Investigating the dynamics of antibacterial drug resistant bacteria within the pig and poultry industries

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by

Evelyn J Pleydell

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I dedicate this thesis to all interested stakeholders. I hope that its contents add, in some way, to the understanding of this important topic, and that people can work together without prejudice in order to ensure the future efficacy of antibacterial drugs.

Preface

This PhD has spanned eight years and three continents, although at the outset it was only envisaged that it would take three to four UK-years. The duration of that journey has involved an unending steep learning curve, with the need to acquire skills across a range of disciplines: microbiology, molecular microbiology, individual chicken identification, epidemiology, statistical analysis and modelling, Bayesian statistics..., multivariate analysis and data mining, a variety of complex and ever-evolving software packages, and eventually (the one I was trying to avoid) subsistence living.

Working in the field of resistance also presented exciting challenges in terms of communicating the (sometimes less than optimal) results of this work to a spectrum of stake holders, such as: government representatives and policy makers, the pharmaceutical industry, livestock industries, veterinary societies, advisers and specialists in organic farming, as well as scientific peers. I was struck by the openness with which all these people listened, and the intelligent and searching nature of the questions that were asked. On these occasions I gained insights into the way in which science can act to objectively inform society, and also the opportunities for science to play a central and cohesive role in bringing groups of people of diverse skills and interests together in order to address an issue.

The first four research chapters of this thesis are based on data that I collected on pig and poultry farms in southern and central England for a project that was funded by a research grant from the Department for Environment, Food and Rural Affairs. At the outset, it was envisaged that longitudinal data would be collected over three years on a number of conventional and organic farms in order to ascertain the occurrence and persistence of antibacterial drug resistant commensal bacteria on farms of contrasting management styles. All the farm information was collected by personal interviews with the farm managers, and I undertook the majority of sampling visits and laboratory work myself.

As the work progressed, the unexpectedly high level of detection some resistant bacteria seen on all farms, regardless of the level of use of antibacterial drugs (ABDs), coupled with a number of the smaller independent farms going out of business or changing the nature of their enterprises, caused a rethink regarding project design. In consequence of this, a series of intensive longitudinal studies were embarked upon using two poultry farms, with the aim of looking more closely at resistance dynamics on a farm

over time, and to try to relate that to farm management events. Again I personally undertook all aspects of this work, from farm sampling, laboratory processing, data entry and, eventually, analysis.

My transfer to New Zealand, then provided the opportunity to examine resistance in a very different country where the poultry industry used ABDs in a very different manner to the British companies. I arrived at a time when a report from a group of experts convened by the New Zealand Food Safety Authority had highlighted that it was particularly difficult to assess the risks posed by the presence of ABD resistant bacteria in New Zealand livestock as very little resistance data had been collected within the country. A grant from the Poultry Industry Association of New Zealand helped to fund a pilot project to look at ABD resistance in bacteria isolated from chicken carcasses. This work forms the basis of the final two manuscripts in this thesis; it provides a real contrast to the UK data as well as insights into the dynamics of resistance at the livestock company level. At this point I was promoted from the laboratory workbench to the lecture theatre, and Lynn Rogers undertook all aspects of the laboratory work associated with this study and provided wise counsel when some of the results were a little unexpected.

At the end of these eight years there are still many questions unanswered and still more have arisen over the course of time; however, this thesis does document progression within my own understanding of antibacterial drug resistance on livestock farms and I hope that I can usefully impart some of that understanding to other interested parties.

Abstract

This body of work sought to investigate factors influencing the patterns of antibacterial drug resistance associated with pig and poultry farms. Collecting data on convention and organic farms in the UK, and from broiler chickens in New Zealand, enabled comparisons to be drawn across a range of farming styles.

Direct links were seen between the use of enrofloxacin on pig farms and the isolation of fluoroquinolone-resistant *Escherichia coli*, and between the prophylactic use of lincomycin-spectinomycin in young chicks and the shedding of vancomycin-resistant *Enterococcus faecium*: both bacteria of potential public health concern. However, increases in the frequency of detection of other antibacterial drug (ABD) resistant bacteria were associated with the oral administration of non-specific ABDs, including therapeutic drugs used for prophylaxis, and sub-therapeutic or growth promoting drugs. Furthermore, frequent use of ABDs was also associated with increases in multidrug resistance (MDR) within individual *E. coli* or *Enterococcus*. Furthermore, using prophylactic ABDs in young chicks appeared to limit the frequency of isolation of susceptible strains of *E. coli* throughout the rearing period of that flock.

The use of multivariate analytical techniques allowed for an assessment of the associations between all farm-level covariates, which was not possible using regression modelling alone. This work indicated that host and bacterial factors also influenced ABD resistance, such as: animal age or production status, feed-type, disease, and mortality rate. Furthermore, finding less clear associations between farm practices and the detection of ampicillin-resistant *E. coli* and erythromycin-resistant *E. faecium* suggests that ABD resistance on a farm is also influenced by external factors, such as other human activities within that region.

Nevertheless, the frequent application of oral antibacterial drugs on some livestock farms was associated with more frequent detection of resistant bacteria, higher counts of ABD-resistant faecal *E. coli*, and a higher degree of multidrug resistance. Therefore, with clinical ABD resistance on the rise, the veterinary profession must increase its understanding of the biology behind ABD resistance, and work closely with the livestock industries to develop farming practices that maintain animal health and productivity, whilst limiting the potential for the development and maintenance of intractable reservoirs of resistance genes on farms.

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Fortunately, the esoteric nature of the modelling was superbly balanced by the down-to-earth (or more appropriately down-to-litter) approach of Dr Rob Davies. Rob's vast knowledge and practical experience of foodborne zoonoses, antibacterial resistance and the UK livestock industries has been indispensable, as was his skill in acquiring research funding.

Thanks must also be offered to Professor Martin Woodward, whose numerous chats over a cup of tea helped me to understand some of the more fundamental scientific aspects of ABD resistance, and whose enthusiasm for science was contagious and really inspired me to aim high.

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Many people at the Veterinary Laboratories Agency also provided a lot of support to help me make the transition from practising veterinarian to research scientist: Ian McLaren hid all traces of amusement as I fumbled to open a universal with one hand or streaked agar plates inelegantly; Dr Ernesto Liebana provided a much needed transfer

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"We cannot solve problems by using the same kind of thinking we used when we created them."
Albert Einstein

Chapter 1

An assessment of the risk factors for the emergence, maintenance, and persistence of antibacterial drug resistant bacteria on pig and meat chicken farms

1.1 The history of antibacterial drug use on farms

Prior to the advent of the widespread clinical use of penicillin in the 1940s, Alexander Fleming had already demonstrated that bacteria that were resistant to penicillin could be induced in the laboratory by altering either the concentration of the drug used or the conditions under which the bacteria were grown (Levy, 1992). Thereafter, the first published reports of clinical isolates of penicillin-resistant *Staphylococcus aureus* appeared as early as 1944 (Barber and Rozwadowskadowzenko, 1948; Kirby, 1944). For two decades, however, science appeared to be ahead of the game as a large number of antibacterial drugs (ABDs) were discovered and patented, revolutionising the treatment of bacterial diseases in human and, subsequently, veterinary medicine.

In the 1950s, in addition to their use as veterinary therapeutic agents, the practice of incorporating sub-therapeutic doses of ABDs into animal feed was introduced in many countries in order to enhance livestock productivity (Dibner and Richards, 2005; Feighner and Dashkevicz, 1987; Knarreborg et al., 2004). Although there are a number of mechanisms contributing to the growth-promoting effects of sub-therapeutic doses of ABDs, the predominant cause is the increase in food conversion efficiency that results from decreased competition for nutrients between the host animal and enteric bacterial populations (Dibner and Richards, 2005). However, this practice came under scrutiny in the 1960s, as strains of *Salmonella enterica* that were resistant to one or more ABDs were isolated from diseased calves at increasing frequency (Anderson, 1968; Gibson, 1965; Wray and Sojka, 1977).

In response, the UK Government commissioned a Joint Committee to report on the use of antibacterial drugs in animal husbandry and veterinary medicine. The resultant Swann Report, published in 1969, concluded that administering ABDs to animals at sub-therapeutic levels presented risks to human and animal health (Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, 1969). However, this committee surmised that these risks could be minimised by only utilising drugs of low therapeutic value for the purposes of growth enhancement. In light of this review, the European Commission imposed the 1974 ban on the use of penicillin and tetracycline as agricultural growth promoting agents across the European Union (EU Directive 70/524/EEU).

Nonetheless, a number of drugs not deemed as important in human medicine at that time (such as avilamycin, avoparcin, salinomycin, spiramycin and virginiamycin) retained their licences as in-feed, sub-therapeutic, growth promoters in Europe. That is, until reservoirs of vancomycin-resistant enterococci (VRE) were discovered on some livestock farms in the 1990s (Bates et al., 1994), an association was found between the presence of VRE in livestock and the use of avoparcin as a growth promoting agent (Aarestrup, 1995). Furthermore, molecular characterisation of vancomycin-resistant *Enterococcus faecium* (VREF) from animal and human sources suggested that the vancomycin-resistance genes were moving horizontally between human and animal-adapted strains of *E. faecium* (Jensen et al., 1998; Stobberingh et al., 1999). This prompted the EU to impose the 1997 ban on the administration of avoparcin to livestock (Directive 97/6/EC), and the manufacturer withdrew the drug from the global market in 1999.

Subsequent to this, in-line with recommendations from the World Health Organisation (WHO) on the prudent use of antibacterial drugs on livestock farms (World Health Organisation 1997), two further EU directives were implemented (EC Regulation 2821/98 and 1831/2003). Together, these directives resulted in a total ban on the use of ABDs as digestive enhancers or growth promoters within Europe from January 2006. The implementation of the final directive occurred amid much scientific controversy, controversy that continues to this day.

The proponents of the withdrawal claim that administering sub-therapeutic doses of an antibacterial drug enhances the selection of resistant strains of bacteria (Florea and Nightingale, 2004). They argue that the most effective way to reduce resistance in bacteria of animal origin is to reduce the need for antibacterial drugs via alterations in animal husbandry practices (Khachatourians, 1998; van den Bogaard and Stobberingh, 1999; Witte, 2000).

The opponents, on the other hand, claim that there is insufficient evidence that removing growth enhancing ABDs does reduce resistance problems in human medicine, and that the largest drivers for these problems is the medical use of ABDs (Bywater, 2004; Davies and Roberts, 1999; Phillips, 1999). They also suggest that removing the

use of non-clinically relevant growth promoters could actually result in an increase in the administration of clinically-relevant therapeutic agents and thus could potentially enhance resistance not reduce it (Casewell et al., 2003).

These discussions remain pertinent today because, in contrast to the situation in the EU, at least 17 classes of antibacterial drugs remain licensed for use as growth-promoting and prophylactic agents in the USA (Mathew et al., 2007). Notably, avoparcin has never been used within the American agricultural industry; however, the US list of drugs licensed for in-feed use does include agents that are identical or closely related to drugs used in human medicine such as penicillin, chlortetracycline, lincomycin and colistin (Mathew et al., 2007; Shea, 2004). However, in response to a growing public awareness, and rejection, of the extensive use of ABDs on farms, some major US food companies are now requesting meat from farms that have not used in-feed antibacterials thus indirectly forcing some producers to change their management policies (Dibner and Richards, 2005). Furthermore, some US producers have voluntarily reduced or removed the use of sub-therapeutic drugs on their farms in order to exploit the burgeoning market for 'antibiotic free production' (Baker, 2006).

Despite the disagreements regarding the potential effects of banning the use of sub-therapeutic growth promoting ABDs, there is a general agreement that there is a need for more data in order to ascertain the actual risks, and benefits, of this form of drug use. In-line with recommendations from the Food and Agriculture Organization of the United Nations, the World Health Organisation, and the World Organisation for Animal Health an increasing number of countries are undertaking the routine surveillance of resistance in bacteria isolated from animals (Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials, 2007).

One of the pioneering programmes was the Danish Integrated Antibacterial Resistance Monitoring and Research Programme (DANMAP). Established in the early 1990s, DANMAP provides statistically valid surveillance of antibacterial drug resistance in bacteria sourced from humans and animals at the national level (Bager et al., 2000). National surveillance systems generally monitor resistance in food-borne, zoonotic organisms, such as *Campylobacter* species and *Salmonella enterica* serovars, but they also monitor resistance in less pathogenic indicator species, such as non-type-specific *Escherichia coli* and *Enterococcus* species. Commensal bacteria, such as the majority of *E. coli* strains and enterococci, can act as markers for resistance in a given environment (Sunde and Sorum, 1999). They also act as potential vectors for resistance genes for more pathogenic bacteria; and in themselves, some strains are opportunistic pathogens.

In recent years, several surveillance systems have also begun to monitor the consumption of antibacterial drugs alongside resistance within bacteria. Due to the availability of centrally recorded prescription data, the DANMAP system allows for the detailed analyses of drug use in the medical and veterinary fields (Bager et al., 2000). In most other countries the systematic recording of drug use is rare, and drug sales

data is commonly used as a proxy for drug use. Nonetheless, comparing the dynamics of ABD resistance in natural populations of bacteria to trends in ABD consumption or sales can be insightful.

1.2 Links between the use of growth promoters and resistance

The various European bans on the use of in-feed, growth promoting drugs for rearing livestock are allowing for the observation of the subsequent dynamics of resistance in pathogenic and commensal bacteria.

1.2.1 The withdrawal of tetracycline as a sub-therapeutic agent

Tetracycline was in common use as a sub-therapeutic growth promoter within the UK pig industry for 17 years until the implementation of a national ban on this practice in 1971. Analysis of faecal samples collected from pigs at market in 1970 and 1972, suggested that there had been a decrease in the proportion of faecal *E. coli* that were tetracycline-resistant in 1972; nonetheless, the percentage of pigs that were shedding tetracycline-resistant *E. coli* (TREC) remained high at 93% (Smith, 1973). After this, the annual percentage of pigs that were shedding TREC actually rose to 100% by 1975 (Smith, 1975). Smith postulated that the lack of decrease in the number of positive pigs was likely to be due to the continued widespread use of tetracycline as a full-dose therapeutic agent.

However, molecular laboratory studies demonstrated that the percentage of isolates that were capable of transferring tetracycline-resistance determinants to susceptible *E. coli* recipient strains was lower for the 1972 isolates compared with the strains collected in 1970 (Smith, 1975). Smith also observed that this decrease was reversible, because supplying extraneous transfer factors resulted in the remobilisation of resistance determinants in 60% of the non-transmitting resistant strains. Therefore, even though the plasmids carrying resistance genes persisted under these changing drug selective pressures, conjugation of this plasmid-borne resistance appeared to impair bacterial fitness, and horizontal gene transfer mechanisms were therefore lost from the population.

However, in the 21st century, tetracycline drugs remain one of the most commonly used classes of ABDs in veterinary medicine across the globe, and the isolation of TREC from animals remains commonplace (de Jong et al., 2009; Literak et al., 2009).

1.2.2 The withdrawal of avoparcin

The Danish withdrawal of avoparcin occurred in 1995, two years ahead of the general EU ban. Data from the DANMAP surveillance programme showed that, 73% of *E.*

faecium isolated from broiler chickens at slaughter in 1995 were resistant to vancomycin, but by 1998 this had fallen to 8% (Aarestrup et al., 2001). Furthermore, the predicted probability of isolating vancomycin-resistant *E. faecium* (VREF) from retail poultry meat also decreased significantly between 1995 and 2001 (Emborg et al., 2003).

In contrast, the proportion of *E. faecium* resistant to vancomycin that were isolated from Danish pigs at slaughter did not decrease between 1995 and 1997, with VREF representing a steady 20% of *E. faecium* isolated per annum (Aarestrup et al., 2001; Bager et al., 1999). Genotyping work using pulsed-field gel electrophoresis (PFGE) indicated that co-selection, due to the use of ABDs other than avoparcin, was influencing the persistence of VREF on pig farms (Aarestrup, 2000). The porcine *vanA*-positive VREF isolates all showed concurrent macrolide resistance due to the carriage of the *ermB* gene, and the two genes were transferred simultaneously from donor to recipient strains.

Indeed, the macrolide drug tylosin was in common use on Danish pig farms at that time, both as a therapeutic agent and in sub-therapeutic doses as an in-feed growth enhancer. The annual quantities of tylosin administered to pigs in Denmark ranged from 52,000 kg to 68,000 kg between 1995 and 1997 (Aarestrup et al., 2001). However, after the withdrawal of the licence for the use of tylosin at sub-therapeutic doses in 1999 the annual consumption of the drug within the Danish pig industry fell to 1,800 kg within a year. In tandem, the percentage of *E. faecium* isolated from Danish pigs that were resistant to vancomycin also fell to 6% (Aarestrup et al., 2001).

The Danish story, as presented above, would seem to suggest that the predominant influence upon the persistence of VREF on farms was the use of ABDs. In fact, observations regarding the persistence of VREF on Norwegian broiler farms appeared to contradict this. In Norway, VREF continued to be detected on broiler farms that had previously administered avoparcin for several years after the withdrawal of the drug (Borgen et al., 2000a,b). During this time the only ABD used within the Norwegian poultry industry was a relatively small amount of zinc bacitracin, although most farms were using ionophor anticoccidial drugs which also show a degree of antibacterial activity.

The Danish and Norwegian studies of VREF persistence are not directly comparable, however, because they were measuring different aspects of the persistence of ABD resistance. The DANMAP programme collected caecal or cloacal samples from birds at slaughter, the samples were plated onto *Enterococcus*-selective media and resultant colonies showing typical *E. faecium* morphology were submitted for confirmation of identity and susceptibility testing (DANMAP 1998). In contrast, the Norwegian poultry farmers submitted faecal material collected from the floors of the poultry-houses that were plated directly onto selective media containing 50 μg/ml vancomycin. Consequently, the Danish surveillance results were reflecting a decrease in the proportion of the intestinal *E. faecium* population of poultry that were vancomycin-

resistant, but were not indicative of the number of farms on which birds were still shedding VREF, albeit at lower levels than previously. In contrast, the Norwegian direct-plating methods revealed that VREF were still detectable on the Norwegian broiler farms that had previously used avoparcin, but supplied limited information about the level of VREF persistence within those farms.

In fact, direct-plating methods demonstrated that five years after the withdrawal of avoparcin, VREF were still detectable in 104 of 140 Danish broiler flocks that had been previously exposed to the drug (Heuer et al., 2002a). Similarly, five years after the withdrawal of avoparcin in the UK, VREF were detectable using direct plating methods on some conventionally managed broiler farms (Garcia-Migura et al., 2005).

1.2.3 The withdrawal of virginiamycin

Another growth-promoting drug that can directly select for resistant bacteria of potential public health concern is the streptogramin drug: virginiamycin. Streptogramin-resistant enterococci are resistant to quinupristin-dalfopristin (QD), which is a drug used in human medicine where it is particularly important in the treatment of nosocomial VREF infections (Smith et al., 2003). Virginiamycin is still in use as a growth promoter in some countries, and there is considerable medical, scientific and political debate regarding the public health risks associated with this use of the drug.

What is clear is that the use of virginiamycin as a growth promoter does select for streptogramin-resistance within the enteric *Enterococcus* populations of farm animals. Danish surveillance revealed that from 1995 to 1997 the amount of virginiamycin consumed by food animals in the country had increased four-fold to more than 10,000 kg per annum (DANMAP 1998). During this period the proportion of *E. faecium* isolated from broiler chickens that were resistant to virginiamycin (VMREF) also increased from 27% to 66%. After the Danish prohibition of all antibacterial (AB) growth-promoting agents, in January 1998, the proportion of VMREF isolated fell, reaching 34% by the year 2000 (Aarestrup et al., 2001). Thereafter, VMREF fluctuated between 10–30% until 2004 (DANMAP 2007). Once again, co-selection of VMREF due to the use of other ABDs was responsible for this persistence. The majority of VMREF were concurrently resistant to penicillin, and as the amount of amoxicillin used in the industry came down (partly due to decreasing clinical efficacy of the drug on broiler farms) the proportion of *E. faecium* that were VMREF dropped to practically zero from 2005 onwards (DANMAP 2008).

Comparative assessments of country-level drug administration practices and ABD resistance surveillance data can also provide insights into trends in ABD use and resistance. In the late 1990s, when VMREF were frequently isolated from Danish poultry, these bacteria were less frequently isolated from Danish pigs and Finnish poultry, and never isolated from Norwegian pigs or poultry (Aarestrup et al., 2000c). These patterns of isolation reflected the frequency of application of virginiamycin

within the livestock sectors of those countries, with the highest use occurring in the Danish poultry industry, and no use of the drug at any time in Norway. This study also found similar associations between higher drug consumption and higher proportions of enterococci expressing resistance to other growth promoters: avilamycin, zinc bacitracin, and tylosin at sub-therapeutic levels.

Whilst, inter-country studies can provide broad assessments of drug use and resistance, farm-level studies allow for a more detailed examination of patterns of streptogramin-resistant *E. faecium* over time and in relation to drug use. One experimental study looked at the effects of dosing consecutively reared flocks of broiler chickens with virginiamycin upon quinupristin-dalfopristin-resistant *E. faecium* (QDREF) isolated from cloacal swabs (McDermott et al., 2005). Although QDREF emerged in the initial flock within five weeks of the commencement of administration of virginiamycin, birds in the second and third flocks were already shedding over 75% QDREF by 7 days of age, suggesting that there was carry-over of QDREF in the environment between flocks. Furthermore, a fourth flock that was reared without in-feed virginiamycin still shed QDREF until seven weeks of age, after which time no more QD-resistant isolates were detected. This work implies that the long-term maintenance of this resistance within chickens, at least at readily detectable levels, requires the continuous application of virginiamycin.

The use of virginiamycin can also potentially influence resistance to erythromycin. Streptogramin drugs, are composed of two different molecules (group A and group B) and full resistance requires two separate mechanisms. Although the streptogramin B molecules are structurally unrelated to the macrolide and lincosamide drug families, they do share the same mechanism of action. Therefore, the macrolide-lincosamide-streptogramin (MLS) resistance gene *ermB* jointly facilitates resistance to erythromycin and the streptogramin B component of virginiamycin (Bozdogan and Leclercq, 1999).

A field study demonstrated this phenomenon using farm-level information on drug use collected from the ante-mortem health certificates that accompany Danish broiler flocks arriving at a processing plant (Emborg et al., 2004). Generalised linear mixed effects modelling (GLMM) showed that there was a 92% chance of selecting an *E. faecium* isolate that was resistant to erythromycin on farms using virginiamycin. After the withdrawal of virginiamycin, the probability of selecting an erythromycin-resistant *E. faecium* fell to 20% on many farms within four years. The authors suggest that, because other macrolide drugs such as tylosin and spiramycin were not in common use on Danish broiler farms, the decrease in erythromycin-resistance was most likely to be associated with the withdrawal of virginiamycin.

1.2.4 The withdrawal of tylosin as a sub-therapeutic agent

Tylosin is a macrolide drug in the same class as erythromycin. In a similar manner to the tetracycline story in the UK, the cessation of use of tylosin at sub-therapeutic doses

in Denmark resulted in an initial decrease in the proportion of *E. faecium* isolated from pigs at slaughter that were resistant to erythromycin (EREF). However, the Danish pig industry still administers 10,000 kg of tylosin per year at full therapeutic dose, and erythromycin-resistance continues to be identified in 30 to 50% of porcine isolates (DANMAP 2008). In contrast, the Danish broiler industry only use 10 kg of macrolide drugs a year, but EREF is persisting in this livestock sector at approximately 13% of isolates a year, implying that other factors are maintaining this relatively low level of resistance.

A Swiss study showed similar results, with the percentage of *Enterococcus* isolates cultured from porcine faeces decreasing after tylosin was withdrawn as a growth promoter (Boerlin et al., 2001). However, this work also showed that there was a concurrent decrease in tetracycline-resistant enterococci around the same time, which the authors also attributed to the fall in the use of tylosin.

1.2.5 The withdrawal of avilamycin

The withdrawal of avilamycin as an in-feed growth promoter within the EU created a lot of controversy because there are no analogues of this drug used in human medicine due to uncertainties over their safety as therapeutic agents (Shryock, 2001). Therefore, the manufacturers of avilamycin suggested that the use of this drug on farms represented a lower public health risk than the use of many therapeutic ABDs.

In terms of assessing resistance to avilamycin itself, the DANMAP figures show a familiar picture, with resistance to avilamycin in broiler-derived *E. faecium* populations rising in line with increasing drug use prior to the 1998 withdrawal, and subsequently falling after the ban (Aarestrup et al., 2001). There was, however, an unexplained spike in 2006 when 13% of *E. faecium* isolates were avilamycin-resistant compared to 2% in 2005 and 3% in 2007, this spike appeared to be species-specific as no *Enterococcus faecalis* isolates were avilamycin-resistant from 2005 though to 2007 (DANMAP 2006).

Farm-level studies also show a link between avilamycin use and avilamycin-resistance in enterococci. A nationwide case-control study of *E. faecium* isolated from broiler chickens at slaughterhouses in France, for instance, found that the odds of isolating an *E. faecium* that was resistant to avilamycin (AREF) was significantly higher when the broilers originated from farms that were using the drug (Chauvin et al., 2005a). Likewise, a 1998 Danish study showed that 72% of *E. faecium* isolated from eight farms that had administered avilamycin in 1996 and 1997 were avilamycin-resistant compared with 23% from ten farms that had not (Aarestrup et al., 2000b). Nonetheless, AREF were still isolated on seven of the ten farms that had not administered avilamycin in 1996 and 1997. The authors did not report whether the drug had been used on these farms prior to 1996.

1.2.6 Resistance in bacteria other than enterococci

Taken together, the results of the studies described above have provided evidence that very low doses of drugs administered in animal feed for prolonged periods can, and do, exert influential selective pressures upon the resistance expressed by populations of enterococci within the intestinal tracts of the treated animals. *Enterococcus* species are the most commonly used indicator organisms for resistance to these growth-promoting antibacterials because these drugs mainly exert their effects upon the Gram-positive members of the enteric bacterial populations (Butaye et al., 2003). In terms of assessing the overall risks associated with using ABDs at sub-therapeutic doses, it would also be of interest to know whether this pattern of ABD use influences resistance in other enteric bacteria, including pathogenic species. There is, however, a relative paucity of published data in this area.

One experimental trial investigated the effects of administering in-feed avilamycin upon the emergence and persistence of antibacterial resistance within a number of different enteric bacteria isolated from pigs (Delsol et al., 2005). This work showed that avilamycin-resistant *Enterococcus faecalis* could be isolated from the pigs during the three-month drug administration period and for one week after the drug was withdrawn from the feed. In contrast, resistance in *E. coli*, *Campylobacter* species and *Salmonella enterica* serovar Typhimurium did not alter during the course of the study; although a higher proportion of tetracycline-resistant *E. coli* were carrying the *tetB* gene rather than *tetA* in the treatment group relative to the controls. This study looked at a single rearing cycle of pigs in isolation, which allowed for the control of extraneous confounding factors that are inherent within on-farm studies. However, it is difficult to extrapolate the results obtained from such simplified models to a farm that is applying sub-therapeutic levels of ABDs routinely to consecutive groups of animals. As shown by the US chicken model of quinupristin-dalfopristin-resistance in *E. faecium*, resistance dynamics can show different patterns in consecutive groups of animals (McDermott et al., 2005).

1.3 Links between the veterinary use of ABDs and resistance

The Scandinavian countries have reported that due to the removal of growth promoting feed additives, the total quantities of ABDs that are being administered to animal populations have decreased. However, the veterinary professions in these countries still rely upon ABDs to treat, and sometimes to prevent, clinical disease. As all ABD use will select for bacteria that are able to resist the effects of the drug, it is important understand the effects that different methods of administering ABDs have upon bacterial drug resistance.

1.3.1 Changes in therapeutic ABD use after the withdrawal of ABGPs

Livestock industries initially adopted the use of antibacterial growth promoters (ABGPs) because they enhanced farm productivity. However, their antibacterial properties also contributed to the maintenance of good health, and some animal production experts predicted that the withdrawal of ABGPs would have adverse effects upon animal health and welfare. One consequence of deteriorating animal health could be a rise in the quantities of therapeutic ABDs used, and these drugs are more likely to be directly related to, and sometimes identical with, drugs used in human medicine (Shryock, 2001).

The removal of ABGPs across Europe has allowed for the assessment of their impact on animal health and therapeutic drug use. In 1986, Sweden became the first country to ban the use of ABGPs. The Swedish broiler industry anticipated that there would be a post-ban increase in the incidence of necrotic enteritis due to *Clostridium perfringens* infections. For the first two years following the ban, farmers administered therapeutic doses of virginiamycin prophylactically throughout the rearing period; however, for economical reasons this practice gave way to the implementation of a two-day course of penicillin as soon as the first signs of enteritis appeared (Wierup, 2001). Over time, it emerged that husbandry practices, such as reducing dietary protein levels and providing adequate airflow, helped to decrease a farm's dependence upon ABD use. The use of a penalty-based classification system within the industry encouraged best practice, and by 1995 the amount of ABDs used within the Swedish poultry industry had become negligible.

There was a similar spike in ABD use within the Norwegian broiler industry after the withdrawal of ABGPs in 1995 (Grave et al., 2004). The mean estimated percentage of chickens treated with ABDs from 1990–1994 was 3.4%, in the second half of 1995 that rose to 11.3%, before falling to 5% in 1996, and then stabilising at 3.9%. The 1996 decrease coincided with 90% of poultry producers changing to the newly licensed anticoccidial drug narasin. Narasin also has antibacterial properties (Butaye et al., 2003) and it is effective against *C. perfringens* (Brennan et al., 2001). Narasin is also in common use within the Swedish broiler industry, and it is likely to be contributing to the low-level of other ABDs used in that country.

In contrast, the Swedish pig industry, encountered longer-term problems than the broilers (Wierup, 2001). Whilst the removal of growth promoters did not cause clinical problems in the older growing and finishing animals, the incidence of post-weaning diarrhoea escalated. Initially, farms relied heavily on the use of olaquindox (an ABD), and then zinc oxide was used as a feed supplement to mitigate the problems. However, husbandry practices again had a role to play, in particular, segregating groups of weaners and raising them in deep-bedding systems was associated with a substantial decrease in the use of medicated feed. Dietary adjustments were also helpful, and by

1999 (13 years after the ban) only 17% of herds were using in-feed zinc oxide, although further 5% were still relying on the prophylactic use of ABDs due to structural and logistical restraints present on those farms.

A retrospective, pharmaco-epidemiological study in Switzerland showed that an increase in ABD use after the withdrawal of ABGPs is not inevitable. Before the 1999 EU ban of four ABGPs, the prescribed daily doses (PDD) per population of pigs in the St Gall region fell from 6.1 in 1996 to 3.6 in 1999 (Arnold et al., 2004). After the ban, the PDD per population remained relatively stable at 3.3 in 2000 and 3.4 in 2001. However, the annual kg of active agents administered had actually risen from 708 kg in 1999 to 956 kg in 2001. These figures reflected a change in prescribing habits of ABDs; there had been a decrease in the use of high-potency drugs, like tylosin, and a corresponding increase in the use of lower potency drugs, such as potentiated sulphonamides, which required higher doses. The reduction in the use of tylosin was a result of the successful efforts of the Swiss pig industry to control porcine respiratory disease via improvements in hygiene and husbandry practices. Nonetheless, problems were once again encountered raising groups of weaner piglets in the absence of ABGPs and there was a post-ban increase in the quantity of colistin administered to low weight pigs.

The Swiss findings were corroborated by a Danish case-crossover study of therapeutic ABD use on 68 farrow-to-finish pig farms. This study highlighted that diarrhoeal disease was the only group of porcine diseases for which there was a significant increase in the daily risk of treatment after the withdrawal of ABGPs (Vigre et al., 2008). However, the number of pigs treated at any given time on a single farm had increased after ABGP withdrawal, and there were notable differences in treatment frequency between farms implying that other farm factors were influencing levels of diarrhoeal diseases and treatment. Nonetheless, DANMAP surveillance data shows that the quantities of tetracycline, macrolides, lincosamides and tiamulin more than doubled after the countrywide withdrawal of ABGPs (DANMAP 2008). Likewise, a study comparing ABD use in human and veterinary medicine in France, documented an annual rise in the sales of macrolides in veterinary medicine immediately following the 1999 EU ABGP ban (Moulin et al., 2008).

Nonetheless, despite the increase in the use of particular ABDs to treat diarrhoeal diseases in weaner pigs, the overall consumption of ABDs by Danish livestock fell by 35% after the withdrawal of ABGPs, even though the number of pigs in the country increased by 20% during the same period (Grave et al., 2006). In Norway too, the total consumption of veterinary ABDs decreased by 40% after the withdrawal of ABGPs (Grave et al., 2004).

1.3.2 Quantities and methods of therapeutic ABD use and resistance

The relative quantities of ABDs used in human and veterinary medicine is also a matter of debate. A recent paper from France demonstrates that the measurement used to assess drug use will influence the interpretation of the results that are obtained (Moulin et al., 2008). Comparing the annual patterns of drug sales data in human and veterinary medicine from 1999 to 2005 showed that all classes of ABDs were used within both disciplines, and both also showed a trend towards lower usage during the study. However, there were differences between how medics and vets were using ABDs. In 2005, higher quantities of drugs were purchased for veterinary purposes, around 60% of the total ABDs sold. However, the biomass of animals in France was estimated to be over four times higher than that of humans, which suggested that in 2005 the human population actually consumed 2.4 times more ABDs per kilogram liveweight than animals. Therefore, the veterinary profession was purchasing greater quantities of ABDs, but the medical profession was administering them more intensively.

However, the manner by which drugs are commonly administered to livestock is highlighted as a cause for concern with respect to enhancing ABD resistance. The French study also revealed that 92% of the total tonnage of ABDs purchased by veterinarians was for use in food animal species, and that more than 88% of the drugs purchased for livestock were administered via the oral route (Moulin et al., 2008). The reason for this is that it is not usually feasible to identify and treat subsets of sick animals within large groups; instead, ABDs are administered to whole groups of animals via their food or water. This means, however, that it is not possible to regulate the dose that an individual animal is (or is not) receiving, and all animals in a treated group are consuming ABDs regardless of an individual's disease status. Furthermore, administering ABDs via the oral route applies a selective pressure across vast numbers of bacteria that are living under reasonably optimal conditions in terms of temperature and nutrition: conditions that are also highly conducive to horizontal gene transfer between bacteria (Blake et al., 2003; Scott, 2002). Therefore, oral ABD administration may not only select for resistant subsets of bacteria that are already carrying resistance genes, but may also enhance the spread of resistance genes through the enteric bacterial populations.

For these reasons, it is important that the relationships between the methods of veterinary use of ABDs and resistance are examined and monitored. A Canadian study of ABD use on pig farms found that treating groups of pigs of any age by the oral route was associated with higher risks of detecting ABD resistant *E. coli* in finisher pigs (those closest to slaughter) in comparison with treating individual pigs with injectable ABDs (Dunlop et al., 1998). A more recent study in Canada reported an apparent protective effect of using injectable ABDs, i.e. there was a lower risk of isolating an ABD resistant *E. coli* from a faecal sample on pig farms using injectable ABDs than on those that were not (Varga et al., 2009). The authors struggled to put

a biological explanation to this observation, but this effect was seen for two different injectable preparations (potentiated sulphonamides and tetracyclines), and occurred for resistance to five different ABDs. One explanation could be that this observation was not so much due to a protective effect of the injectable ABDs, but rather farms using injectables were not relying so heavily on in-feed ABD preparations, which were strongly associated with an increased risk of resistance. However, even when controlling for in-feed use in a multivariable model the lower risk ratios for the injectable preparations remained significant.

Some livestock producers also use oral ABDs prophylactically, whereby a drug is administered to every group of animals reared on the farm for a specified period as a method of disease prevention. A French paper from the 1980s looked at the effects of such a practice upon trimethoprim-resistant *E. coli* shed by broiler chickens (Chaslus-Dancla et al., 1987). This farm administered a prophylactic cocktail of chloramphenicol, dihydrostreptomycin, neomycin and tetracycline to the birds at ten days of age. Although trimethoprim was not in use on the farm, the proportion of *E. coli* that were resistant to trimethoprim was 1-5% from all faecal samples. Furthermore, 70 of 78 trimethoprim-resistant isolates were able to transfer trimethoprim-resistance genes to susceptible *E. coli*, with eight of these isolates co-transferring ampicillin resistance along with the trimethoprim and showing an unusual biotype in that they produced atypical biochemical test results. Therefore, the use of ABDs appeared to be co-selecting for resistance to other drugs, and possibly selecting for unusual biotypes of ABD-resistant *E. coli*.

Co-selection of resistance to one ABD due to the use of a different ABD has been reported in other studies of resistant *E. coli* on pig farms. In Japan, the use of tetracyclines and beta-lactams in particular were associated with increased proportion of *E. coli* showing resistance to those and other classes of drug (Harada et al., 2007, 2008). This work also demonstrated regional differences in the multidrug-resistance (MDR) phenotypes that were isolated, such that the drugs to which resistance was co-selected by ABDs differed between regions. Similar findings were seen in a Canadian study of *E. coli* of porcine origin, which found that the detection of resistance to five of sixteen ABDs investigated was associated with the use of pharmacologically unrelated ABDs (Rosengren et al., 2007).

1.3.3 Studies of total ABD withdrawal

Some of the people raising the strongest concerns about the impacts of ABD resistance on farms advocate the complete withdrawal of ABDs from agricultural use. This would certainly leave veterinarians with limited means of treating bacterial diseases in livestock, and would therefore severely compromise animal welfare, but would it actually minimalise resistance?

In the 1980s, the University of Kentucky was experiencing difficulties obtaining

ABD-resistant-free weanling piglets suitable for use in ABD feeding studies. More than 20% of the faecal coliform populations of piglets brought onto the farm, regardless of source, were resistant to tetracycline (Langlois et al., 1983). Therefore, they withdrew the use of all ABDs from their own breeding-herd, and regularly monitored resistance in coliforms for ten years. The proportion of coliforms that were resistant to sulfamethoxazole and ampicillin fluctuated between 6-92% and 0-18% respectively. Streptomycin-resistance decreased slowly, whilst tetracycline resistance initially decreased but then increased again seven years later. Comparing the resistance phenotypes of isolates collected in the first 13 months after ABD withdrawal with those obtained after 126 months, showed that the number expressing resistance to two or more ABDs had fallen from 42% to 24%, but over 8.5% of the 126-month isolates were still resistant to four or more drugs (Langlois et al., 1988a). These multidrug resistant coliforms were more likely to be isolated from pigs under seven months of age, and resistance was more commonly associated with pigs in the finishing units compared to those on pasture (Langlois et al., 1988b).

Other studies have also shown that the age and production status of pigs affects ABD resistance in *E. coli* (Brun et al., 2002; Mathew et al., 2001). This implies that in order to compare resistance between farms, or even between repeated visits to a single farm, samples should either be collected from livestock of comparable age and production status, or analytical methods that incorporate age as an influencing variable are required.

