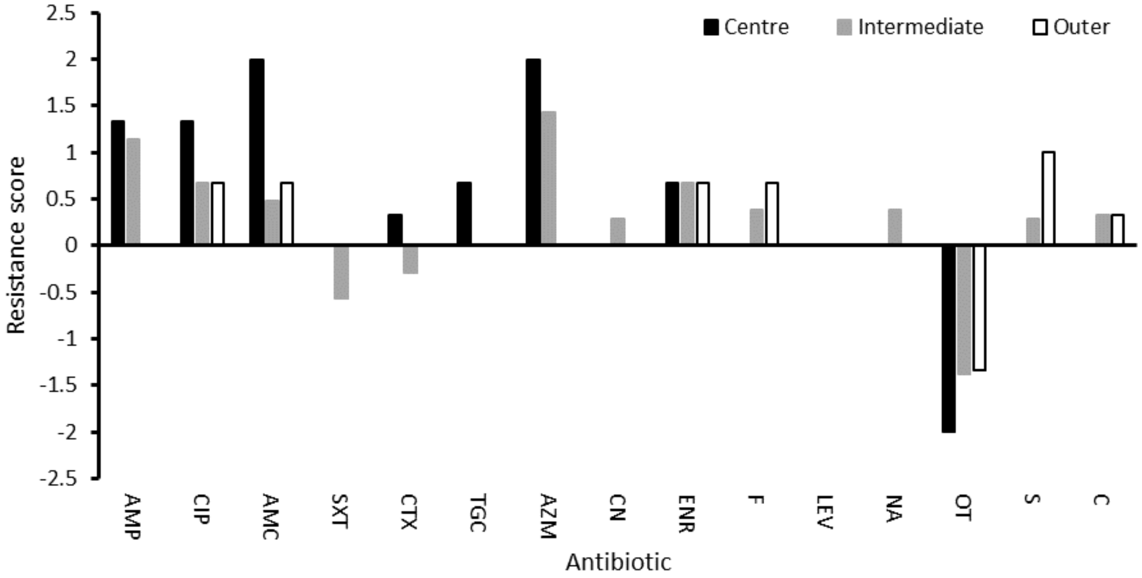

***Figure 5.16:** The changes in resistance scores of lactose-positive isolates between day 5 and day 100 (centre and outer positions) and day 0 and day 100 (intermediate position) for each antibiotic.*

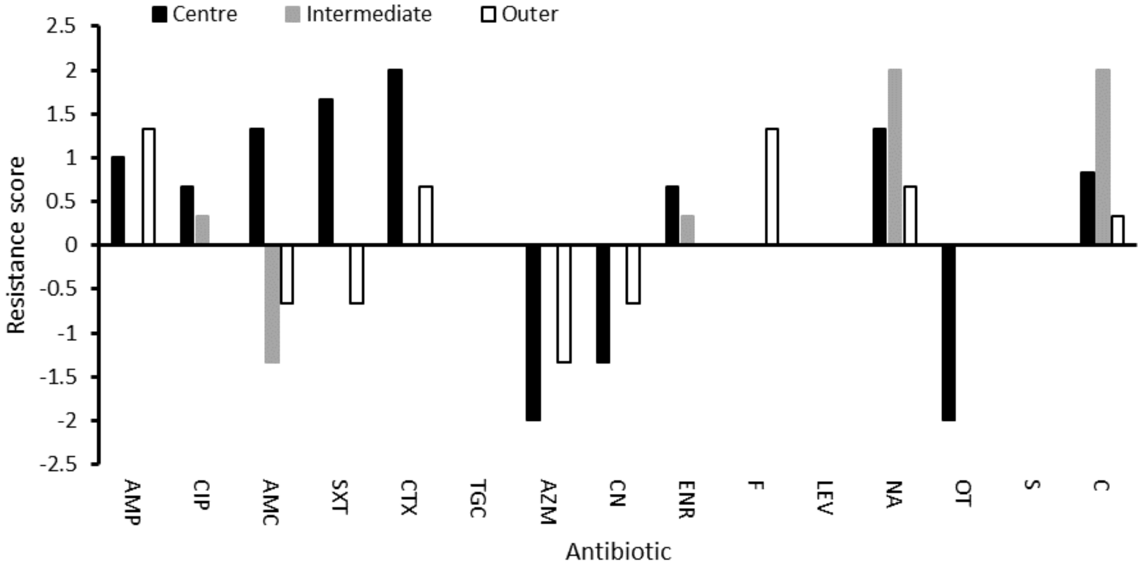

***Figure 5.17:** The changes in resistance scores of lactose-negative isolates between day 5 and day 100 (centre and outer positions) and day 0 and day 100 (intermediate position) for each antibiotic.*