In an extension of their previous work, the Kentucky group looked at the percentage of resistant isolates taken from their ABD-free herd 154-months after ABD withdrawal, compared to those from two other closed-herds operating differing ABD policies (Gellin et al., 1989). In one herd the administration of ABDs only occurred in response to clinical signs of disease; nonetheless, over 64% of the pigs in this herd had received at least one course of ABD treatment. The third herd applied sub-therapeutic doses of chlortetracycline continually to the growing animals, all lactating sows received neomycin, and piglets under 15 kg received sub-therapeutic penicillin. For many of the drugs studied, a decreasing trend was seen in the proportion of coliform isolates that expressed resistance to that drug in relation to decreasing levels of ABD use in the herd: from the high-use farm to medium-use farm to the zero-use farm. However, over 10% of coliforms in the zero-use herd showed resistance to seven of twelve drugs, and cephalothin-resistance was actually highest in this herd (16% compared with 8-9%). Furthermore, tetracycline resistance remained high in the medium-use herd at 67-80% despite no reported use of tetracycline on that farm, and 96-99% of *E. coli* were resistant to sulfisoxazole in all three herds, implying that factors other than ABD use were responsible for the maintenance of resistance to this drug.

Thus, whilst the withdrawal of the use of ABDs on livestock farms is likely to be associated with decreases in the numbers of resistant enteric coliforms, not all such

bacteria will decrease uniformly and some may persist at high levels in the absence of any ABD use. Prior to the discontinuation of ABD administration in the pig herd, the group in Kentucky had collected environmental samples from the farm to look for the presence of tetracycline-resistant coliforms (Langlois et al., 1978). This work had shown that, 5% of soil, 11% of feed and 17% of water samples were positive for tetracycline-resistant-coliforms. The presence of resistant bacteria in the farm environment and feedstuffs offers one possible explanation for the continued shedding of resistant bacteria from animals in ABD-free environments.

Looking to the medical literature, restricting prescription practices of doctors does sometimes result in decreases in resistance in clonal pathogens, such as *Streptococcus pnuemoniae* (Arason et al., 1996; Molstad and Cars, 1999), but has been less successful at reducing plasmid-mediated resistance in clinical isolates of *E. coli* for instance (Enne, 2010; Enne et al., 2001; Sundqvist et al., 2010).

1.3.4 Resistance on organic farms

Organic farms present another opportunity to assess resistance on farms operating under restricted ABD use. A registered organic farm will be committed to operating under a set of standards that aim to minimise the use of drugs such as ABDs. However, the exact definitions of organic, and the standards that such farms practise under, differ between countries. Nonetheless, the need to minimise ABD therapies underpins all organic farming standards and all prohibit the use of ABDs for prophylactic reasons.

One study of conventional and ABD-free pig farms found that *Salmonella* (of undetermined serovars) were isolated from a higher percentage of faecal samples collected on the ABD-free farms than the conventional (Gebreyes et al., 2006). Furthermore, a higher frequency of *Salmonella* isolation occurred from carcasses of ABD-free animals at the abattoir relative to animals reared conventionally. Other groups have also found that food-borne pathogens can be more readily isolated from organic farms compared to conventional (Luangtongkum et al., 2006; Young et al., 2009). Indeed, some scientists point to studies such as these and warn that the removal of ABDs from livestock rearing could actually have adverse public health effects due to an increase in zoonotic enteric diseases of food-borne origin (Miller et al., 2006; Singer et al., 2007).

Assessing the resistance profiles of *Salmonella* isolated from conventional and organic pig farms found that a significantly higher percentage of isolates from the conventional pigs showed resistance to eight of ten drugs (Gebreyes et al., 2006). However, over 85% of isolates were resistant to tetracycline on both farms. There were also higher percentages of multidrug resistant (MDR) isolates seen on the conventional farm, although the standard penta-resistance pattern of 'ACSSuT'[1] generally associated with *Salmonella* Typhimurium was more frequent on the ABD-free farm.

[1] Ampicillin, chloramphenicol, streptomycin/spectinomycin, sulphonamides, tetracycline.

Another study ascertained the minimum inhibitory concentrations (MICs) of non-haemolytic *E. coli* and *Salmonella* (92% of which were *S.* Typhimurium group B) on conventional and organic pig herds (Mathew et al., 2001). Whilst non-type specific *E. coli* on the organic farms had generally lower MICs than those from conventional herds, with respect to the *Salmonella* significantly higher MICs from the conventional farm isolates were only seen for ceftiofur and oxytetracycline. This work implies that for a clonal pathogen, like *Salmonella*, factors other than ABD use contribute to the presence of MDR strains on a farm.

Turning to *Campylobacter* on conventional and organic chicken meat farms, for both *C. jejuni* and *C. coli*, a significantly higher proportion of isolates from the conventional birds showed resistance to eight of nine drugs, with the exception being gentamicin to which no isolates were resistant from either farm type (Luangtongkum et al., 2006). Ciprofloxacin-resistance showed the greatest contrast between farm types, with 53% and 66% of *C. jejuni* and *C. coli* isolates from the conventional operations expressing resistance, compared to less than 1% of the organic isolates. The authors put this difference down to a complete lack of use of fluoroquinolones on the organic farms as well as the persistence of fluoroquinolone-resistant *Campylobacter* on conventional farms that had used such drugs in the past. This study also reported that a relatively high number of organic isolates were resistant to tetracycline although this drug had never been used on those farms (52% of *C. jejuni* and 61% of *C. coli*); however, this was still significantly lower than the conventional isolates (89% for both species).

1.3.5 Resistance in developing countries

ABD resistant pathogens are also on the increase in developing countries where problems of poor sanitation and unregulated sales of human and veterinary ABDs by untrained people can exacerbate the problem (Okeke et al., 2007; Vlieghe et al., 2009; Yang et al., 2004). However, the true extent of the situation in these countries, in both human and veterinary fields, is not fully apparent, as surveillance of resistance is often rudimentary or non-existent. Nonetheless, due to the international transportation of bacteria that can occur as humans (Mendez Arancibia et al., 2009) and animals (Sato et al., 2009) cross borders, increases in resistant, and multidrug resistant, bacteria in developing countries are of global concern as well as local.

A paper from China reported that 100% of *E. coli* ($n = 160$) isolated from young pigs with diarrhoea and diseased chickens had been resistant to nalidixic acid, and 79% were resistant to ciprofloxacin (Yang et al., 2004). Furthermore, most isolates showed multidrug resistance with over 70% identified as resistant to tetracycline, trimethoprim/sulfamethoxazole, ampicillin and/or streptomycin. The authors reported that the prophylactic use of ABDs was common on pig and poultry farms in China, and that ciprofloxacin in particular was in frequent use on poultry farms, although there were increasing reports within the country of the decreasing effectiveness of FQs

against clinical infections in livestock.

Another Chinese study showed that resistance in *E. coli* ($n = 212$) isolated from two large pig farms had increased between 2002 and 2007 (Tian et al., 2009). There had also been a significant increase in the detection of strains that were producing extended-spectrum beta-lactamases (ESBLs): from 2% of isolates in 2002 to 11% in 2007 ($p = 0.02$). The two ESBL-producing isolates from 2002, which originated from the same farm, were carrying bla_{SHV} beta-lactamase genes (SHV-2 and SHV-11); however, all 13 isolates from 2007 were carrying $bla_{\text{CTX-M}}$ genes, and all 13 isolates had been isolated from sick or recently deceased animals. The authors postulated that following approval for the use of the third-generation cephalosporin ceftiofur in animals in China in 2002, the frequent use of this drug on both farms was likely to be the main driver for this emergence of ESBL-resistant *E. coli*.

A report from Korea describes high levels of resistance in *Enterococcus* species to eight ABDs commonly used in the country as feed-additives for growth promoting reasons (Hwang et al., 2009). The list of eight drugs included five therapeutic agents used in human medicine. Resistance was most common in *E. faecalis* with over 80% of isolates (from both pigs and poultry) showing resistance to each drug, with the exception of penicillin and flavomycin. Resistance was lower in *E. faecium* and there was more variation between different drugs, but at least 25% of isolates still showed resistance to each drug.

Information from the African continent is sparser. A study of *Salmonella enterica* serovars associated with chicken carcasses in Senegal found that 28% isolates were resistant to furan drugs, a figure that is high in comparison to those reported from other countries (Bada-Alambedji et al., 2006). The authors reported that furan drugs were in common use on rearing and breeding farms in Senegal and that farm managers were often self-administering the drugs without consulting a veterinarian. In South Africa, although a national surveillance programme for ABD resistance has recently been established, there are currently no restrictions on the use of ABDs as growth promoting or therapeutic agents and farmers can obtain ABDs without a prescription (Oguttu et al., 2008). In one study, over 70% of poultry-derived *E. coli* showed resistance to the drugs in common use within the South African poultry industry: tetracyclines, fluoroquinolones, penicillins, fosfomycin and sulphonamides. Furthermore, 47% of *E. coli* isolated from abattoir workers and 35% of isolates from veterinary students also showed resistance to fosfomycin, even though the drug is not used in human medicine in the country. The authors suggested that this was evidence for the transfer of fosfomycin resistance between poultry and human populations of *E. coli*.

1.4 Farm factors that may influence resistance other than ABD use

The observation that resistance is not necessarily eliminated from a farm if ABDs are withdrawn can not always be related to co-selection by the use of other drugs. This could imply that there are other farm-associated factors that are contributing to the maintenance of resistance on a farm.

1.4.1 The use of disinfectants

One possibility is presented by other biocides, such as disinfectants, that are in common use on many farms. Because disinfectants have antibacterial effects, their use could theoretically select for disinfectant-resistant bacteria and this may increase the number of multidrug resistant bacteria on farms via shared mechanisms of resistance or linked resistance genes. To date, however, the peer-reviewed literature contains no field studies that have conclusively demonstrated that this is a practical problem either on livestock farms or in hospital environments (Aarestrup and Hasman, 2004; Alonso-Hernando et al., 2009; Sheldon, 2005; Weber and Rutala, 2006).

One reason for the lack of an obvious link between the two is that the biocidal effects of disinfectants do not target specific receptors or sites within bacteria; instead, they cause more general problems such as the disruption of cell membranes and proteins (Ioannou et al., 2007). Therefore, bacteria develop resistance to disinfectants using nonspecific mechanisms that include: decreasing the uptake of the biocide into the bacterial cell, detoxification of the biocide within the cell, and increased extrusion of the biocide from the cell (Russell, 2000).

Whilst there is a dearth of literature about disinfectant-resistant bacteria on farms, there are a number of papers describing laboratory experiments with such bacteria (Karatzas et al., 2007; Randall et al., 2004, 2007). The majority of disinfectant-resistant bacteria show over-expression of multidrug efflux pumps, such as AcrAB (Levy, 2002). Efflux is the method by which bacteria actively pump toxic chemicals out of the cytoplasm into the surrounding environment. Whilst many of these efflux pumps are chromosomally encoded and intrinsic to bacteria, mutations in regulatory genes can result in the presence of an increased number of pumps crossing the bacterial membrane. One such regulatory locus is the *mar* (multiple-antibiotic-resistance) locus in Enterobacteriaceae (Levy, 2002). Activation of, or mutations in, *mar* result in, among other things, an up-regulation in the expression of AcrAB. The AcrAB efflux pump facilitates the removal of disinfectants (such as triclosan, quaternary-ammonium-compounds and chlorhexidene) as well as several ABDs (such as tetracyclines, beta-lactams, chloramphenicol and quinolones) and other toxic chemicals found naturally in the environment of enteric bacteria, such as bile salts (Levy, 2002; Randall et al., 2007). So could up regulation of AcrAB be a means by which disinfectants might select

for ABD resistant Enterobacteriaceae on farms?

A number of laboratory studies suggest that this is a possibility. In one study, serovars of *S. enterica* grown in sub-inhibitory concentrations of triclosan or phenolic disinfectants did show 4-fold increases in MICs to ampicillin, chloramphenicol, tetracycline and ciprofloxacin (Randall et al., 2007). The majority of these mutants also showed increased resistance to cyclohexane, a chemical solvent used as a marker for efflux related low-level multidrug resistant strains. However, another study of *S. enterica* isolates that originated from Danish poultry houses found no evidence that serovars capable of persisting in poultry houses were more resistant to five commonly used disinfectants than non-persisting strains (Gradel et al., 2005). Nor did this particular study find any link between up regulation of MAR-type efflux pumps and resistance to any of five disinfectants tested. Although other laboratory-based work has shown that prolonged exposures to quaternary ammonium compounds (consisting of seven days of serial passages in broths containing subinhibitory concentrations) can select for stable *S.* Typhimurium variants with reduced susceptibility to multiple antibacterial drugs (Karatzas et al., 2007).

One reason for MAR mutant strains not predominating in farm environments could be an accompanying decrease in fitness. Proteomics work has shown that, relative to their parent strains, *S.* Typhimurium MAR mutants showed increased protein synthesis and alterations in the production of proteins associated with virulence (Karatzas et al., 2008). Correspondingly, during *in vitro* studies these mutant strains displayed decreased growth, decreased motility and decreased invasiveness. Furthermore, in an animal model study, *S.* Typhimurium MAR mutants were out competed by their parent strains as assessed by comparative concentrations of parents and mutants in the caecal contents of chickens that had been co-infected with both types (Randall et al., 2008). In a further experiment, parent strains were transmitted more rapidly between infected birds and naïve birds inhabiting the same pen than the mutant strains.

Therefore, it seems that disinfectant-selection of ABD resistant bacteria remains a theoretical possibility that is demonstrable in the laboratory, but currently, there is no strong evidence that disinfectants are exerting a large influence upon ABD resistance in the field.

1.4.2 The use of anticoccidial drugs

The ionophores are anticoccidial drugs that are incorporated into poultry feed in many countries in order to prevent coccidiosis, an enteric disease caused by parasitic protozoal species within the genus *Eimeria.* The ionophores are derived from *Streptomyces* species, and commercial drugs available for use with livestock species include monensin, narasin, lasalocid and salinomycin (Butaye et al., 2003). The ionophor drugs are not used in human medicine; and only salinomycin is classified as a growth-promoting drug, and is therefore no longer permitted for in-feed use in the EU.

As well as their anticoccidial activity, the ionophores, particularly narasin, also show activity against *Clostridium perfringens*, the causative bacterium of necrotic enteritis, another common enteric disease (Brennan et al., 2001). This combination of anticoccidial and anticlostridial activity has been cited as one of the reasons that some countries have managed to effectively control necrotic enteritis after the withdrawal of in-feed growth promoters (Grave et al., 2004; Wierup, 2001).

Studies of resistance in *C. perfringens* of poultry origin, have not found evidence of the development of narasin resistance in this species. In 2004, the MICs for narasin were assessed for *C. perfringens* isolates ($n = 102$) from Swedish, Norwegian and Danish poultry (Johansson et al., 2004). This work showed that, even though the poultry industries within the Nordic countries had been using narasin for several years, none of the isolates were expressing narasin resistance. Similar findings have also been obtained in Belgium and Brazil (Martel et al., 2004; Silva et al., 2009).

While, at this time, the clinical efficacy of narasin against necrotic enteritis seems to be secure, narasin-resistance does occur in species of enterococci. A paper from Belgium in 1999, estimated the MICs for *E. faecium* ($n = 199$) and *E. faecalis* ($n = 154$) to a panel of in-feed ABDs that included narasin (Butaye et al., 1999). The isolates originated from pets, farm animals and foods. There is no internationally accepted breakpoint MIC for narasin, in the Belgian study $\geq 1\,\mu g/ml$ was chosen due to a strong bimodal distribution of MICs. Therefore, 17% of *E. faecium* isolates and 2% of *E. faecalis* isolates were deemed resistant, or at least less susceptible. However, the breakpoint set by the Norwegian and Swedish surveillance programmes is $\geq 4\,\mu g/ml$ (NORM/NORM-VET 2006; SVARM 2007), using this breakpoint 5% and 1% of the Belgian *E. faecium* and *E. faecalis* isolates would have been designated resistant.

The common use of narasin also offered a hypothesis for the persistence of vancomycin-resistant *E. faecium* (VREF) on broiler farms in Norway. Norwegian and Swedish broiler chickens do commonly shed narasin-resistant *E. faecium*, 73% of isolates were narasin-resistant in Norway in 2006 and 89% in Sweden in 2007 (NORM/NORM-VET 2006; SVARM 2007). One study that compared narasin resistance in vancomycin-resistant (VREF) and vancomycin-susceptible *E. faecium* (VSEF) isolated from broilers, found that 50% of VREF were narasin resistant compared to 80% of VSEF (Sorum et al., 2004). Given the small numbers of isolates per group (70 VREF and 20 VSEF) and the ambiguity in the breakpoint for narasin-resistance, it is hard to ascertain if the between group differences are statistically valid.

Interestingly, a clone of VREF has re-emerged in Sweden in recent years even though avoparcin was withdrawn from use in this country in the early 1980s (Nilsson et al., 2009). All VREF isolates ($n = 384$) from 2001 belonged to a single multi-locus sequence typing (MLST) group, all were carrying the *vanA* gene on identical Tn*1546* transposons, and all were concurrently resistant to narasin. The authors state that because narasin-resistance is also common amongst populations of vancomycin-

susceptible *E. faecium*, narasin is unlikely to be the primary selective factor in the emergence of this clone.

1.4.3 The use of heavy metals

The links between resistance to heavy metals and resistance to ABDs are more definite. Heavy metals such as zinc, cobalt and copper are essential micronutrients for bacteria (Nies, 1992; Outten et al., 2000), and they acquire them from their surrounding environments using nonspecific membrane transport systems. However, all heavy metals are toxic to bacteria at high concentrations; therefore, bacteria living in environments of high heavy metal concentrations need to decrease the cytoplasmic concentration of metal cations by either forming metal complexes, or actively pumping the metals out of the cell. The majority of active efflux mechanisms are plasmid-encoded, and metal-resistance genes can occur on large plasmids that are concurrently carrying ABD resistance genes (Hasman and Aarestrup, 2002). Heavy metal resistant bacteria, including pathogenic species, have been identified in industrial, marine, clinical and agricultural environments (Aguiar et al., 1990; Bass et al., 1999; Choudhury and Kumar, 1996; Stepanauskas et al., 2005).

The agricultural industries use heavy metals in a variety of ways. Copper sulphate, for instance, is used as:

- a therapeutic agent within foot baths for microbial foot disorders in livestock.
- a general disinfectant.
- and a food additive to assist with the control of enteric disorders in growing animals.

Therefore it may not be surprising that copper-resistant *E. faecium* have been isolated from a number of livestock species. In *E. faecium* isolates originating from Danish pigs, a gene encoding for a heavy-metal transporter (*tcrB*) has been identified on conjugative plasmids. Furthermore, in 31% of copper-resistant porcine *E. faecium* ($n = 45$) the plasmid carrying *tcrB* was also carrying *ermB* and *vanA* genes, rendering these strains simultaneously resistant to macrolides and vancomycin (Hasman and Aarestrup, 2002).

The emergence of copper-resistant *E. faecium* is likely to be related to the common practice in some European countries in the late 1990s, of adding copper to the diet formulations of pigs to control diarrhoeal diseases. Copper was added at 25–175 ppm, with the highest levels being administered to weaner animals. A study investigating resistance in enterococci demonstrated that, at that time, copper-resistance was carried by 56–75% of *E. faecium* from pigs in high copper-using countries (Aarestrup et al., 2002). By comparison, in Sweden, where the maximum acceptable level of copper sulphate in diets for pigs of all ages was 35 ppm, only 6% of *E. faecium* isolates collected from slaughterhouses showed copper-resistance. In this study *tcrB* was identified in

the copper-resistant strains, but not all strains of *E. faecium* carrying *tcrB* showed simultaneous resistance to vancomycin.

In fact, despite the links between resistance to copper and vancomycin, trends observed within the DANMAP surveillance program suggest that copper is only a weak selective agent for VREF (Hasman and Aarestrup, 2005). Between 1997-2003, all VREF isolated from pigs were concurrently resistant to copper; however, the prevalence of copper resistance oscillated between 45% and 75% of porcine *E. faecium* isolates, whilst vancomycin-resistance within declined from 23% to 3% of isolates. Therefore, during a period in which the use of macrolides was decreasing, the continued administration of in-feed copper to young pigs was not enough to maintain levels of VREF in slaughter-age animals.

Copper-resistance also occurs within Gram-negative bacteria, and mechanisms in *E. coli* and *Salmonella* Typhimurium include inducible efflux pumps (Lim et al., 2002). A comparative study of six bacterial species from Denmark found that 100% of *Salmonella* isolates showed high MICs to copper sulphate, while *E. coli* isolates showed slightly lower MICs (Aarestrup and Hasman, 2004). In this study, the copper MIC distributions of the enterococci showed a bimodal pattern: an indication of acquired resistance mechanisms. Therefore, the authors concluded, that copper-resistance was intrinsic to *Salmonella* serovars, and they hypothesised that *Salmonella* may actually have an advantage over more susceptible bacteria on pig farms administering in-feed copper at high incorporation concentrations.

1.4.4 Farm husbandry practices

While several research groups are studying the influence of the use of antimicrobial agents other than ABDs upon ABD resistance, fewer studies are available regarding the impact of other farm husbandry practices upon resistance. If altering farm husbandry practices enables farms to maintain productivity in the absence of ABGPs (Wierup, 2001), then can alterations in non-drug-related farm husbandry practices, also affect resistance on a farm? There seems to be little published research in this area with respect to pig and poultry farms; however, research on cattle farms suggests that farm management decisions may influence ABD resistance.

One study examined the resistance to ABDs expressed by environmental contaminants isolated from bulk milk-tank samples collected from approximately 400 dairies (Kirk et al., 2005). Cluster analysis of ABD resistance phenotypes identified four groups of contaminants. Using these four clusters as dependent variables, multinomial regression models were fitted to data regarding farm husbandry practices as recorded in interview-based questionnaires. Practices associated with clusters containing ABD resistant isolates included the choice of bedding material, the number of days bedding was left in situ before it was replaced, and not drying udders in the milking parlour before placing the milking clusters on the teats. However, there were also differences in

the constituent bacterial species within the clusters, so the between cluster differences in ABD resistance could be due to differences in the structure of the bacterial populations present in different types of bedding, rather than a selection for resistant bacteria per se. Furthermore, the largest relative risks for being in the two predominantly streptococcal resistant clusters were associated with the use of ABDs to treat diseases other than mastitis.

Differences in patterns of bacterial resistance in relation to farm husbandry practices have also been reported in bacteria isolated from veal calves at slaughter (Di Labio et al., 2007). Farm-level factors associated with high proportions of ABD resistant *E. coli* included: bringing purchased calves on to the farm, purchasing calves from a small number of suppliers, feeding medicated feed to incoming calves upon arrival, and not participating in a quality-assurance programme. Potential risk factors for a high proportion of ABD resistant *Campylobacter* included providing outdoor access to the calves, maintaining larger groups of finishing animals, and feeding milk by-products. None of the management practices studied were associated with either increased or decreased proportions of resistant *E. faecium*; although, injecting incoming calves with antibacterial drugs upon arrival was associated with a decreased risk of erythromycin-resistance being expressed by *E. faecalis* isolates.

To date, there do not seem to be equivalent studies on pig and poultry farms, where the predominant selective pressures on resistance are the frequent application of ABDs. However, the ease with which certain resistant bacteria can be isolated from pig and poultry farms administering little or no ABDs may imply that other farm husbandry practices can have a significant impact upon resistance within these livestock species.

1.4.5 Environmental contamination

Regardless of the primary factors involved in the emergence of resistance on a farm, if resistant bacteria are able to survive in the farm environment even after the removal of those factors, then the environment itself could become a reservoir for resistance.

The ability of *Enterococcus* species to survive under adverse conditions contributes to their success as opportunistic, nosocomial pathogens; and it is well documented that enterococci can survive for several months in hospital environments (Kramer et al., 2006). Research has also shown that ICU patients placed in rooms in which VREF were detected on the surfaces were at higher risk of becoming colonised with VREF during their hospitalisation (Martinez et al., 2003). Therefore, the persistence of VREF on broiler farms in the absence of obvious selective pressures, as has been documented in Denmark and Norway (Borgen et al., 2000b; Heuer et al., 2002a), could potentially be enhanced in environmentally hardy strains of *E. faecium* that had acquired *vanA* genes.

In hospitals, rigorous cleaning programmes have to be implemented to successfully control endemic, as well as epidemic, nosocomial VREF infections (Dancer, 2009;

Hayden et al., 2006). Therefore, the cleaning and disinfecting protocols used on a farm could offer one route to VREF decontamination. On European broiler farms, the litter is usually removed from the sheds between each flock of birds and the sheds are thoroughly cleaned prior to the arrival of the next flock, but farms differ in the precise protocols and chemicals that they use. Currently, few reported studies have assessed the impact of different cleaning regimes on resistance on a farm. In the study of VREF persisting on Danish broiler farms, the VREF-negative control farm included formalin fogging at high temperatures within the cleaning protocol, a procedure that was not practised on any of the four VREF-positive farms (Heuer et al., 2002b). While no absolute conclusions are possible from a study with just one control farm, it would be interesting to test whether high-temperature, formalin fogging is a potential intervention measure for decreasing VREF contamination on farms.

Recent studies in Sweden also suggest that the degree of environmental contamination on broiler farms can influence the dynamics of VREF shed by growing birds (Nilsson et al., 2009). An intensive study of three VREF-positive broiler farms found that the proportions of environmental samples that were positive for VREF showed considerable between-farm variation at 42%, 64% and 94%. After the placement of the birds on each farm, floor samples were collected throughout the rearing cycle using disposable, swab overboots. On the farm with the lowest carry-over of VREF in the house environment, VREF-positive floor samples were first obtained in two of four houses, 21 days after the chicks arrived. Whereas, on the two farms with the higher levels of environmental contamination, VREF positive floor samples were obtained from houses within two-weeks of the arrival of the chicks. Furthermore, the same trend seen with the environmental contamination was seen in VREF isolation from caecal samples of birds at slaughter, with 0%, 55% and 77% of samples positive, respectively. The paper did not specify the cleaning regimes that were utilised on each of the farms.

Pest species, such as flies and rodents, represent another potential vehicle for the carry-over of resistance between consecutive groups of livestock, and such species have previously been implicated in the carry-over of foodborne pathogens (Gregory et al., 1997; Liebana et al., 2003). A study in the Czech Republic characterised and compared *E. coli* isolates originating from pigs, rodents (eight species were identified) and the common housefly, *Musca domestica*, present on two pig farms (Literak et al., 2009). This work found that there was some conservation of resistance phenotypes of *E. coli* between pigs, rodents and flies within a farm. However, although there were differences between the PFGE genotypes carried by pigs and rodents on a farm, there was more overlap in genotypes carried by pigs and flies. The authors hypothesised that the flies could be acquiring resistant strains of *E. coli* from the pigs, horizontal gene transfer could be occurring between pig-adapted *E. coli* and *E. coli* strains carried by sympatric rodents. An alternative explanation could be that ABDs within the farm environments were directly exerting selective pressures upon the rodent-adapted *E. coli* populations.

A series of experimental studies demonstrated dissemination of resistant bacteria between various animal species sharing a barn (Marshall et al., 1990). Resistance plasmids were introduced into strains of *E. coli* of livestock origin that were marked by nalidixic acid resistance, and these laboratory-modified strains were inoculated into small groups of livestock (cattle and pigs). Also located within the barn were livestock that were not in direct contact with the inoculated animals, and mice and chickens housed in cages. Insects and wild birds had free access to the barn. The resistant strain was isolated from the inoculated animals throughout each experiment, as well as the caged mice in the same pen, flies throughout the barn, and the people that were tending to the animals. If the animal attendants used clothing dedicated to each pen and disposable plastic over boots, then resistant strains were not detected in uninoculated livestock. However, with no such biosecurity measures, the resistant strain was detected in neighbouring pens within a few days of inoculation. This work clearly demonstrated that resistant bacteria can move between animal species, including humans, within a farm-like environment, although whether the primary vectors were flies and wild birds or humans is less clear.

1.5 Ecological factors influencing ABD resistance

Aside from the direct impact of farm practices upon resistance, it is possible that factors outside the control of the farm manager could also influence ABD resistance on a farm. Such factors could include: the number and type of other farms in the surrounding area, heavy metals in the soil, elevation and climate, contaminated water sources, nearby hospitals or industrial sites. In fact, the factors that could theoretically influence resistance at a given location are numerous, and for this reason there have been recent calls to expand the view of ABD resistance from a clinical and agricultural problem to a wider ecological issue (Aminov, 2009; Fajardo et al., 2009; Pallecchi et al., 2008; Singer et al., 2006).

However, it is currently unclear as to which, if any, of these factors are actually operating at influential levels on farms, and trying to include an array of ecological factors in the design and analysis of resistance studies would incur significant increases in economic, logistic and analytic expense. In order to assess whether such expense would be justified, is there any evidence in the published literature to suggest that such ecological factors are influencing resistance in populations of commensal bacteria at appreciable levels?

1.5.1 Resistance in isolated populations of humans

Studies conducted in two geographically remote villages in South America found that surprisingly high proportions of faecal *E. coli* carried by the inhabitants were expressing ABD resistance. In an isolated community of Guaran Indians in Bolivia, 67% of faecal

E. coli showed resistance to one or more drugs, and 92% of isolates from a Chayahuita Indian village in Peru were expressing resistance to at least one ABD (Bartoloni et al., 2004, 2009). Both communities were only accessible on foot, a very limited number of people ever left either village to travel to urban centres, very few inhabitants in either community had ever received ABD therapy, and neither village had ever administered ABDs to livestock. Furthermore, these two villages were geographically and climatically distinct from one another and, therefore, the potential ecological selective pressures would be expected to be different in each. However, the ABDs to which resistance was expressed by faecal *E. coli* were actually very similar in both communities: tetracycline, ampicillin, potentiated sulphonamides and chloramphenicol (in order of decreasing prevalence).

Genetic characterisation of the *E. coli* isolates from both villages found that the resistance genes present were identical to those commonly identified in communities that are more frequently exposed to ABDs, not novel genes as might be expected in relation to novel environmental selective pressures (Bartoloni et al., 2009; Pallecchi et al., 2007). Furthermore, comparing faecal *E. coli* sourced from the nearest urban centre to the Chayahuita village isolates found that both populations contained a high diversity of *E. coli* strains, but the resistance phenotypes in each location were analogous, and identical resistance genes and mobile genetic elements were present (Bartoloni et al., 2009). Therefore, the presence of ABD resistance in these remote populations appeared to be related to the occasional movement of villagers to and from the nearest towns, and once resistance genes are introduced into an isolated community they moved horizontally through the resident *E. coli* populations.

1.5.2 Resistance in populations of wild animals

Studies of resistance in wild populations of mammals and birds provide another means for assessing the potential for ecological risk factors to exert measurable influences upon ABD resistance, and a number of studies have reported higher than expected levels of ABD resistance in communities of wild animals.

Some of the highest reported levels of ABD resistance in wild animal populations were seen in Enterobacteriaceae isolated from mice that were resident in British woodland (Gilliver et al., 1999). Over 90% of isolates were resistant to amoxicillin, amoxicillin-clavulanic acid and cefuroxime (a second generation cephalosporin). Another study looking at bacteria isolated from marine vertebrates off the north east coast of USA found that 58% of bacterial isolates ($n = 287$, across several taxa) were resistant to one or more ABD; however, for *E. coli* isolates the levels were lower at 16% of isolates showing ABD resistance (Rose et al., 2009). Thirty-five percent of faecal *E. coli* isolated from a variety of wild animals in Portugese national parks showed resistance to tetracycline, and 19–22% showed resistance to ampicillin, streptomycin and potentiated sulphonamides. Multidrug resistance (defined as the simultaneous

expression of resistance to three or more ABDs) was present in 26% isolates. (Costa et al., 2008).

However, resistant bacteria are not so readily isolated from all populations of wild animals. In Finland, just one *E. coli* isolate in a collection of 98 that had been isolated from wild moose, deer and voles expressed any resistance at all and this was to streptomycin (Osterblad et al., 2001). In comparing their results to those of the British woodland rodent study, the authors suggested that the virtual lack of resistance in *E. coli* from Finnish wildlife reflected the lower ABD consumption in the country compared to the UK, due to lower densities of both humans and livestock in Finland. This theory is supported by a report of zero resistance in Enterobacteriaceae isolated from Gentoo penguins on the Antarctica peninsula, one of the most isolated regions in the world (Bonnedahl et al., 2008).

Other studies also support the existence of a relationship between ABD resistance and proximity to human populations. Assigning a resistance score to faecal *E. coli* originating from six different animal and human populations showed increasing scores with increasing human population density (Skurnik et al., 2006). *Escherichia coli* isolated from animals in Antarctica and Central Gabon had resistance scores of zero, wildlife in the Pyrenees and Fontainebleau forest scored 5% and 7%, respectively, livestock in the Pyrenees scored 11%, and the joint highest scores were seen for pet animals and healthy humans in France at 19%. Furthermore, the proportions of *E. coli* carrying integrons were highest for humans and pets (16%), followed by farm animals (7%), and no integrons were detected in *E. coli* isolated from wildlife.

Similar findings were seen in a study of resistance in three Nepalese communities located at increasing distances from Kathmandu (Walson et al., 2001). This work showed that the proportion of lactose-fermenting, Gram-negative coliforms from human faecal samples expressing resistance to one or more drugs decreased with increasing distance from the capital. Furthermore, no trends were seen when the resistance in a region was compared to the ABD histories of the individual volunteers.

Differences in resistance relative to location from human activities are also apparent on more localised scales. A study of three troops of wild baboons in Kenya in the 1980s found that 94% of faecal Gram-negative bacteria shed by the troop who regularly visited a tourist lodge and scavenged through the rubbish bins showed resistance to at least one of four drugs tested (Rolland et al., 1985). Resistance in the two more isolated troops was lower at 36% and 47%. Furthermore, 65% of samples from the lodge-associated troop contained multidrug resistant (MDR) bacteria (defined as resistance to $\geq$ three ABDs), and these MDR strains were able to transfer the resistance genes they were carrying in recipient-donor laboratory experiments. In contrast, only 5-8% of samples from the two isolated groups contained MDR strains, and transfer experiments with these strains were not successful.

Taken together, these studies do support the existence of ecological influences on

ABD resistance in populations of humans and animals. Thus far, however, there is only strong evidence for the inclusion of some estimation of human population density or human activities into epidemiological studies of resistance. Of course, this variable in itself may simply be a proxy for other influential ecological variables, such as the intensity of ABD use, or industrialisation, or water source contamination; but, to date, the actual extra-farm influences on resistance, and the details of how these interact with the, undeniably, strong influence of the level of ABD use on the farm itself remain to be elucidated.

1.5.3 Farms as environmental sources of resistance genes

Part of the effect of increasing human population density could be due to increasing agricultural density. If the local density of farms acted to influence the bacteria present across all livestock populations in the area, then this could help to explain the presence, and persistence, of resistant bacteria on organic farms using minimal amounts to no ABDs.

Wildlife studies do suggest that bacteria shed by wildlife living close to livestock farms are more likely to express resistance. One study of free-living Canada geese followed four populations in contrasting habitats in North Carolina and Georgia (Cole et al., 2005). One population were resident in a recreational theme park, one at a reservoir for a steam-operated power-generation plant, one around a horticultural research station, and the fourth on a wastewater lagoon on a pig farm. The majority of *E. coli* isolates originated from the two agricultural sites, and 72% of isolates from the pig lagoon flock expressed resistance to one or more ABDs, compared to 19% of the horticultural station flock. Furthermore, 36% of *E. coli* isolates from the pig farm flock were carrying class 1 integrons, whilst these were not detected in any *E. coli* from the other flocks.

In a similar manner, another study compared resistance in *E. coli* isolated from species of small mammals (mice, shrews and voles) living around pig farms with *E. coli* isolated from mammals residing in what the authors referred to as natural areas (Kozak et al., 2009). Resistant *E. coli* were more common in the farm-caught rodents (36% isolates) compared to the wild-caught animals (8% of isolates). Tetracycline was the drug to which there was the highest number of resistant isolates from rodents, and 83% of the faecal samples collected from pigs on the farms ($n = 125$) also contained tetracycline-resistant *E. coli*.

Inevitably, the method by which a livestock farm disposes of animal waste will have an impact upon the presence and persistence of resistance in the surrounding environment. All livestock farms have to dispose of manure, with the largest farms handling vast quantities of animal-related waste. Several studies have investigated the potential impacts on ABD resistance from the practice of spreading manure upon farmland.

One study cultured and characterised bacteria of different species from soil samples collected around a small number of pig farms, dairy farms and non-agricultural sites in the USA and Canada (Ghosh and LaPara, 2007). Every pig farm was administering sub-therapeutic doses of in-feed chlortetracycline, and the three largest farms administered an additional two to three ABDs at sub-therapeutic levels at intervals throughout the rearing periods. In contrast, the dairy farms were only using ABDs at full therapeutic doses in response to clinical disease. The three large pig farms each finished over 1000 pigs a year (range 1200 to 3000). The manure management on these farms involved storing the faecal waste in underground pits for 6–12 months prior to injecting it approximately 15 cm into the soil. The fourth pig farm, finishing just 40 animals a year and only administering chlortetracycline sub-therapeutically, had no specific waste disposal protocol. In fact, on this farm the faecal waste simply overflowed from the animal pens and remained in situ.

The highest percentages of tetracycline-resistant bacteria were isolated from soil samples originating from two of the pig farms: one of the large farms and the smallest farm. Furthermore, on the smallest pig farm a greater number of species of bacteria were carrying a greater diversity of *tet* genes compared to bacteria from soil samples from the other farms; suggesting that lateral transfer of *tet* genes was occurring between soil bacteria that were exposed to a constant supply of manure.

During the course of the study, the small pig farm ceased operating, but the heaps of dung remained. Sequential soil samples collected from this site for a further 18 months demonstrated that tetracycline resistance was persisting in soil bacteria, with the highest levels of resistance seen in soil samples collected in closest proximity to the disused pig pens. Thus, the manure spilling out of the pig pens on this farm appeared to be acting as a hotspot for tetracycline resistance within the soil bacteria.

1.6 Bacterial factors influencing resistance

It is clear that once resistance has emerged within a population of bacteria it may persist for longer than expected even in the absence of the primary selective agent. Aspects of fundamental bacteriology can help to explain these observations.

Firstly, the notion that bacteria are independent, discrete units that respond in predictable ways to selective pressures is no longer tenable. Bacteria in a given environment are in communication with each other via chemical signalling systems (Reading and Sperandio, 2006; West et al., 2006). They frequently interact antagonistically with each other and have developed an array of mechanisms to allow them to compete with sympatric strains and species (Brown et al., 2009; Kirkup and Riley, 2004; Van Melderen and De Bast, 2009). Moreover, studies of bacteria in biofilms have shown that they are capable of living in coordinated synergistic communities (Burmolle et al., 2006; Clutterbuck et al., 2007). Furthermore, if their environment

changes in an extreme and adverse manner, they are able to respond by relaxing their genetic control systems, a phenomenon known as the SOS response (Janion, 2008). The SOS response increases the rates of spontaneous mutation and horizontal gene transfer; and by doing so enhances the ability of that species or strain to adapt to changing circumstances, such as the introduction of an ABD into the bacterial environment (Beaber et al., 2004; Dorr et al., 2009; Dwyer et al., 2009).