5.3.4 Analyses to determine potential factors influencing changes in antibiotic resistance during composting

Total antimicrobial resistance score data, featuring isolates from every position and both lactose-positive and lactose-negative groups, was potentially influenced by three main PCs. PC1 accounted for 33.31% of variation in the data, PC2 for 18.35%, and PC3 for 12.98%. A PC1 and PC2 plot revealed that some pairs of antibiotics clustered together, such as enrofloxacin and ciprofloxacin, streptomycin and azithromycin, and tigecycline and levofloxacin (which remained near 0 for both axes in all plots). Oxytetracycline, gentamicin, ampicillin and amoxycillin/clavulanic acid were rather distant from other antibiotics, however. Only azithromycin, streptomycin, gentamicin and oxytetracycline were negatively driven by PC1, and oxytetracycline also the only antibiotic negatively driven both PC1 and PC2 (Figure 5.18). For the plot of PC1 and PC3, exceptionally close clustering was seen between azithromycin and oxytetracycline, amoxycillin/clavulanic acid and chloramphenicol, and enrofloxacin and ciprofloxacin (clustering at 0,0 alongside tigecycline and levofloxacin). As such, no fluoroquinolone antibiotics (enrofloxacin, ciprofloxacin and levofloxacin) were driven by PC1 or PC3. Ampicillin and cefotaxime were also much closer in this plot (Figure 5.19). In the PC2 and PC3 plot, ciprofloxacin and enrofloxacin continue to cluster, gentamicin and nalidixic acid cluster, and oxytetracycline, chloramphenicol and nitrofurantoin very loosely cluster (Figure 5.20). From all of the plots it could be determined that the only PC driving the results for enrofloxacin and ciprofloxacin was PC2, and largely the only PC driving CTX was PC1, both of which were positive drives (Figures 5.18, 5.19 and 5.20).

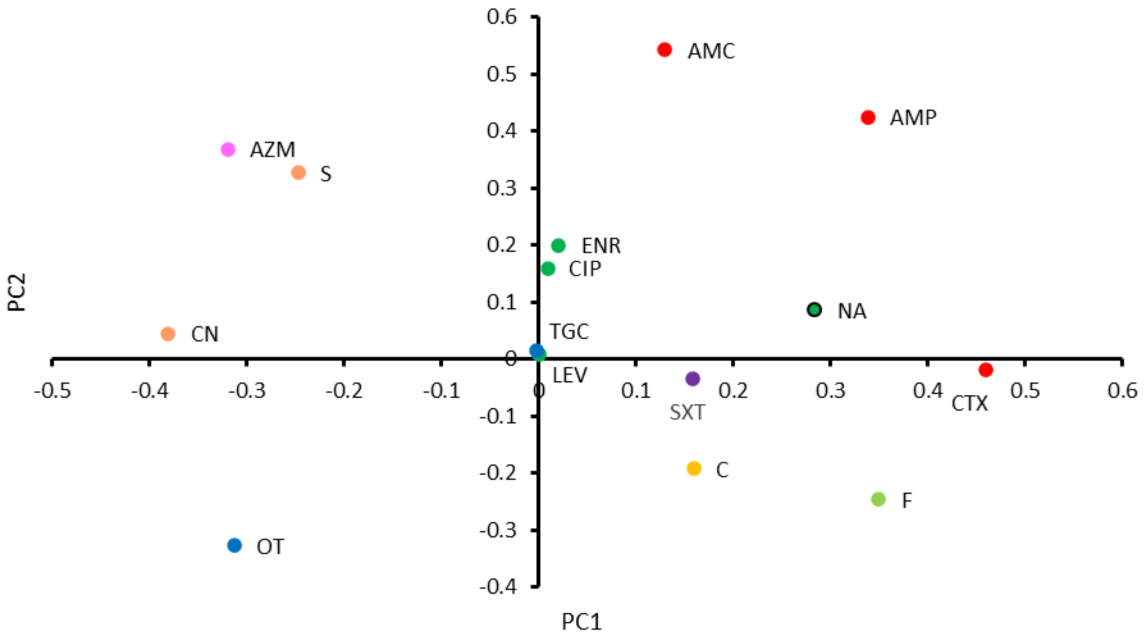

Figure 5.18: *PC1 and PC2 loadings from the principal component analysis of antibiotic resistance scores across the sampling period. Red = beta-lactam, dark green = fluoroquinolone, dark green with black border = quinolone, dark blue = tetracycline, light green = nitrofuran, yellow = phenicol, purple = sulfonamide, orange = aminoglycoside, pink = macrolide.*

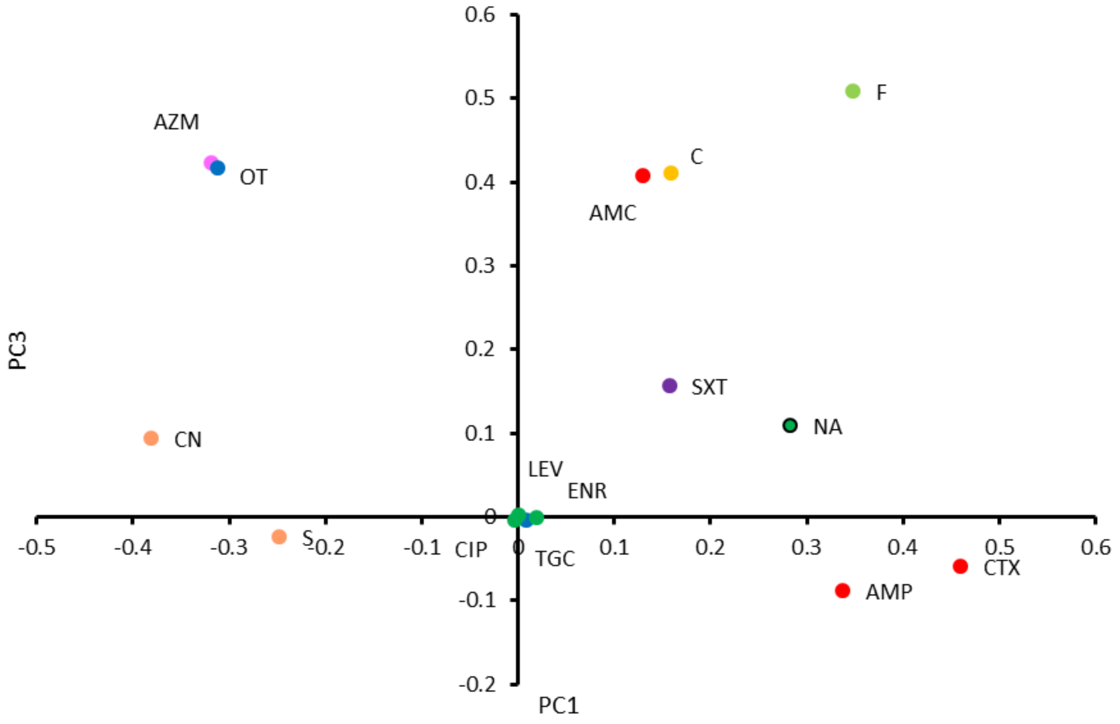

Figure 5.19: *PC1 and PC3 loadings from the principal component analysis of antibiotic resistance scores across the sampling period. Red = beta-lactam, dark green = fluoroquinolone, dark green with black border = quinolone, dark blue = tetracycline, light green = nitrofuran, yellow = phenicol, purple = sulfonamide, orange = aminoglycoside, pink = macrolide.*

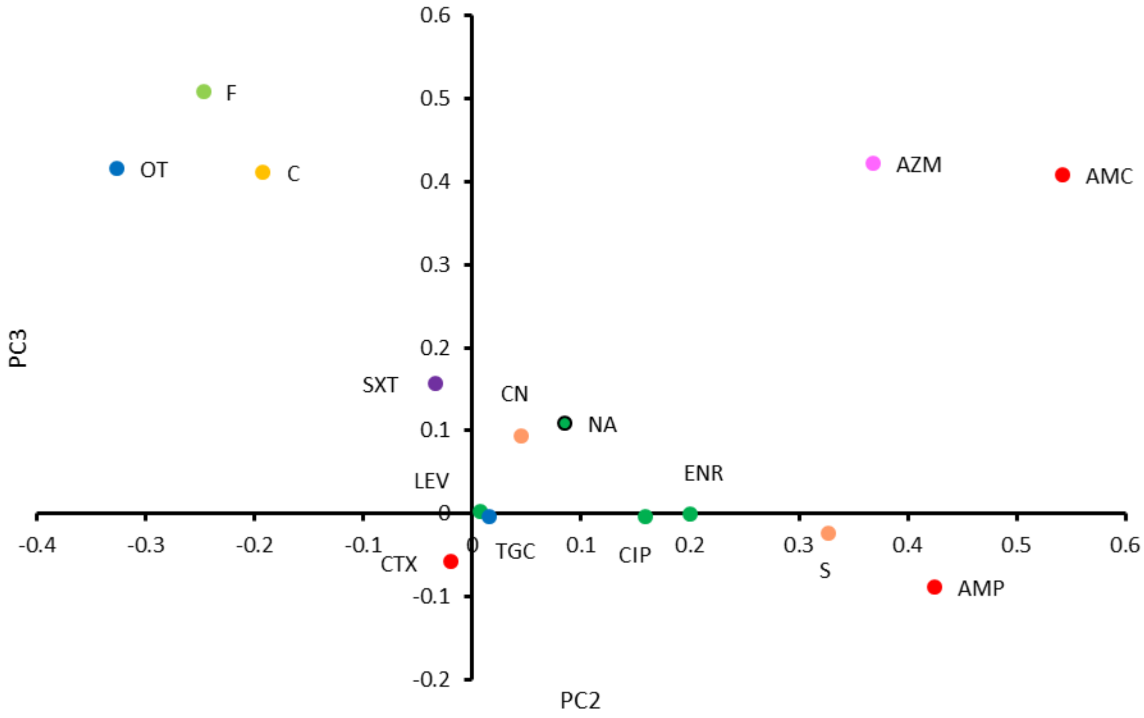

Figure 5.20: *PC2 and PC3 loadings from the principal component analysis of antibiotic resistance scores across the sampling period. Red = beta-lactam, dark green = fluoroquinolone, dark green with black border = quinolone, dark blue = tetracycline, light green = nitrofuran, yellow = phenicol, purple = sulfonamide, orange = aminoglycoside, pink = macrolide.*

Analyses to determine the impact of the known factors on the results seen for each antibiotic resistance were carried out. As these analyses used the measurements of the zones of inhibition rather than resistance scores, note that a negative relationship indicates that antibiotic resistance is increase (i.e., the zones of inhibition are shrinking), and a positive relationship indicates a decrease in antibiotic resistance (as zones of inhibition are getting larger). For time, only relationships of a moderate or high strength were of special consideration, and the cutoff point for this classification was designated as an *r* value of 0.5 or -0.5. Following this, resistance to the antibiotics ampicillin, cefotaxime, enrofloxacin, nalidixic acid, oxytetracycline, and trimethoprim/sulfamethoxazole had a notable relationship with time (Table 5.1). From these, only oxytetracycline had a positive relationship, and so it is probable that only oxytetracycline resistance notably experienced a decrease due to time alone.