An increasing understanding of the fundamental microbiology of resistant bacteria has the potential to contribute to the design and interpretation of research into resistance on farms and to inform more holistic risk assessments. Some of the recent advances in bacterial science with particular reference to ABD resistance are summarized below.

1.6.1 Biological fitness of bacteria carrying resistance mechanisms

The concept of resistant strains of bacteria decreasing in frequency towards extinction in the absence of anthropogenic ABD administration requires there to be a decrease in biological fitness associated with the maintenance of resistance mechanisms in the absence of drug use. The replication and expression of genes is a biologically costly process; however, bacteria are remarkably adaptive, and adaptations can occur with extraordinary rapidity.

In the UK, the licensed indications for the combination agent co-trimoxazole, a potentiated sulphonamide, were restricted due to hypersensitivity reactions in some human patients. Within eight years the use of this drug had fallen to 25% of the pre-restriction levels but, over the same time period, the percentage of clinical isolates of *E. coli* from an east London hospital that were resistant to sulphamethoxazole had actually increased slightly (Enne et al., 2001). The nature of sulphonamide resistance within clinical *E. coli* had also changed with an increase in the proportion of isolates that were carrying the *sul2* gene, and in 73% of these isolates, *sul2* was located on a conjugative plasmid containing multiple resistance genes.

The persistence of sulphonamide-resistant *E. coli* could have been an instance of co-selection, with the use of other ABDs selecting for the plasmids that were also carrying *sul2*. However, growth competition studies revealed that whilst bacteria carrying three of four *sul2*-bearing plasmids suffered a loss in fitness, a relatively small plasmid (*p9123*) carrying *sul2* and two streptomycin-resistance genes actually conferred a 4% fitness advantage upon both its natural carrier and a laboratory strain of *E. coli* to which the plasmid was transferred (Enne et al., 2004). DNA sequence analysis of *p9123* did not reveal any known virulence genes concurrently present upon the plasmid, and the authors concluded that there may be strains of *E. coli* that are wholly adapted to, and even advantaged by, carrying certain resistance plasmids.

Another study of *E. coli*, found that inducing the expression of the *tetA* gene (encoding for a tetracycline efflux pump) in the absence of tetracycline imposed a

high fitness cost, and this cost increased in a linear manner with the level of gene expression (Nguyen et al., 1989). However, in a tetracycline-free environment, gene regulation mechanisms that resulted in the repression of expression of *tetA* ameliorated these fitness costs. This regulation of gene expression meant that strains bearing inducible tetracycline resistance operons were no longer disadvantaged in a tetracycline-free environment even if they were carrying multiple copies of a tetracycline-resistance plasmid.

Adaptive alterations in conjugation rates also occur, such that in the absence of selective pressures for the expression of genes carried on a plasmid the rate of conjugation decreases (Dahlberg and Chao, 2003). Simulation modelling suggests that decreasing the conjugation rates for large plasmids would increase the competitiveness of the host bacterium (Haft et al., 2009).

The practical implications of these studies are that under appropriate selective pressures, such as the presence of ABDs, the acquisition of a multidrug resistance plasmid would enhance the survival of the bacteria in that environment. However, if the selective ABD is removed, compensatory mutations in combination with adaptations of gene expression can act to stabilise the plasmid within the population.

However, some ABD resistant bacteria remain less fit than the corresponding susceptible wild-type strains. One laboratory-based study demonstrated that a fluoroquinolone-resistant *S.* Typhimurium mutant grew more slowly than the wild-type strain, and it showed reduced ability to invade intestinal epithelial cells rendering it less pathogenic than the wild-type strain (Faberga et al., 2009). When grown in a fluoroquinolone-free environment there was a partial reversion of FQ-resistance and the MIC of the strain decreased even though the mutations in *gyrA* were still present. In fact, although nalidixic acid resistant strains of *S.* Typhimurium are isolated reasonably commonly, fluoroquinolone-resistance is generally rare within this serovar.

Actually measuring bacterial fitness, however, can be complicated and the methods chosen can affect the study results. In particular, discrepancies in results can be obtained from *in vitro* and *in vivo* experiments as demonstrated by one study investigating ABD resistant *E. coli.* The investigators inserted various plasmids and transposons into a wild-type strain of *E. coli* originally isolated from pig faeces (Enne et al., 2005). Some of the *in vitro* work suggested that there was a decrease in fitness associated with the carriage of some of the genetic elements; however, these same costs were not seen in the *in vivo* models. Therefore, the authors concluded that the fitness costs incurred by that particular strain of *E. coli* carrying resistance-elements within the pig gut were low.

1.6.2 Plasmid addiction and postsegregational killing systems

Postsegregational killing (PSK) systems are another compensatory mechanism that decreases the fitness costs related to the maintenance of plasmids in a bacterial popu-

lation. The genes encoding for the toxin-antitoxin PSK systems are located on large, low copy number plasmids such as *pSM*19035 in *Streptococcus pyogenes* (Zielenkiewicz and Ceglowski, 2005). Strains bearing PSK systems actively eliminate plasmid-free segregants from the surrounding environment, thus removing the competition and increasing the frequency of plasmid-carrying strains. This gives rise to 'plasmid addiction' because loss of the plasmid during cell division would render the antitoxin-free daughter cells susceptible to the toxins of plasmid-bearing strains. PSK-positive plasmids can displace PSK-negative plasmids from hosts, suggesting that PSK systems evolved due to plasmid competition for host cells (Cooper and Heinemann, 2000).

PSK systems may be contributing to the long-term persistence of VREF on farms in the absence of obvious drug-related co-selection (Johnsen et al., 2005). A Norwegian characterisation study of VREF used hybridisation techniques to show that 68 of 70 isolates originating from broiler chickens and broiler farmers were carrying a putative PSK system that showed structural and functional homology with a *S. pyogenes* PSK-system (Sorum et al., 2006). Further work showed that *vanA* bearing plasmids in two strains of *E. faecium*, one isolated from poultry and one from a poultry farmer, were carrying PSK systems on the *vanA* plasmids (Sletvold et al., 2007).

The active suppression of PSK-free strains of *E. faecium* within the farm environment would enhance the likelihood that incoming chicks would become colonised with PSK-positive VREF. Within the adventitious environment of the intestines of growing birds the VREF multiply and are then be shed back into the house environment at relatively high levels: approximately 5×10^6 cfu/g faeces in the Norwegian study (Sorum et al., 2006). If the cleaning and disinfection protocol does not decrease VREF in the environment beneath a putative threshold level then the next batch of chicks become colonised, and a cycle of colonisation-multiplication-contamination of the environment ensues.

1.6.3 Other functions of antibiotics

In clinical settings, relatively high concentrations of antibacterial drugs are administered in order to kill or inhibit the growth of pathogenic bacteria. However, it has been proposed that antibiotics produced by environmental bacteria may have different effects and roles at different concentrations (Aminov, 2009; Fajardo and Martinez, 2008):

- At high concentrations the toxins are inhibitory to competitors.
- At low concentrations these chemicals have alternative functions such as signalling mediators within bacterial communication systems.

As evidence for this theory, the proponents point out that bacteria respond to challenges from ABDs via a comprehensive series of adaptations including increases in the rates of genetic mutation, recombination, horizontal gene transfer, and resistance-gene expression (Fajardo and Martinez, 2008). Furthermore, these responses show

inter- and intra-specific specificity, i.e. different bacteria respond to different drugs in different ways. This specificity implies that there are complex pathways involved in the response of bacteria to ABDs. As further evidence for alternative functions of antibiotics, it has been shown that well-recognised signalling molecules in *Pseudomonas aeruginosa* appear to have antibacterial properties if present in high concentrations (Kaufmann et al., 2005; Ueda et al., 2010).

Advocates of the theory that the production of some antibiotics may have evolved for non-biocidal reasons support their arguments by quoting recent advances in the understanding of bacterial populations in soils (Martinez et al., 2009). Until recently, many of these soil bacteria were difficult to study as it is not possible to culture them in the laboratory; however, metagenomic methods are now allowing for the exploration of resistance genes present within populations of bacteria in soil. This work has shown that soil communities represent substantial reservoirs of a vast array of resistance genes that are not currently recognised in pathogenic species (D'Costa et al., 2007; Riesenfeld et al., 2004). Even in environments that are remote from the anthropogenic use of ABDs, the soil-based resistance reservoir (the soil resistome) contains an enormous diversity of resistance genes (Allen et al., 2009). What is more, within an average sample of soil there are bacteria that are actually dependent upon environmental antibiotic substances for their survival, utilising these antibiotics as a source of carbon (Dantas et al., 2008). These antibiotic utilising strains are typically multidrug resistant and some are related to pathogenic species.

Phylogenetic studies of the evolutionary histories of ABD resistance genes have revealed an ancient ancestry stretching back way beyond the human use of antibacterial drugs (Aminov and Mackie, 2007). Beta-lactamase enzymes, for instance, have been evolving for over two billion years and for much of this time they have been associated with plasmids (Hall and Barlow, 2004). However, the rapid dissemination of some resistance genes between multiple strains and species of bacteria can be seen as a much more recent event that is likely to be related to increases in horizontal gene transfer rates in response to the human use of ABDs over the past 60-70 years (Aminov and Mackie, 2007).

Taken together, if antibiotics play important physiological roles in bacteria, and if antibiotic resistance genes code for mechanisms that do more than impart resistance upon a bacterium, then the elimination of an established clinically-relevant resistance gene after the withdrawal of the anthropogenic use of that ABD is not guaranteed, particularly if the resistance mechanism is encoded for by a gene on a plasmid.

1.7 ABD resistance in foodborne pathogens

That there are links between the use of ABDs and ABD resistance expressed by bacteria resident within that environment seems beyond doubt. To this effect, resistant

commensal bacteria have been proposed to act as both markers of resistance in a given environment and as reservoirs of resistance genes for other, potentially more pathogenic, bacteria. But is this in fact true, are there more likely to be resistant pathogens on farms using higher quantities of ABDs? Enhancing ABD resistance within the two commonest foodborne pathogens, *Campylobacter* and *Salmonella*, could have serious implications for public health and healthcare associated costs (Fraser et al., 2009).

1.7.1 ABD resistant *Campylobacter* species on farms

Emergence of resistance in *Campylobacter* species

Just two drug families are available for treating severe cases of human campylobacteriosis: fluoroquinolones (FQs) and erythromycin. Therefore, the potential emergence of FQ-resistant *Campylobacter* due to the administration of FQs to meat chickens has received some attention. Studies have shown that FQ-resistant *Campylobacter* emerges rapidly after enrofloxacin is administered via the drinking water to chickens housed individually (van Boven et al., 2003) and in small groups (Luo et al., 2003; Takahashi et al., 2005). Similarly, FQ-resistant *Campylobacter coli* emerges rapidly if three-week old piglets are dosed with oral enrofloxacin (Delsol et al., 2004a). In all cases, mutations in the quinolone-resistance-determining region (QRDR) of the *gyrA* gene formed the basis of the observed resistance.

Interestingly, one study following *Campylobacter* after dosing chickens with FQs did not see a concurrent rapid increase in FQ-resistance within the enteric *E. coli* population of chickens (van Boven et al., 2003). The differential rates of emergence of FQ-resistance between *Campylobacter* and *E. coli* are due to different mechanisms of FQ-resistance. *Escherichia coli* classically develop FQ-resistance via stepwise increases in minimum inhibitory concentration (MIC) corresponding to an increasing number of mutations in the *gyrA* gene and a concurrent over expression of efflux pumps. In contrast, *Campylobacter* are capable of developing full resistance following a single point-mutation in the QRDR of *gyrA*, in conjunction with a constitutively expressed CmeABC efflux pump (Lin et al., 2002; Pumbwe and Piddock, 2002). Furthermore, DNA microarray studies of *C. jejuni* have shown an increased expression of the mutation frequency decline (*mfd*) gene in response to ciprofloxacin (Han et al., 2008). The *mfd* gene is involved in DNA repair, and over-expression results in an increase in the frequency of spontaneous mutations to FQ-drugs. Thus, hypermutable strains of *Campylobacter* emerge under FQ-drug selective pressures enhancing the likelihood of the emergence of spontaneous-mutant FQ-resistant strains.

In a UK study, the recent use of FQ drugs on pig farms was indeed strongly associated with the detection of FQ-resistant *Campylobacter* (Taylor et al., 2009). However, multivariable logistic regression analysis showed that other farm factors were also associated with detecting FQ-resistant *Campylobacter*. Both buying-in growing animals from other farms and providing brushes with boot-dips increased the likelihood

of detection of FQ-resistant *Campylobacter*. Whereas, the lowest levels of detection were associated with farms that had high biosecurity scores and that only allowed visitors onto the site if they had been free of pig contact for two-days prior to the visit.

The study also looked at FQ-resistant *E. coli* on the same farms. FQ-use and pig-free visitors were again positively and negatively (respectively) associated with resistance, but there was also a strong seasonal effect, with higher detection rates in the summer months compared with the winter, as well as an increase in likelihood of detection if there were other pig farms within a mile of the study farm (Taylor et al., 2009).

Macrolide-resistance does not emerge so rapidly in *Campylobacter*. Macrolide-resistance is mediated via point-mutations affecting the drug target region of the 23s rRNA subunit in conjunction with active efflux mechanisms (Luangtongkum et al., 2009). Two levels of erythromycin-resistance can arise: single-step mutations affecting the ribosomal proteins infer low to intermediate macrolide-resistance (MIC 8–64 μg/ml), whilst step-wise mutations in 23S rRNA infer high-level resistance (MIC $>$ 512 μg/ml). However, the mutation frequency rates for macrolide-resistance in *Campylobacter* are 10,000-fold lower than those for FQ-resistance, and the emergence of high-level macrolide-resistance therefore requires longer periods of drug exposure.

What this means in practical terms, with respect to the use of macrolides in broiler production, has been investigated using chicken models. In one study, chickens received oral doses of macrolide-susceptible *Campylobacter* and five-days later the birds were either given a three-day course of tylosin at a therapeutic dose via the drinking water, or were fed sub-therapeutic levels of tylosin for the rest of the rearing cycle (Lin et al., 2007). No erythromycin-resistant *Campylobacter* isolates were recovered from the three-day treatment group; however, erythromycin-resistant *Campylobacter* were isolated after administering in-feed sub-therapeutic tylosin for 17 to 35 days, with the high-level resistant strains requiring the longest periods of drug administration prior to emergence.

A separate study showed similar results, in this work erythromycin-resistance emerged with highest frequency (83% of isolates) in *C. coli* isolated from the caeca of chickens fed prolonged courses of subtherapeutic tylosin (Ladely et al., 2007). This study also highlighted between-species differences in the emergence of macrolide resistance, with the lowest frequency of resistance (8% of isolates) seen in *C. jejuni* isolated from birds that had received a short course of tylosin at therapeutic doses.

On a clinical note, although the majority of problems caused by clinical resistance in *Campylobacter* currently occur in medical fields, resistance can also emerge in veterinary pathogens. A recent paper from the USA has reported that *Campylobacter jejuni* has replaced *Campylobacter fetus* subspecies *fetus* as the major cause of *Campylobacter*-associated abortions in sheep (Sahin et al., 2008). Three different genotyping techniques found that the majority of the US isolates studied (66 of 71)

belonged to a single clone, and although the clone was predominantly susceptible to a panel of drugs tested, it was resistant to tetracycline - the only drug licensed for use in cases of ovine *Campylobacter*-associated abortions. The authors concluded that using in-feed tetracycline as a prophylactic measure against such abortions was no longer likely to be efficacious.

Persistence of ABD resistance in *Campylobacter* species

A national survey of *Campylobacter* in Danish broiler chickens found that the levels of FQ-resistant *Campylobacter* varied markedly between farms, ranging from detection in zero to nearly 100% of samples from a single farm (Pedersen and Wedderkopp, 2003). PFGE genotyping patterns were obtained for *C. jejuni* and *C. coli* isolates from four of the *Campylobacter*-positive farms. Two of these four farms had not administered FQ drugs for one to five years, nonetheless farm-specific clones of FQ-resistant *Campylobacter* were isolated from consecutive flocks of birds on all four farms, implying carry-over of clones between flocks.

An explanation for the persistence of FQ-resistant *Campylobacter* in the absence of primary selective drug use could be due to an increase in fitness of FQ-mutants. This was demonstrated, by simultaneously inoculating chickens with two strains of FQ-resistant *Campylobacter*, which differed only in the presence or absence of a single-point mutation in the *gyrA* gene. It was seen that, even though no FQ drugs were administered, the resistant mutant actually out-competed and completely displaced the susceptible strain within three days of inoculation (Luo et al., 2005).

In contrast, macrolide-resistance in *Campylobacter* is less stable in the absence of drug-selective pressures (Caldwell et al., 2008). Low-level resistant strains, due to alterations in the ribosomal proteins, were maintained in chickens that were fed in-feed tylosin, but within two weeks of tylosin being withdrawn from the diet susceptible isolates had replaced the resistant ones. However, high-level resistant strains were much more stable and 100% of isolates recovered from cloacal swabs were still expressing high-level resistance 44 days after the oral inoculation of birds with no exposure to ABDs.

1.7.2 ABD resistant *Salmonella enterica* serovars on farms

In general, animal-related salmonellae are highly clonal, with outbreaks of particular strains within serovars occurring across wide geographic areas, and a succession of strains seen over time (Butaye et al., 2006; Lan et al., 2009). In some serovars these clones can be multidrug-resistant (MDR), and the number of MDR strains in circulation are increasing.

Selection of ABD resistant strains of *Salmonella enterica*

Between 1996 and 2006 the veterinary-related ABD consumption in Denmark showed a 250% increase in the quantities of ABDs (largely tetracycline) that were administered to Danish livestock (mainly pigs) (Emborg et al., 2008). Of *Salmonella* isolated from pigs at slaughter, only *S.* Typhimurium showed a concurrent increase in resistance during the same period. However, the increase in resistance was not due to the emergence of new resistance genes within established clones, rather it reflected changes in the relative frequencies of the various phage types of *S.* Typhimurium with fewer isolations of the largely ABD susceptible DT12, and more frequent isolations of phage types expressing a greater degree of multidrug resistance, such as: DT120, DT170 and DT104. This result confirmed that of an earlier study that had investigated farm-level influences on the likelihood of obtaining a tetracycline-resistant *S.* Typhimurium from a pig at slaughter. A positive relationship was seen between the isolation of tetracycline-resistant *S.* Typhimurium and the quantity of tetracycline used on the farm from which the pig had originated, but the phage type of the isolate was also strongly influential (Emborg et al., 2007).

A characterisation study of multidrug resistant (MDR) *S.* Newport isolated from humans, cattle and the environment, compared strains isolated since 1998 to previously circulating strains (Berge et al., 2004). There were significant alterations in PFGE genotypes and ABD resistance phenotypes between the pre-1998 and post-1998 isolates, again implying that there had been a shift in the genotypes present rather than alterations in the resistance of resident strains. In addition, the post-1998 isolates showed a stronger degree of clonality with one predominant PFGE-ABDR cluster in circulation, which expressed resistance to cephalosporins. In contrast, the pre-1998 strains were predominantly susceptible to cephalosporins and, therefore, the use of the cephalosporin drug ceftiofur on dairy farms offers a potential hypothesis for the increase in cephalosporin resistance. However, despite the common use of neomycin and spectinomycin in dairy calves there had been a decrease in aminoglycoside resistance in the post-1998 isolates. The authors suggest that this opposes the hypothesis that the use of ABDs in the dairy industry is responsible for the emergence of MDR strains of *S.* Newport. It is difficult to assess the validity of this claim using the data published, because only 16 of the *S.* Newport isolates are identified as originating from dairy calves, all bovine isolates had been analysed as a single group, and figures concerning drug use were not provided.

An *in vivo* animal model that administered a variety of strains of *S.* Typhimurium DT104 to groups of five-week-old pigs also indicated that the emergence of resistant salmonellae during ABD treatment is due to the selection of strains that are already expressing resistance (Delsol et al., 2004b). Administering oral enrofloxacin for five days selected for a strain that was already resistant to nalidixic acid and cyclohexane, and the increased shedding of this FQ-resistant DT104 persisted for 2–5 weeks beyond the end of

course of treatment. In a similar manner, dosing young pigs with tetracycline-resistant *S.* Typhimurium DT104 followed by a seven-day course of in-feed chlortetracycline at full therapeutic dose increased faecal concentrations of *Salmonella* for up to six-weeks post-treatment (Delsol et al., 2003). Comparative analysis of *E. coli* shed by the pigs showed that the proportion of the faecal *E. coli* populations that were resistant to tetracycline also increased for up to two-weeks post-treatment.

The effects of administering enrofloxacin using different routes were investigated using an *in vivo* model. Within 24 hours of oral administration the percentage of *Salmonella* shed that were nalidixic acid-resistant had increased, but administering the drug via the intra-muscular route did not increase faecal shedding of resistant strains when compared to the untreated control group (Wiuff et al., 2003). Increasing the oral dose of enrofloxacin to up to six times the recommended dose resulted in decreased shedding of resistant strains. These differences were not seen for the general coliform flora, and resistant strains increased rapidly to nearly 100% of isolates after enrofloxacin administration by either route. Furthermore, increasing the dose of drug used did not prevent this rise in resistance within the coliform populations.

Persistence of ABD resistant *Salmonella enterica*

One group monitored the effect of the 1974 EU ban on the use of tetracycline as a subtherapeutic growth promoter in The Netherlands. The proportion of *Salmonella* ser. Typhimurium isolates obtained from pigs and humans that were resistant to tetracycline, dropped from approximately 90% and 80%, respectively, of isolates in 1974, to 34% and 25% in 1980 (van Leeuwen et al., 1982). However, whilst mono-resistance to tetracycline did fall from 18% to approximately 5% in cattle, the multi-resistant phenotype ACKTTm (ampicillin, chloramphenicol, kanamycin, tetracycline, trimethoprim) increased dramatically from 6% in 1975 to 60% in 1980. The authors suggested that this was due to the large-scale use of other ABDs, particularly trimethoprim, to treat severe infections caused by highly virulent strains of *S.* ser. Dublin and *S.* ser. Typhimurium that were infecting veal calves at that time.

Evidence for the conservation of *Salmonella* resistance phenotypes within integrated livestock systems was provided by a study that took place on 37 finishing pig units belonging to three livestock companies in Brazil (Bessa et al., 2007). Characterisation of *S.* Typhimurium ($n = 66$) found that none were resistant to ciprofloxacin, but over 60% were resistant to tetracycline, sulphonamide and streptomycin. Resistance phenotypes of isolates from farms belonging to the same company were more similar than those that had come from farms belonging to different companies. Furthermore, the most common phenotype from one company expressed resistance to seven drugs, compared to two and three drugs in the other companies. Unfortunately the authors could not access any specific drug use data for the farms that had been sampled.

Whilst ABD use is likely to play a role in the persistence of MDR strains on a

farm or in a region (Glynn et al., 2004), other farm factors have also been shown to influence both persistence of MDR strains and their transmission between farms. In one study, the greatest risk factor for the introduction of MDR *Salmonella* onto a dairy farm was the practice of rearing replacement heifers off-site in loose aggregates of animals originating from a variety of farms (Adhikari et al., 2009). There was also an association between increasing herd size and an increased risk of the introduction of a new strain of MDR Salmonella. Meanwhile, an intervention study showed that separating hospital pens from maternity pens was effective in controlling an outbreak of MDR *S.* ser Newport within a farm (Cobbold et al., 2006).

The persistence and transmission of MDR *Salmonella* are testaments to the biological fitness of these strains, but the basis of this fitness is less clear. Resistance mechanisms that evolve due to chromosomal mutations, such as resistance to streptomycin or fluoroquinolones, usually (but not always) incur a cost in fitness on the mutant strains in terms of the ability to colonise and grow within a host or for transmission between hosts (Zhang et al., 2006). However, compensatory mechanisms evolve at other sites in the chromosome that alleviate these fitness costs (Maisnier-Patin et al., 2002; O'Regan et al., 2010). What is more, these compensatory mutations are deleterious to the mutants if the resistance mechanism is lost, thus the re-emergence of susceptibility in compensatory-adapted strains is unlikely (Zhang et al., 2006). Therefore, compensatory mechanisms can act to stabilise a resistance within the population, which over time could lead to increases in the degree of multidrug resistance expressed by the predominant strains that are in circulation.

However, many of the resistance genes expressed by MDR Salmonella are located on large plasmids bearing multiple genes (Michael et al., 2006). The carriage and replication of these plasmids in themselves incurs an energetic cost upon the host and this would be expected to impede the competitive ability of the MDR strains (Zhang et al., 2006). To-date, little work has been published regarding how *Salmonella* offset these costs and extrapolations have to be made from the growing literature studying resistance plasmids in species such as *E. coli.* Given the increasing emergence and spread of MDR *Salmonella* strains, it seems likely that compensatory mechanisms are acting to stabilise these MDR phenotypes. Therefore, it would seem sensible to make efforts to try to prevent more of these MDR strains from emerging.

1.8 Concluding summary

The mobile resistance genes currently circulating in pathogenic and indicator bacteria are likely to have emerged from soil bacteria. The size and diversity of the soil resistome indicates that for every new ABD brought onto the market, it is likely that previously unrecognised resistance genes will be mobilised from this source, and under the appropriate conditions, these novel genes will be able to spread horizontally through

bacterial populations. The strongest influences upon the occurrence and spread of resistance within enteric bacteria on a farm are associated with the use of ABDs, with the most potent selection pressures applied by feeding ABDs to groups of livestock for prolonged periods. Other farming practices, such as adding heavy metals to animal feed, and the use of ionophor anticoccidial drugs, can also influence resistance. However, there is little field evidence that the use of disinfectants on a farm represents a strong selective pressure for the emergence or persistence of ABD resistant bacteria.

In order to develop efficacious strategies for controlling ABD resistance on farms, it is important to understand resistance on levels other than a simple response to the administration of a specific ABD. On a wider scale, ecological factors also influence resistance within a geographical region. The exact natures of those influences are not yet clear; however, the proportions of bacteria expressing resistance that are shed by animals and humans that are not consuming ABDs, increase with increasing proximity to areas of high human population density: a phenomenon that could help to explain the presence of resistant bacteria on livestock farms that do not administer ABDs. On the microbial scale, the ability of bacteria to adapt to the fitness costs involved in carrying and expressing the mechanisms needed for resistance means that the withdrawal of the use of ABDs is unlikely to result a decline in resistance to zero. Plasmid-borne resistance is particularly difficult to eliminate once it is present, and the presence of multiple resistance genes on the same plasmid leads to the commonly observed phenomenon of co-selection: where the use of one drug maintains resistance to other pharmacologically unrelated drugs.

Nonetheless, preventing the practice of administering sub-therapeutic quantities of ABDs not only decreases the total quantity of ABDs administered within a country, but also results in decreased numbers of bacteria expressing resistance to those drugs. In some countries, there have been increases in the quantities of therapeutic ABDs administered after the withdrawal of ABGPs; however, alterations to farm husbandry practices have been shown to effectively decrease a farm's dependence upon ABDs. The possible exception to this trend is post-weaning diarrhoeal disease in pigs, which is proving hard to control under modern farming conditions without the use of ABDs.

The potential risks to public health posed by the occurrence of high prevalences of resistant bacteria on some farms are uncertain. However, given the remarkable speed at which bacteria can evolve, waiting until we have all the data needed to develop accurate risk models before acting to decrease resistant bacteria on farms may result in irreversible changes in the ecology of bacterial populations associated with livestock. It is clear that farming practices do influence the number of resistant bacteria present on a farm, and the degree of multidrug resistance expressed by these bacteria; therefore, ongoing research and monitoring should aim to inform the appropriate adjustments of agricultural practices needed to minimise the potential health risks posed to humans and animals by high prevalences of increasingly multi-resistant bacteria on farms.

1.9 The aims of this thesis

To this end, the aims of this body of work were to generate and analyse data regarding patterns of ABD resistance on pig and poultry farms, with a view to assessing potential associations between farm management practices and resistance.

Pig and poultry farms were chosen as the most heavily medicated sectors of the British livestock industry, and farms were recruited that illustrated a variety of drug use protocols. Besides collecting data on ABD use, other farm management factors were also recorded, such as: size of farm, feeding protocols, approach to cleaning and disinfection, type of housing, and disease problems present.

In the course of the work a number of fundamental questions arose and were addressed:

1. What are the most appropriate methods for measuring resistance?
2. How does one estimate drug use on a farm?
3. What analytical techniques are available for data of these types?

The exploration of these questions forms the foundation of much of the work described within this thesis.

"The purpose of learning is growth, and our minds, unlike our bodies, can continue growing as we continue to live." *Mortimer Adler*

Chapter 2

Associations between farm-level estimates of antibacterial drug use and the detection of ampicillin-resistant *Escherichia coli* in faecal samples

Abstract

The quantities of antibacterial drugs (ABDs) administered on 25 pig and poultry farms over a two-year period were estimated using retrospective drug use data. The presence of ampicillin-resistant *Escherichia coli* (AREC) in pooled faecal samples was assessed using chromogenic agar incorporating 8 μg/ml ampicillin. The quantities of ABDs used varied markedly between farms. The heaviest users were conventional pig farms, where oral tetracyclines accounted for 70% of ABD use; whilst, many of the organic farms administered no ABDs at all. Despite the differences in ABD use, for the majority of farm visits, AREC were detected in over half of the faecal samples collected on both conventional and organic farms. Nonetheless, positive relationships were seen between annual ABD use and the level of AREC detection, with the highest proportions of positive samples occurring on farms administering oral ABDS routinely to every group of growing animals. Regression modelling confirmed that AREC were more frequently detected on the farms feeding antibacterial growth promoting drugs (ABGPs) compared with the farms that were not. Furthermore, the simple drug use variable: number of days of routine oral ABD medication, was found to fit the data as closely as the calculated estimates of the number of animal daily doses (ADD) administered. This suggests that a survey of routine oral ABD use may be adequate for estimating ABD selective pressures in countries that lack standardised systems for recording drug use at the farm-level.

2.1 Introduction

International guidelines recommend that countries monitor resistance to antibacterial drugs (ABDs) within livestock species (ECDC / EFSA / EMEA / SCENIHR, 2009). An important aspect of such a resistance surveillance system is the concurrent monitoring of national levels and patterns of ABD use (FAO / WHO / OIE Expert Meeting, 2003; Franklin et al., 2001). Collecting data on both the ABD resistant bacteria present and the quantities of ABDs used, is necessary to provide evidence-based direction to 'prudent-use' guidelines for prescribing veterinarians (Aarestrup, 2005). In addition, such data are also crucial for the continued development of microbial risk assessments of the potential impacts of veterinary and agricultural ABD use upon public health (Snary et al., 2004). For this purpose, countries around the world have implemented active surveillance systems to monitor the trends in ABD resistance within selected bacterial species isolated from livestock animals (Hammerum et al., 2007). The bacteria monitored typically include organisms that can cause foodborne human ailments, such as *Salmonella* and *Campylobacter*, and also indicator species that may act as sentinels for, and reservoirs of, resistance genes present in a given environment, such as none-type-specific *Escherichia coli* and *Enterococcus* species (Sorum and Sunde, 2001). However certain ABD resistant indicator species, such as ampicillin-resistant *E. coli* (AREC), appear to be ubiquitous on many livestock farms, and their ready detection on farms where the use of ABDs is greatly restricted, or even zero (Bunner et al., 2007; Hoyle et al., 2006; Pleydell et al., 2007; Sato et al., 2005), suggests that such ABD resistant bacteria are not simply related to recent ABD use.

Whilst collecting the microbiological data necessary for resistance surveillance poses various logistic issues, collating ABD use data can be particularly challenging, especially at the national level. One source of such data is sales figures from the pharmaceutical industries or drug wholesalers (Grave et al., 1999). However, the lack of information regarding the species to which the purchased drugs were actually administered places limitations on the use of drug sales to examine the associations between veterinary drug consumption and ABD resistance. In Denmark, to address this issue, a national electronic monthly monitoring system has been put into place to collect ABD usage data at the farm-level (Stege et al., 2003). Danish veterinarians are now obliged to record and report all the drugs that they personally administer to animals as well as those that they sell to their clients for continuing treatments. However, in countries without an infrastructure for recording drug use, producing reliable estimates of the quantities of ABDs used is not an easy task in either veterinary (Chauvin et al., 2005b) or human medicine (Stichele et al., 2006). Furthermore, the actual measurements used to quantify drug use have to be suitable for assessing links with ABD resistance; and, if international comparisons are to be made, then these measures also need to be standardised. For instance, simply calculating the kilograms of active agents used does not account for differences in potency between different drugs, nor does it provide

information on the intensity of drug use in relation to the numbers of animals medicated (Chauvin et al., 2001; Jensen et al., 2004). To address these issues, measures in-line with the Defined Daily Doses used in human medicine have been adopted in veterinary pharmaco-epidemiological research (Chauvin et al., 2005b; Pol and Ruegg, 2007; Timmerman et al., 2006; Vieira et al., 2009).

This study aimed to estimate the annual quantities of ABD consumption across a range of commercial pig and poultry farms that were utilising a variety of management and drug-use policies in a country without a national system for recording ABD use at the farm-level. Data were collected on the use of therapeutic classes of ABDs as well as antibacterial growth promoting agents (ABGPs) that were incorporated into the animal feed at sub-therapeutic doses. The drug-use estimates were then used to investigate whether associations could be seen between the proportion of faecal samples in which ampicillin-resistant *E. coli* (AREC) were detected and various aspects of ABD use.

2.2 Materials and methods

2.2.1 Data collection

During the summer of 2001, twenty-five commercial pig and poultry farms were recruited on to the study by approaching specialist veterinary practices or utilising links that had been already established with researchers in other fields. The farms recruited encompassed a variety of farming practices and were located across southern and central England. Three of the organic poultry units also reared turkeys, and therefore a conventional turkey farm was also recruited. Farm sampling was conducted from December 2001 through to November 2003. Forty-five farm visits were carried out and, whenever possible, each farm was visited at least once in the first sampling year and once in the second year.

On each visit an average of 60 pooled-faecal-samples were collected. The pooled-samples comprised of approximately 1 g of faeces taken from each of eight fresh droppings that were located within the specified area being sampled. If it was not possible to identify individual faecal droppings within a house or pen, then a wand swab was inserted into the slurry in eight different spots within a given area. The aim on each visit was to collect samples from all the age groups or production sectors present on the farm on that day, and the number of samples collected from each area of the farm was chosen to reflect the proportion of the total stock that was present in that area. All samples were held at 4 °C during transportation and prior to processing in the laboratory, which occurred within 24 hours of sample collection.

2.2.2 Laboratory methods

Detection of resistant bacteria within the samples was carried out by diluting each pooled-faecal sample using an equal weight to volume of buffered peptone water (BPW) and then homogenising the resultant mixture using a vortex mixer. Ten μl of the homogenised mixture was streaked onto CHROMagar ECC® agar for *Escherichia coli* (CHROMagar, Paris, France) incorporating ampicillin at 8 μg/ml: a breakpoint chosen to harmonise with those used by the Public Health Laboratory Service (PHLS, UK) in 2001. The ampicillin concentration in the plates was checked daily using bacterial strains of known minimum inhibitory concentrations (MICs) and plates containing ampicillin were discarded if not used within 48 hours of pouring. After incubation a plate was scored as positive if there were colonies showing morphology typical of *E. coli* growing outside the initial area of inoculation. Details of the control strains used and the validation work undertaken to determine whether this method selected for ampicillin-resistant *E. coli* have been described previously (Pleydell et al., 2007).[1]

2.2.3 Estimating annual drug usage

Data were collected regarding the amounts of ABDs administered to the livestock species of interest on that farm over the 12 months preceding the first visit of each year. These data were collected by face-to-face discussion with the farm manager, with concurrent reference to written farm records and veterinary invoices wherever possible. To enable estimations to be made of the amounts of ABGPs and in-feed therapeutic ABDs administered, data were collected regarding the drug incorporation rates and the ages of livestock to which medicated feed was administered. Wherever possible, feed labels were collected to verify the incorporation rates.

In order to estimate the total annual feed intake of livestock during the period over which in-feed ABDs were administered, a series of growth and feed intake curves were constructed using the production figures from each farm in combination with standard production data (Anonymous, 1995; Carr, 2006; Wallenstein Feed and Supply Ltd, Ontario, 2006). Thereafter, using the individual farm incorporation rates, kilograms of active ABDs consumed per year per farm were calculated for each farm-drug combination.

The use of kilograms of active agent as a primary measure of drug use on farm does not adequately reflect the intensity of drug use on that farm (Chauvin et al., 2001). For instance, the apparent selective pressure would be biased to large farms, and small farms administering high quantities of drugs to all growing animals may appear to be relatively low users of ABDs due to size alone. Furthermore, because there are differences in the potencies of different drugs, farms relying mainly on lower potency drugs (such as amoxicillin) would appear to be heavier ABD users than farms that used

[1]This work is described in Chapter 4 on page 102

higher potency drugs (such as tylosin).

To avoid these biases, drug use can be standardised by calculating the number of doses of ABDs that are administered to a stated number of animals. Human pharmaco-epidemiological studies use internationally standardised doses called Defined Daily Doses (DDD), which represent the average maintenance dose per day for each drug. In veterinary medicine doses have to be defined on a species basis, and there are no internationally recognised standardised doses for veterinary species (Jensen et al., 2004). Therefore, a set of livestock-species-specific animal daily doses (ADD_{sp}) were derived for the ABDs used in this study, by determining the average maintenance dose in mg/kg for each agent for the typical indications in that livestock species. Separate ADD_{sp} were used for oral and injectable preparations of the same compound to account for the different dose rates associated with the two routes of administration.

Thus, the number of standardised doses of therapeutic ABDs administered per 1000 standard-weight animals per day was calculated for each drug used on each farm:

$$\frac{\text{total quantity active therapeutic drug used on farm in 1 year (mg)}}{ADD_{sp}\,(\text{mg/kg}) \times SW_{50}\,(\text{kg}) \times 365\,\text{days} \times \text{animals finished per year}} \times 1000\,\text{finished animals} \tag{2.1}$$

SW_{50} refers to the weight of animal at half the standard finished live weight for that species and this was set to 50 kg for pigs, 1 kg for broiler chickens and 8 kg for turkeys. Using a standard weight in this way will invariably under estimate the amounts of ABDs applied to lighter animals and over estimate the amounts of ABDs applied to heavier animals; but the age of animal at the time of dosing was not readily available on some farms so the mid-point weight was chosen to represent an average weight of a growing animal on the farm. These same issues are encountered in human medicine when trying to assess drug consumption in children using Defined Daily Doses, which are specified for adults (Chauvin et al., 2001).

As it is not meaningful to calculate standard therapeutic maintenance doses for drugs administered as sub-therapeutic growth promoters, a standardised measure of exposure to such drugs was calculated instead. Estimates were made of the total quantities of ABGPs that a bird or post-weaning pig would consume if all the food it ate over the course of its life incorporated the growth promoter at the standard incorporation rate. Average daily doses of ABGPs were then derived by dividing the estimates of total quantity by the total numbers of days of ABGP consumption these would represent to give species-specific figures that were termed 'animal growth promoting days' (AGD_{sp}). For each growth promoter used on each farm, the daily number of AGD_{sp} administered per 1000 finished animals was calculated:

$$\frac{\text{total quantity of active growth promoter used on farm in 1 year (mg)}}{AGD_{sp} \times 365\,\text{days} \times \text{animals finished per year}} \times 1000\,\text{finished animals} \tag{2.2}$$

In addition to the estimates of actual quantities of ABDs administered, the length of

time over which oral ABD drugs were routinely administered to livestock being reared on the farms was also calculated. Therefore, the numbers of days for which ABGPs were incorporated within the feed and the numbers of days for which therapeutic ABDs were routinely administered were calculated and combined into a single measure.

2.2.4 Data analysis

Trellis plots of bar charts showing the estimated annual quantities of ABDs used on the study farms were produced using the **barchart** function within the **lattice** library (Sarkar, 2008) in R Version 2.9.2 (R Development Core Team, 2009). Comparative violin plots were used to display the percentage of pooled faecal samples collected during each visit from which AREC were isolated for each category of farm studied. Violin plots comprise a box-and-whiskers plot combined with a kernel density smooth to highlight the underlying frequency distribution of the data. The violin plots were produced using a **panel.violin** function within the **bwplot** function also in the **lattice** library in R. The bandwidth selection method used to estimate the kernel densities for the violin plots was the Sheather-Jones 'solve-the-equation' estimator (Sheather and Jones, 1991). The box plots of the proportion of samples positive for AREC against various drug covariates were also produced in R using the **boxplot** function in the **base** package.

Multi-level generalised linear mixed effects models were constructed to allow for the simultaneous comparison of the effects of administering therapeutic ABDs and AB growth promoters upon the proportion of pooled faecal samples from which AREC were detected. To account for the clustered nature of the data, a three-level hierarchy was considered with faecal samples (k) clustered within visits (j) which in turn were clustered within farms (i). The outcome variable (y_{ijk}) was coded as a binary variable that equated to the detection (1) or not (0) of AREC within each pooled faecal sample. An interaction term between therapeutic ABD use and ABGP use was incorporated to allow for the possibility that the effect of each class of drugs upon the frequecy of detection of resistance may vary according to the quantity of the other class t.

Thus, the probability that faecal sample k collected during visit j to farm i would contain AREC was related to a linear predictor of fixed and random effects by a logit transform:

$$\begin{aligned} P(y_{ijk} = 1) &= p_{ijk} \\ \text{logit}(p_{ijk}) &= \beta_0 + \beta \mathbf{X}_{ijk} + \alpha_i + \alpha_{ij} \\ \alpha_i &\sim N(0, \sigma^2_{\alpha_i}) \\ \alpha_{ij} &\sim N(0, \sigma^2_{\alpha_{ij}}) \end{aligned}$$

Here $\mathbf{X}_{ijk}$ is a vector of fixed effects that includes the covariates for age/status, method of sample collection, species, therapeutic ABD use, ABGP use and the

interaction term between therapeutics and growth promoters. β_0 is the mean logit transformed probability of detecting resistance when the covariates are at their default values, and β is the vector of regression parameters for the fixed effects. The random effects for farm (α_i) and visit (α_{ij}) are both assumed to be normally distributed with means of 0 and variances of $\sigma^2_{\alpha_i}$ and $\sigma^2_{\alpha_{ij}}$ respectively.

Initial model development was undertaken in R using the `lmer` function in package `lme4` (Bates and DebRoy, 2004). The final models were then fitted using Bayesian inference via Gibbs sampling as implemented in WinBUGS 1.4.2 (Spiegelhalter et al., 2007). To reflect the lack of prior knowledge in this study, non-informative distributions were sought for all the prior probability distributions, but this is complicated by the distorting effect of the logistic transform. For example, a noninformative (flat) prior for β_0 would actually imply an informative (U-shaped) distribution for the corresponding probability for detecting resistance (p_{ijk}). This problem was approached by specifying noninformative priors for the probability of detecting resistance at selected covariate values, and using these to impute priors on the model parameters β_0 and β. A similar approach has been used by Garabed et al. (2008). For example, a prior of:

$$\beta_0 \sim N(0, 0.5)$$

gives a reasonable approximation to a uniform prior on the probability of detecting resistance when the covariates are at their default values. We can then induce an appropriate prior on the coefficient for a particular categorical variable (β_{CAT}) by defining:

$$\beta_0 + \beta_{\text{CAT}} \sim N(0, 0.5)$$

which corresponds to an approximate uniform prior on the probability of detecting AREC in that category when other covariates are at their default values.

For the continuous variables the prior distributions (β_{CONT}) were similarly set by specifying the prior probability of resistance at the mean value of each covariate:

$$\beta_0 + \beta_{\text{CONT}}\bar{x}_{\text{CONT}} \sim N(0, 0.5)$$

For the interaction term the prior distribution (β_{INT}) was set by specifying the prior probability of resistance with each continuous covariate set at its mean value:

$$\beta_0 + \beta_1\bar{x}_1 + \beta_2\bar{x}_2 + \beta_{\text{INT}}\bar{x}_1\bar{x}_2 \sim N(0, 0.5)$$

The models were compiled using three chains, and three sets of realistic initial values were specified for the chains. The model was run for 20,000 iterations using a thinning interval of 50 iterations; after a burn-in period of 5000 iterations the chains were sampled between iterations 5,000–20,000. Convergence of each model was assessed using the Gelman-Ruben statistic (Brooks and Gelman, 1998) and the fit of each

model was assessed and compared using the Deviance Information Criteria (DIC) (Spiegelhalter et al., 2002).

2.3 Results

2.3.1 Patterns of antibacterial drug use

The quantities of antibacterial drugs (ABDs) used varied widely between the farms. Figure 2.1 on page 52 shows that the highest annual quantities were being administered on some of the conventional pig units: with the two largest breeding-finishing units administering approximately 500 kg of active ABDs over a 12-month period. However, when farm size is taken into account, Figure 2.2 shows that some of the smaller conventional farms were using ABDs more intensely, in terms of the quantities of ABDs administered per growing animal, than some of the larger ones. Note the difference in scales of the y axes between the two types of livestock in these figures; on average a conventional pig farm was administering 5.6 times the quantity of ABDs per kilogram live weight finished animal than a conventional broiler unit (Wilcoxon rank sum test with continuity correction: $W = 36, p = 0.019$).

Tables 2.1 and 2.2 on page 54 highlight the ABDs that were used most frequently. Across the pig units, tetracycline drugs accounted for 70% of the total weight of ABDs used; and more than 99% of the total doses of tetracycline were administered orally. This data was collected before the European Commission withdraw the licenses from the remaining four ABGPs in 2006 and, therefore, the ABGP avilamycin accounted for a further 23% of kilograms of drugs used. The only other growth promoter used during this study was salinomycin, and it accounted for less than 0.5% of total drug use as it was only used on a single finishing unit. Ten farms used beta-lactam drugs over the course of the two years, including three of the organic farms that were using injectable beta-lactams to treat individual, clinical cases; however, beta-lactams only accounted for 3% of the total kilograms of ABD use on the pig farms. Although the weight of tylosin used was equal to that of the beta-lactams, due to the higher potency of the drug this corresponded to a higher number of doses of tylosin than beta-lactams, and oral tylosin use across three farms actually accounted for 12% of the total Animal Daily Doses (ADD) administered over two years. The remainder of the drug use on the pig farms consisted of lower quantities of a further 14 ABDs across nine farms.

Table 2.2 shows that the broiler units used a narrower range of agents than the pig units, and that a greater proportion of drug use was due to the in-feed growth promoter avilamycin which accounted for 58% of the total weight of ABDs used. The most commonly used therapeutic agent was amoxicillin at 26% of the total weight of drugs used, followed by the combination agent lincomycin-spectinomycin (8% of total use) and tylosin (7%). All ABDs used on the broiler farms were administered orally in the feed or water. Only one of the organic poultry farms used ABDs at any point

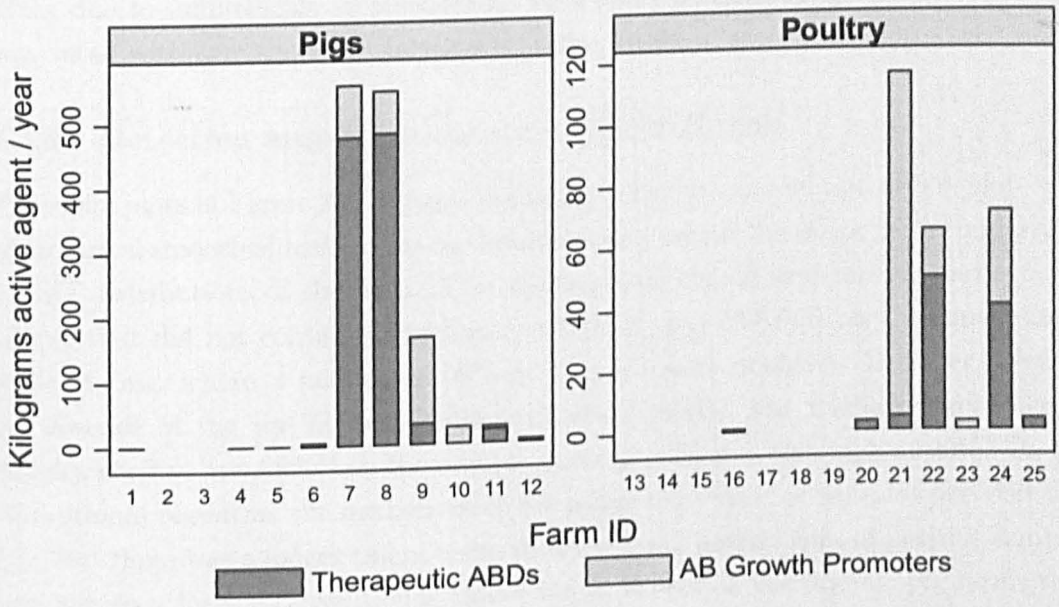

Figure 2.1: The mean annual quantities of antibacterial drugs (ABDs) administered on each of the study farms measured in kilograms of active agents.

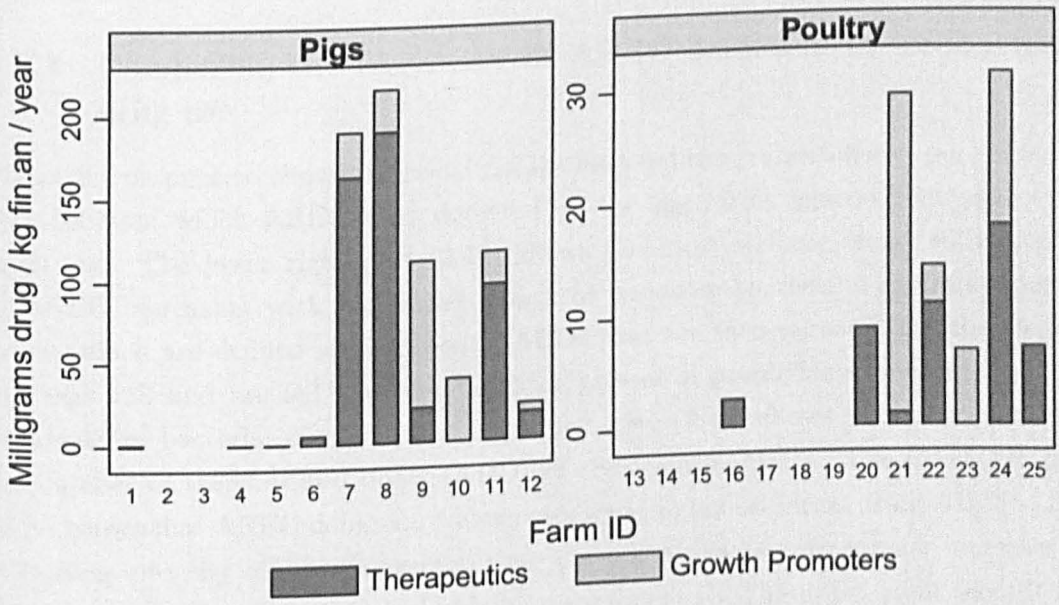

Figure 2.2: The mean annual quantities of antibacterial drugs (ABDs) measured in milligrams of active agents administered per kilogram of finished animals produced over a 12 month period.

Farms 1–5 and 13–19 are organic farms.
Note the difference in the scales on the y-axes between the two types of livestock.

during the study and this was to treat four groups of newly delivered chicks that were dying due to omphallitis: opportunisitic bacterial infections of the yolk sacs usually associated with contaminated hatcheries (Kahn, 2008).

2.3.2 Detecting ampicillin-resistant faecal *E. coli*

The violin plots in Figure 2.3 on page 55 combine the data summation of box plots with symmetrical smoothed histograms to simultaneously reveal the shape of the underlying density distributions of the data. This figure shows that it was rare to find a faecal sample that did not contain ampicillin-resistant *E. coli* (AREC) on the conventional broiler farms, where a median of 99% of samples were positive. However, even in the absence of the use of beta-lactams or other ABDs, the median percentage of samples positive for AREC on the organic poultry units was also high at 90%. On the conventional pig farms the median value for percentage positive samples per visit was 92%, but there was a longer tail of visits showing lower percentages of positive samples than was seen for the conventional broiler units. It was on the organic pig farms that the lowest percentages of positive samples were detected; these units showed a median value of 75% positive samples and none of the visits to organic pig farms yielded 100% of samples positive. However, even on these farms the interquartile-range was 62%–91% positive samples and the widest part of the density distribution occurred above the median value.

2.3.3 Comparing the frequency of AREC detection to antibacterial drug use

Figure 2.4 on page 56 shows the relationships between the percentages of pooled faecal samples from which AREC were detected on the pig farms against four aspects of ABD use. The lower right plot (2.4d) shows the most striking trend with median % AREC increasing with increasing length of exposure to routine oral therapeutic drugs, which are defined as therapeutic ABDs that are incorporated into the feed at the feed mill and are fed to every batch of pigs as a prophylactic measure against outbreaks of bacterial disease. The upper left plot (2.4a) shows a similar trend for the number of standardised doses (ADD) of therapeutic ABDs. The lower left plot (2.4c) shows that AREC detection frequencies were higher on farms using ABGPs, but increasing intensity of growth promoter use did not correlate with further increases in the percentage of samples in which AREC were detected. The upper right plot (2.4b) does not show any association between the quantity of beta-lactams used, which was relatively low on all farms, and % AREC.

The relationships between the frequencies of AREC detection on the poultry farms and aspects of ABD use are explored in Figure 2.5 on page 57. Even in the absence of ABD use the median proportion of positive samples is very high at 90%; nonetheless, as the quantity of therapeutic ABD use on the farms increased there was an upward

Table 2.1: A ranked list of the most commonly administered antibacterial drugs (ABDs) on 12 pig farms in the UK over a two year period from 2002 to 2003. The drugs are ranked according to the quantities that were administered.

Drug	Used by n farms	Quantity (kg active agent)	Oral therapeutics (ADD_{pig})[a]	Injectable therapeutics (ADD_{pig})[a]	Growth promoters (AGD_{pig})[b]
Tetracyclines	5	1868	9.3×10^7	3.5×10^5	-
Avilamycin	5	613	-	-	7.7×10^6
Beta-lactams	10	69	3.2×10^6	1.1×10^6	-
Tylosin	3	69	1.4×10^7	6×10^1	-
Other ($n_D = 14$)[c]	9	39	1.7×10^6	9.9×10^5	1.5×10^5
Total	10[d]	2658	1.1×10^8	2.4×10^6	7.9×10^6

[a] ADD_{pig} = pig-specific Animal Daily Doses (see page 48).
[b] AGD_{pig} = pig-specific Animal Growth promoting Days.
[c] n_D represents the total number of other drugs used that are not listed in the table.
[d] Two of the five organic farms did not administer any ABDs over the course of the study.

Table 2.2: A ranked list of the most commonly administered antibacterial drugs (ABDs) on 13 meat chicken farms in the UK over a two year period from 2002 to 2003. The drugs are ranked according to the quantities that were administered.

Drug	Used by n farms	Quantity (kg active agent)	Oral therapeutics (ADD_{chkn})[a]	Growth promoters (AGD_{chkn})[b]	Growth promoters (AGD_{turk})[c]
Avilamycin	4	382	-	1.6×10^8	6.4×10^7
Amoxicillin	6	173	1.1×10^7	-	-
Linco/Spect[d]	4	51	1.1×10^5	-	-
Tylosin	1	43	4.3×10^5	-	-
Other ($n_D = 3$)[e]	3	12	5.1×10^5	-	-
Total	7[f]	661	1.2×10^7	1.6×10^8	6.4×10^7

[a] ADD_{chkn} = chicken-specific Animal Daily Doses.
[b] AGD_{chkn} = chicken-specific Animal Growth promoting Days.
[c] AGD_{turk} = turkey-specific Animal Growth promoting Days.
[d] Combination agent lincomycin-spectinomycin.
[e] n_D represents the total number of other drugs used that are not listed in the table.
[f] Six of the seven organic farms did not administer any ABDs over the course of the study.

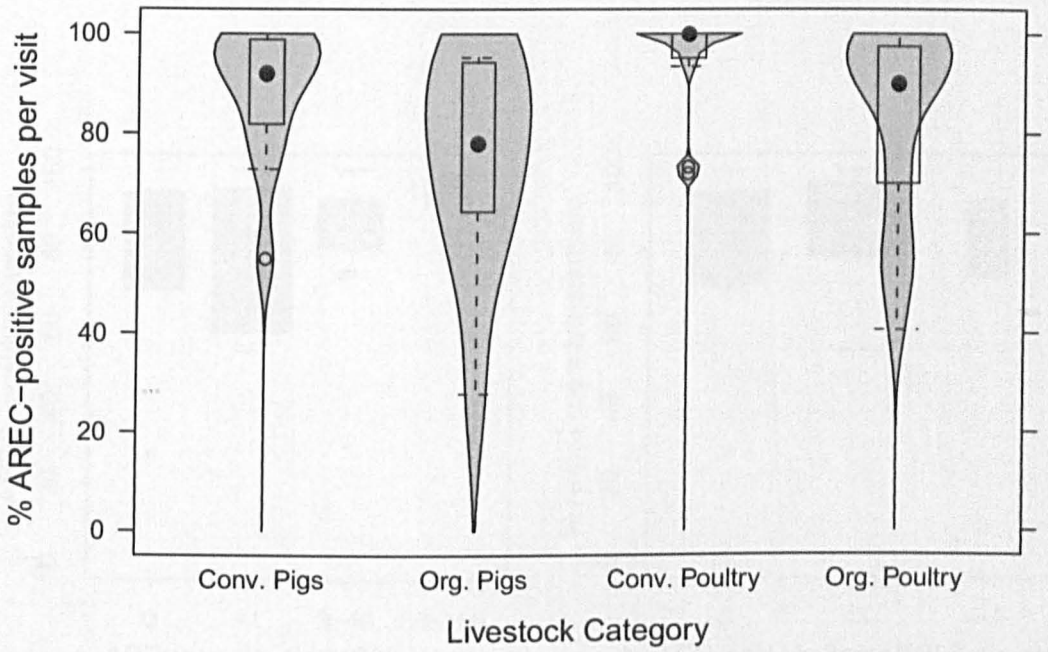

Figure 2.3: Violin plots showing the percentages of faecal samples collected per visit from which ampicillin-resistant *E. coli* were isolated for each of the four types of farm studied.

Conv. = conventional farms; Org. = organic farms.

Violin plots incorporate box-and-whiskers plots with kernel density smooths to highlight the frequency distributions of the underlying data: closed circles = median values; boxes = interquartile ranges; whiskers = data ranges; open circles = outlier values; grey-shading = kernel density smooths.

trend in the median values and a narrowing of the data ranges (see upper left plot, 2.5a). The lower left plot (2.5c) shows that AREC were isolated from 100% faecal samples collected on broiler units using ABGPs regardless of the actual intensity of growth promoter use. The lower right plot (2.5d) combines growth promoter use and routine therapeutic ABD use into a single variable of days of routine medication. Any farm using 18 days of more of routine medication was associated with 100% AREC. In contrast to the pig farms, a trend in increasing % AREC was discernable on farms administering higher numbers of doses of amoxicillin (upper right plot, 2.5b), which was the most commonly utilised therapeutic ABD.

2.3.4 Regression models of the pig data

Table 2.3 on page 60 shows the results of the two generalised linear mixed regression models that most closely fitted the pig data. The two models differed only in the measures of ABD use that were fitted:

Model 1 used the natural logarithms of the standardised measures of daily doses of therapeutic ABDs and exposure-days of ABGPs per 1000 finished pigs per year.

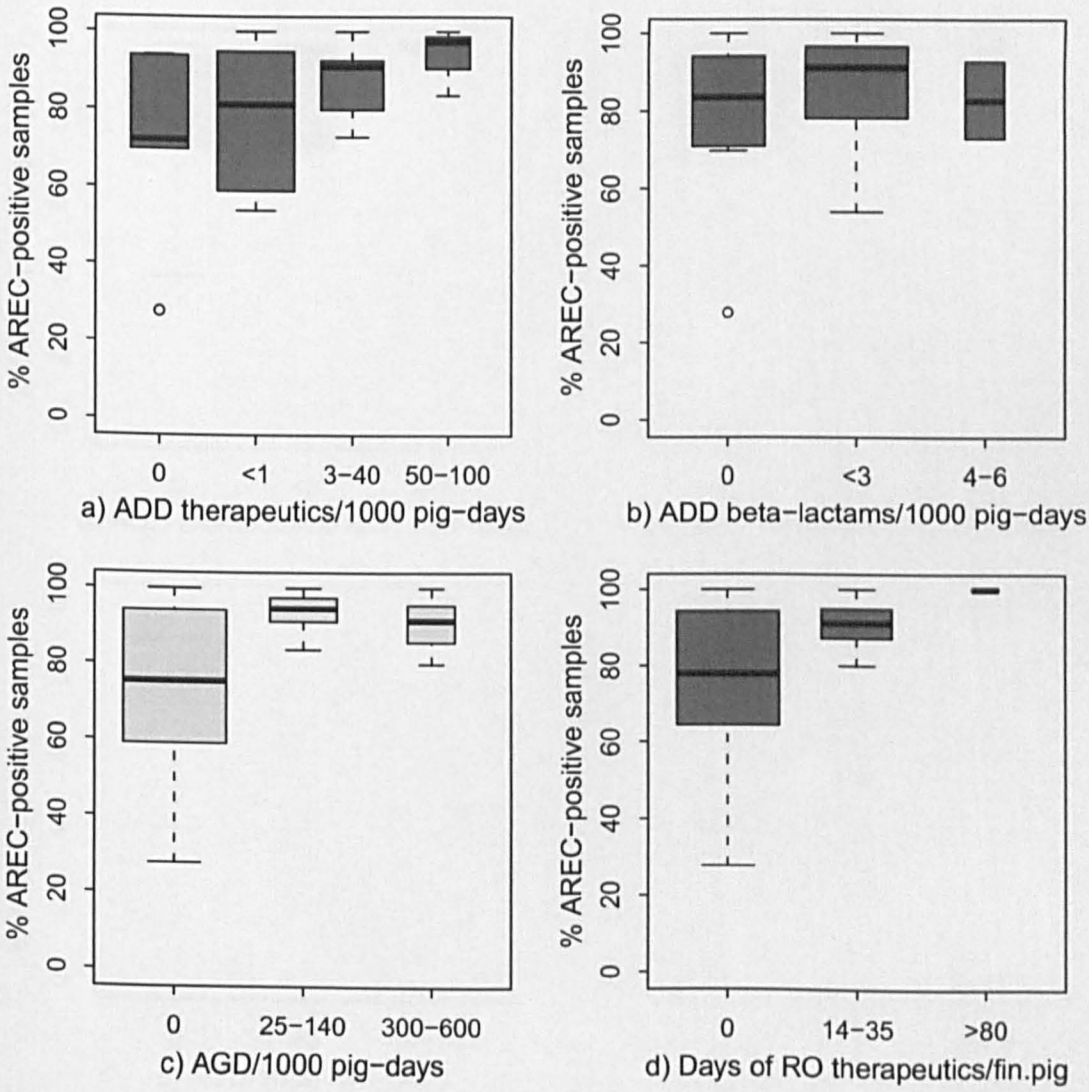

Figure 2.4: Box-and-whiskers plots of pig-farm data showing various antibacterial-drug-use variables against the percentage of pooled faecal samples collected per visit from which ampicillin-resistant *E. coli* were isolated.

ADD = pig-specific standardised Animal Daily Doses (see page 48).
AGD = pig-specific standardised Animal Growth promoting Days.
RO = Routine Oral; Days of RO therapeutics/fin.pig = the number of days for which a finished pig would have been routinely medicated with oral therapeutic antibacterial drugs (i.e. days of prophylactic medication).

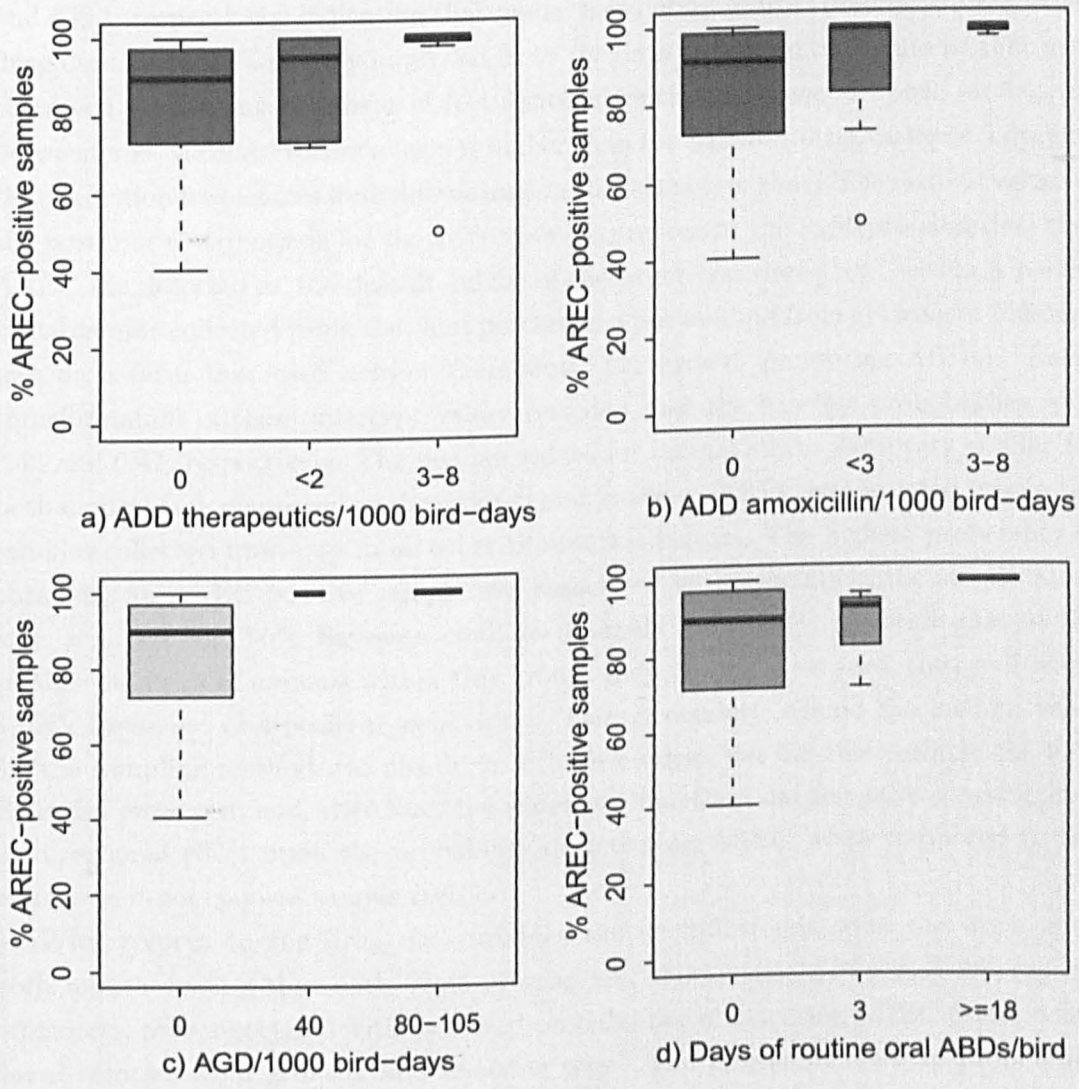

Figure 2.5: Box-and-whiskers plots of poultry-farm data showing various antibacterial-drug-use variables against the percentage of pooled faecal samples collected per visit from which ampicillin-resistant *E. coli* were isolated.

ADD = species-specific standardised Animal Daily Doses (see page 48).

AGD = species-specific standardised Animal Growth promoting Days.

Days of routine oral ABDs/bird = the number of days for which a finished bird would have been routinely medicated with oral therapeutic ABDs and AB growth promoters as a combined value.

Model 2 used the numbers of fortnights of routine oral ABD use; i.e. the length of time that every individual growing pig on a given farm was exposed to in-feed ABDs regardless of actual clinical indications within each batch.

The deviance information criterion (DIC) values for these two models were 636.0 and 636.1, respectively, indicating that model using standardised dose measurements of drug used provided a slightly improved fit to the data compared to the model that used the much simpler measurement of fortnights of routine drug use. In both models the between-visit variance within a farm is higher than the between-farm variance, implying that detection frequencies were fluctuating on the farms over time. The median values of the posterior distributions for the intercepts (β_0) represent the logit(probabilities) that AREC are detected at the default values of the other covariates (i.e. within a pooled faecal sample collected using the eight pinches in a pot method from growing or finishing pigs on a farm that used neither therapeutic nor growth promoting ABDs). Back-transformation of these intercept values revealed that the baseline probabilities were 0.44 and 0.47, respectively. The median values for animal status were very similar for both models and, compared to the growers and finishers, AREC was isolated from more samples collected from pigs in all other production sectors. The highest probability of obtaining an AREC positive sample was associated with animals in the service areas and, although the 95% Bayesian credible intervals (95% BCI) are wide due to the smaller numbers of animals within this group, they do not cross zero, thus indicating a high likelihood of a positive association. The uncertainty around the median value for the sampling method was also high in both models, but for this variable the 95% BCIs did cross zero and, therefore, the wand-swab method did not have a predictable unidirectional effect upon the probability of detecting AREC when compared to the pinches-in-a-pot, pooled sample method.

With respect to the drug use variables the overall trends were the same with both models finding that both types of drug use, therapeutic ABDs and AB growth promoters, were associated with increased probabilities of detecting AREC from pooled faecal samples from growing and finishing pigs. The largest increase in probability (0.78) was seen for the administration of 14 days of routine oral therapeutics. The average length of time for which the farms administered prophylactic ABDs in this way was 30 days, and the model predicts that the probability of detecting AREC on a farm that administered routine oral therapeutic drugs for this period of time would be rather higher at 0.94. Fourteen days of in-feed growth promoters was associated with a smaller increase in probability of AREC compared to the equivalent length of time of therapeutic use, and the 95% BCI just stretched across 0 indicating that there was a small possibility that 14 days of ABGPs had no effect upon AREC detection. Nonetheless, with the average length of ABGP administration at 103 days, using the median of the posterior distribution to predict the probability of detecting AREC on farms using this period of ABGP administration returned a value of 0.95. The

interaction term was negative in both models, and for Model 2 the BCI did not cross zero. A negative interaction term implies that administering both therapeutic and growth promoting drugs did not result in a simple additive effect upon AREC detection, but instead the importance of either variable as a predictor for increased AREC decreased as the level of use of the other increased.

2.3.5 Regression models of the poultry data

The results for two generalised linear mixed models of the detection of AREC in faecal samples collected on poultry-meat farms are shown in Table 2.4 on page 62. Both models included the age of birds sampled and the method of sample collection; however, it was not possible to also control for the species sampled (chicken or turkey) because species and age were themselves associated. The two drug use variables that were fitted were:

Model 3 used the natural logarithms of the standardised measures of daily doses of therapeutic ABDs and exposure-days of ABGPs per 1000 finished birds per year.

Model 4 used the average number of three-day periods of in-feed therapeutic ABD and ABGP administration that a bird would receive during the rearing cycle.

The incorporation of an interaction term between the therapeutic and growth promoting drug variables prevented the poultry models from converging, potentially due to the relative lack of between-farm variation in drug use patterns compared to the pig data.

Table 2.4 on page 62 shows that the poultry model of best fit (lowest DIC) was the model that used the measure of three-day courses of routine in-feed ABD medication. Once again the median variance between visits within farms was higher than between farms for both models, although the 95% BCI were wide. The intercept for both models was similar, with back-transforming showing a probability of 0.6 for detecting AREC in a faecal sample from a meat chicken that was under three weeks of age and being reared on a farm that used neither therapeutic ABDs nor ABGPs. Both models suggested that there was an increased probability of detecting AREC for birds over 80 days of age, although the credible intervals included zero indicating the possibility of no age effect within this dataset. However, for the poultry dataset, using a wand-swab to collect the sample was also associated with an increased probability of AREC detection compared to the pot method.

With respect to the ABD covariates, although the use of therapeutic ABDs was associated with an increased probability of detecting AREC, the 95% BCI around the estimates of the parameter were wide and contained zero, implying that there was a high degree of uncertainty around this result and there was a reasonable probability that the effect of therapeutics upon AREC detection was less than zero when controlling for the use of ABGPs. However, in both models the use of ABGPS was definitely associated

Table 2.3: Results of two generalised linear mixed models of the probability of detecting ampicillin-resistant E. coli in pooled faecal samples collected on 12 pig farms. The two models differed only in the measures of drug use that were fitted.

Variables and parameters	**Model 1** Log(ADD_{pig}) and log(AGD_{pig})[a]			**Model 2** Fortnights of routine in-feed ABDs[b]		
	Median[c]	95% BCI[d]	Probability[e]	Median	95% BCI	Probability
DIC[f]	636.0			636.1		
Farm-level variance	0.70	0.04–2.39	-	0.81	0.05–2.62	-
Visit-level variance	1.46	0.91–2.41	-	1.43	0.86–2.41	-
Intercept	−0.24	−1.24–0.61	0.44	−0.14	−1.19–0.70	0.47
Animal status						
Growers and finishers	Ref			Ref		
Farrowers and weaners	**0.96**[g]	**0.43–1.50**	**0.67**	**0.94**	**0.41–1.48**	**0.69**
Dry sows and gilts	**1.00**	**0.31–1.73**	**0.68**	**0.97**	**0.29–1.70**	**0.70**
Service area	**1.58**	**0.75–2.48**	**0.79**	**1.55**	**0.73–2.44**	**0.80**
Therapeutic ABDs	**0.81**	**0.01–1.73**	**0.64**	**1.39**	**0.32–3.28**	**0.78**
AB growth promoters	**0.68**	**0.14–1.26**	**0.61**	0.42	−0.04–0.89	0.57
AB_{ther}*AB_{gp} interaction	−0.24	−0.50–0.02	-	−0.25	−0.53– −0.005	-
Sampling method						
Pot	Ref			Ref		
Swab	−0.25	−0.91–0.42	0.38	−0.20	−0.87–0.47	0.42

[a] Model 1 fitted the natural logarithms of the Animal Daily Doses and Animal Growth promoting Days per 1000 pig-days.
[b] Model 2 fitted the period for which routine antibacterial drugs (ABDs) and antibacterial growth promoters (AGPDs) were incorporated into the pig feed as measured in fortnights.
[c] Median values of the posterior distributions.
[d] 95% Bayesian Credible Intervals.
[e] Probability of detecting AREC in a faecal sample.
[f] DIC = Deviance Information Criterion; the lower the value the better the fit of the model to the data.
[g] The model parameters highlighted in bold are those for which the 95% BCI do not cross zero, i.e. there is strong evidence for an association between that covariate and the detection of AREC within this set of data.

with an increased probability of detection of AREC. The probability of detecting AREC in a faecal sample on a farm after three-days of AGBP had risen to 0.71 in the absence of use of therapeutic drugs; however, the average length of time ABGPs were fed to chickens in this study was 34 days and the probability of detecting AREC after this duration of administration was 0.998.

2.4 Discussion

The quantities of antibacterial drugs (ABDs) used varied greatly across the 25 farms that were studied; which is in accordance with other studies that have attempted to measure ABD use at the level of the farm or the group (Chauvin et al., 2002; Timmerman et al., 2006). In the study described here, some farms appeared to be acting as 'super-users' of ABDs whilst other farms were administering a range of lower quantities, including eight of the twelve organic farms that were administering no ABDs at all.

The definition of an organically reared animal differs between countries. In the US, for instance, any animal that has been treated with ABDs permanently loses its organic status (The National Organic Program, 2007). In the UK, however, if it is clinically necessary to treat an organic animal with ABDs, that animal will regain its organic status after twice the length of the withdrawal period of the specific drug used has elapsed (Department for Environment, Food and Rural Affairs, 2006). Despite this greater degree of flexibility within the UK organic standards, this study found that the quantities of ABDs used on twelve organic farms were low to non-existent, with only one farm resorting to medicating groups of animals in the face of a severe outbreak of disease.

However, marked differences in ABD use were not only seen between organic and conventional producers, but also between the individual conventional farms. The participating conventional farms included independently owned farms and also farms that were operated by livestock companies. In the UK in 2006, approximately 60% of the 1,681 indoor poultry flocks on the national register were owned by a company and more than half of these company-owned premises were operated by 11 large companies (RADAR Veterinary Surveillance Strategy, 2006). Therefore, the ABD use protocols of these large, integrated, livestock companies are likely to have a major influence on the pattern of ABD use within the UK broiler industry at the national level. In the UK pig industry too, the increasing trend is towards farms operating within integrated companies. In this study, four conventional pig companies were represented and one conventional farm was independently operated.