***Table 5.1:** The results of ANOVA analyses for the potential relationship of the antibiotic resistances with time. Red shading indicates a resistant zone of inhibition measurement, and green shading indicates a susceptible measurement.*

Antibiotic resistance	F-value ($F_{10,184}$)	p-value	*r* value	ZoI measurement (mm) day 10
AMC	2.21	0.019	-0.10	18.9
AMP	8.28	<0.001	-0.76	8.8
AZM	3.06	0.001	0.30	19.2
CIP	5.09	<0.001	-0.13	25.9
CN	4.94	<0.001	0.38	19.8
CTX	8.55	<0.001	-0.72	17.1
ENR	5.74	<0.001	-0.56	23.3
LEV	4.46	<0.001	-0.20	26.4
NA	6.39	<0.001	-0.70	17.8
OT	12.71	<0.001	0.75	22.7
SXT	2.70	0.004	-0.52	16.2
TGC	4.09	<0.001	0.017	24.3

Relationships with average temperature were much weaker, and so the cutoff for consideration was lowered to 0.2 or above, and -0.2 or below. With this, ampicillin, gentamicin, cefotaxime, enrofloxacin, nalidixic acid, and oxytetracycline remained (Table 5.2). The only antibiotic resistances of these with positive relationships, and therefore the only resistances that are notably decreasing as a result of increasing temperature, are oxytetracycline and gentamicin.

***Table 5.2:** The results of ANOVA analyses for the potential relationship of the antibiotic resistances with average temperature. Red shading indicates a resistant zone of inhibition measurement, yellow shading indicates an intermediate resistant measurement, and green shading indicates a susceptible measurement.*

Antibiotic resistance	F-value ($F_{31,163}$)	p-value	*r* value	ZoI measurement (mm) highest average temperature (30.22°C)
AMC	2.54	<0.001	-0.036	13.7
AMP	4.41	<0.001	-0.29	7
AZM	1.58	0.037	-0.0007	11.5
C	2.31	<0.001	-0.035	16.2
CIP	2.57	<0.001	-0.065	26.8
CN	1.98	0.003	0.23	19.2
CTX	3.51	<0.001	-0.19	14.7
ENR	2.79	<0.001	-0.28	21.7
LEV	2.43	<0.001	-0.13	24.8
NA	3.28	<0.001	-0.20	16.8
OT	5.04	<0.001	0.20	19.7
SXT	2.94	<0.001	-0.046	12.5
TGC	2.36	<0.001	-0.0024	19.7

Despite the effect of temperature, the heap position had little effect, with only amoxycillin/clavulanic acid and trimethoprim/sulfamethoxazole being influenced ($F_{2,192}$=7.01, p=0.001 and $F_{2,192}$=3.25, p=0.041, respectively). For amoxycillin/clavulanic acid, the intermediate position was associated with having the most resistance, followed by the centre, then outer (which was associated with susceptibility). For trimethoprim/sulfamethoxazole, isolates of the centre position were the most resistant, followed by intermediate, then outer, although these were all associated with susceptibility. Relationships with lactose-fermenting ability were the opposite, with the majority of antibiotic resistances possessing a significant relationship (Table 5.3). The only antibiotic resistances to not possess significant relationships were amoxycillin/clavulanic acid, ampicillin, enrofloxacin, and tigecycline. For those possessing significant relationships, whether it was lactose-positives or lactose-negatives which had the lowest zone of inhibition was fairly equal (6 and 5 instances, respectively).

Table 5.3: *The results of ANOVA analyses for the potential relationship of the antibiotic resistances with the ability to ferment lactose. Red shading indicates a resistant zone of inhibition measurement, yellow shading indicates an intermediate resistant measurement, and green shading indicates a susceptible measurement.*

Antibiotic resistance	F-value ($F_{1,193}$)	p-value	ZoI measurement (mm) lactose-negatives (1 d.p.)	ZoI measurement (mm) lactose-positives (1 d.p.)
AZM	37.65	<0.001	19.5	14.5
C	27.33	<0.001	16.3	20.5
CIP	10.52	0.001	30.1	28.0
CN	57.57	<0.001	20.3	16.8
CTX	36.23	<0.001	17.7	23.1
F	113.91	<0.001	9.1	15.4
LEV	10.77	0.001	29.2	27.6
NA	7.55	0.007	19.8	21.7
OT	10.27	0.002	18.0	14.7
S	38.81	<0.001	15.8	12.3
SXT	11.28	<0.001	15.7	18.3