A higher intensity of ABD use was seen on pig farms compared with poultry. Other European countries collecting data around the same time period also found that pig farms were the heaviest users of veterinary ABDs (Stege et al., 2003), and that

Table 2.4: *Results of two generalised linear mixed models of the probability of detecting ampicillin-resistant E. coli in pooled faecal samples collected on 13 meat poultry farms. The two models differed only in the measures of drug use that were fitted.*

Variables and parameters	Model 1 Log(ADD_{chkn}) and log($AGD_{chkn/turk}$)[a]			Model 2 Duration of administration of ABDs[b]		
	Median[c]	95% BCI[d]	Probability[e]	Median	95% BCI	Probability
DIC[f]	590.9			590.5		
Farm-level variance	0.70	0.02–2.89	-	0.66	0.01–2.76	-
Visit-level variance	1.79	1.15–2.96	-	1.86	1.20–3.06	-
Intercept	0.39	−0.80–1.34	0.60	0.38	−0.83–1.35	0.59
Age of birds						
1–21 days	Ref			Ref		
22–42 days	0.42	−0.30–1.15	0.69	0.41	−0.32–1.14	0.69
43–80 days	0.37	−0.39–1.07	0.68	0.39	−0.33–1.09	0.68
≥80 days	1.24	−0.13–2.57	0.84	1.22	−0.17–2.58	0.83
Therapeutic ABDs	1.31	−0.39–3.40	0.85	1.10	−0.35–3.08	0.81
AB growth promoters	**1.14**[g]	**0.34–2.27**	**0.82**	**0.51**	**0.19–0.95**	**0.71**
Sampling method						
Pot	Ref			Ref		
Swab	**0.95**	**0.30–1.64**	**0.79**	**0.93**	**0.28–1.62**	**0.79**

[a] Model 1 fitted the natural logarithms of the Animal Daily Doses and Animal Growth promoting Days per 1000 bird-days.
[b] Model 2 fitted the period for which antibacterial drugs (ABDs) and antibacterial growth promoters (AGPDs) were incorporated into the poultry feed as measured in courses of length three-days.
[c] Median values of the posterior distributions.
[d] 95% Bayesian Credible Intervals.
[e] Probability of detecting AREC in a faecal sample.
[f] DIC = Deviance Information Criterion; the lower the value the better the fit of the model to the data.
[g] The model parameters highlighted in bold are those for which the 95% BCI do not cross zero, i.e. there is strong evidence for an association between that covariate and the detection of AREC within this set of data.

tetracyclines were the most commonly used preparations (DANMAP 2002; MARAN 2002);(Goodyear, 2006). To attempt to relate the quantities of ABDs used on the highest consuming pig farm to a more familiar human pattern of ABD use, we could conceive of a course of ABD treatment whereby a standard tablet of a generic ABD contained 250 mg of active agent and the directions were to take one tablet four times a day for five days. In such a scenario a 100 kg finishing pig on farm 8 would have received the equivalent of approximately 80 tablets, or four courses of treatment, during its 22 week life. One explanation of such a high level of drug use, is that during the course of this study the British pig industry was in the midst of an epidemic of Post-weaning Multisystemic Wasting Syndrome (PMWS). All seven conventional farms in this study were affected to varying degrees, with small numbers of suspect pigs being noted on three of the organic units as well. Conventional farms 7 and 8 (see Figures 2.1 and 2.2 on page 54) belonged to the same company and it was particularly badly affected. In fact, the level of ABDs used on these farms became commercially nonviable, and the company depopulated the farms and disinfected the premises in an attempt to control the disease. In the first few months following repopulation the quantities of ABDs used on these farms dropped considerably to 10% and 22% respectively of the previous levels.

Irrespective of the large between-farm differences in drug-use practices, the frequencies of AREC detection across all 25 farms were generally high, with a median value of over 70% of faecal samples positive across all four farm-types. The lowest frequencies of AREC detection in this study were found on some of the organic pig units with less than 30% of samples positive on one particular visit. There are several potential reasons for the differences in AREC results on organic pig versus prganic poultry farms. Exposure of the faecal material to UV, heat and desiccation may be a factor in the lower frequencies of AREC detection on the outdoor pig farms. Whereas, organic chickens are housed together at night, which allows for the easy collection of fresh droppings from the houses in the morning, but also potentially facilitates the spread of resistant bacteria through a flock. Furthermore, at the time of this study, there were no large-scale organic breeding poultry flocks in the UK, and the majority of birds reared on organic farms were sourced from conventional breeding systems. When the organic farmers in this study were questioned, they could provide no information on the ABD practices occurring within those breeding flocks or at the hatcheries. Similarly, although there was also a lack of organic pig breeding herds, the organic pig herds were semi-closed breeding-finishing units and relatively few animals (usually just the boars and occasional replacement gilts) were brought on to an established farm.

Even though AREC were readily detected on most of the farms, nevertheless, relationships were seen between increasing intensities of ABD use and an increasing percentage of AREC positive faecal samples on both the pig and poultry farms. On the pig farms no relationship was evident between the use of beta-lactam drugs and the

frequency of AREC detection, and only 3% of recorded ABD use was ascribed to drugs of this class. Furthermore, although the conventional poultry units were commonly administering therapeutic courses of amoxicillin, fitting this as a separate term within a generalised linear mixed model did not fit the data as well as either of the two models presented. Therefore, the results of the modelling suggest that farms using higher quantities of ABDs per se are, either directly or indirectly, selecting for populations of *E. coli* that contain more ampicillin-resistant strains.

This study was carried out prior to the 2006 EU ban on the use of the four remaining growth promoting ABDs (Regulation (EC) 1831/2003). Therefore, the incorporation of separate terms for the use of therapeutics and growth promoters within the regression models allowed for an initial estimation of the relative importance of these two drug use covariates in terms of potential associations with the frequency of AREC detection on a farm. For both pigs and poultry, there was a higher degree of uncertainty around the parameters for therapeutic agents, which probably reflects the varying effects of different drugs. Nonetheless, the results of the models that assessed the length of time over which a pig or bird consumed ABDs (Models 2 and 4 in Tables 2.3 and 2.4, respectively) suggested that the selective force of a course of therapeutic ABDs was at least double that of a growth promoting agent administered for an equivalent length of time. However, using the model results to predict the probability of detecting AREC on a farm administering ABGPs for a representative length of time, found that the probability increased to over 0.9. Therefore, this work suggests that, when controlling for therapeutic ABD use, a farm administering growth promoters over a prolonged period of time is still exerting a significant selective pressure for the faecal shedding of AREC.

This seeming relationship between farms using growth promoters and an increase in the frequency of detection of AREC is particularly intriguing because avilamycin is thought to exert its actions largely upon the Gram-positive enteric flora, not Gram-negative species such as *E. coli* (Butaye et al., 2003; Treede et al., 2003). Therefore, the effects of using avilamycin are generally studied using bacteria such as the enterococci (Butaye et al., 2005). One model-based study incorporated avilamycin at the manufacturer's recommended rate into the feed of a group of five-week old pigs for three months (Delsol et al., 2005). No alterations were seen in the MICs of *E. coli* isolates to ampicillin during this period, but the MICs to tetracycline did increase in the treated pigs compared to the controls. Therefore, although the *E. coli* isolated throughout the experiment were all intrinsically resistant to avilamycin (MIC $> 128\,\mu g/ml$), the administration of avilamycin did appear to be indirectly affecting the *E. coli* population. Together these experimental and field observations may be related to shifts in the balance between Gram-positive and Gram-negative enteric bacteria, allowing for the proliferation of background strains of resistant *E. coli.* Alternatively, it is possible that the use of growth promoters could be acting as a proxy variable for

other aspects of management on the farm that have not been modelled here.

The regression modelling also found that the number of days that oral ABDs were administered fitted the data just as well as the estimates of the actual quantities of ABDs administered. This is an interesting finding for two reasons. Firstly, it suggests that a major selective force for increased frequencies of detection of faecal AREC on a farm is the length of time that ABDs are administered in the animals' food or water. The implication of this is that treating animals with ABDs only in the face of the clinical expression of disease would be less likely to select for an increased detection of faecal AREC across the farm as a whole, compared to routinely administering oral ABDs to all growing animals; unless the level of clinical disease was high on a farm, which would result in frequent ABD treatments. Secondly, this finding has implications for the monitoring of ABD use on farms in those countries where drug consumption data is not routinely gathered, because it suggests that recording the drugs that are routinely administered to livestock via the oral route along with the length of time over which they are administered may be sufficient in terms of looking for correlations between drug use and the detection of resistant bacteria.

Attempting to estimate the actual quantities of ABDs administered is logistically challenging. Face-to-face interviews and examination of farm records would be less feasible for a larger study, and standardising the data was difficult due to the different formats in which the drug use data was available on each farm, namely: treatment record books, veterinary invoices, personal recall by the farm manager, and feed labels. Other studies have also encountered difficulties in obtaining accurate estimations of ABD use on farms. A study from France, assessed the validity of farmers' declarations regarding drug use on pre-slaughter documentation against veterinary invoices and showed that the level of discrepancy between the two varied from 0% to 12% (Chauvin et al., 2005b). Another study sent questionnaires to veterinary practices employing pig veterinarians requesting details about the last group-level prescription that they had made, the study met with a 37% response rate (Chauvin et al., 2002). A Belgian study used face-to-face interviews to gather data on group medications administered to the pigs at slaughter age on the farm at the time of the visit. This work suggested that 6%–20% of drug use on the farms was unrecorded in the farm records (Timmerman et al., 2006). The reliability of farm records of ABD use have also been examined by collecting all empty drug bottles, tubs and sachets on farms using an in situ rubbish bin. This work also found that the drug records on five of 34 farms were inaccurate, and most of the farmers involved indicated that they would find the recording of individual drug use over time inconvenient (Dunlop et al., 1998).

In conclusion, this study found that even though AREC can be readily detected on livestock farms using no ABDs there are, nonetheless, relationships between increasing ABD use and increasing frequency of AREC detection. The strongest selective pressure appeared to be the use of routine oral ABDs, both therapeutic classes and growth

promoters, and this may, over prolonged periods of time, adversely influence ABD resistance in species of bacteria that are not directly targeted by the drug itself. A study of a larger number of farms using ABDs in a variety of ways, could help to further examine the differential selective pressures associated with use of ABDs for clinical, prophylactic or growth promoting reasons, by decreasing the width of the credible intervals of the posterior distributions and increasing the certainty around these model predictions. The suggestion that the use of a reasonably simple method of measuring ABD use (recording the number of days of routine oral medication) would increase the logistic and economic feasibility of a larger study.

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"Everything is vague to a degree you do not realize until you have tried to make it precise."
Bertrand Russell

Chapter 3

Using multiple correspondence analysis and hierarchical clustering to investigate the associations between farm management practices and antibacterial drug resistant faecal bacteria on pig and poultry farms.

Abstract

Multivariate statistical techniques were used to describe the farm-level factors associated with resistance to five antibacterial drugs (ABDs) in *E. coli* (ampicillin, gentamicin, ciprofloxacin) and *E. faecium* (vancomycin, erythromycin) isolated from pigs and poultry on 25 commercial livestock farms. Multilevel logistic regression modelling was utilised to investigate the statistical significance of the covariate patterns that were elucidated. In general, negative faecal samples (those from which ABD resistant bacteria were not isolated), occurred more commonly on farms that were not administering any ABDs. For two categories of bacteria of direct public health concern, vancomycin-resistant *E. faecium* in poultry and ciprofloxacin-resistant *E. coli* in pigs, there were strong links between detection on a farm and the use of specific ABDs: lincomycin-spectinomycin and fluoroquinolones, respectively. However, gentamicin-resistant *E. coli* was detected on both pig and poultry farms despite little or no use of aminoglycosides, and for both livestock species detection appeared to be associated with farms using a greater number of different ABDs over the course of a year. Ciprofloxacin-resistant *E. coli* were sporadically detected in flocks on company-operated, meat chicken farms even though fluoroquinolones were not used on the rearing farms themselves. Whilst, ampicillin-resistant *E. coli* and erythromycin-resistant *E. faecium* were commonly isolated from all participating farms, including those not

administering ABDs. For these two bacteria, factors associated with increased detection included: the use of ABDs as oral prophylactic and growth promoting agents, the production status of the animals sampled, concurrent disease and mortality, and type of feed. Given the diversity of factors influencing the detection of ABD resistant indicator bacteria on livestock farms, a single set of guidelines would be unlikely to minimise all such bacteria. Nonetheless, the highest frequencies of detection of resistant bacteria were associated with farms administering the highest quantities of ABDs; therefore, it would be prudent for farm managers and veterinarians to work together to monitor and limit the emergence and persistence of ABD-resistant bacteria on farms that are routinely administering oral ABDs to livestock.

3.1 Introduction

Commensal bacterial species of mammals and birds are purported to act as markers of resistance within given environments and as reservoirs of antibacterial drug (ABD) resistance genes (Blake et al., 2003; Sabate et al., 2006; Sunde and Sorum, 1999; Yates et al., 2006). *Escherichia coli* and *Enterococcus* species are generally classified as commensal bacteria because they readily colonise humans and animals without causing disease and their presence can be detected in a wide variety of environments (Johnson et al., 2004; Kuhn et al., 2003; Mallon et al., 2002; Souza et al., 1999). However, certain strains of these species can cause clinical infections in susceptible hosts; notably as nosocomial infections due to their ability to persist in hospital environments (Rice, 2009; Sanchez et al., 2002; Top et al., 2008), but community acquired infections are seen too (Ball et al., 2008; Moor et al., 2008; Woodford et al., 2004). In recent years, these species have been the focus of several studies of ABD resistance in the environment as they are relatively easy to isolate and, due to their propensity to acquire, carry and transfer genes encoding for resistance to ABDs, the detection of multidrug resistant (MDR) strains is increasingly common (Garcia-Migura et al., 2007a; Macovei and Zurek, 2006; Nogrady et al., 2006; Sunde and Norstrom, 2006). In light of this, many countries have now established ongoing resistance surveillance programmes monitoring both pathogenic and commensal bacteria associated with livestock species (de Jong et al., 2009; Gilbert et al., 2007; Hammerum et al., 2007).

A number of studies have shown links between alterations in ABD use in hospital or farm environments and alterations in the balance of resistance within the bacteria within those environments (D'Agata et al., 2007; Emborg et al., 2004; Lauderdale et al., 2007; Willemsen et al., 2009). In view of this, many countries now suggest that ABDs should be used 'prudently'. However, it is evident that ABD resistant bacteria can be isolated from farms that are administering restricted quantities of ABDs, and even those that are applying no ABDs at all (Hoyle et al., 2004a; Pleydell et al., 2007; Roesch

et al., 2006; Sato et al., 2005). Therefore, factors other than the direct, local use of ABDs must be contributing to the presence and persistence of resistance on livestock farms.

The management of ABD resistance in hospitals relies upon the identification of critical control points in the transfer and persistence of resistance within an individual establishment (Safdar and Maki, 2002; Sample et al., 2002; Souli et al., 2009; Walker et al., 2004). Similarly, the identification of farming practices that enhance or impede the development and persistence of ABD resistant bacteria could help to elucidate possible critical control points for decreasing resistance on livestock farms. National surveillance programmes, however, generally sample animals at abattoirs and provide data on the national trends in resistance. In order to more fully understand the biological processes involved in the occurrence and persistence of ABD resistant bacteria on farms the collection of farm-level data is required. Furthermore, different resistant bacteria exhibit different behaviours and the results of studies on one sort of bacterium (such as the widely studied ampicillin-resistant *E. coli*) may not be applicable to other bacteria, thus necessitating the collection of data on a variety of bacteria.

However, the collection of farm-level data for a number of different bacteria is labour-intensive and expensive, and the resultant dataset is likely to contain a large number of covariates across a more limited number of observations. This sort of structure can cause problems if relying on classical analytical methods, such as regression modelling, due to factors such as interaction, confounding, multicollinearity and a lack of statistical power related to sample size (Cortina, 1993; Dohoo et al., 1996). Studies using a questionnaire-based approach to collecting data are also prone to these difficulties, and descriptive, multivariate analyses are often utilised in such cases (Crocker et al., 2007; Greenacre, 2002; Poitras et al., 2007; Ribbens et al., 2008). One such approach, that is particularly suited to this sort of data, is multiple correspondence analysis (MCA): a technique that maps the associations between multiple variables within Euclidean space (Dohoo et al., 1996; Greenacre, 2007).

This study sought to undertake detailed studies of ABD resistant commensal bacteria across a panel of farms utilising a variety of farming practices. MCA and subsequent hierarchical clustering were utilised as exploratory methods in order to evaluate farm-level influences upon resistance in aerobic commensal bacteria isolated from fresh faecal samples. Associations between resistance and farm practices highlighted by the MCA models were then explored further using multi-level regression models to estimate the degree of uncertainty around the associations between variables.

[0]See Chapters 2, 4 and 5

3.2 Materials and methods

3.2.1 Data collection

In 2001, twenty-five commercial pig and poultry farms located across southern and central England were recruited on to the study as described in Chapter 2 on page 46. Each farm was visited at least once in 2002, and again in 2003. On each visit, data were collected regarding animal husbandry and ABD usage practices that were employed on the farm, and an average of 60 pooled-faecal-samples were collected across all the livestock sectors present on the farm at that time. Pooled faecal samples were of two types: if individual fresh faecal masses were discernible, then 1 g pinches were taken from eight separate droppings and placed into a single pot; where this was not possible sterile, charcoal wand-swabs were inserted into the faecal slurry in eight separate places. All samples were held at 4 °C during transportation and prior to processing in the laboratory, which occurred within 24-hours of sample collection. (For full details of the sampling strategy see page 46)

If ciprofloxacin-resistant *E. coli* were detected on a poultry farm, a follow-up visit was made to the positive flocks to collect pre-slaughter samples from the previously positive flocks.

3.2.2 Laboratory methods

The direct-plating methods used to detect ABD resistant bacteria within the samples have been described in Chapter 2 on page 47. Five bacterial-drug combinations were investigated: ampicillin, gentamicin and ciprofloxacin resistance in populations of *Escherichia coli*; and erythromycin and vancomycin resistance in populations of *Enterococcus faecium*. Selective agar media containing ABDs at breakpoint concentrations were used to allow for the detection of resistant bacteria within the faecal samples: CHROMagar ECC® for *E. coli* (CHROMagar, Paris, France); Slanetz and Bartley for *E. faecium* (Oxoid, Basingstoke, United Kingdom). The ABD concentrations in the prepared plates were checked daily using bacterial control strains of known MICs (see Tables A.1 and A.2 in Appendix A on pages 191 and 199). The breakpoints utilised for *E. coli* were chosen to harmonise with those used by the Public Health Laboratory Service (PHLS, UK) in 2001. In the absence of PHLS breakpoints for erythromycin resistance in *E. faecium*, the breakpoints adopted for this species were those used by the Danish Veterinary Laboratories (DVL) in 2001. Ten microlitres of a homogenised 1:1 weight-to-volume mixture of faeces and buffered peptone water was streaked onto a plate in order to obtain single colonies. Each plate was divided into two equal halves to allow for two samples to be plated on a single plate. The streaked plates were incubated at 37 °C for 16 to 18 hours for *E. coli*, and 42 °C for 32 to 48 hours for *E. faecium*. Bacterial growth on the plates was recorded in a semi-quantitative manner: no growth outside the area of inoculation = zero, < 100 well-spaced colonies present

on half a plate = one, profuse growth with > 100 colonies per half-plate = two.

The process for confirming the identity of the *E. coli* isolates in general, and ampicillin-resistant *E. coli* in particular, has been detailed in Chapter 4 on page 102. Previous MIC work undertaken at the VLA to validate the methods used to select ciprofloxacin-resistant *E. coli* have been published elsewhere (Taylor et al., 2008). The identity of the presumptive *E. faecium* isolates was confirmed using real-time PCR as published previously (Garcia-Migura et al., 2005), and the vancomycin-resistant strains were shown to be carrying *vanA* genes using a multiplex PCR assay as published in the same paper.

3.2.3 Estimating annual drug usage

Estimates were made of the kilograms of active therapeutic ABDs and antibacterial growth promoters (ABGPs) that were administered on each farm over the 12-month period preceding the first visit of each year. The raw quantities of therapeutic agents were then adjusted to take into account the size of the farm and the varying potencies of the individual drugs used, and the adjusted quantities were expressed in numbers of species-specific Animal Daily Doses (ADD_{sp}) per 1000 finished animals per year. For the ABGPs an average daily dose was calculated for each livestock species and used as a constant term in order to derive the number of species-specific Animal Growth promoting Days (AGD_{sp}) per 1000 finished animals per year. These methods have been previously described in full detail (see Chapter 2, page 48).

3.2.4 Data analysis

Preliminary data analysis was carried out using stacked bar plots to visualise the proportions of faecal samples from which the five varieties of antibacterial-resistant bacteria were isolated. The bar plots were stratified by livestock species (pig or poultry) and farming system (conventionally managed or organic), and were produced in R version 2.9.2 (R Development Core Team, 2009).

3.2.5 Multiple correspondence analysis and cluster analysis

Multivariate analysis using multiple correspondence analysis (MCA) and subsequent hierarchical clustering allowed for a simultaneous assessment of associations between the numerous potential explanatory variables and the five resistance response variables. The raw data were coded as indicator matrices with columns corresponding to each individual category of each variable (hereafter termed elements) and rows representing the results from each farm visit. In order to retain the variation within the data, continuous variables were converted to dummy variables using non-disjunctive (fuzzy) coding by placing hinges at natural breaks within the data (Murtagh, 2005, pgs. 80–85). The data from the pig and poultry farms were analysed separately, and the potential

explanatory variables that were considered for each species are shown in Table 3.1 on page 74 and Table 3.2 on page 75.

MCA models calculate the χ^2 distance between the profile of a column or row and the respective weighted average column or row profile. These distances are transformed, to enable mapping of the profile points in multi-dimensional Euclidean space. Eigen-reduction then finds a lower dimensionality subspace that approximates the true positions of the profile points, and the model outputs eigenvalues for every column and row profile within each dimension of this subspace. Knowing how many dimensions to retain for subsequent analysis is often an arbitrary decision on the part of the researcher who is trying to identify those dimensions that contain useful information about associations between elements, and to discard dimensions containing non-meaningful noise within the dataset. In this study a systematic approach was undertaken, whereby, for every MCA model fitted to actual data, a series of ten MCA models were fitted to randomly permuted data, and only those dimensions showing higher eigenvalues than the random data were analysed further, as per the methods of Ciampi et al. (2005). The random data sets were produced using a volatile Excel spreadsheet (Office Excel 2003, Microsoft Corporation, Redmond, USA) that retained the structure of the original data frame but complied with a hypothesis of independence between farm visits (rows) and the variable elements (columns).

Outlier elements in a MCA model are those that are poorly represented and as such they disproportionately contribute to the model inertia (variance), which can distort the apparent associations between other elements (Ciampi et al., 2005). In order to identify such outliers, a series of MCA models were initially fitted to:

1. the resistance elements alone.

2. the combination of resistance elements and drug use elements.

3. the combination of resistance elements and farm management elements.

The quality values (the sums of the squared correlations across the selected n-dimensions) were examined to identify covariates for which all constituent elements had quality values less than 500 (i.e. were less than 50% represented by the selected n-dimensions), and these underrepresented variables were discarded from further analysis.

A synthesis MCA model was then fitted that included resistance, drug use and farm management variables, but excluded the identified outlier covariates. The coordinates of the projections of the elements onto the selected n-dimensional subspace were extracted from the synthesis model and hierarchical clustering of the coordinates was undertaken using the methods of Murtagh (2005, pgs. 46–57). These methods involve weighting the squared Euclidean distances by the column (elemental) masses, constructing a nearest neighbour chain from an arbitrary starting point and using a minimum variance, weighted agglomerative clustering algorithm to produce crisp clusters. Elements of low mass are those that occur infrequently, whilst elements of

Table 3.1: Details of the full set of variables that were assessed for the pig data, including the abbreviations and category definitions used in the multiple correspondence analyses, and the hinges used to transform the continuous variables into fuzzy-coded categories.

Variable	Description	Levels (elements)		
Response		**0**	**1**	**2**
AR	Ampicillin-resistant *E. coli*	no growth	< 100 col.[a]	> 100 col.
CR	Ciprofloxacin-resistant *E. coli*	no growth	growth	-[b]
ER	Erythromycin-resistant *E. faecium*	no growth	< 100 col.	> 100 col.
GR	Gentamicin-resistant *E. coli*	no growth	growth	-
Categorical		**0**	**1**	**2**
cdhd	Closed herd	-	-	-
dip	Number of boot dips on farm	none	some	many
dpop	Depopulation in last six months	no	yes	-
feed	Type of feed	-	pelleted	other
onhd	Open herd	-	-	-
pmws[c]	PMWS present on farm	no	yes	-
wat	Source of water for stock	-	mains	other
Continuous[d]		**0**	**1**	**2**
amg	Aminoglycosides[e]	0	0.5	-
bla	Beta-lactams[e]	0	0.12	4.58
dexr	Days exposed to routine ABDs[f]	0	75	150
dft	Number of times disinfect/y	0	1	4
fq	Fluoroquinolones[e]	0	0.5	-
gpd	Growth promoters[g]	0	100	300
inj	Injectable ABDs[e]	0	0.14	1.01
mls	MLS drugs[e h]	0	1	50
ndg	Number of ABDs used/y	0	4	8
oral	Oral therapeutic ABDs[e]	0	14.06	94.76
tadd	Therapeutic ABDs[e]	0	0.24	66.46
tet	Tetracyclines[e]	0	9.07	66.88
		1	**2**	**3**
fmsz	Number of pigs finished/y	405	4,250	29,800
mort	Annual herd mortality (%)	1	2.5	4

[a] The number of colonies present on half an agar plate. [b] - = This level was not utilised for this variable. [c] Postweaning multisystemic wasting syndrome. [d] Continuous variables and counts. The values shown depict the values at which the hinges were placed to produce the non-disjunctively coded categories. [e] All variables related to therapeutic antibacterial drugs (ABDs) were measured using Animal Defined Daily doses per 1000 pigs finished per year (ADD_{pig}/1000 pigs/y). For details of how these were calculated see page 48. [f] ABDs = antibacterial drugs. [g] The antibacterial growth promoting drugs (ABGPs) were measured using Animal Growth promoting Days per 1000 pigs finished per year(AGD_{pig}/1000 pigs/y). [h] Macrolides, lincosamides and streptogramins.

Table 3.2: Details of the full set of variables that were assessed for the poultry data, including the abbreviations and category definitions used in the multiple correspondence analyses, and the hinges used to transform the continuous variables into fuzzy-coded categories.

Variable	Description	Levels (elements)		
Response		**0**	**1**	**2**
AR	Ampicillin-resistant *E. coli*	no growth	< 100 col.[a]	> 100 col.
CR	Ciprofloxacin-resistant *E. coli*	no growth	growth	-
ER	Erythromycin-resistant *E. faecium*	no growth	< 100 col.	> 100 col.
GR	Gentamicin-resistant *E. coli*	no growth	growth	-
VR	Vancomycin-resistant *E. faecium*	no growth	growth	-
Categorical		**0**	**1**	**2**
ccln	External cleaning company used	-	-	-
dft	Disinfect between flocks	no	yes	-
dp	Boot dips at entrance to houses	no	yes	-
ent	Enteritis a problem on farm[b]	no	yes	-
flw	Water lines flushed between flocks	no	yes	-
gmb	Games birds seen around farm	no	yes	-
htyp	Type of housing used	conv.[c]	mobile	old building
ls	Lincomycin-spectinomycin used	no	yes	-
mage	Multiple ages of birds on farm	-	-	-
msp	Multiple species on farm	-	-	-
resp	Respiratory disease on farm[b]	no	yes	-
rng	Birds are free-range	no	yes	-
sage	Single-age, all-in-all-out farm	-	-	-
scln	Farm staff clean houses	-	-	-
sept	Septicaemia a problem on farm[b]	no	yes	-
ssp	Single species on farm	-	-	-
tur	Poultry species sampled	chicken	turkey	-
wat	Source of water	-	mains	other
Continuous[d]		**0**	**1**	**2**
bla	Beta-lactams[e]	0	0.91	7.05
dsd	Age when dosed with ABDs[f] (days)	0	7	21
emp	Empty time between flocks (days)	0	7	14
gpd	Growth promoters[g]	0	17.07	104.1
ndg	Number ABDs used/y[e]	0	3	7
tadd	Therapeutic ABDs[e]	0	1.16	7.21
		1	**2**	**3**
fmsz	Number of birds finished/y	1,230	35,000	1,609,500
hage	Age of houses (months)	25	114	384
mort	Average flock mortality (%)	1.95	5.95	12.28

[a] The number of colonies present on half an agar plate. [b] In the opinion of the farm manager. [c] Purpose-built, static, conventional poultry house. [d] Continuous variables and counts. The values shown depict the values at which the hinges were placed to produce the non-disjunctively coded categories. [e] Measured using Animal Defined Daily doses per 1000 pigs finished per year (ADD_{pig}/1000 pigs/y). [f] Antibacterial drugs. [g] Measured using Animal Growth promoting Days per 1000 birds finished per year($AGD_{chkn/turk}$/1000 birds/y).

high mass occurred frequently. Therefore weighting by the column masses prevents the dendrogram from being unduly influenced by the low frequency elements.

Deciding where to cut the dendrogram resulting from a hierarchical cluster analysis determines the number of clusters that will arise from that analysis. This decision can often be arbitrary, however there are a variety of methods available that attempt to identify and validate the number of clusters within a dataset (Halkidi et al., 2002a,b; Handl et al., 2005). In this study, 14 different cutting distances were assessed by partitioning the elements into two to fifteen clusters. Cluster validation was undertaken using silhouette coefficients[1], which provided easy visualisation of the results at the level of both the clusters and the individual elements, and thus enabled assessments to be made of the stability of the assignment of each element to a cluster (Rousseeuw, 1987). Variables consisting of elements that were inappropriately assigned over multiple cluster solutions were identified as outliers, removed and a final MCA model was fitted to the refined dataset.

To enable visualisation of the results of the analyses, symmetrical 2D maps were used to display the elemental projection coordinates onto the two principal dimensions (axes) of the optimised MCA models. The two principal axes are those with the highest eigenvalues and they therefore describe the largest sources of variation within the data. However, in order to visualise the associations between elements across all the dimensions that were identified as containing meaningful information, the clusters obtained from the hierarchical cluster analysis of the eigenvalues were superimposed onto the 2D MCA maps, with those elements of uncertain classification being shown as potentially belonging to two clusters.

The non-disjunctive coding, multiple correspondence analyses, factor analytical interpretations and weighted hierarchical clustering were carried out in R 2.9.2 (R Development Core Team, 2009) based on the code of Murtagh (2005). The silhouette values were calculated in R using the `cluster` package (Maechler et al., 2005). The 2D maps were produced in R and the identified clusters across the n-chosen dimensions were superimposed using Powerpoint (Office Powerpoint® 2003, Microsoft Corporation, Redmond, USA).

3.2.6 Multi-level regression modelling

The potential associations between variables that were highlighted by the multiple correspondence analyses and hierarchical clustering were then fitted using multi-level logistic regression models. The data were analysed at the sample level, and continuous covariates were initially fitted as continuous fixed effects. The binary resistance outcomes (ciprofloxacin- and gentamicin-resistant *E. coli* and vancomycin-resistant *E. faecium*) were modelled using multi-level logistic regression models as described in Chapter 2 on page 49. The more commonly occurring resistant bacteria (ampicillin-

[1]For details on the calculation and utilisation of silhouette values see page 191 in Appendix A.

resistant *E. coli* and erythromycin-resistant *E. faecium*) were modelled as three-level categorical outcome variables using unordered, multinomial regression models whereby the outcomes of both resistance ($r = 1$) and profuse resistance ($r = 2$) were each individually compared to zero resistance ($r = 0$). An unordered model was chosen in order to assess which covariates were associated with each of the denoted levels of resistance in comparison to the hypothetical ideal of no resistance. The multinomial model can be expressed as:

$$P(Y_{ij} = r|\mathbf{x}_{ij}) = \frac{e^{g_r(\mathbf{x}_{ij})}}{\sum_{s=0}^{2} e^{g_s(\mathbf{x}_{ij})}} \tag{3.1}$$

$$g_r(\mathbf{x}_{ij}) = \beta_{r0} + \sum_{p=1}^{n} \beta_{rp} x_{ijp} + \alpha_{ijr} \tag{3.2}$$

$$\alpha_{ijr} \sim N(0, \sigma_r^2) \tag{3.3}$$

where $P(Y_{ij} = r|\mathbf{x}_{ij})$ is the conditional probability of resistance outcome r in faecal sample j collected during visit i given the vector of input covariates $\mathbf{x}_{ij}$; and $g_r(\mathbf{x}_{ij})$ is the linear predictor for resistance outcome r, which is a scalar function of the vector of parameters and the vector of p input covariates. The regression coefficients for the r^{th} level of resistance are designated $\beta_{r0},\ldots,\beta_{rp}$; s denotes the levels of the resistance outcomes with values from zero to two; and α_{ijr} is the random effect for the j^{th} sample from the i^{th} visit and the r^{th} resistance outcome. The random effects were assumed to be normally distributed with a mean of 0 and variance σ_r^2. Although there were repeat visits within farm, the median number of visits per farm was two and, therefore, there were insufficient numbers of within-farm visits to reliably calculate a variance at that level. Furthermore, the hierarchical modelling reported in Chapter 2 on page 58 had already suggested that, for AREC at least, variance between visits was greater than variance between farms. Therefore, farm was not included within the random effects hierarchy for these models.

To control for the method of sample collection (pot or swab) this variable was fitted within every model. In a similar manner, to account for potential age-related differences in resistance, attempts were made to incorporate the production status of the pigs and the age of birds being sampled within all models.

Using the results of the MCA and cluster analysis as a guide to variable selection, the models were fitted using Bayesian inference via Markov chain Monte Carlo (MCMC) methods as implemented in WinBUGS1.4.2 (Spiegelhalter et al., 2007). Assumed noninformative prior distributions were used for each of the fixed effects and variance parameters σ_{ijr}^2 (as described in Chapter 2 on page 50). Samples were obtained from three chains with dispersed initial values. Thinning intervals of 20–100 were sufficient to reduce autocorrelation within the Markov chains to acceptable levels for the various resistance-bacterium combinations that were modelled. Suitable burn-in

periods and run-lengths were determined using MCMC diagnostic algorithms that assess convergence of the chains (Brooks and Gelman, 1998; Geweke, 1992) and consistency of the model estimations (Heidelberger and Welch, 1983; Raftery and Lewis, 1992), as implemented within the `coda` package (Plummer et al., 2006) in R. For every iteration of the model, the probability of detecting ABD resistant bacteria within a faecal sample was calculated, given the covariates that had been fitted; therefore, the model outputs included the posterior distributions and 95% Bayesian Credible Intervals (BCI) around these estimated probabilities of detecting resistance.

3.3 Results

The general trends in the proportions of pooled faecal samples from which each of the five varieties of resistant bacteria were grown are shown in Figure 3.1 on page 79. Ampicillin-resistant *E. coli* (AREC) was the most commonly isolated resistant bacterium in all four livestock groups, followed by erythromycin-resistant *E. faecium* (EREF) for all groups except the conventional pigs, where gentamicin-resistant *E. coli* (GREC) was more commonly detected. Ciprofloxacin-resistant *E. coli* (CREC) was more commonly isolated on the conventional farms for both livestock species, whereas vancomycin-resistant *E. faecium* (VREF) were commonly isolated on some conventional broiler farms but virtually undetected in any other group. The proportions of samples yielding profuse growth of resistant bacteria (> 100 colonies per half-plate) were low for all types of bacteria on all types of farm. There were no profuse growth plates for CREC, and < 2% of plates showed profuse growth for GREC and VREF; therefore, these three varieties of bacteria were subsequently analysed as binary variables. For the more commonly occurring AREC and EREF, 2–8% of samples per farm type showed profuse growth and these variables were modelled as tertiary outcomes with levels zero to two.

Although none of the poultry rearing farms were administering fluoroquinolone drugs, ciprofloxacin-resistant *E. coli* were sporadically detected in faecal samples within certain flocks on a farm. Follow-up visits to these farms found that the same flocks were still shedding this bacterium at the end of the rearing cycle prior to slaughter.

3.3.1 MCA model of the pig data

The initial porcine MCA models identified two variables for which all elements were outliers: annual herd mortality, and the number of times the houses on the farm were disinfected in 12 months; and these two variables were removed from the final model.

Therefore, the refined MCA model of the pig data utilised 21 dimensions to represent 57 categories of data (elements) within 22 variables. Variance within a MCA model is represented by the inertia of the model, and this was high at 1.12. The 2D map from this model (Figure 3.2 on page 80) shows that the first principle axis corresponded to 28%

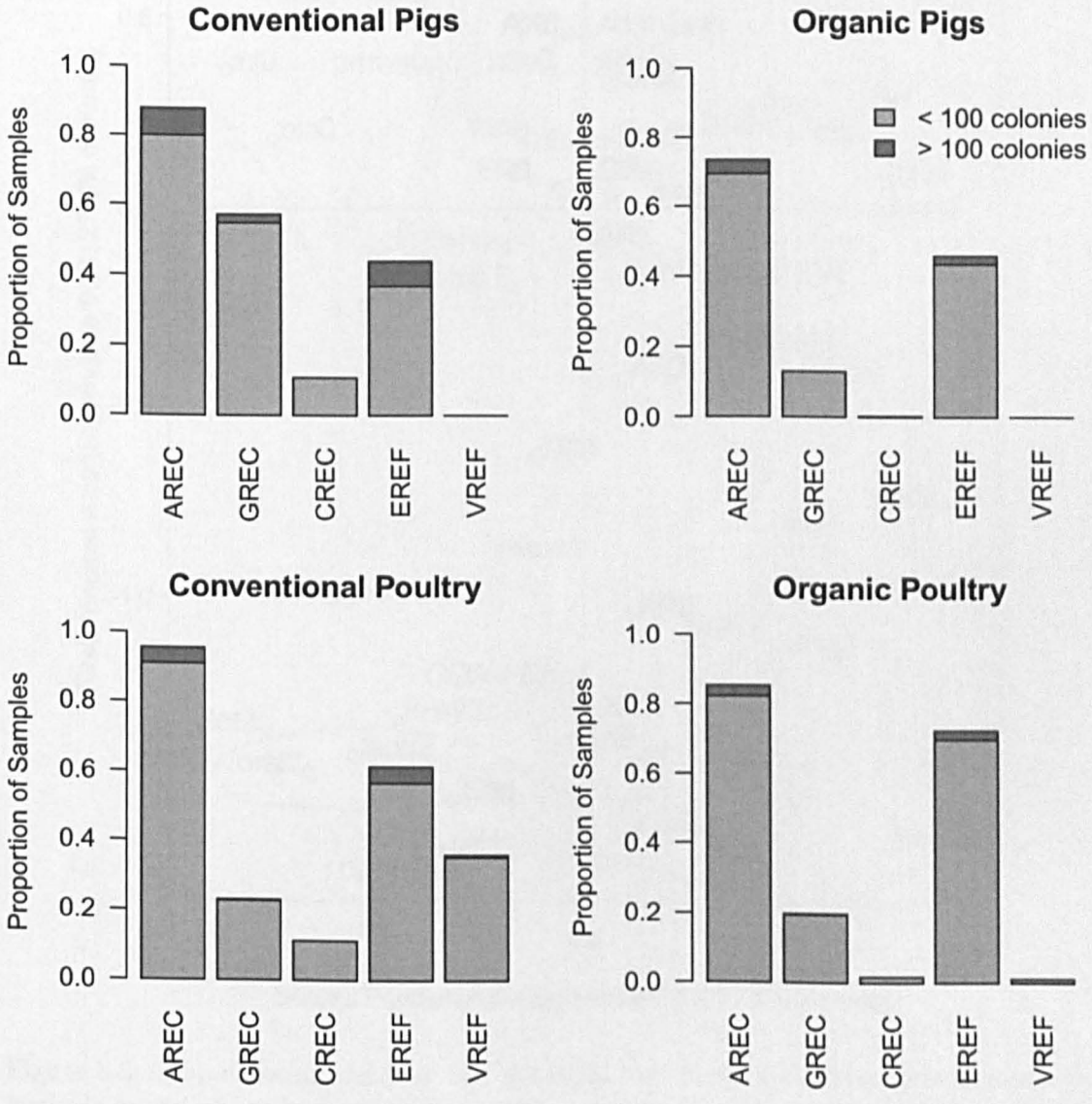

Figure 3.1: Stacked bar plots showing the proportion of pooled faecal samples from which each of five types of antibacterial drug (ABD) resistant bacteria were isolated for four different categories of livestock.

AREC = ampicillin-resistant *E. coli*; GREC = gentamicin-resistant *E. coli*; CREC = ciprofloxacin-resistant *E. coli*; EREC = erythromycin-resistant *E. faecium*; VREC = vancomycin-resistant *E. faecium*.

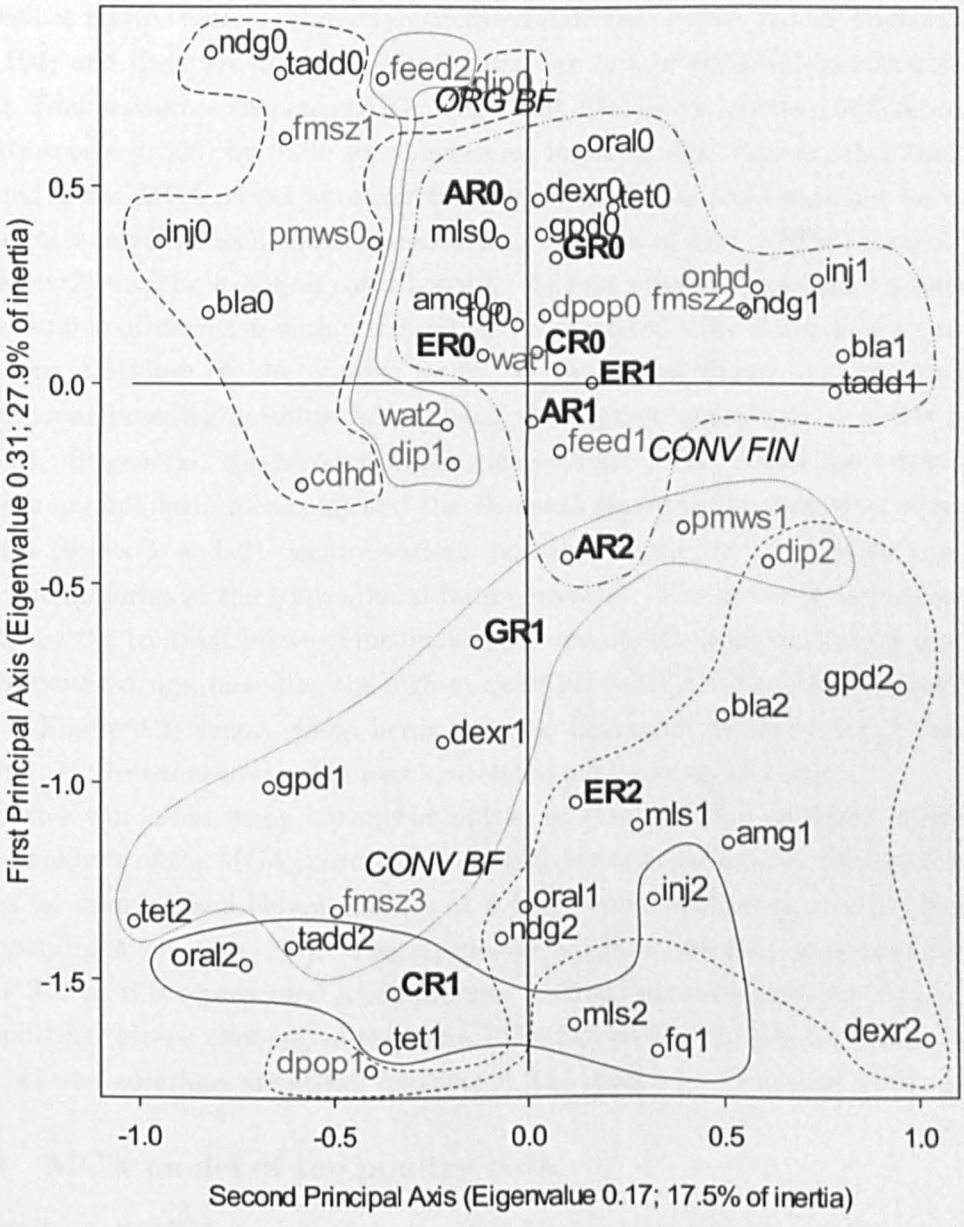

Figure 3.2: Map showing the first two principal axes from a multiple correspondence analysis model of antibacterial drug (ABD) resistant *E. coli* and *E. faecium* isolated from fresh faecal samples on 12 pig farms alongside ABD use and farm management practices. Superimposed onto the map are six clusters derived from a hierarchical cluster analysis of the data projections in the first six dimensions of the MCA model.

The ABD resistance variables are represented in black print; the drug use variables in dark grey; and the other farm related variables in lighter grey. The key for the variable names can be found in Table 3.1 on page 74.

Three supplementary variables that were not included in the MCA model or the cluster analysis are shown in italics: ORG BF = organic breeding-finishing farm; CONV BF = conventional breeding-finishing farm; CONV FIN = conventional finishing unit.

The different clusters of variables are delineated using lines of differing type. Variables with silhouette coefficients < 0.05 are shown as members of two neighbouring clusters. For full details of the variables within the clusters see Table A.3 on page 196 in Appendix AP1.

of the total model inertia and the second a further 18%. In total, six dimensions showed eigenvalues above those of randomly generated data (see Figure A.1 in Appendix A on page 194) and these six dimensions accounted for 77% of the total inertia within the model. Four resistance elements (AR1, AR2, ER0, ER1) were less than 50% represented (quality scores < 500) by these six dimensions, implying that factors other than those included in the MCA model were contributing to these two ABD resistant bacteria.

Factor analytical techniques revealed that the use of oral ABDs (element labels dexr0-dexr2) was the strongest contributor to the first principal axis and, therefore, the largest source of variation within the dataset. Correlated with this axis is a gradation from zero ABD use on the organic farms (at the top of Figure 3.2) to the largest conventional breeding-finishing farms using the highest quantities of ABDs (at the bottom). In general, the zero-resistance elements are found above the origin (along with the organic farm elements) and the elements denoting the detection of resistant bacteria (levels 1 and 2) occupy various positions along the axis below the origin (with the majority of the conventional farm elements). The second principal axis was defined by the contrast between medium-sized, open herds using mid-range quantities of therapeutic drugs, including the highest quantities of injectable beta-lactams (to the right of Figure 3.2) versus closed herds using no injectable ABDs or beta-lactams (to the left). No resistance elements were correlated with this second axis.

Cluster validation using silhouette widths to examine the weighted, hierarchical cluster analysis of the MCA coordinates, showed peaks in the overall average silhouette widths for two, six and eleven clusters at 0.4, 0.41 and 0.49 respectively (Figure A.2 in Appendix A on page 195). The six-cluster solution has been superimposed onto Figure 3.2, as this represented a compromise between extreme lumping (two clusters) and splitting (eleven clusters) of the data. (The full results of both the six-cluster and eleven-cluster solutions are shown in Table A.3 in Appendix A on page 196.)

3.3.2 MCA model of the poultry data

The preliminary MCA models of the poultry data identified three variables as outliers: game birds seen around poultry houses, type of house, and whether the farmer considered respiratory disease to be a problem on the farm. A further four variables were identified as outliers using cluster analysis: the farmers' perceptions of enteritis and septicaemia as problems on their farms, the use of fluoroquinolones within the 12 months prior to sampling, and the species of poultry sampled (chicken or turkey). All of seven outlier variables were removed from the final MCA model.

The optimised model utilised 28 dimensions to represent 57 elements within 23 variables. At 1.04, the total model inertia was lower than that of porcine model and this is reflected in the tighter bunching of elements in Figure 3.3 on page 82 compared to Figure 3.2. The first principle axis of the poultry model accounted for 33% of the total model inertia and the second a further 14%. Eight of the 28 dimensions were

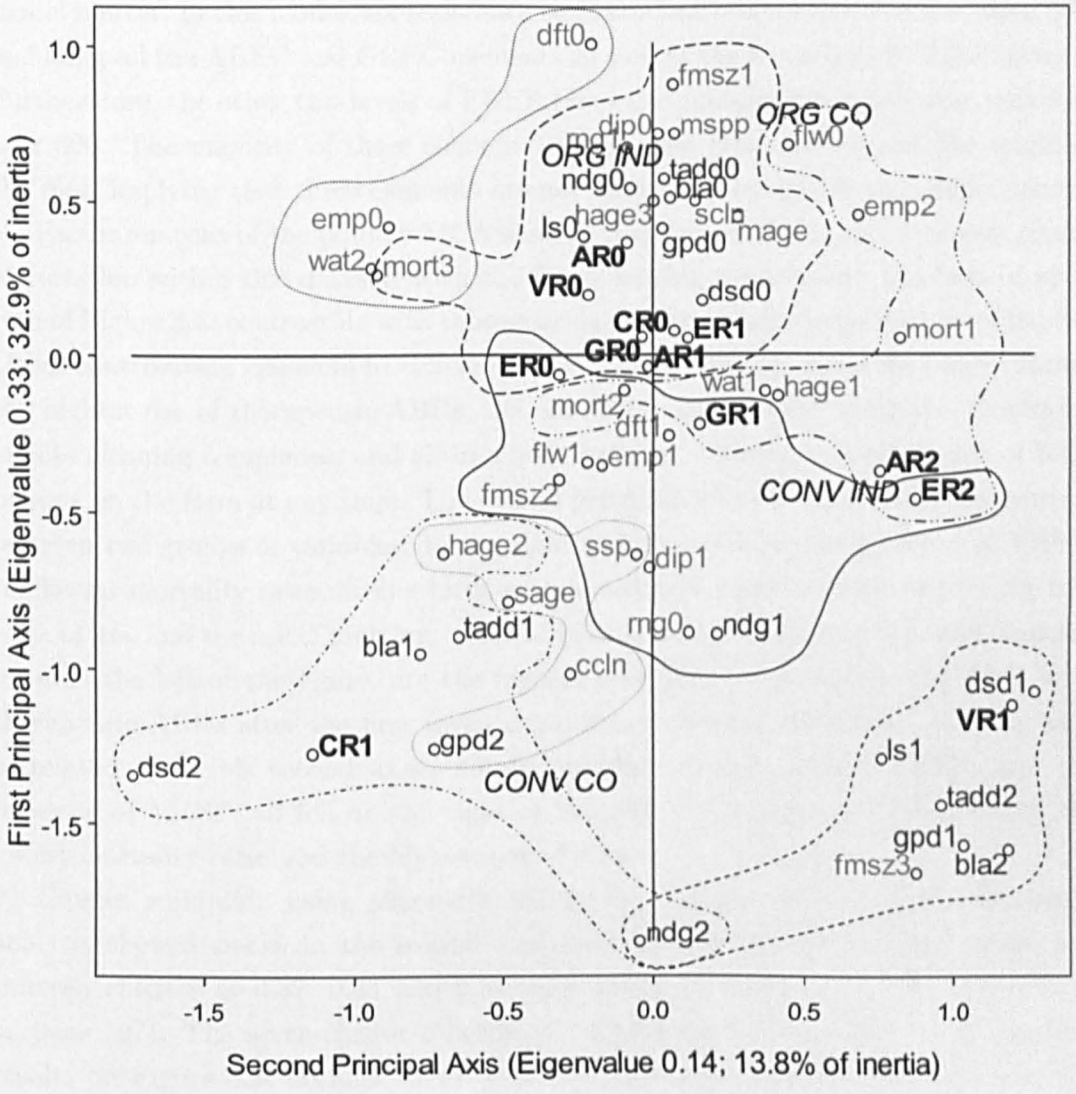

Figure 3.3: Map showing the first two principal axes from a multiple correspondence analysis model of antibacterial drug (ABD) resistant *E. coli* and *E. faecium* isolated from fresh faecal samples on 13 poultry farms alongside ABD use and farm management practices. Superimposed onto the map are seven clusters derived from a hierarchical cluster analysis of the data projections in the first eight dimensions of the MCA model.

The ABD resistance variables are represented in black print; the drug use variables in dark grey; and the other farm related variables in lighter grey. The key for the variable names can be found in Table 3.2 on page 75.

Four supplementary variables that were not included in the MCA model or the cluster analysis are shown in italics: ORG IND = independent, organic farm; ORG CO = organic farm belonging to an integrated livestock company; CONV IND = independent, conventional farm; CONV CO = conventional farm belonging to an integrated livestock company.

The different clusters of variables are delineated using lines of differing type. Variables with silhouette coefficients < 0.05 are shown as members of two neighbouring clusters. For full details of the variables within the clusters see Table A.4 on page 198 in Appendix AP1.

found to have eigenvalues above that of randomly generated datasets (see Figure A.1 in Appendix A on page 194) and these eight dimensions accounted for 86% of the total model inertia. In this model, six resistance elements had quality scores of less than 500, including all the AREC and GREC elements as well as the top levels of EREF growth. Furthermore, the other two levels of EREF (zero and mid-level growth) were only just over 500. The majority of these elements can be seen clustered around the origin of the plot, implying that these elements are not deviating strongly from independence.

Factor analysis of the poultry MCA showed that farm size was the strongest source of variation within this dataset, with the farms rearing the smallest numbers of birds (top of Figure 3.3) contrasting with those rearing the largest numbers (bottom of figure). Other contributing elements to this axis were traits associated with the largest farms: the highest use of therapeutic ABDs, the use of lincomycin-spectinomycin, employing off-site cleaning companies, and all-in-all-out policies resulting in a single age of birds present on the farm at any time. The second principle axis was formed by the contrast between two groups of variables: to the right of Figure 3.3 are the presence of VREF, the lowest mortality rates, dosing birds with lincomycin-spectinomycin within the first week of life, and the use of high quantities of beta-lactams. Opposing this, and depicted towards the left of the figure, are the highest mortality rates, and dosing birds with therapeutic ABDs after the first seven days of age. Several resistance elements were correlated with this second axis: notably profuse AREC, profuse EREF, and the presence of VREF, all fell on the right of the plot corresponding to farms with the lowest mortality rates and the highest use of ABDs.

Cluster validation using silhouette widths for the weighted, hierarchical cluster analysis showed peaks in the overall average silhouette widths for two, seven and thirteen clusters at 0.37, 0.35 and 0.37 respectively (see Figure A.3 in Appendix A on page 197). The seven-cluster solution was chosen to represent the cluster analysis results on Figure 3.3, because fewer elements were misclassified under this solution. (The full results of both the seven-cluster and thirteen-cluster solutions are shown in Table A.4 in Appendix A on page 198.)

3.3.3 Ciprofloxacin-resistant *E. coli*

Ciprofloxacin-resistant *E. coli* (CREC) were not commonly isolated from any category of farm (Figure 3.1), however they were isolated more frequently from the conventional pig and poultry farms (11% of samples for both) compared to the organic pig and poultry farms (< 1% for pigs and 2% for poultry).

The porcine MCA model found that the detection and non-detection of CREC correlated with opposing sides of the third principle axis, which was formed by the contrast between farms using fluoroquinolones (FQs) and the highest quantities of macrolides (detection), and those farms that had been depopulated and cleaned within the preceding six months (non-detection). Figure 3.2 shows that cluster analysis also

Table 3.3: The posterior distribution statistics from two multi-level Bayesian logistic regression models of the presence of ciprofloxacin-resistant *E. coli* (CREC) in pooled faecal samples collected on pig and broiler poultry farms.

Variable names	Variable categories	Posterior distributions of model parameters		Posterior probabilities of detecting CREC	
		Mean	SD[a]	Median	95% BCI[b]
Model 1: Pigs					
Random effect	visit ($n = 22$)	1.30	0.84	-	-
Intercept		-4.45	0.60	-	-
Fluoroquinolones	not used in last 12m[c]	Ref[d]	-	0.01	0.004–0.02
	used in last 12m	**3.12**[e]	**0.78**	0.20	0.015–0.25
Sampling method	pot	Ref	-	0.07	0.05–0.08
	swab	-0.48	0.62	0.01	0.02–0.06
Model 2: Poultry					
Random effect	visit ($n = 30$)	4.19	2.36	-	-
Intercept		-1.33	0.91	-	-
Fluoroquinolones	not used in last 12 m	Ref	-	0.05	0.04-0.07
	used in last 12 m	1.32	1.51	0.15	0.08-0.24
Livestock on farm	single species[f]	Ref	-	0.12	0.09–0.16
	multiple species	**-3.74**	**1.20**	0.04	0.03–0.06
Sampling method	pot	Ref	-	0.06	0.05–0.08
	swab	0.50	0.67	0.03	0.01–0.05

[a] Standard deviation about the mean. [b] 95% Bayesian Credible Intervals. [c] FQs not used on farm for at least 12 months prior to sampling visit. [d] Baseline reference category for that covariate. [e] The parameters highlighted in bold are those for which the mean of the posterior distribution was more than twice the value of the SD. These parameters were deemed to be of a high degree of certainty, and therefore represented the covariates that showed the strongest associations with the detection of resistance. [f] Dedicated poultry farm.

grouped the detection of CREC with the use of FQs, along with the highest use of macrolides (which were predominantly tylosin, see Chapter 2 page 54), as well as the highest use of therapeutic drugs in general. Logistic regression modelling confirmed that there was a strong association between the use of FQs on a farm and the detection of CREC: with a median probability of detection of 0.2 on a farm administering FQs compared to 0.01 on a farm not using such drugs (Table 3.3 on page 84).

In contrast, none of the poultry farms administered FQ drugs during the course of the study. Furthermore,only a single farm had used FQs within the 12-months preceding the first sampling visit, and this was an organic farm where the attending vet had administered enrofloxacin to two batches of young chicks that were suffering high mortality rates due to omphalitis (bacterial yolk sac infections). Nonetheless,

CREC were detected on five farms during the sampling period, including the farm where the drug had been used previously. Within a given visit, this bacterium was only detected in certain houses on a farm, but when it was detected in a house it was persistently shed by that flock until slaughter. The MCA model showed that CREC showed the highest correlations with the first and second principle axes. Along the first axis the detection of CREC fell on the side of the largest conventional farms, and along the second it was associated with farms that were not administering therapeutic ABDs prophylactically within the first seven days of life and the highest mortality rates. Cluster analysis also grouped CREC with farms not dosing birds within the first week as well as the use of the highest number of different ABDs, although the latter element was split between two clusters. In contrast, logistic modelling suggested that a more general farm-type variable provided the best fit to the data (Table 3.3), with a far lower probability of detecting CREC on farms rearing multiple livestock species (0.04) compared to the dedicated broiler farms (0.12). This model also included a covariate for the use of FQs, but the standard deviation of the mean was high due the use of this drug on only one farm.

3.3.4 Gentamicin-resistant *E. coli*

GREC were most commonly isolated from conventionally-managed pig farms, where 57% of samples were positive, compared with 13–23% across the other types of farm (see Figure 3.1). For the pig data, GREC were correlated with the first principle axis of the MCA model, where detection fell on the side of the largest farms using the highest numbers and quantities of ABDs, and non-detection fell on the side of zero oral ABD use. Cluster analysis also grouped GREC with large farms, the presence of PMWS on a farm, mid-level use of routine oral drugs and high-level use of tetracyclines (Figure 3.2). However, the variable that provided the best fit to the data in the logistic regression model was the routine use of oral ABDs: a variable that incorporated the use of both growth promoters and therapeutic drugs (Table 3.4 on page 86). The model estimated that the probability of detecting GREC on farms not using routine oral drugs was 0.13, compared to 0.54-0.61 on those farms routinely administering oral ABDs for four or more weeks.

For GREC on poultry farms the picture was not clear, and this was in line with the low MCA quality score for this variable. The highest correlations were found with axes six and seven. Axis seven was formed by the farms using the highest number of different ABDs (including the highest quantities of AB growth promoters) versus those with the highest mortality rates; with the detection of GREC falling on the drug use side. The variables that contributed to the sixth axis were not disinfecting houses nor leaving them empty for a while between flocks of birds (the side on which GREC detection aligned) versus farms with mid-level mortality rates. The silhouette coefficient for the detection of GREC returned by the cluster analysis was < 0.05, implying that it was

Table 3.4: The posterior distribution statistics from two multi-level Bayesian logistic regression models of the presence of gentamicin-resistant *E. coli* (GREC) in pooled faecal samples collected on pig and broiler poultry farms.

Variable names	Variable categories	Posterior distributions of model parameters		Posterior probabilities of detecting GREC	
		Mean	SD[a]	Median	95% BCI[b]
Model 3: Pigs					
Random effect	visit ($n = 22$)	2.02	0.48	-	-
Intercept		-1.69	0.46	-	-
Status	grower & finishers	Ref[c]	-	0.32	0.29–0.36
	farrowers & weaners	0.10	0.28	0.45	0.40–0.50
	dry sows & gilts	0.54	0.37	0.30	0.23–0.37
	service area	0.72	0.47	0.18	0.10–0.28
Routine oral ABDs[d]	weeks exposed[e]	**0.16**[f]	**0.08**	-	-
	zero	-	-	0.13	0.10–0.16
	4–5 weeks	-	-	0.61	0.69–0.77
	9–20 weeks	-	-	0.54	0.60–0.66
Sampling method	pot	Ref	-	0.33	0.30–0.36
	swab	0.47	0.31	0.42	0.37–0.48
Model 4: Poultry					
Random effect	visit ($n = 30$)	1.94	0.41	-	-
Intercept		-2.74	0.45	-	-
House disinfection	between flocks	Ref	-	0.19	0.17–0.22
	rarely or never	**1.48**	**0.38**	0.28	0.24–0.32
Number of drugs	ABDs used/y[e]	**0.42**	**0.20**	-	-
	zero	-	-	0.17	0.15–0.20
	1–2 drugs	-	-	0.18	0.15–0.22
	3–7 drugs	-	-	0.36	0.30–0.42
Sampling method	pot	Ref	-	0.19	0.17–0.21
	swab	**1.1**	**0.29**	0.35	0.29–0.40

[a] Standard deviation about the mean. [b] 95% Bayesian Credible Intervals. [c] Baseline reference category for that covariate. [d] A variable combining oral therapeutic antibacterial drugs administered prophylactically to all growing pigs and sub-therapeutic growth-promoting drugs. [e] Fitted within the model as a continuous variable, but in order to calculate comparative posterior probabilities the variable was split into three levels. [f] The parameters highlighted in bold are those for which the mean of the posterior distribution was at least twice the value of the SD. These parameters were deemed to be of a high degree of certainty, and therefore represented the covariates that showed the strongest associations with the detection of resistance.

split between two clusters, as illustrated in Figure 3.3. However, in line with the MCA model, the logistic regression modelling suggested that GREC were detected on farms using three or more different ABDs in a 12-month period and those that were not practising disinfection (Table 3.4). Furthermore, the sampling method also came up as significant in this model with an increased probability of detection from wand swab samples compared to pinches of faeces in a pot.

3.3.5 Ampicillin-resistant *E. coli*

AREC were isolated from at least 70% of samples collected from all four categories of farm. All three levels of AREC (zero, mid-level and profuse growth) were associated with the first principle axis in the porcine MCA model: non-detection occurred on the side of no oral ABD use, whilst mid- and high-level growth occurred on the side of the large farms using high quantities of drugs. Mid-level growth and non detection of AREC were strongly correlated with the fifth axis where the strongest contributors were the use of commercially pelleted feed and the provision of mains water (associated with the detection of AREC) versus alternative feed and water sources (associated with the non detection of AREC). These associations were not so easy to see using the clustering scheme, which placed all three levels of AREC within the large group at the centre of the map, although profuse AREC is also grouped with the presence of PMWS on a farm, and the highest use of tetracyclines (Figure 3.2). The multinomial model highlighted pig status as an important factor with positive associations between the growth of AREC at both levels and samples collected from farrowers and weaners, and adults in the service areas (Table 3.5 on page 88). The model also found a positive association between mid-level AREC growth and the presence of PMWS, whilst the use of non-pelleted food was associated with a decreased probability of detection. The probabilities of detecting AREC and profuse AREC were higher (0.84 and 0.08–0.09 respectively) on farms routinely administering oral ABDs compared to those that weren't (0.67 and 0.04); however, the standard deviations for the coefficients for this variable were high.

Profuse growth of AREC from poultry faeces was correlated with the second principle axis of the MCA model, and it fell on the same side of the axis as the detection of VREF and the use of lincomycin-spectinomycin. Mid-level growth and non-detection of AREC were both correlated with the seventh axis, which was formed by the juxtaposition of highest mortality rates versus highest number of different drugs and highest quantities of growth promoters used. Non-detection fell on the high mortality side, whilst mid-level growth remained relatively close to the axis origin. Cluster analysis placed non-detection and mid-level growth in the same large central cluster containing the small farms using no ABDs, whilst high-level growth clustered with profuse growth of EREF and detection of GR1 (although these two resistance elements were split between clusters) on farms with low mortality and the newest houses (Figure 3.3). The multinomial regression results in Table 3.6 on page 89 show positive

Table 3.5: The posterior distribution statistics from a multi-level Bayesian, multinomial regression model of the presence of ampicillin-resistant *E. coli* (AREC) in pooled faecal samples collected on pig farms.

Variable names	Variable categories	Posterior distributions of model parameters		Posterior probabilities of detecting AREC at the given level	
		Mean	SD[a]	Median	95% BCI[b]
Ampicillin-Resistance Level 0					
Baseline reference level of zero resistance[c]					
Ampicillin-Resistance Level 1					
Random effect	visit	1.29	0.36	-	-
Intercept		0.42	0.42	-	-
Status	grower & finishers	Ref[d]	-	0.72	0.68–0.76
	farrowers & weaners	**0.60**[e]	**0.29**	0.74	0.69–0.80
	dry sows & gilts	0.67	0.39	0.83	0.76–0.90
	service area	**1.05**	**0.46**	0.73	0.61–0.83
Routine oral ABDs[f]	zero	Ref	-	0.67	0.63–0.71
	4–5 weeks	0.30	0.82	0.84	0.77–0.90
	9–20 weeks	0.88	0.70	0.84	0.79–0.89
PMWS[g]	not present on farm	Ref	-	0.65	0.60–0.69
	present on farm	**1.68**	**0.55**	0.78	0.74–0.81
Feed provided	commercial pellets	Ref	-	0.77	0.74–0.79
	other	-0.82	0.43	0.51	0.43–0.59
Sampling method	pot	Ref	-	0.65	0.58–0.72
	swab	-0.38	0.34	0.74	0.70–0.77
Ampicillin-Resistance Level 2					
Random effect	visit	2.83	1.41	-	-
Intercept		-1.56	0.66	-	-
Status	grower & finishers	Ref	-	0.04	0.02–0.06
	farrowers & weaners	**1.45**	**0.52**	0.11	0.07–0.15
	dry sows & gilts	0.15	0.71	0.04	0.01–0.08
	service area	**2.73**	**0.75**	0.12	0.06–0.21
Routine oral ABDs	zero	Ref	-	0.04	0.02–0.06
	4–5 weeks	**0.78**	**0.17**	0.09	0.06–0.15
	9–20 weeks	0.55	1.05	0.08	0.05–0.12
PMWS	not present on farm	Ref	-	0.03	0.02–0.06
	present on farm	0.28	0.74	0.09	0.06–0.11
Feed provided	commercial pellets	Ref	-	0.03	0.02–0.06
	other	**-1.97**	**0.97**	0.02	0.005–0.05
Sampling method	pot	Ref	-	0.04	0.02–0.05
	swab	**1.91**	**0.53**	0.18	0.13–0.24

[a] Standard deviation about the mean. [b] 95% Bayesian Credible Intervals. [c] The unordered multinomial model actually estimates two models: mid-level resistance relative to zero resistance, and profuse resistance relative to zero resistance. [d] Baseline reference category for that covariate. [e] The parameters highlighted in bold are those for which the mean of the posterior distribution was more than twice the value of the SD. These parameters were deemed to be of a high degree of certainty, and therefore represented the covariates that showed the strongest associations with the detection of resistance. [f] Oral therapeutic antibacterial drugs administered prophylactically to all growing pigs. [g] Post weaning multisystemic wasting syndrome.

Table 3.6: The posterior distribution statistics from a multi-level Bayesian, multinomial regression model of the presence of ampicillin-resistant *E. coli* (AREC) in pooled faecal samples collected on poultry farms.

Variable names	Variable categories	Posterior distributions of model parameters		Posterior probabilities of detecting AREC at the given level	
		Mean	SD[a]	Median	95% BCI[b]
Ampicillin-Resistance Level 0					
Baseline reference level of zero resistance[c]					
Ampicillin-Resistance Level 1					
Random effect	visit	1.71	0.36	-	-
Intercept		0.44	0.46	-	-
Growth promoters	log(AGD)[d]	**1.51**[e]	**0.49**	-	-
	zero use	-	-	0.83	0.80–0.85
	used on farm	-	-	0.95	0.92–0.97
Mortality[f]	percentage[g]	**0.22**	**0.09**	-	-
	$< 6\%$	-	-	0.84	0.81–0.86
	$\geq 6\%$	-	-	0.88	0.85–0.90
Sampling method	pot	Ref[h]	-	0.86	0.84–0.88
	swab	**0.93**	**0.34**	0.83	0.78–0.88
Ampicillin-Resistance Level 2					
Random effect	visit	1.37	0.53	-	-
Intercept		-0.12	0.60	-	-
Growth promoters	log(AGD)	**1.59**	**0.50**	-	-
	zero use	-	-	0.03	0.02–0.04
	used on farm	-	-	0.05	0.03–0.07
Mortality	percentage	**-0.40**	**0.13**	-	-
	$< 6\%$	-	-	0.06	0.04–0.08
	$\geq 6\%$	-	-	0.008	0.003–0.02
Sampling method	pot	Ref	-	0.03	0.02–0.04
	swab	1.08	0.56	0.06	0.03–0.09

[a] Standard deviation about the mean. [b] 95% Bayesian Credible Intervals. [c] The unordered multinomial model actually estimates two models: mid-level resistance relative to zero resistance, and profuse resistance relative to zero resistance. [d] Natural logarithm of Animal Growth promoting Days per 1000 finished birds per year (see page 48). [e] The parameters highlighted in bold are those for which the mean of the posterior distribution was more than twice the value of the SD. These parameters were deemed to be of a high degree of certainty, and therefore represented the covariates that showed the strongest associations with the detection of resistance. [f] Average flock mortality. [g] Fitted within the model as a continuous variable, but in order to calculate comparative posterior probabilities the variable was split into two levels. [h] Baseline reference category for that covariate.

Table 3.7: The posterior distribution statistics from a multi-level Bayesian logistic regression model of the presence of vancomycin-resistant *E. faecium* (VREF) in pooled faecal samples collected on poultry farms.

Variable names	Variable categories	Posterior distributions of model parameters		Posterior probabilities of detecting VREF	
		Mean	SD[a]	Median	95% BCI[b]
Random effect	visit	2.06	0.74	-	-
Intercept		-4.63	0.57	-	-
Linco-spect[c]	not used in last 12 m	Ref[d]	-	0.01	< 0.01–0.02
	used in last 12 m	**5.28**[e]	**0.90**	0.59	0.54–0.64
Sampling method	pot	Ref	-	0.15	0.17–0.18
	swab	0.42	0.42	0.09	0.12–0.15

[a] Standard deviation about the mean. [b] 95% Bayesian Credible Intervals.
[c] Lincosamide-aminoglycoside combination agent lincomycin-spectinomycin. [d] Baseline reference category for that covariate. [e] The parameters highlighted in bold are those for which the mean of the posterior distribution was more than twice the value of the SD. These parameters were deemed to be of a high degree of certainty, and therefore represented the covariates that showed the strongest associations with the detection of resistance.

associations between farms using ABGPs and the detection of AREC at both levels. This model also confirmed relationships between AREC detection and mortality rates with increasing mortality associated with increased AREC detection, but decreased a probability of detecting profuse resistance.

3.3.6 Vancomycin-resistant *E. faecium*

VREF were not isolated from faeces on any of the pig farms. However, 36% of samples from the conventional poultry farms and 1% of samples from organic poultry were positive for this bacterium. The detection of VREF was a strong contributor to the second principle axis of the poultry MCA model. Cluster analysis returned a silhouette value of 0.65 (the highest for any of the poultry and pig clusters) firmly grouping VREF detection with the largest farms using the highest quantities of therapeutic ABDs and dosing chicks with lincomycin-spectinomycin in the first week of life (Figure 3.3). Logistic regression also found a strong positive association between the use of lincomycin-spectinomycin and the presence of VREF, with a probability of 0.59 for the detection of VREF on a farm administering this drug compared to 0.01 on a farm that was not (Table 3.7 on page 90).

3.3.7 Erythromycin-resistant *E. faecium*

EREF was the second most frequently detected bacterium in this study, with higher frequencies of detection on the poultry farms compared to the pig units. However, equal or higher proportions of samples were positive on the organic farms (46% of pig samples

and 73% of poultry) compared to the conventional farms of the same species (44% and 62% respectively). Furthermore, it was hard to ascertain associations between EREF and the farm-level covariates measured in this study, and many of the regression models that were fitted struggled to converge.

The pig MCA model found that, like AREF, non-detection of EREF was correlated with the fifth axis, which was formed by the contrast between pelleted feed and mains water versus non-pelleted feed and non-mains water, with non-detection of EREF associated with the side of alternative feed and water. Non-detection also showed some correlation with axis two, where it aligned with the closed herds using no drugs. Cluster analysis grouped non-detection and mid-level growth within the same cluster along with the zero drug-use variables, whilst profuse growth of EREF clustered with higher drug use variables and also those farms that had depopulated and disinfected within six months of the sampling visit (Figure 3.2). Table 3.8 on page 92 shows the results of the multinomial model that drew closest to convergence. This model (and indeed all models fitted) highlighted the status of the pigs that had been sampled as the strongest influence on the detection of EREF in a sample: with all groups showing higher probabilities (0.43–0.47) of mid-level resistance than the growers and finishers (0.37), and the breeding animals in the service areas also showed a higher probability (0.11) of detecting profuse-level resistance compared to the growers and finishers (0.02). However, even after running the model for seven days and 90,000 iterations some covariates had not achieved stationarity (as assessed using the method of Heidelberger and Welch).

The poultry MCA model showed a correlation between profuse growth of EREF and the second principle axis, where it fell on side of the axis determined by: the detection of VREF, the use of lincomycin-spectinomycin, the highest quantities of beta-lactams used, and the lowest mortality rates. Cluster analysis struggled to group the elements of this variable into clean clusters, although mid-level growth did cluster with the negative resistance elements and zero drug use elements. Multinomial regression analysis also highlighted an association with mortality, with profuse growth of EREF grouping with farms showing the lowest mortality rates (Table 3.9 on page 93).

3.4 Discussion

This study took a multi-faceted analytical approach to investigate patterns of association within a multivariate dataset of antibacterial drug resistance on livestock farms. The drivers influencing resistance are complex and go beyond a simple cause and effect response to the use or withdrawal of ABDs (da Costa et al., 2009; Martinez et al., 2009; Wagner et al., 2008; Zhou et al., 2009), and this work has confirmed that it is not possible to generalise from the selective pressures influencing a specific drug-bacterium combination to all other resistant bacteria.

Table 3.8: The posterior distribution statistics from a multi-level Bayesian, multinomial regression model of the presence of erythromycin-resistant *E. faecium* (EREF) in pooled faecal samples collected on pig farms.

Variable names	Variable categories	Posterior distributions of model parameters		Posterior probabilities of detecting EREF at the given level	
		Mean	SD[a]	Median	95% BCI[b]
Erythromycin-Resistance Level 0					
Baseline reference level of zero resistance[c]					
Erythromycin-Resistance Level 1					
Random effect	visit	0.91	0.21	-	-
Intercept		-0.82	0.36	-	-
Status	grower & finishers	Ref[d]	-	0.37	0.33–0.42
	farrowers & weaners	**0.63**[e]	**0.22**	0.43	0.37–0.49
	dry sows & gilts	**1.19**	**0.30**	0.47	0.37–0.56
	service area	**0.94**	**0.38**	0.43	0.31–0.52
Boot dips	none on farm	Ref	-	0.35	0.29–0.42
	some on farm	-0.06	0.43	0.36	0.31–0.40
	many on farm	0.83	0.45	0.52	0.46–0.57
Sampling method	pot	Ref	-	0.42	0.38–0.45
	swab	-0.22	0.24	0.35	0.28–0.42
Erythromycin-Resistance Level 2					
Random effect	visit	5.93	3.83	-	-
Intercept		-1.21	0.77	-	-
Status	grower & finishers	Ref	-	0.02	0.01–0.04
	farrowers & weaners	0.50	0.55	0.06	0.04–0.09
	dry sows & gilts	1.08	0.61	0.11	0.06–0.17
	service area	**2.03**	**0.78**	0.11	0.06–0.17
Boot dips	none on farm	Ref	-	0.01	0.002–0.04
	some on farm	-1.73	1.43	0.02	0.008–0.04
	many on farm	-0.78	0.93	0.13	0.09–0.16
Sampling method	pot	Ref	-	0.06	0.04–0.08
	swab	-0.51	0.76	0.02	0.005–0.04

[a] Standard deviation about the mean. [b] 95% Bayesian Credible Intervals. [c] The unordered multinomial model actually estimates two models: mid-level resistance relative to zero resistance, and profuse resistance relative to zero resistance. [d] Baseline reference category for that covariate. [e] The parameters highlighted in bold are those for which the mean of the posterior distribution was more than twice the value of the SD. These parameters were deemed to be of a high degree of certainty, and therefore represented the covariates that showed the strongest associations with the detection of resistance.

Table 3.9: The posterior distribution statistics from a multi-level Bayesian, multinomial regression model of the presence of erythromycin-resistant *E. faecium* (EREF) in pooled faecal samples collected on poultry farms.

Variable names	Variable categories	Posterior distributions of model parameters		Posterior probabilities of detecting EREF at the given level	
		Mean	SD[a]	Median	95% BCI[b]
Erythromycin-Resistance Level 0					
Baseline reference level of zero resistance[c]					
Erythromycin-Resistance Level 1					
Random effect	visit	2.02	0.36	-	-
Intercept		1.99	0.37	-	-
Mortality[d]	< 5%	Ref[e]	-	0.75	0.71–0.79
	5–8%	**-1.72[f]**	**0.60**	0.56	0.53–0.59
	> 8%	-0.92	0.79	0.76	0.69–0.82
Sampling method	pot	Ref	-	0.65	0.62–0.67
	swab	-0.009	0.25	0.62	0.56–0.68
Erythromycin-Resistance Level 2					
Random effect	visit	2.09	0.76	-	-
Intercept		-0.56	0.60	-	-
Mortality[f]	< 5%	Ref	-	0.11	0.08–0.14
	5–8%	**-3.36**	**0.90**	0.009	0.004–0.02
	> 8%	-2.20	1.24	0.007	0.001–0.03
Sampling method	pot	Ref	-	0.04	0.03–0.05
	swab	-1.04	0.59	0.02	0.01–0.05

[a] Standard deviation about the mean. [b] 95% Bayesian Credible Intervals. [c] The unordered multinomial model actually estimates two models: mid-level resistance relative to zero resistance, and profuse resistance relative to zero resistance. [d] Average flock mortality. [e] Baseline reference category for that covariate. [f] The parameters highlighted in bold are those for which the mean of the posterior distribution was more than twice the value of the SD. These parameters were deemed to be of a high degree of certainty, and therefore represented the covariates that showed the strongest associations with the detection of resistance.