Significant time × compost heap position interactions were evident for only a handful of antibiotic resistances, and the patterns seen as a result of this interaction vary (Table 5.4). With amoxycillin/clavulanic acid, the lowest zone of inhibition measurement at day 100 belonged to centre-derived isolates, followed by outer and then intermediate isolates. This corresponded to resistance for centre isolates, but susceptibility for the others. For this antibiotic resistance, the regressions produced match this pattern, with the strongest negative relationship belonging to the centre (i.e., time may be having the strongest effect on centre isolates in terms of increasing resistance) (*r* =-0.44), followed by an extremely weak positive relationship for the outer (*r* =0.017), and stronger positive relationship for the intermediate (*r* =0.27). The pattern of the zones of inhibitions and the pattern of the regressions were not analogous for the other resistances experiencing this time-position interaction, however. For ampicillin resistance, isolates of the intermediate position had the lowest zone of inhibition, followed by centre and outer with the same measurement, although all of these measurements corresponded to resistance regardless. Despite this, the centre position had the strongest negative relationship with time (*r* =-0.73), albeit for intermediate it was only marginally weaker (*r* =-0.70). The outer position had a rather weak relationship (*r* =-0.25). For both chloramphenicol and trimethoprim/sulfamethoxazole resistance, the outer position had the strongest relationship with time (*r* =-0.44 and *r* =-0.58, respectively), followed by the centre position and then intermediate, which comparably had much weaker regressions. The zone of inhibition measurements at day 100 were different across the two antibiotic resistances however; the lowest measurement with chloramphenicol resistance was for intermediate isolates (intermediate resistance), then centre (intermediate resistance) and outer (susceptible), whilst for trimethoprim/sulfamethoxazole resistance it was centre isolates (intermediate resistance), followed by outer (susceptible) and then intermediate (susceptible).

Table 5.4: *The results of two-way ANOVA analyses for the potential interaction between time and position for the antibiotic resistances. Red shading indicates a resistant zone of inhibition measurement, yellow shading indicates an intermediate resistant measurement, and green shading indicates a susceptible measurement. The darkness of the blue shading represents strength of the potential relationship.*

Antibiotic resistance	F-value ($F_{19,163}$)	p-value	*r* value - centre	*r* value - intermediate	*r* value – outer	ZoI (mm) day 100 - centre	ZoI (mm) day 100 – intermediate	ZoI (mm) day 100 – outer
AMC	2.01	0.01	-0.44	0.27	-0.017	17.3	20.0	19.5
AMP	2.11	0.007	-0.73	-0.70	-0.25	9.5	7.3	9.5
C	2.61	<0.001	-0.077	-0.0031	-0.44	16.3	14.0	18.8
SXT	2.49	0.001	-0.21	0.056	-0.58	12.8	18.2	17.7

For the time × lactose-fermenting ability interaction, ten antibiotic resistances were implicated (Tables 5.5 and 5.6), and all but one resistance (trimethoprim/sulfamethoxazole) experienced an average temperature × lactose-fermenting ability interaction (Tables 5.7 and 5.8).

It is apparent that for the time × lactose-fermenting ability interaction in resistances that increased over time, lactose-positive isolates were largely the most affected by time in terms of increasing antibiotic resistance. Lactose-negative isolates being more readily impacted by time rather than lactose positives in terms of increasing resistance was found in cefotaxime resistance only. Furthermore, lactose-positive isolates were most often associated with having resistance at day 100, whilst the opposite was true for lactose-negatives (Table 5.5). For resistances that decreased with time, lactose-positive isolates were again most associated with a smaller zone of inhibition measurement at day 100, and lactose-negatives the most strongly impacted by time in terms of decreasing resistance. The only time this was to the contrary was for oxytetracycline, where it was lactose-positives that were potentially the most strongly impacted, although the smallest zone of inhibition measurement still belonged to lactose-positives (Table 5.6).

For the average temperature × lactose-fermenting ability interaction (for resistances which increased with average temperature), again it was lactose-positives who were the most impacted by this factor for increasing resistance. Despite this, however, it was lactose-negatives that tended to have more resistant final zone of inhibition measurements (Table 5.7). For resistances which decreased with rising average temperature, oxytetracycline and gentamicin were distinct in terms of whether lactose-positive or lactose-negative isolates were the most impacted (Table 5.8).

Table 5.5: *The results of two-way ANOVA analyses for the potential interaction between time and lactose-fermenting ability for the antibiotic resistances that increased over time. Red shading indicates a resistant zone of inhibition measurement, yellow shading indicates an intermediate resistant measurement, and green shading indicates a susceptible measurement. The darkness of the blue shading represents strength of the potential relationship, with darker associated with stronger.*

Antibiotic resistance	F-value ($F_{10,173}$)	p-value	*r* value – lactose-negatives	*r* value – lactose-positives	ZoI (mm) day 100 – lactose-negatives	ZoI (mm) day 100 – lactose-positives
AMC	3.00	0.002	0.0046	-0.4562	21	16.9
CIP	4.00	<0.001	0.044	-0.63	28.56	23.33
CTX	4.51	<0.001	-0.69	-0.45	13.2	20.9
ENR	3.81	<0.001	-0.015	-0.77	24.8	21.8
LEV	4.14	<0.001	0.074	-0.72	28.4	24.4

Table 5.6: *The results of two-way ANOVA analyses for the potential interaction between time and lactose-fermenting ability for the antibiotic resistances that decreased over time. Red shading indicates a resistant zone of inhibition measurement, and green shading indicates a susceptible measurement. The darkness of the blue shading represents strength of the potential relationship, with darker associated with stronger.*