Simultaneously examining several drug-bacterium combinations allowed this study to take a wider view of ABD resistant bacteria on livestock farms, as well as enabling comparisons to be made between different bacteria. However, the increase in breadth came with a corresponding decrease in depth, due to the inflated sample processing times and costs. Therefore, the final dataset was of a relatively small sample size in terms of numbers of participating farms and visits undertaken within an individual farm, and many of the farm-level covariates were related to each other, such as, access to range and low to zero use of ABDs, or large flock size and higher use of ABDs. Fitting such collinear variables into a single regression model can prevent convergence and inflate the standard deviations, which may contribute to spurious results (Dohoo et al., 1996). The use of descriptive multivariate techniques, however, facilitated the exploration of broader relationships within the data by investigating whether the various resistant bacteria were associated with groups of variables identifiable with different styles of farming; and this also allowed for a reduction in the number of variables that were taken forward into the regression modelling (Poitras et al., 2007; Thomsen et al., 2007)

The three analytical techniques were used in this study were: factor analysis of MCA results, hierarchical cluster analysis of MCA results, and multi-level regression modelling. For the strongest associations within the dataset (the detection of CREC on pig farms using fluoroquinolones, and the detection of VREF on broiler farms using lincomycin-spectinomycin) the results of the three techniques were in close agreement. These general results are also in accordance with other studies; for instance, in a large cross-sectional study of UK pig farms, the detection of fluoroquinolone-resistant *E. coli* and *Campylobacter* was strongly associated with the use of FQ drugs (Taylor et al., 2009). Whilst, a study in Denmark showed that after the ban of the glycopeptide growth promoter avoparcin, the linkage of vancomycin and erythromycin resistance genes was responsible for the persistence of VREF on pig farms that were administering tylosin (Aarestrup et al., 2001). However, the use of MCA provided more information than the regression modelling alone, by showing that the pig farms using fluoroquinolones were also those that were likely to be administering the highest quantities of therapeutic drugs in general, including the highest quantities of macrolide use. This association is supported by the use of fluoroquinolone drugs as second-line agents that are administered when the cheaper first-line drugs are not deemed to be sufficient. In a similar manner, the poultry MCA model also highlighted that VREF were predominantly found on the farms that had the lowest mortality rates and that were routinely administering the lincomycin-spectinomycin to incoming chicks as a prophylactic measure.

For the other categories of resistant commensals that were studied, no direct links were seen between the use of a specific ABD and the detection of resistance to that drug; and for these bacteria, there were more discrepancies between the results obtained

from the three analytical techniques. In particular, hierarchical cluster analysis of the MCA coordinates from multiple dimensions did not manage to separate non-detection from detection for the commonest resistant bacteria: ampicillin-resistant *E. coli* and erythromycin-resistant *E. faecium*. One possible reason for this is that the clustering method used was trying to define crisp, well-isolated clusters. Given the complexity of influences upon resistance at the bacterial, animal and farm levels, a fuzzy clustering method that probabilistically assigned individual elements to each of a number of different clusters, may have provided different insights (Bezdek et al., 1984). Another potential problem has been noted with the commonly used two-tier approach of undertaking MCA and then clustering the results, because MCA seeks to find a low-dimensional representation of the data, and this may not always accurately represent the clusters present within the full dimensionality of the original data (Hwang et al., 2006). To try and circumnavigate this issue, methods have been proposed that attempt to combine data reduction and cluster analysis within a single step (Desarbo et al., 1991; Hwang et al., 2006).

A further reason for the lack of clarity resulting from the cluster analysis for AREC and EREF, could be related to the low quality scores obtained for these elements from the MCA model. These low scores imply that the farm management variables were less strongly associated with resistant bacteria than they were with themselves. In fact, regression modelling showed that for both resistant bacteria, the pig-level variable of status was influential, with growing and finishing animals (i.e. those closest to the consumer) showing lower frequencies of detection than other groups. However, the porcine MCA models for these two bacteria, suggested that a higher percentage of negative samples were obtained on farms using alternatives to commercially-prepared pellets, such as fermented feed. This could be due to dietary-related influences upon enteric bacterial populations in general, or resistant bacteria in particular. However, as prophylactic ABDs and growth promoters are incorporated into pelleted feed at the feed mills these two variables are not completely independent. Such collinearity could be responsible for inflated standard deviations around the posterior means for these two variables when fitted in the same model.

In a similar manner, trying to fit age within the poultry regression models was hampered by the strong association between the age of a bird and the different types of farm studied, which resulted in regression models that struggled to converge. However, all three analytical techniques highlighted that samples yielding profuse recovery of both AREC and EREF were more commonly obtained from the poultry farms with the lowest mortality rates, which also happened to be those applying the highest quantities of ABDs.

For gentamicin-resistant *E. coli* (GREC), there were closer associations between its isolation and non-specific drug-use variables, than the specific use of aminoglycoside drugs. Associations have been previously reported between the use of the aminogly-

coside drug apramycin on pig farms and resistance to gentamicin in *E. coli* (Jensen et al., 2006; Kim et al., 2005a). However, less than 1% of the standardised doses of ABDs administered on the pig farms in this work were aminoglycosides, and yet GREC were commonly isolated on the conventional pig farms. However, both the pig and the poultry MCA models showed correlations between the farms using the highest number of drugs and detection of GREC; and this relationship was also elucidated by the poultry regression model, although exposure to routinely administered oral ABDs fitted the data more closely in the pig model. These associations could reflect the co-selection of GREC due to linked-genes on mobile genetic elements such as plasmids and transposons. Gentamicin was used historically within the poultry industry, particularly in some hatcheries where unhatched eggs were dipped in the drug and sometimes newly hatched birds were injected directly. In countries where these practices occurred, GREC (including strains causing clinical infections) were commonly isolated from birds (Altekruse et al., 2002; Dubel et al., 1982). Thus, these practices could have increased the numbers of GREC within the industry, with persistence occurring due to co-selection by other currently used drugs.

The fluoroquinolones, were another class of drugs to which resistance was sporadically seen on some poultry farms despite a lack of use. The MCA suggested that detection of CREC was associated with large farms using external cleaning companies, and the regression model highlighted a higher probability of detection on single species farms. Taken together, these results support the previously published observation that CREC can be disseminated within integrated poultry companies (Petersen et al., 2006). What was also seen in the study reported here is that a CREC-shedding flock shed this bacterium persistently right up until slaughter, and therefore these bacteria were being taken into the processing plants.

In summary, this study used an array of analytical methods to investigate the influential farm-level selective pressures for resistance to five antibacterial drugs in two species of enteric bacteria. In general, aspects of the use of ABDs were the greatest influences on the frequency of detection of ABD resistance, but other husbandry factors and animal-level factors were also associated with the most commonly detected resistant bacteria: ampicillin-resistant *E. coli* and erythromycin-resistant *E. faecium*. It was also seen that the poultry farms with the lowest mortality rates were those using ABDs as prophylactic agents, and that this pattern of use was associated with vancomycin-resistant *E. faecium* and profuse growth of ampicillin-resistant *E. coli* and erythromycin-resistant *E. faecium* from samples. Therefore, in order to safeguard public health and to provide clinically relevant information to farm managers and veterinarians it is recommended that antibacterial drug resistance is monitored on farms that are routinely applying ABDs to livestock.

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"We shall never cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time."
Thomas Stearns Eliot

Chapter 4

Sources of variation in the faecal ampicillin-resistant *Escherichia coli* concentration shed by organic meat chickens[1]

Abstract

Currently, there is limited published data on the population dynamics of antibacterial drug (ABD) resistant commensal bacteria. This study was designed to evaluate both the proportions of the *Escherichia coli* populations that are resistant to ampicillin at the level of the individual chicken on a commercial broiler farm, as well as the feasibility of obtaining repeated measures of faecal *E. coli* concentrations. Short-term temporal variation in the concentration of faecal *E. coli* was investigated, and a preliminary assessment was made of potential factors involved in the shedding of high numbers of ampicillin-resistant *E. coli* by growing birds in the absence of the use of ABDs. Multi-level linear regression modelling revealed that the largest component of random variation in log-transformed faecal *E. coli* concentrations was seen between sampling occasions for individual birds. The incorporation of fixed effects into the model demonstrated that the older, heavier birds in the study were significantly more likely ($P = 0.0003$) to be shedding higher numbers of ampicillin-resistant *E. coli*. This association between increasing weight and high shedding was not seen for the total faecal *E. coli* population ($P = 0.71$). This implies that, in the absence of the administration of ABDs, the proportion of faecal *E. coli* that were resistant to ampicillin increased as the birds grew. This study has shown that it is possible to collect quantitative microbiological data on broiler farms, and that such data could make valuable contributions to risk

[1] A paper based on the work detailed in this chapter has been published as E. J. Pleydell, P. E. Brown, M. J. Woodward, R. H. Davies, and N. P. French. Sources of variation in the ampicillin-resistant *Escherichia coli* concentration in the feces of organic broiler chickens. *Applied and Environmental Microbiology*, 73(1):203–10, 2007

assessments concerning the transfer of resistant bacteria between animal and human populations.

4.1 Introduction

Within a given environment, the commensal bacterial populations of birds and mammals act as useful markers of antibacterial drug (ABD) resistance (AubryDamon et al., 2004; Okeke et al., 2000; Vatopoulos et al., 1998). In the nutrient-rich environment of the intestinal tract commensal micro-organisms are present in high numbers. Many of these species of bacteria are adept at both carrying resistance genes (Gulay et al., 2000; Pallecchi et al., 2004), and exchanging genetic material with members of their own, and other, species (Blake et al., 2003; Scott, 2002). For these reasons intestinal bacteria may constitute important reservoirs of ABD resistance (Sunde and Sorum, 2001). Furthermore, investigations of commensal organisms in healthy host populations and ABD-free environments have found that resistance genes can persist in commensal bacteria in the absence of ABD selective pressures (Khachatryan et al., 2004; Sato et al., 2005). Thus, research into ABD resistance in commensal populations can provide valuable insights into the panoptic dynamics of ABD resistance (Boerlin, 2004; Summers, 2002).

When studying ABD resistance, the microbiological methods utilised will strongly influence the interpretation of the results gained. This has been aptly demonstrated by two Danish studies of vancomycin-resistant *Enterococcus faecium* (VREF) on broiler chicken farms following the 1995 Danish ban of the use of avoparcin as an in-feed growth-enhancing agent. One study used Danish surveillance data whereby the proportion of VREF amongst *E. faecium* isolates cultured from broiler samples had been ascertained. Analysis of this dataset showed a highly significant decline in the proportion of VREF amongst isolates that had been cultured between 1995 and 1998 ($P < 0.00001$)(Bager et al., 1999). However, the second study utilised isolation methods that directly selected for VREF from the broiler samples. This alternative technique showed that there was no significant decrease in the proportion of VREF positive flocks in Denmark between 1998 and 2001 ($P > 0.1$), and VREF were still detected within 74.3% of conventional broiler flocks five years after the withdrawal of avoparcin (Heuer et al., 2002a).

Furthermore, if one is aiming to elucidate the dynamics of populations of ABD resistant bacteria then quantitative microbiological techniques are required. Currently, quantitative data of this nature are limited for commensal bacterial populations. Such data gaps may be impeding the development of comprehensive epidemiological models and quantitative microbial risk assessments (Humphry et al., 2002), and models of this nature could make valuable contributions to the debate regarding the significance of the use of ABDs in the livestock industries with respect to the transmission of resistant

bacteria to human populations (Snary et al., 2004).

The aims of this field study were to test the suitability of sampling and laboratory protocols that were designed to generate quantitative microbiological data for investigating ABD resistance on broiler farms; to use the data produced to examine the within-bird over-time dynamics of ampicillin-resistant *E. coli* (AREC) for broiler chickens that are not exposed to ABDs; and to evaluate the relative contribution of a variety of potential components of variation to the concentration of faecal *E. coli* that are ampicillin-resistant in an environment where exogenous ABDs are not administered.

4.2 Materials and methods

4.2.1 Farm details

The study took place over five consecutive days in October 2003, on a well-established, organic, mixed-species livestock farm in southern England. The outdoor chicken unit comprised of 20,000 birds aged between 21–77 days. The birds were housed in groups of 1500 in adjacent mobile barns, and they had unrestricted access to an area of range surrounding each barn during daylight hours. In the four years preceding this study, ABD therapy had been administered to poultry on just one occasion. This had occurred in 2001, when 3,000 birds less than seven days of age had been dosed with enrofloxacin due to an outbreak of yolk sac infections in chicks arriving from a commercial hatchery.

4.2.2 Sampling protocol

Two male and two female birds were randomly chosen in each of six mobile barns. Each barn housed birds of a single age, and three different ages of birds were studied: 30-days, 57-days and 70-days. Dark-coloured stock marker was used to enable individual identification of the study birds within each house. Sampling took place between 10:00 and 12:30 on each morning of the study. The marked birds were caught, weighed and then placed inside portable pet-carriers lined with fresh paper for a period of up to ten minutes. After this time, faecal droppings voided within the pet carrier were collected from the lining paper. If the birds did not defecate within the pet carrier they were released and observed until defecation occurred, whereby the droppings were immediately collected from the house floor or field surface. The faecal samples were scored for consistency, colour and volume, and were held at 4 °C during transportation to the laboratory. All samples were processed within 12-hours of collection.

4.2.3 Laboratory methods

Each faecal sample was weighed, and an equal amount (volume to weight) of buffered peptone water (BPW) was added prior to mixing using a vortex mixer. A 1:10 dilution was obtained by homogenising 4 g of the faecal suspension with 16 ml BPW using

a Stomacher 400 Circulator (Seward, Norfolk, UK). A ten-fold dilution series down to 1:10,000 was produced using maximum recovery diluent (MRD). Presumptive *E. coli* counts were obtained by plating the faecal dilutions onto CHROMagar ECC® agar plates (CHROMagar, Paris). A Whitley Automatic Spiral Plater (WASP1, Don Whitley Scientific Limited, Whitley, UK) was used in the logarithmic mode to dispense 50 or 100 l of the 1:10,000 or 1:100 dilutions respectively onto the CHROMagar ECC® plates. In order to assess variation in *E. coli* counts due to laboratory techniques two dilution series were prepared from one sample per house per day, and all dilutions from all samples were plated in duplicate.

The total presumptive *E. coli* counts were obtained by plating onto plain CHROMagar ECC®, whilst presumptive ampicillin-resistant *E. coli* (AREC) counts were obtained by plating onto CHROMagar ECC® incorporating 8 mg/L ampicillin. This concentration of ampicillin was chosen to correspond to the breakpoint minimum inhibitory concentration (MIC) of the British Society of Antimicrobial Chemotherapy. After inoculation the plates were incubated at 37 °C for 18–24 hours. The plates were counted manually after the identities of inoculated plates had been blinded by a separate member of the laboratory staff. Presumptive *E. coli* were selected using colony morphology.

The CHROMagar ECC® agar plates and the BPW and MRD diluents were prepared by the biological products unit at the Veterinary Laboratories Agency following VLA standard operating procedures in line with ISO9001/2000 accreditation systems. Control organisms NCTC 10418, ATCC 25922 and two internal VLA controls of known ampicillin MICs (S/28/99 and LR22) were plated on to each batch of media in order to check the performance of the selective media. Plates containing ampicillin that had not been used within 48-hours of preparation were discarded.

For the purpose of this study AREC were defined as those colonies of typical morphology growing on CHROMagar ECC® media containing 8 mg/l ampicillin. The accuracy of this definition was examined using real-time PCR techniques on a subset of 141 presumptive AREC isolates from the study. To confirm whether these isolates were *E. coli*, the glutamate decarboxylase *gadA* gene was detected using TaqMan real-time PCR (Applied Biosystems, USA) based on the methods of Meiland et al. (2003). The same panel of isolates was also screened for the presence of *bla*TEM beta-lactamase genes using the LightCycler® 2.0 System (Roche Diagnostics, Basel, Switzerland). The primers (TEMf 5′TCG TGT CGC CCT TAT TCC CTT TTT; TEMr 5′GCG GTT AGC TCC TTC GGT CCT C) were designed using DNASTAR software (DNASTAR, Madison, WI), and were based upon a published sequence of a *bla*TEM-1 gene located on a plasmid carried by a strain of *Klebsiella pnuemoniae* (GenBank data accession number AF309824).

4.2.4 Data analysis

Trellis scatter plot matrices were produced using S-PLUS 4.6 (MathSoft Incorporated, Cambridge, Massachusetts). Box and whisker plots were produced using the boxplot function in R (R Development Core Team, 2009).

Due to the longitudinal nature and hierarchical structure of the study, multi-level, mixed-effects, linear regression models were used to assess the sources of variation in log-transformed *E. coli* concentrations. Separate models were run for total *E. coli* (TEC) concentrations and AREC concentrations. Repeated samples obtained from the same bird across time were denoted as bird-days, and these were nested within birds, which in turn were nested within houses. To allow an assessment of variation due to laboratory procedures the hierarchy was extended so that the replica aliquots were nested within the double dilutions that were plated, which in turn were nested within the replica ten-fold dilution series that were constructed and nested within bird-days. The structure of the random effects hierarchy is shown in Figure 4.1.

House: $i = 1$–6
↪ Bird: $j = 1$–24
↪ Bird-day: $k = 1$–5
↪ Dilution series: $l = 1$ 2
↪ Dilution: $m = 1$–2
↪ Aliquot: (residual) $= 1$–2

Figure 4.1: The structure and notation used to describe the random effects hierarchy in the preliminary multi-level regression models

Logarithmic transformations of *E. coli* concentrations in colony-forming units per gram were approximately normally distributed; therefore, the following mixed-effects model was fitted by restricted maximum likelihood (REML) using the `lme` function from the `nlme` library in R (Pinheiro et al., 2005). Writing Y_{ijklm} to refer to the log of the observed concentration of *E. coli* in a plate poured from dilution m of dilution series l from bird j in house i on day k, we have:

$$Y_{ijklm} \sim N\left(\mu + \mathbf{X}_{ijklm}\beta + A_i + B_{ij} + C_{ijk} + D_{ijkl},\ \tau^2\right), \tag{4.1}$$

where, μ is the mean intercept and $\mathbf{X}_{ijklm}$ is a vector of fixed effects with regression coefficients β. The random effects A_i, B_{ij}, C_{ijk}, and D_{ijkl} are independent, normally distributed random variables, each with a mean of zero and variances σ_A^2, σ_B^2, σ_C^2 and σ_D^2 respectively. The residual random error incorporating variation at the plate level is denoted by τ^2.

Initially, the contributions of the different levels of variation were explored using intercept-only models (i.e. without the incorporation of any fixed effects X_{ijklm}). Those levels of the hierarchy that were found to be contributing minimally to the overall

variance were collapsed in all subsequent models. After refining the random effects portion of the model, separate fixed effects models were fitted by adding each covariate individually in order to assess their significance, and determine which of the fixed effects variables were suitable for inclusion within the multivariable models. The final model incorporated the significant covariates and a collapsed three-level hierarchy of random effects. To obtain 95% credible intervals for the random effects, this final model was then fitted using Markov chain Monte Carlo (MCMC) sampling within a Bayesian framework as implemented by WinBUGS Version 1.4.1 (Spiegelhalter et al., 2007). Noninformative priors were used for both fixed and random effects. Convergence was assessed by running multiple chains and examining sample paths. After a burn-in period of 10,000 iterations, the posterior distributions were sampled between iterations 10,001–30,000 using a thinning interval of 50. The results of the MCMC fit were compared with those of the REML fit.

In order to assess the relationship between the proportion of *E. coli* within a single faecal sample that were resistant to 8 mg/l ampicillin and the weight of a bird, logit-transformed proportions were fitted using an additional REML mixed-effects model. The proportions fitted within this model were derived by dividing the mean concentration of AREC by the mean concentration of TEC for each dilution series plated per sample. Therefore, the random effects hierarchy for the proportion models was only of four-levels: house, bird and bird-day with faecal dilution series incorporated within the residual random error.

4.3 Results

4.3.1 Data summary

A total of 115 faecal samples were analysed. The median concentration of TEC for all the samples was 4.0×10^6 colony forming units per gram (CFU/g) [6.6 $\log_{10}$ CFU/g] within a range extending from 6.0×10^3 to 2.1×10^8 CFU/g [3.8 to 8.3 $\log_{10}$ CFU/g]. The median concentration of AREC was 10-fold lower than the median concentration of TEC at 2.1×10^5 CFU/g [5.3 $\log_{10}$ CFU/g]. However, the range of AREC counts was of similar dimensions to that for the TEC counts, extending from 1.0×10^3 to 1.7×10^8 CFU/g [3.0 to 8.2 $\log_{10}$ CFU/g].

Figure 4.2 on page 105 shows a trellis scatterplot matrix detailing the daily concentrations of both TEC and AREC for each of the 24 marked birds. The plots show that there is considerable variation in the faecal *E. coli* concentrations both between birds and between sampling days for an individual bird. Likewise, the proportion of the total *E. coli* population that are ampicillin-resistant also varies markedly between and within birds. Note that bird 19 was mistakenly removed during thinning of the flock prior to the visit on the second morning, and bird 18 developed severe enteritis during the study and it was impossible to collect a faecal sample from him on the final

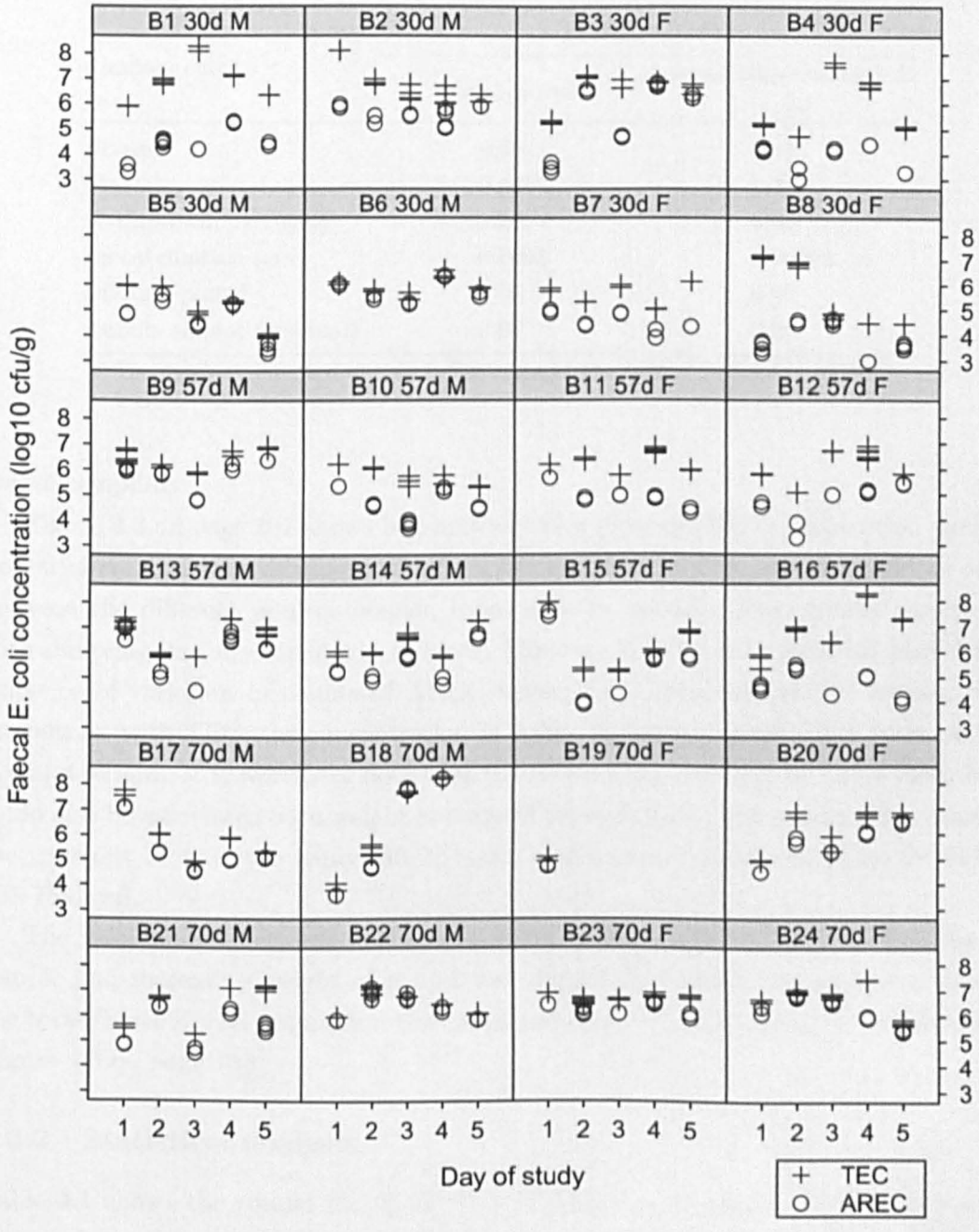

Figure 4.2: A trellis scatter plot matrix showing the daily faecal concentrations of total *E. coli* (+) and *E. coli* resistant to ≥ 8 mg/l ampicillin (○) for each of 24 birds over 5 consecutive days.

Bn denotes the individual birds; nd denotes the age of the bird on the first day of the study; and M\F denotes the sex of the bird.

Each row of the plot represents birds from the same house. The age of the birds increases from the top to the bottom of the figure.

Table 4.1: Two intercept-only, random-effects linear regression models of $\log_e$ concentrations of total and ampicillin-resistant *E. coli* in faecal samples collected from organic broiler chickens

Random effect	Variance (σ^2)	
	Total *E. coli*	Ampicillin-resistant *E. coli*
House	0.59	0.81
Bird	0.0003	**1.23**[a]
Within-bird, over-time	**3.55**	**3.68**
Faecal dilution series	0.0003	0.00002
Dilution plated	0.05	0.07
Sample aliquot (residual)	0.06	0.06

[a] The numbers reported in bold indicate marked sources of variation (variance >1).

day of sampling.

Figure 4.3 on page 107 shows box-and-whiskers plots of TEC concentration against potential conditioning variables. Whilst some variation in TEC counts could be seen between the different poultry houses, there were no unidirectional trends associated with sampling-day, age, or weight of birds. Likewise, figure 4.4 on page 107 also shows evidence of variation in counts of AREC between the different poultry houses, but, in contrast with TEC, the concentration of AREC increased as the birds increased in age and weight. It is worthy of note that the house-level variation in AREC seen here could also be associated with weight and age of birds, because houses 1 and 2 contained the youngest birds in the study (30–35days), and houses 5 and 6 contained the eldest (70–75 days).

The relationship between an increasing concentration of AREC within a faecal sample and increasing weight of a bird was due to an increase in the proportion of the total faecal *E. coli* population that were resistant to 8 mg\l ampicillin as shown in Figure 4.5 on page 108.

4.3.2 Statistical analysis

Table 4.1 shows the results for the TEC and AREC intercept-only linear regression models fitted using REML (i.e. random-effects models without the incorporation of fixed-effect covariates). Within the random-effects hierarchy, in the absence of fixed effects, the greatest source of variation for both TEC and AREC was seen within an individual bird over time (σ^2 = 3.55 and 3.68 respectively). In these models, house-effects also contributed to the variation within both the total population and the ampicillin-resistant sub-set (σ^2 = 0.59 and 0.81). However, whilst between-bird variation was negligible for the TEC concentrations (σ^2 = 0.0003), between-bird variation in AREC concentrations was sizeable (σ^2 = 1.23). For both TEC and AREC

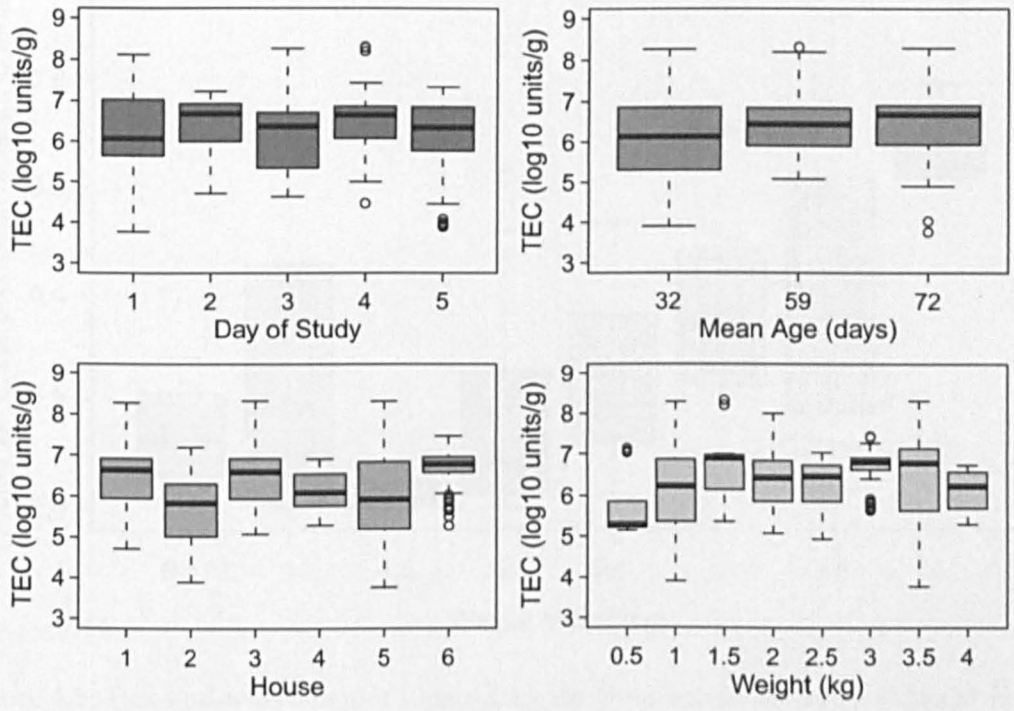

Figure 4.3: Box-and-whiskers plots illustrating total faecal *E. coli* (TEC) concentrations against potential explanatory variables.

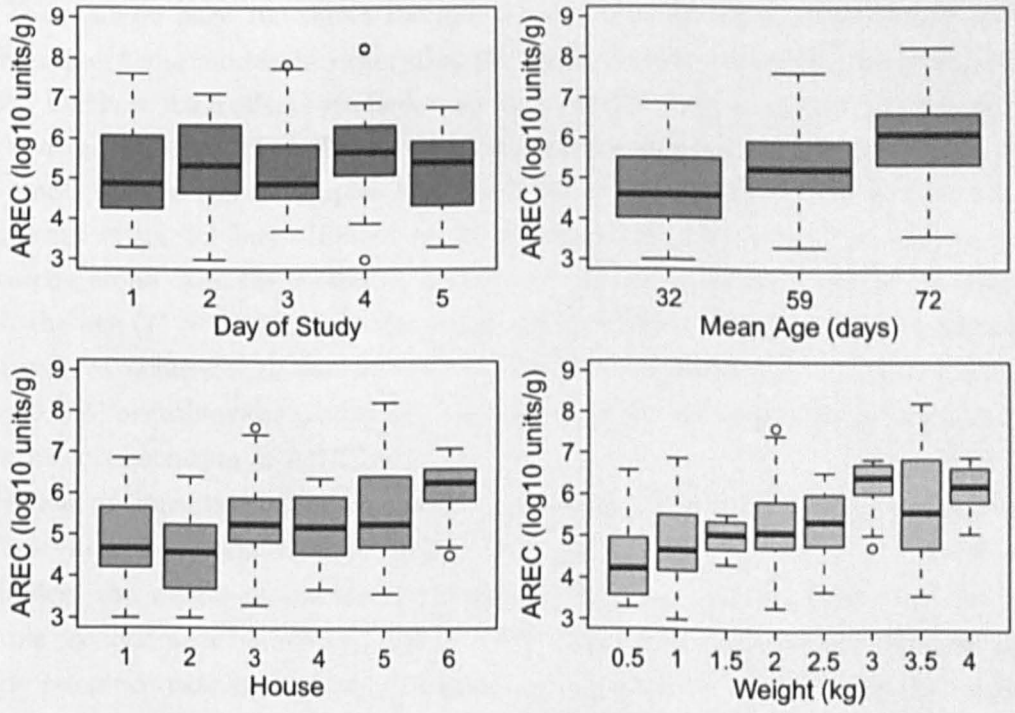

Figure 4.4: Box-and-whiskers plots illustrating the concentrations of the subsets of *E. coli* that were resistant to $\geq$ 8 mg\l ampicillin (AREC) against potential explanatory variables.

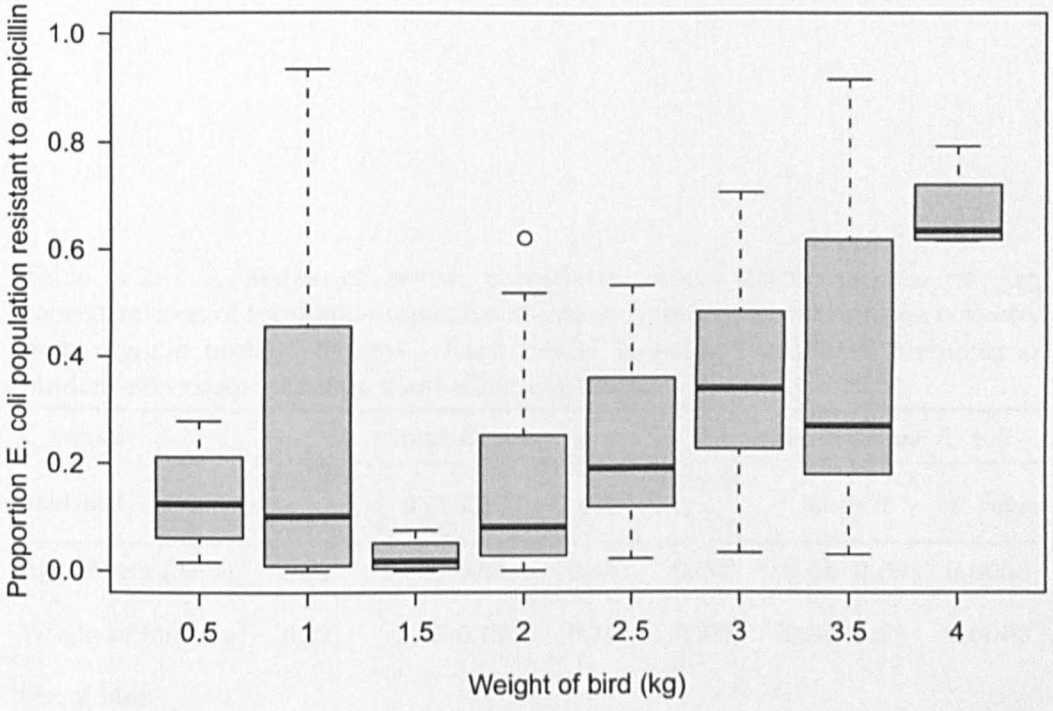

Figure 4.5: Box-and-whiskers plot illustrating the proportions of the total faecal *E. coli* population that were resistant to $\geq$ 8 mg\l ampicillin against weight of bird.

the levels of variation in the measured concentrations due to laboratory effects were extremely low.

Table 4.2 on page 109 shows the individual results for seven mixed-effects models, with each of these models incorporating the random effects hierarchy and a single fixed effect. Of those fixed effects studied, none were significantly associated ($P \leq 0.05$) with the concentration of TEC. There was a suggestion of a negative association between the small volume faecal samples and the TEC concentration; however, this was not significant at the 5% level. Therefore the null model, incorporating the random effects hierarchy alone, was the model of best fit for the TEC concentrations. In contrast, both the age ($P = 0.0008$) and the weight ($P = 0.0003$) of the bird were significantly positively associated with the concentration of AREC. Furthermore, a highly significant ($P = 0.005$) negative relationship was also seen between the small volume faecal samples and the concentration of AREC.

Model optimisation using comparative strategies found that the multivariable model of best fit for log-transformed AREC concentration incorporated two fixed effect variables: the weight of the bird as a centred variable, and the volume of the faecal sample recoded as a binary variable by combining normal and profuse samples into a single reference category. After the incorporation of these fixed effects, the variance at the level of the house was seen to be negligible ($\sigma^2 = 0.002$). Thus the final random effects hierarchy incorporated: bird effects, within-bird-over-time effects, and the laboratory effects collapsed into a single level. The optimised model was then fitted

Table 4.2: A series of seven univariate, mixed-effects models of $\log_e$ concentrations of total and ampicillin-resistant *E. coli* in faecal samples collected from organic broiler chickens. Each model included the 5-level hierarchy of random-effects and a single fixed-effect covariate.

Covariate	Total *E. coli*			Ampicillin-resistant *E. coli*		
modelled	Coeff[a] (β)	95% CI	*P* value	Coeff (β)	95% CI	*P* value
Age of bird (days)	0.02	-0.03–0.06	0.44	**0.06**[b]	**0.03–0.09**	**0.0008**
Weight of bird (kg)	0.12	-0.53–0.78	0.71	**1.05**	**0.50–1.59**	**0.0003**
Sex of bird						
Male	Ref[c]			Ref		
Female	-0.20	-0.92–0.52	0.59	-0.97	-2.06–0.12	0.10
Source of sample						
Pet carrier	Ref			Ref		
House floor/field	-0.41	-1.31–0.48	0.36	0.13	-0.89–1.14	0.81
Faecal colour						
Dark brown	Ref			Ref		
Light brown	0.43	-0.55–1.42	0.39	-0.22	-1.28–0.85	0.69
Red/brown	0.38	-0.60–1.37	0.45	-0.06	-1.11–0.10	0.92
Faecal consistency						
Well-formed	Ref			Ref		
Loose	-0.17	-0.91–0.57	0.65	-0.22	-1.04–0.60	0.59
Liquid	0.52	-0.72–1.76	0.41	0.36	-0.97–1.70	0.60
Faecal volume						
Average	Ref			Ref		
Scanty	-0.78	-1.64–0.08	0.08	**-1.33**	**-2.23– -0.42**	**0.005**
Profuse	-0.13	-0.98–0.72	0.76	-0.15	-1.05–0.76	0.75

[a] Coeff = coefficient values for the covariates.
[b] The numbers reported in bold indicate marked effects ($P < 0.05$).
[c] Ref indicates the reference categories for the categorical covariates.

Table 4.3: A comparison of the estimates derived from classical (REML) and Bayesian (MCMC) fits of a mixed-effects linear regression model in which the $\log_e$ concentration of ampicillin-resistant *E. coli* in poultry faeces depends on the covariates listed and a hierarchy of nested random-effects

Variable	Restricted maximum likelihood				MCMC[a]	
	Var[b] (σ^2)	Coeff[c] (β)	95% CI	*P* value	Median[d]	95% BCI[e]
Random-effects						
Bird	0.90				0.74	0.007–2.29
Within-bird, over-time	3.32				3.52	2.60–4.91
Residual[f]	0.22				0.22	0.18–0.27
Fixed-effects						
Faecal volume						
Average to profuse		Ref[g]			Ref	
Scanty		-1.27	-2.13 – -0.42	0.003	-1.19	-2.05 – -0.33
Weight of bird		0.98	0.45–1.50	0.0004	0.99	0.47–1.52

[a] MCMC = model fitted by Markov chain Monte Carlo methods using Gibbs sampling.
[b] Var = variance values for the random effects.
[c] Coeff = coefficient values for the covariates.
[d] Median values of the posterior distributions of the mixed effects.
[e] 95% BCI = 95% Bayesian credible intervals around the median.
[f] The residual variance incorporates the variance associated with the laboratory methods.
[g] Ref indicates the reference category for categorical covariates.