Antibiotic resistance	F-value ($F_{10,173}$)	p-value	*r* value – lactose-negatives	*r* value – lactose-positives	ZoI (mm) day 100 – lactose-negatives	ZoI (mm) day 100 – lactose-positives
AZM	9.52	<0.001	0.62	-0.38	25.9	12.6
CN	2.79	0.003	0.53	0.031	23.44	16.11
OT	2.23	0.018	0.55	0.81	27.6	17.8
TGC	5.18	<0.001	0.28	-0.55	27.7	20.9
S	3.92	<0.001	0.18	-0.086	19.0	8.3

Table 5.7: *The results of two-way ANOVA analyses for the potential interaction between average temperature and lactose-fermenting ability for the antibiotic resistances that increased with average temperature. Red shading indicates a resistant zone of inhibition measurement, yellow shading indicates an intermediate resistant measurement, and green shading indicates a susceptible measurement. The darkness of the blue shading represents strength of the potential relationship, with darker associated with stronger.*

Antibiotic resistance	F-value ($F_{30,132}$)	p-value	r value – lactose-negatives	r value – lactose-positives	ZoI (mm) at highest temperature (30.22°C) – lactose-negatives	ZoI (mm) at highest temperature (30.22°C) – lactose-positives
AMC	2.30	<0.001	-0.020	-0.017	11.0	15.3
AMP	1.56	0.045	-0.16	-0.28	7.0	7.0
AZM	5.23	<0.001	0.0092	-0.038	11.3	11.7
C	2.00	0.004	-0.0061	-0.045	13.0	19.3
CIP	2.13	0.002	0.0039	-0.25	29.3	24.3
CTX	2.87	<0.001	-0.11	-0.21	9.0	20.3
ENR	1.87	0.009	-0.12	-0.25	22.0	21.33
F	2.07	0.003	-0.056	0.065	7.0	17
LEV	2.15	0.002	-0.0090	-0.20	26.0	23.7
NA	2.04	0.003	-0.084	-0.23	15.0	18.7
S	2.13	0.002	0.0083	-0.052	13.7	10.7
TGC	2.36	<0.001	0.0044	-0.049	19.3	20.0

Table 5-8: *The results of two-way ANOVA analyses for the potential interaction between average temperature and lactose-fermenting ability for the antibiotic resistances that decreased with average temperature. Red shading indicates a resistant zone of inhibition measurement, and green shading indicates a susceptible measurement. The darkness of the blue shading represents strength of the potential relationship, with darker associated with stronger.*

Antibiotic resistance	F-value ($F_{30,132}$)	p-value	r value – lactose-negatives	r value – lactose-positives	ZoI (mm) at highest temperature (30.22°C) – lactose-negatives	ZoI (mm) at highest temperature (30.22°C) – lactose-positives
CN	1.81	0.012	0.20	0.10	21.0	17.3
OT	1.92	0.007	0.092	0.28	20.0	19.3

5.4 DISCUSSION

5.4.1 The effect of composting on enteric bacteria levels

The levels of enteric bacteria of all positions and lactose-fermenting ability fluctuated throughout the composting period, but nevertheless increased (compared to day 31) by the end of the period (day 100) (Figures 5.1 and 5.2). It appears that the growth of lactose-negative enteric bacteria was especially promoted, and enteric bacteria of the outer position proliferated the most successfully. The lactose-negative enteric bacteria of the centre position and lactose-positive of the outer position were unique (including from each other) in terms of the factors that drove their changes in CFU g^{-1} F Wt., whilst the other groups were far more similar in terms of what may have affected them (Figures 5.3, 5.4 and 5.5). The time spent within the composting environment was an increasing factor for enteric bacteria of all depths within the compost heap, likely due to the lack of challenge by temperature from day 51 onward and the probable anaerobic conditions within the heap (as was explained in 3.4.3), the latter of which enteric bacteria would naturally be suited to. However, it does seem that the growth of the enteric bacteria of the centre position was hindered slightly by the temperatures of the compost heap (indicated from the ANOVA analyses). This is likely due to the heat of the heap being generated at the depths where the centre position was located, but nevertheless the enteric bacteria proceeded to grow, undoubtedly due to the inconsistency of higher temperatures.

These results are in opposition to what is found for other composting studies, where enteric bacteria such as *Clostridium perfringens*, *Enterococcus* species, *Staphylococcus* species, and *Escherichia coli* all decrease, often to levels below the detection limits employed (Macklin *et al.*, 2006; Thomas *et al.*, 2020; Subirats *et al.*, 2020). This is undoubtedly due to the insufficient thermophilic phase, probable anaerobic environment, and potentially even the length of the experimental period (thus enabling bacteria to proliferate further for a greater amount of time). Indeed, as seen in the study by Wilkinson et al. (2011), *E. coli* in poultry litter heated to both 35°C and 45°C (reflective of the uppermost temperatures achieved in this study) are able to persist at a vastly greater capacity than when exposed to temperatures of 55°C and above (as ideally should be reached during a thermophilic composting stage). It is worth noting, however, that this was under lab-based conditions. Although decreases were seen in the study by Macklin *et al.* (2006) despite the study's brief thermophilic phase and lack of aeration (as in this study), the difference was measured after two weeks; if the composting period was terminated in this study at a much earlier stage (i.e., day 36), decreases would have been seen also (Figures 5.1 and 5.2). As such, the aforementioned study cannot be used as a reliable comparison. Instead of being comparable to other chicken manure composting studies, the data from the present study are instead more similar to that of chicken litter "storage" studies, due to the lack of aeration in this study and its subsequent effects. Amongst studies that were conducted over a longer period (as to be more comparable to ours), staphylococci and enterococci remained at high levels after 120 days of storage, and *E. coli* levels also remained at high levels or increased after 12 weeks (Graham *et al.*, 2009; Wilkinson *et al.*, 2011).

5.4.2 The effect of composting on antibiotic resistance

The impact of the unsustained thermophilic phase and lack of aeration extends to the results seen for antibiotic resistance also. The resistance of isolates to the majority of the antibiotics employed in this study (10) increased over 100 days (although only significantly for three of these), but decreased to four (only significantly for two). Antibiotic resistances will therefore be examined based on whether they increased between day 0 and day 100, or decreased (according to resistance score). Overall, however, it can be said that composting appeared to have affected each antibiotic resistance in fairly unique way (based on PCA plots), with the exception of ciprofloxacin and enrofloxacin, who persistently clustered and so were likely affected much in the same way due to composting.

5.4.3 Antibiotic resistances that increased during the composting process

Ampicillin, ciprofloxacin, amoxycillin/clavulanic acid, cefotaxime, nitrofurantoin, enrofloxacin, nalidixic acid, and chloramphenicol all increased during the composting process, with the latter three significantly so. Azithromycin was also reported as increasing according to its change in resistance score between day 0 and 100, but has less straightforward results, and so will be given its own separate focus. Tigecycline is not included also, as only one isolate was found to be resistant in the entire sampling period. As interpreted by the ANOVAs performed, all but two (chloramphenicol and nitrofurantoin) may have increased as a result of time, particularly in the case of ampicillin, cefotaxime, and nalidixic acid. There could be multiple underlying reasons for this. One cause could be the general proliferation of bacteria with resistances to these antibiotics (which is supported by the increasing enteric bacteria levels discussed previously), thus increasing their prevalence in samples and the instances of them being isolated. Another reason could be the horizontal transfer of ARGs on MGEs, particularly in the case of integrons and transposons, as *intI1* and certain transposon genes have been shown as difficult to remove through composting (Xie *et al.*, 2016; Peng *et al.*, 2018; Wang *et al.*, 2019; Zhou & Yao, 2020; Subirats *et al.*, 2020). A further reason could be the increasingly anaerobic conditions as the composting process went on, and resistant bacteria were those able to thrive in such conditions.

The relationship with time was influenced by the position from which the isolates were derived in the cases of ampicillin, amoxycillin/clavulanic acid, and chloramphenicol. This influence appears to be derived from the fact that the centre position was associated with higher resistance, as well as time having a stronger impact on isolates from the centre position for ampicillin and amoxycillin/clavulanic acid resistance, and on the outer position for chloramphenicol and trimethoprim/sulfamethoxazole resistance on their increasing resistance levels. Perhaps this could be indicative that in this study, isolates possessing ampicillin and amoxycillin/clavulanic acid resistance tended to be facultatively anaerobic, whilst for the other two resistances they tended to be aerobic. It could also be that some other, unmeasured factor dictated this difference, such as the nutrients available at the different positions promoting the growth of different bacteria.

The ability of isolates to ferment lactose also impacted the degree to which resistances increased over time. Overwhelmingly it was lactose-positive isolates that were associated with resistance and an increase in resistance over time, except for in the case of cefotaxime resistance. Unfortunately, there is not a very large volume of other studies investigating the role of lactose-fermenting ability in antimicrobial resistance, particularly in this context. However, from a study based around *E. coli* of differential lactose-fermentation in a clinical setting, some comparisons can be made. Amoxicillin/clavulanic acid resistance was at a slightly higher level in lactose-positive isolates, agreeing with our findings, but all other results differ. Ciprofloxacin resistance was found at higher levels in lactose-negative isolates, and cefotaxime resistance higher in lactose-positives (Gajdács *et al.,* 2020). In a *Salmonella*-centred study in a much more appropriate setting however, lactose-positive isolates were associated with higher antibiotic resistance levels (Alexan, 2017). A study examining *Klebsiella pneumoniae* also found this, including streptomycin resistance as was also found in this study (Cabelli & Pickett, 1953).

Whilst significant for many resistances, the exceptionally weak relationships with average temperature indicate it was unlikely to realistically have had a large impact on the development of resistances, particularly in comparison to time.

Azithromycin resistance is unique from the other resistances in that, despite increasing from before composting to its completion for all positions according to resistance score, time actually had a deleterious effect on the level of resistance, and it could be found clustering with other resistances that decreased with time in two of the three PCA plots. These results are due to the other aspect that makes this resistance unique; there is an exceptional polarisation, depending on lactose-fermenting ability, of whether the resistance increases or decreases with time. Lactose-negative isolates decreased in resistance over time and were likely quite heavily impacted by this factor (and potentially the other factors it can entail, such as nutrient, moisture and oxygen level changes), whilst lactose-positives harshly increased. The potential explanation for this may be because of the existence of plasmids that confer both lactose fermentation and resistance genes, as introduced in the previous chapter (4.4.2) (Williams Smith & Parsell, 1976; Carey *et al.,* 1983; Walia *et al.,* 1987). As such, as time passed in the experiment, any such plasmids would have been able to propagate and spread, partially explaining the impact of time on increasing resistance. Of course, it is also entirely possible that the persistence and increase in resistance observed was in part due to these resistances belonging to mesophilic bacteria, which were able to survive and even proliferate within the heap due to the absence of a prolonged thermophilic phase to successfully remove them, and favourable conditions thereafter. Again calling back to the study by Wilkinson et al. (2011), this is illustrated with the slower removal of *E. coli* from poultry litter at temperatures under 55°C.

As with enteric bacteria levels, and also likely due to the unstable higher temperatures achieved and extended length of composting permitting growth, results were contradictory to what is found for other chicken litter/manure composting studies. ARGs associated with the previously listed antibiotics of this study (that is, those that increased) all generally decreased from as a result of composting in other studies, with the exception of some chloramphenicol resistance genes (Cui *et al.,* 2016; Xie *et al.,* 2016; Qian *et al.,* 2018; Zhou & Yao, 2020; Esperón *et al.*, 2020). As discussed before however, a decrease in certain ARGs may not signify that phenotypic resistance would also decrease. For stored poultry litter, results varied; over 50% of *Enterococcus faecium* isolates were resistant to ciprofloxacin after storage for 120 days in one study (Graham *et al.*, 2009), but levels of ciprofloxacin-resistant *Enterobacteriaceae* decreased after 63-day storage in another (Moraru *et al.,* 2012). ARGs to several antibiotic classes decreased during 60-day storage in a further study, but not in the bottom layer of the stockpile. Furthermore, a number of ARGs to all antibiotic classes included were persistent throughout the study, and ARG richness increased greatly (Gupta *et al.,* 2021).

5.4.4 Antibiotic resistances that decreased during the composting process

Trimethoprim/sulfamethoxazole, gentamicin, oxytetracycline and streptomycin decreased in resistance over the composting period (according to resistance scores), with the latter two doing so significantly. Only gentamicin and oxytetracycline resistance decreased as a result of time as a single factor, with oxytetracycline resistance in particular being heavily impacted by time, especially for lactose-positive isolates when considering a time and lactose-fermenting ability interaction. For the other resistances however (gentamicin and streptomycin) it was lactose-negatives that were the most impacted. For every antibiotic resistance that decreased as a result of time, lactose-positive isolates were associated with being more resistant than lactose-negatives, perhaps, again, due to the existence of the previously described plasmids. Interestingly, trimethoprim/sulfamethoxazole resistance was found to increase in the centre position, and only amongst lactose-negative isolates; perhaps, then, a high proportion of enteric bacteria belonging to this group (lactose-negative bacteria of the centre of the heap) were *P. aeruginosa*, as it has intrinsic resistance to trimethoprim/sulfamethoxazole due to an efflux system and would be able to survive in the centre as a facultative anaerobe (Köhler et al., 1996). Lactose-positive isolates from the intermediate and outer positions actually increased in resistance to gentamicin (intermediate position isolates only) and streptomycin, and so clearly some mechanism amongst lactose-positive enteric bacteria providing aminoglycoside resistance had likely occurred.

It is unknown why these antibiotic resistances decreased whilst so many other increased. Perhaps enteric bacteria harbouring these resistances prior to/at the very beginning of composting were simply unsuited to the conditions created within the compost heap, through being especially heat-sensitive, or sensitive to the changing nutrients, pH and moisture, which would have occurred.

5.5 CONCLUSION

The results of this study highlight the importance of proper aeration during composting to enable the successful removal of both enteric bacteria and antibiotic resistance in chicken litter, in order to make it safe for use as fertiliser. If farmers instead choose to store chicken litter, or do not carefully maintain aerated composting conditions, it can be seen through this study that the resulting product could be environmentally hazardous, even more so than fresh chicken litter itself. The study also shows that there is some importance in the position/depth of the composting heap in terms of changes in antibiotic resistance throughout composting, as some antibiotic resistances received very different results depending on where the isolates were derived from. Whether isolates were lactose fermenters or not also had a large role to play in terms of the resistance seen and its change over time.

6 FINAL CONCLUSIONS

Several conclusions can be drawn as a result of the research completed in this study, the most prominent of which are that non-commercial, antibiotic-free chicken litter can harbour high levels of resistance to a multitude of different antibiotics and antibiotic classes, and that aeration of a compost heap is of utmost important in order for compost heaps to achieved an extended thermophilic phase, and subsequently effectively remove enteric bacteria and antibiotic resistant bacteria of chicken litter. Insights were given into the severity of what can occur if this aeration is not achieved, through the increase of resistance to the majority of the antibiotics tested. The impact of compost heap location/depth on different factors was also revealed, with composting temperatures closely resembling one another in terms of pattern, and the antibiotic resistances of enteric bacteria only rarely being impacted in any capacity by the location/depth from which they were isolated. This study is also one of few to employ phenotypic methods to determine resistance in this research area, which may be more important to include than genotypic methods alone in order to capture the true profile and level of resistance in chicken litter and compost.

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