Table 4.4: The estimates derived from a REML-fitted, mixed-effects linear regression model of the logit-transformed proportion of faecal *E. coli* that are resistant to $\geq$ 8 mg/liter ampicillin

Variable	Variance (σ^2)	Coefficient (β)	95% CI	*P* value
Random-effects				
House	0.40			
Bird	1.41			
Within-bird, over-time	2.14			
Residual[a]	0.38			
Fixed-effect				
Weight of bird		1.04	0.31–1.76	0.007

[a] The residual variance incorporates the variance associated with the double dilution series.

using both REML and MCMC techniques. Table 4.3 on page 110 shows the estimates from the two fits; there was good general agreement between the two fits for both the random and fixed effects.

Table 4.4 on page 110 shows the results of the models of logit-transformed proportions of faecal *E. coli* that were resistant to ampicillin, confirming that there was a significant relationship between increasing proportion of AREC and increasing weight of bird ($P = 0.007$). In contrast to the log-linear models of AREC concentration, a significant association was not found between the logit-transformed proportion of *E. coli* that were ampicillin-resistant and the volume of the faecal sample tested ($P = 0.07$). Furthermore, incorporation of faecal volume within the mixed effects model did not enhance the model fit and therefore the final mixed-effects model for the logit-transformed proportion data incorporated the centred-weight of bird as a single fixed-effect.

4.3.3 Isolate characterisation

All 141 presumptive AREC that were screened by PCR were found to be *gadA*-positive, and 138/141 were found to be carrying a *bla*TEM beta-lactamase gene. Thus the use of morphological colony characteristics on agar incorporating 8 mg/l ampicillin in order to identify ampicillin-resistant *E. coli* was validated.

4.4 Discussion

This study has suggested that in the absence of antibacterial-drug administration a positive relationship exists between the weight of growing meat chickens and the proportion of the faecal *E. coli* population that are resistant to ampicillin. Single-variable fixed effects linear regression models found that both weight and age of bird were positively and significantly associated with AREC concentration. However when weight and age were included as fixed effects within a single model the standard errors of the regression coefficients increased such that neither variable was declared to be significantly associated with the response variable. Obviously, the weight of a bird is heavily influenced by both its age and its sex; therefore, age and weight are highly correlated variables. A comparison of models determined that the multivariable models giving the best fit were those incorporating weight as a fixed effect; thus, age and sex were not incorporated within the final model. This does makes biological sense as all the birds within a single house are of a single age, whereas each of the individual birds will have a unique weight. Therefore, measuring the weight of the bird also provides within-house distributions for a bird-level variable that are not available if age is used instead. Furthermore, because the birds were reared in single-age groups, age and weight will also be correlated within a house. This relationship would account for the large decrease in random variation at the house-level between the null intercept-

only model ($\sigma^2 = 0.81$) and the 5-level hierarchy mixed-effects model ($\sigma^2 = 0.002$) for AREC.

The largest proportions of the random variation in *E. coli* concentration, both for total and ampicillin-resistant populations, were found to occur between sampling occasions for an individual bird. Furthermore, the variation at this level remained high even after the addition of the fixed effects into the model. This suggests that the enteric bacterial populations of these growing birds are highly dynamic. In contrast, whilst the between-bird random variation had a negligible influence on TEC concentration, it exerted a marked influence on the concentration of AREC. The random variation in AREC concentration decreased with the addition of the fixed effects, but still remained at a notable level ($\sigma^2 = 0.90$). This indicates that at the individual bird-level there are other factors that are playing a role in the proportion of the *E. coli* population that are ampicillin-resistant that have not been explained by the final model presented here.

This work has shown that it is possible to obtain quantitative data at the individual-chicken level for farm-based studies of ABD resistance amongst the aerobic commensal flora of commercial poultry. However, as it is difficult to obtain a usable sample of completely liquid faeces, such as may be produced by birds with severe enteritis, a degree of selection bias could be imposed by these methods. This did occur on one sampling occasion during this investigation. Furthermore, a significant negative association was found between the faecal samples of smallest volumes and the concentration of AREC. Generating serviceable and accurate faecal dilution series can be difficult with very small samples, and it is likely that there is a higher level of laboratory-based errors contained within the bacterial counts obtained from small volume samples. Therefore, it is uncertain as to whether this is a true effect, due possibly to differences in the proportion of AREC that may occur in different areas of the gastro-intestinal tract, or whether this is simply a reflection of laboratory errors. Nonetheless, although these methods were labour intensive and time consuming, the results of the regression modelling showed that actually only a minor proportion of random variation was attributable to the laboratory methods, a result that has allowed for an increase in efficiency in subsequent, larger investigations due to a reduction in the number of replica dilution series constructed for each sampling visit.

There are a number of explanations that could account for a potential relationship between weight of bird and proportion of faecal *E. coli* that are resistant to ampicillin. For instance, many mobile genetic elements transfer more than a single resistance gene between bacteria, and therefore it is possible that genes encoding for beta-lactamases are linked to genes encoding for factors involved with, for example, colonisation and adhesion to the gut wall (Amabile de Campos et al., 2005; Laporta et al., 1986; Martinez and Baquero, 2002). It is also possible that this trend illustrates that there is a degree of active selection for AREC within the growing host. Such active selection of resistant strains could be either be due to inter-bacterial competition causing alterations in the

population structure of the enteric flora (Carlson et al., 2001; Portrait et al., 2000), or it could be the direct result of bacterial-host communication (Sperandio et al., 2003).

Alternatively, the resistance genes themselves may be conferring other properties, besides drug resistance, on those bacteria that carry them. For instance, the carriage of *bla*TEM genes could act to enhance the assembly of peptidoglycans during the production of the bacterial cell wall (Livermore, 1995). There is little published work in this area; however, one study did compare the fitness of streptomycin-sulfadiazine-tetracycline-resistant *E. coli* that had been derived from young calves, with mutant strains that had been generated within the laboratory by knocking out the resistance genes. In this instance, no difference in fitness was seen between the wild-type resistant and mutant susceptible strains, suggesting that the carriage of those three resistance genes was not conferring a fitness advantage (Khachatryan et al., 2006).

Associations between the age of calves and antibacterial-drug-resistant *E. coli* have previously been reported. In these studies, rapid colonisation of neonatal calves with resistant *E. coli* has been observed in the absence of antibacterial drug administration (Hinton et al., 1994; Hoyle et al., 2004a). One study found that the peak prevalence of shedding of AREC by beef calves was seen when the animals were 4-months of age; after this the shedding of resistant *E. coli* declined with increasing age (Hoyle et al., 2004b). Similarly, in another study, in vivo competition experiments demonstrated that strains of streptomycin-sulfadiazine-tetracycline-resistant *E. coli* inoculated into neonatal calves out-competed *E. coli* that were sensitive to those three drugs even in the absence of the administration of ABDs. However, this trend was not seen when the same fitness studies were carried out using older animals; therefore, the authors concluded that calf-adapted *E. coli* were responsible for the maintenance of antibacterial-drug-resistance in dairy calves (Khachatryan et al., 2004). The oldest birds in the study presented here were just 10-weeks of age, and these birds were processed in the week following the study. Therefore, while this work may have highlighted true differences in the dynamics of resistant gut flora between chickens and calves, it is likely that the substantial differences in management practices (such as age of slaughter or number of in-contact animals) are also important drivers in these apparent differences between livestock species. Nonetheless, the results of this study could also support a hypothesis that chicken-adapted strains of *E. coli* that are concurrently bearing antibacterial-drug-resistance genes are acting to maintain resistance on meat farms even in the absence of antibacterial drug use. It would be interesting to ascertain whether the chicken-adapted strains of AREC that have been collected from this work are also capable of predominating within the gut flora of fully mature chickens.

This observational study focussed on a single drug-resistance within a single bacterial species. In order to determine whether these results are likely to be applicable to other resistant bacteria, such as tetracycline-resistant *E. coli* for instance, then a greater degree of phenotypic and genotypic characterisation of the AREC isolates is

required. It currently remains unclear as to whether we are observing the clonal-expansion of resistant strains of *E. coli*; or an increase in numbers of genes or gene vehicles within a more stable resident population of *E. coli* strains. Published molecular studies of AREC isolated from beef farms have demonstrated that distinct genetic strains of antibacterial-drug-resistant *E. coli* spread through cohorts of calves over time in a successive manner (Hoyle et al., 2006, 2005). Assuming that the same phenomena could occur within poultry farms, it would be of interest to determine whether this is principally due to strains freshly acquiring resistance determinants within the farm environment, or whether successions of resistant strains of *E. coli* are either persisting in the farm environment or being repeatedly introduced onto the farm.

It terms of ascertaining the wider ecological implications of these results, it must be remembered that *E. coli* are actually a minority species within the flora of the intestinal tract, which predominantly consists of obligate anaerobic species. As many of these intestinal anaerobes are also capable of carrying and transferring resistance genes (Scott, 2002), *E. coli* only offer a narrow window onto the overall dynamics of the enteric flora. Nonetheless, this study has found that quantitative microbiological techniques can reveal trends within populations of resistant *E. coli*. Using these techniques has revealed that it may be possible for the ampicillin-resistant proportion of the faecal *E. coli* population to increase even in the absence of the use of exogenous ABDs. This observation is worthy of further investigation.

Acknowledgements

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"Habit is habit and not to be flung out of the window by any man, but coaxed downstairs a step at a time." *Mark Twain*

Chapter 5

A comparison of the dynamics of antibacterial drug resistant *Escherichia coli* populations on two meat chicken farms: one conventionally managed and one organic

Abstract

Longitudinal studies were carried out two meat chicken farms in the UK in 2004-2005. Initially, environmental samples were obtained from cleaned poultry houses, and then from paper lining the crates in which the incoming chicks arrived. Thereafter, faecal samples were collected from 24 marked, individual birds throughout the rearing cycles on both farms. The faecal concentrations of total *Escherichia coli*, ampicillin-resistant *E. coli* and chloramphenicol-resistant *E. coli* were ascertained, and *E. coli* isolates were tested against a panel of 17 antibacterial drugs. The majority (87%) of *E. coli* isolates originating from the chick-box liners from both farms showed resistance to zero, one or two drugs only. In contrast, 73% and 23% of isolates originating from cleaned sheds on the conventional and organic farm, respectively, were resistant to three or more drugs. Mean faecal *E. coli* concentrations were approximately one log higher for the conventional birds compared to the organic, and the mean proportions of *E. coli* populations that were resistant to ampicillin and chloramphenicol were also higher on the conventional farm. All incoming chicks on the conventional farm were prophylactically dosed with lincomycin-spectinomycin for the first three days, thereafter, and throughout the rearing cycle, the faecal *E. coli* isolates from this farm were predominantly resistant to streptomycin, spectinomycin and sulfamethoxazole (SSS) in conjunction with resistance to a further one to four drugs. On the organic farm, multidrug resistant isolates were isolated less frequently and SSS phenotypes only sporadically. Nonetheless, on the organic farm (in the absence of antibacterial

drug administration) the proportions of faecal *E. coli* populations that were resistant to chloramphenicol increased from around 35 days of age to slaughter. In this study, cleaned poultry houses on both farms represented a source of resistant *E. coli* for incoming chicks, and on a farm administering prophylactic drugs to day-old chicks the birds shed multidrug resistant *E. coli* for the entire rearing cycle until slaughter.

5.1 Introduction

Studies of antibacterial drug (ABD) resistance on meat chicken farms have shown that resistant, aerobic bacteria can be readily isolated from faecal samples and from the environments in which the birds are reared. Across the globe, resistance to long-established drugs, such as ampicillin and tetracycline, are routinely identified in Gram-negative bacteria of poultry origin (Al-Ghamdi et al., 1999; Dai et al., 2008; Meyer et al., 2008; Pleydell et al., 2007). However, at this time, bacterial resistance to newer drugs, such as some of the extended spectrum beta-lactams, show more restricted geographical patterns (Duan et al., 2006; Greko et al., 2009; Pleydell et al., 2010b)[1]. This may be changing, however, because in many regions there is a trend towards an increasing prevalence of extended-spectrum-beta-lactamase (ESBL) production in Gram-negative bacteria of human and animal origins (Greko et al., 2009; Yuan et al., 2009).

Although the isolation of certain resistant bacteria from meat chickens is often commonplace, the factors driving such high prevalences are still not fully understood, particularly on organic farms where the use of ABDs is restricted. Furthering our understanding of the changes in patterns of resistance on a farm over time, could help to identify the most influential risk factors for the multiplication and persistence of resistant bacteria, and this in turn would help to inform effective, evidence-based, ABD resistance-control strategies (McEwen and Singer, 2006; Miller et al., 2006).

The meat chicken industry encompasses a range of contrasting farming practices. Conventionally managed broiler chickens are reared in large flocks of thousands of birds, in climate-controlled houses, until slaughter at 35 to 42 days. The flocks are managed as a single unit and practices such as the administration of vaccinations and prophylactic or clinical ABDs are applied across the whole group. In contrast, organically managed meat chickens are reared in much smaller groups for a longer period of time (80 days on average) and after reaching a certain age (often 21 days) they have free access to pasture range during daylight hours. Under UK organic regulations, vaccinations are permitted, but the prophylactic use of ABDs is not, although ABDs may be used clinically under veterinary supervision in the face of an outbreak of diagnosed bacterial disease. Given the striking differences between the two farming systems one would expect to see differences in the dynamics of ABR bacteria present.

However, measuring ABD resistance on a livestock farm presents a number of

[1]Chapter 7 reports a lack of ESBLs in *E. coli* of poultry origin in New Zealand.

logistical and statistical difficulties (Davison et al., 2000). Presence/absence data is most readily obtainable, but data of this type may provide insufficient resolution to track the temporal dynamics of resistance. Quantifying the concentration of bacteria within a faecal sample may provide the resolution needed, but the much increased sampling and processing times limit the sample size in terms of numbers of chickens, and numbers bacterium-drug combinations that can be studied. A further option is to study the resistance phenotypes (or patterns of resistance) of bacteria isolated from samples. This method provides a wider screen of the ABD resistances present, but there are still logistical and financial limits to the number of isolates that can be screened and, therefore, low prevalence ABD resistance could go undetected.

This study aimed to use a combination of techniques to describe the dynamics of faecal concentrations and resistance phenotypes of ABD resistant *E. coli* over the entire rearing cycles of birds on two meat chicken farms operating under contrasting management protocols. The background dynamics of resistant *E. coli* on an organic farm not administering ABDs were compared to those on a conventional farm that was administering ABDs prophylactically across all flocks present, as well as treating individual flocks in response to symptoms of enteric disease.

5.2 Materials and methods

5.2.1 Farm details

In 2004 and 2005, longitudinal studies were carried out on two meat chicken farms in the UK. One farm was a dedicated broiler unit that was part of an integrated poultry company using conventional management protocols. Six flocks of 50,000 birds were simultaneously reared in computerised climate-controlled houses, and the farm finished approximately two million birds per year. Between batches of birds, the whole site was depopulated and the litter was removed from the sheds. The empty houses were cleaned using a sanitising agent, and then disinfected using two to three different agents. An empty period of five to seven days was observed before applying the sawdust bedding ready for the next flock of birds. All incoming chicks were prophylactically dosed for three days with lincomycin-spectinomycin, a broad-spectrum ABD therapeutic agent. Flocks were thinned at five weeks of age, when 12,000 cock birds were removed from each shed and sent for processing, whilst the remaining 38,000 birds were reared for a further seven days.

The other study site was part of a large organic farm rearing a number of different livestock species, as well as cultivating horticultural crops. The organic farm finished approximately 12,000 birds a year, in weekly batches of 2,300 birds, and operated its own on-site processing plant. Day-old chicks came onto the farm weekly in groups of 1150. For the first 21 days, the chicks were reared on sawdust bedding, in heated, indoor brooding houses, with access to natural light and outdoor sights and sounds in the form

of a glass-enclosed veranda. At 21 days the birds were transferred to mobile houses in a nearby field where they had unlimited access to pasture during daylight hours. The birds were reared to a maximum of 80 days and no ABDs were administered on this unit during the duration of the study. Individual houses were cleaned using power-washing and an iodophor disinfectant. After the brooding houses were cleaned they remained empty for about seven days between flocks; although (due to the multi-age production system) nearby brooding houses were still occupied. After the finished flock had been sent for processing, the floorless mobile houses in the field were moved to a fresh patch of ground prior to washing and disinfecting the walls and equipment. These houses were then bedded-up with fresh straw later in the same day before introducing the next batch of 21-day old birds the following morning.

5.2.2 Sample collection

Empty houses

Prior to the application of fresh bedding and the arrival of the new flocks of birds, environmental samples were collected from cleaned and disinfected sheds. In order to standardise the sampling procedure, specified numbers of samples were collected from specified sections of equal size within each house. Due to the difference in size between the organic and conventional houses, a maximum of 95 samples per house were collected from the former and 150 samples per house from the latter. Samples were taken from the floors, walls, cracks in the floors and walls, drinkers, feeders, heaters, air vents, pipe-work and other equipment that was present in some houses such as dividing partitions or in situ weighing scales. Samples were also taken from the floors of the anterooms and from the ground outside the doors to the houses.

Surface samples were collected using 30 cm^2 sterile gauze swabs that were moistened with sterile buffered peptone water (BPW). An area of 50 cm^2 was sampled with a single swab and two swabs collected from the same area of the house were placed in a single capped plastic jar containing 200 ml sterile BPW. Cracks in the house surfaces and smaller areas such as nipple drinkers were sampled using wand swabs in a charcoal transport medium. The sampling team wore disposable gloves that were changed between each sample. Four brooding houses were sampled on the organic farm; however, due to the large size of the conventional sheds, two houses were sampled in detail on this farm.

Incoming chicks

Four flocks of birds were followed through the whole rearing cycle on each farm. Samples were obtained of the paper lining the boxes in which the chicks arrived by placing 20 cm^2 of the most heavily soiled area of each liner into 200 ml BPW. On the conventional farm 50 boxes per house were sampled, equivalent to 10% of the total number; all 12

boxes from each house were sampled on the organic farm.

Faecal samples

When the birds were at least two days of age, six individual birds were randomly selected from each of the eight flocks. The selected birds were marked with dark-coloured stock marker; they were weighed and placed in individual pet carriers lined with fresh paper for up to 15 minutes before being released back into the flock. Voided faecal samples were then collected into sterile pots from the paper in the base of the boxes. Each flock on each farm was visited ten times and on each consecutive visit the same birds were sought and fresh droppings were collected. Due to the large number of birds in the conventional flocks it was occasionally difficult to locate a marked individual, particularly when the birds were moulting; therefore, if a previously marked bird could not be found, a replacement bird was recruited, sampled and marked. This happened seven times on the conventional farm, and once on the organic farm due to the death of a young chick in the first week of the study. (The details of the birds that were sampled on each visit are shown in Tables B.1 and B.2 in Appendix B on pages 201 and 202.)

5.2.3 Laboratory methods

Empty houses and incoming chicks

The post-cleaning samples were transported at ambient temperature and incubated overnight at 37 °C; wand swabs were transferred into 10 ml BPW prior to incubation. The following day, 10 μl of incubated broth was streaked onto CHROMagar ECC®*E. coli* chromogenic agar (CHROMagar, Paris). Two samples were streaked onto separate halves of each plate. Each sample was streaked onto plain CHROMagar as well as two further CHROMagar plates that incorporated an ABD at a given breakpoint concentration:

1. 8 μg/ml ampicillin.
2. 16 μg/ml chloramphenicol.

An array of control organisms of known MICs were used to check the levels of ABDs in the plates (for details of the control strains used see Table B.3 in Appendix B on page 203). Streaked plates were incubated at 37 °C for 18 to 24 hours. Plates upon which colonies of appropriate morphology had grown were recorded. From each plain CHROMagar plate, a single colony was selected and sub-cultured onto the same type of media prior to storage at −80 °C on glycerol-coated beads.

Faecal samples

The faecal samples were transported at 4 °C and processing commenced within seven hours of collection. A 1:1 dilution of faeces to BPW was stomached for three minutes

in filtration bags using a Stomacher 400 Circulator (Seward, Norfolk, UK). Of the supernatant obtained, 1 ml was transferred into 9 ml maximum recovery diluent (MRD), this was vortexed for 30 seconds and a ten-fold dilution series was constructed. Two dilution strengths were plated per sample onto the three types of agar (plain ECC, ECC with ampicillin, and ECC with chloramphenicol) using a Whitley Automatic Spiral Plater (WASP1, Don Whitley Scientific Limited, Whitley, UK). For the total *E. coli* (TEC) and ampicillin-resistant *E. coli* (AREC): 50-100 μl of the 1:100 or 1:1000 solutions allowed for accurate assessments of the samples containing high *E. coli* concentrations, whilst 50-100 μl of the 1:10 or 1:100 solutions were necessary for the low concentration samples. However, up to 200 μl of the 1:10 solution was needed to estimate the much lower concentrations of chloramphenicol-resistant *E. coli* (ChREC) that were seen during some visits. The inoculated plates were incubated at 37°C for 18 to 24 hours prior to manual reading. Single colonies selected from the plain CHROMagar ECC plates were subcultured and stored at −80°C.

Susceptibility testing

A panel of 358 isolates was assembled for susceptibility testing: 181 from the conventional farm and 177 from the organic. The panel encompassed isolates from the cleaned houses, the chick-box liners, and the faecal samples from the growing birds. All the isolates in this panel had been cultured originally on the plain CHROMagarECC plates that did not contain ABDs, only one isolate was tested per sample, and these isolates were blindly picked from the trays in which they were being stored at −80°C. (Details of the breakdown of numbers of isolates from each source are shown in Tables B.4 and B.5 on page 203 in Appendix B.) Up to three environmental isolates were selected from each area of each house that was sampled, and up to four faecal isolates from each house on each sampling day. Up to 13 isolates per house were selected from the chick-box liners from the conventional farm, and up to two per house from the organic.

The stored isolates were resuscitated prior to susceptibility testing using nutrient agar plates. Microbroth dilution was performed using 96-well Sensititre plates that had been custom-made by Trek Diagnostic Systems (East Grinstead, England). The plates contained the following 17 ABDs in doubling dilution concentration series within the ranges shown in brackets: ampicillin (1-32 μg/ml); amoxicillin and clavulanic acid (2-32 μg/ml); ceftiofur (0.5-8 μg/ml); chloramphenicol (2-64 μg/ml); florfenicol (2-64 μg/ml); streptomycin (4-64 μg/ml); gentamicin (1-32 μg/ml); apramycin (4-64 μg/ml); spectinomycin (4-64 μg/ml); neomycin (4-128 μg/ml); tetracycline (2-32 μg/ml); sulphamethoxazole (32-512 μg/ml); trimethoprim (4-32 μg/ml); trimethoprim and sulphamethoxazole (1-8 μg/ml); nalidixic acid (4-128 μg/ml); ciprofloxacin (0.03-4 μg/ml); colistin (4-64 μg/ml). The plates were reconstituted with 50 μl of a standardised bacterial suspension in accordance with the manufacturer's protocol; they

were incubated at 37 °C for 16 hours and read manually.

5.2.4 Data analysis

E. coli associated with empty houses and incoming chicks

A lattice of four mosaic plots stratified by type of house was used to visualise the frequencies of isolation of generic *E. coli* (TEC), ampicillin-resistant *E. coli* (AREC) and chloramphenicol-resistant *E. coli* (ChREC) from samples collected from cleaned poultry houses and chick box liners. Mosaic plots are graphical representations of multi-way contingency tables first developed in the early 1980s (Hartigan and Kleiner, 1984) and then extended by Friendly (1994). The plots were produced using the `strucplot` functions within the `vcd` (visualising categorical data) package (Meyer et al., 2006) in R version 2.9.2 (R Development Core Team, 2009).

Faecal *E. coli* concentrations

The dynamics of faecal concentrations of *E. coli* over time within each flock were visualised as loess smoothed scatter plots of changes in concentration with age of birds.

Loess smoothing entails fitting a series of locally-weighted-least-squares polynomial regression models across the data and using a smooth curve to connect the predicted values for each observation (Cleveland, 1979). First degree polynomial regression was used in these analyses. The `loess` function in R is based on the work of Cleveland (1981), and by default it uses tricubic weighting and nearest-neighbour bandwidth adjustments. Bandwidth refers to the width of the bin of observations fitted in each local regression model, and nearest-neighbour adjustment means this bandwidth is adjusted to incorporate m nearest-neighbours within each fit. Therefore, another parameter that needs to be stipulated is the span, which determines the proportion of the total data that is fitted within each model. The span values were selected using a leave-one-out cross-validation (LOOCV) technique based on the prediction sum of squares (PRESS) (Allen, 1974) by means of R code written by Fox (2000, 2005). PRESS cross-validation was performed individually for each of the 24 subsets of data (i.e. the three *E. coli* populations in each of four houses on two farms). The optimal value for span for each subset of data was that which minimised the square of the sum of the errors of prediction (see Figure B.1 in Appendix B on page 200). Two values of span were selected, one for the conventional data and one for the organic, these values were the mean of the optimal spans for all subsets of data originating from that farm (see Table B.6 in Appendix B on page 204). Therefore, for data from the conventional farm a span of 0.39 was selected (i.e. 39% of observations were used in every local regression fit), whilst a span of 0.31 was chosen for the organic farm data.

Plots of the residuals from loess fits using these span values, confirmed that the chosen span values provided a good fit to the data (see Figure B.2 in Appendix

B on page 205) (Cleveland and Devlin, 1988). Furthermore, a visual assessment of the loess fits using the chosen span values suggested that they were preventing overt fluctuations in the regression curves, which could represent non-meaningful noise within the data, but that enough definition was retained to assess potential alterations in *E. coli* population dynamics after management events. The management events considered were: the administration of ABDs, the thinning of the conventional flocks (removal of a sub-section of birds at 35 days), and the transfer of the organic birds from the indoor brooder sheds to mobile houses in the field.

The loess smoothed scatter plots were produced using the **ggplot2** package in R (Wickham, 2009) with data preparation being carried out using the accompanying **reshape** package (Wickham, 2007). The timing of management events on each farm were superimposed onto the smoothed scatter plots using labelled arrows.

Resistance phenotypes

The heterogeneity of resistance phenotypes within and between farms was assessed using standard diversity, pairwise differences and analysis of molecular variance (AMOVA) models as implemented in the software package Arlequin version 3.1 (Excoffier et al., 2005). The phenotype of each isolate was coded as a binary number consisting of 17 digits each representing resistance (1) and susceptibility (0) to a drug. The standard diversity (D) of phenotypes within a farm is equivalent to the probability of selecting different phenotypes if two isolates are chosen at random (Nei, 1987). The estimated pairwise differences ($\hat{\pi}$) between isolates from a single farm is the mean number of differences in resistance and susceptibility between all pairs of isolates from that farm (Tajima, 1993).

AMOVA partitioned the total variance within the set of phenotypes into the covariance components of the stipulated population structure, i.e. between farms, between flocks within a farm, and within a flock. The method of AMOVA, as implemented in Arlequin, incorporates the frequencies of phenotypes at different levels of the population, and the number of differences in the drugs to which resistance was expressed between isolates (Excoffier et al., 1992). Arlequin calculates AMOVA using a non-parametric permutation approach.

Investigations into spatial and temporal patterns of phenotypes were assessed by assigning each isolate to one of seven phenotype-derived groups, the details of which are provided in Table 5.1 on page 130. A preliminary assessment of the distribution of these seven phenotypic groups across different sources and different ages of bird was undertaken using **ggplot2** in R to produce colour-indexed tile plots.

Log-linear modelling

Associations between the phenotypes of bacteria isolated from faecal samples and other variables connected to those samples were assessed using log linear modelling

and extended mosaic plots. Log-linear models, as applied in this work, describe the general associations that are present between categorical variables within a dataset without designating some as dependent variables and others as predictive covariates (Goodman, 1971).

A fully saturated log-linear model for a three-way contingency table of variables A, B and C containing i rows, j columns and k layers can be expressed as:

$$y_{ijk} = \ln(f_{ijk}) = \mu + \hat{\lambda}_i^A + \hat{\lambda}_j^B + \hat{\lambda}_k^C + \hat{\lambda}_{ij}^{AB} + \hat{\lambda}_{ik}^{AC} + \hat{\lambda}_{jk}^{BC} + \hat{\lambda}_{ijk}^{ABC}, \qquad (5.1)$$

where:

$$\hat{\lambda} = \sum_{i,j,k} a_{ijk} y_{ijk}. \qquad (5.2)$$

Here $\ln(f_{ijk})$ is the natural logarithm of the frequency of observations in cell (i, j, k); μ is the mean of the log of the frequencies for all cells; the $\hat{\lambda}$s denote the estimated effects of the variables; and a_{ijk} are constants (Goodman, 1970). The main effect of variable A is denoted by $\hat{\lambda}_i^A$, whilst $\hat{\lambda}_{ij}^{AB}$ denotes the interaction effect of variables A and B, and $\hat{\lambda}_{ijk}^{ABC}$ the effect of the three-way interaction between the variables.

The model described above is fully saturated because each theoretically possible effect has been fitted and thus the expected frequencies derived from this model will exactly match the observed. Different hypotheses regarding the associations between the variables can be tested by setting specified λ effects to zero, fitting the appropriate marginal frequencies and generating maximum likelihood estimates of the expected frequencies using an iterative fitting algorithm. Whether that particular null hypothesis of independence is a likely fit to the data, can then be assessed using the likelihood-ratio chi-square statistic to estimate the residual frequency that is not accounted for by the effects that have been modelled. Therefore, if a model fits the data well, the likelihood-ratio chi-square statistic will be low and the probability of the observed data being obtained under that particular hypothesis of independence will be high. For a three-way table the likelihood-ratio chi-square statistic is calculated as follows:

$$\chi^2 = 2 \sum_{i,j,k} f_{ijk} \ln(f_{ijk} / \hat{F}_{ijk}), \qquad (5.3)$$

where $\hat{F}_{ijk}$ is the estimated expected frequency under the particular hypothesis of independence that has been fitted.

Extended mosaic plots were used to assist with assessing the fit of the models. Extended mosaic plots display the structure of the underlying contingency table whilst simultaneously allowing for the visualisation of the distribution of the likelihood-ratio chi-square statistic over the individual cells of the table (Friendly, 1994). Using shading, the magnitude and direction of the deviance residuals for each individual cell are

depicted. Thus exposing the cells that are departing most strongly from the particular null hypothesis of independence that has been fitted, and suggesting alternative or additional associations that may be present between the variables.

Within the total panel of 358 *E. coli* isolates, 162 *E. coli* had originated from faecal samples of known birds with corresponding *E. coli* concentration data. Of this subset of 162 isolates, 84 originated from the conventional farm and 78 from the organic. Associations were sought between five variables: the resistance phenotypes, the proportion of *E. coli* in the sample that had been resistant to ampicillin, the proportion resistant to chloramphenicol, the age of the bird when the sample was collected and the farm of origin. The three continuous variables were converted to categorical forms.

To begin with all two-way relationships between the five variables were explored using a matrix of bivariate mosaic plots, analogous to a scatter plot matrix for quantitative data (Friendly, 1999). The bivariate plots showed little evidence of an association between the percentage of *E. coli* in a sample that were AREC and the percentage that were ChREC. Therefore, for ease of visual interpretation, sequential log-linear models were fitted to two sets of four variables: one incorporating AREC and the other ChREC. Initially the fully saturated models were fitted and the results of these models, along with the bivariate relationships highlighted in the mosaic matrix, were used as the basis for formulating the first hypotheses of independence. Models were then fitted sequentially in order to find the model of best fit, and this was deemed to be the most parsimonious model that did not depart from the hypothesis of independence that had been fitted, and that, therefore, adequately represented the true associations between the fitted variables.

The log linear models and the associated mosaic plots were produced using the `strucplot` framework within the `vcd` package in R.

5.3 Results

5.3.1 *Escherichia coli* associated with empty houses and incoming chicks

The numbers of samples obtained from cleaned poultry houses and chick-box liners, from which *E. coli* were cultured are shown in the mosaic plots in Figure 5.1 on page 126. It can be seen that the recovery of *E. coli* (tiles shaded dark grey) occurred less frequently from samples collected on the conventional farm compared to the organic. However, there were differences in recovery rates between the two conventional houses, with house D yielding more positive samples (dark grey tiles) than house B. It can also be seen that on both farms, similar proportions of samples yielded recovery of generic *E. coli* (denoted TE in Figure 5.1) and ampicillin-resistant *E. coli* (denoted AR), whilst the recovery of chloramphenicol-resistant *E. coli* (denoted CR) was lower in all houses except for the mobile barns on the organic farm where TEC, AREC and

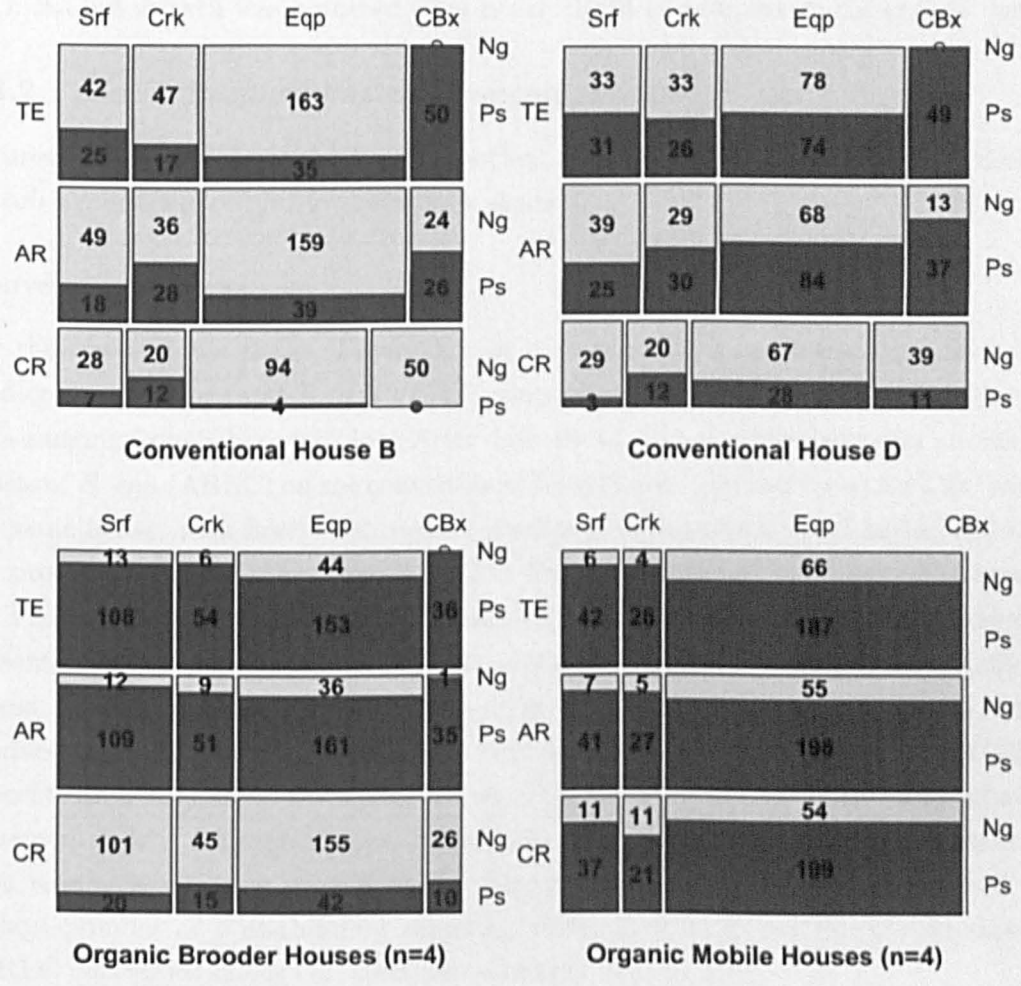

Figure 5.1: A trellis of four mosaic plots displaying the frequencies of growth (Ps, dark grey cells) or no growth (Ng, light grey cells) of three types of *E. coli* (TE, AR, CR) from environmental samples collected from different areas of cleaned poultry houses (Srf, Crk, Eqp) and from the paper lining the boxes in which the chicks were delivered (CBx).

A mosaic plot is a graphical representation of a contingency table, the size of each tile is proportional to the frequency of events in that cell of the underlying table. The actual cell frequencies have been superimposed upon each tile in bold font. Cells with a frequency of zero are represented by a bullet in the appropriate shade of grey.

TE = generic *E. coli*; AR = ampicillin-resistant *E. coli*; CR = chloramphenicol-resistant *E. coli*; Srf = floor and wall surfaces; Crk = cracks in surfaces; Eqp = equipment in house; CBx = chick-box liners; Ng = negative plate (no growth); Ps = positive plate (growth).

The brooder houses were heated sheds in which the organic chicks were reared for 21 days after which time they were moved to floorless mobile houses in the field (hence there are no chick-box liners associated with the organic mobile houses).

ChREC were isolated from similar proportions of samples. With respect to the chick box liners (CBx), *E. coli* were isolated from every sample, but ChREC were isolated from less than half the samples on both farms. AREC isolation varied between batches of chicks but growth was obtained from nearly 100% of samples on the organic farm.

5.3.2 Faecal *Escherichia coli* concentrations

Figures 5.2 and 5.3 show the loess smoothed scatter plots of faecal concentrations of *E. coli* against age of bird for each flock studied.

Conventional flocks

For the conventional flocks (Figure 5.2 on page 128) the range of median flock-level predicted values for total *E. coli* (TEC) concentrations 7.36 to 7.69 log, with the raw data ranging from 5.29 to 9.67 log. After days 10–14, the smoothed plots for ampicillin-resistant *E. coli* (AREC) on the conventional farm closely followed those for TEC within the same house, with flock-level median predicted values of 6.91 to 7.36 log, although the proportion of TEC that were AREC in House A was lower than the other houses.

The sub-populations of chloramphenicol-resistant *E. coli* (ChREC) were generally present in lower concentrations (flock-level median predicted values 5.14 to 6.39 log across the houses; data ranging from 0 to 9.32 log). In the two houses where samples were obtained from three and four day-old birds (houses C and D) ChREC concentration rose from low initial values to a peak around 10 days, after which it remained steady or dropped back slightly. In all four houses, ChREC concentrations then rose to a maximal peak between days 20 and 30. In the two houses with the highest number of post-thinning sampling visits (houses A and B), the decrease in ChREC concentrations is curtailed, or reversed, after thinning.

In two houses (B and D) amoxicillin was administered around day 30 in response to decreases in the appetites of the birds, and increases in the wetness of the litter in the house. During amoxicillin treatment the concentrations of TEC and AREC rose to over 8 log, but returned to pre-treatment levels rapidly after treatment ceased. In house B there was a corresponding peri-treatment peak in ChREC. In house D, however, the ChREC population seemed to decrease after the first three-day course of amoxicillin, whilst the TEC and AREC populations did not decrease until a second course of treatment was instigated. However, with similar decreases in ChREC also occurring in the two flocks that were not dosed with amoxicillin, it is difficult to assess whether the administration of amoxicillin truly affected the ChREC dynamics.

Organic flocks

Figure 5.3 on page 129 shows that the smoothed TEC concentrations on the organic farm were approximately 1 log lower than on the conventional, with flock-level median

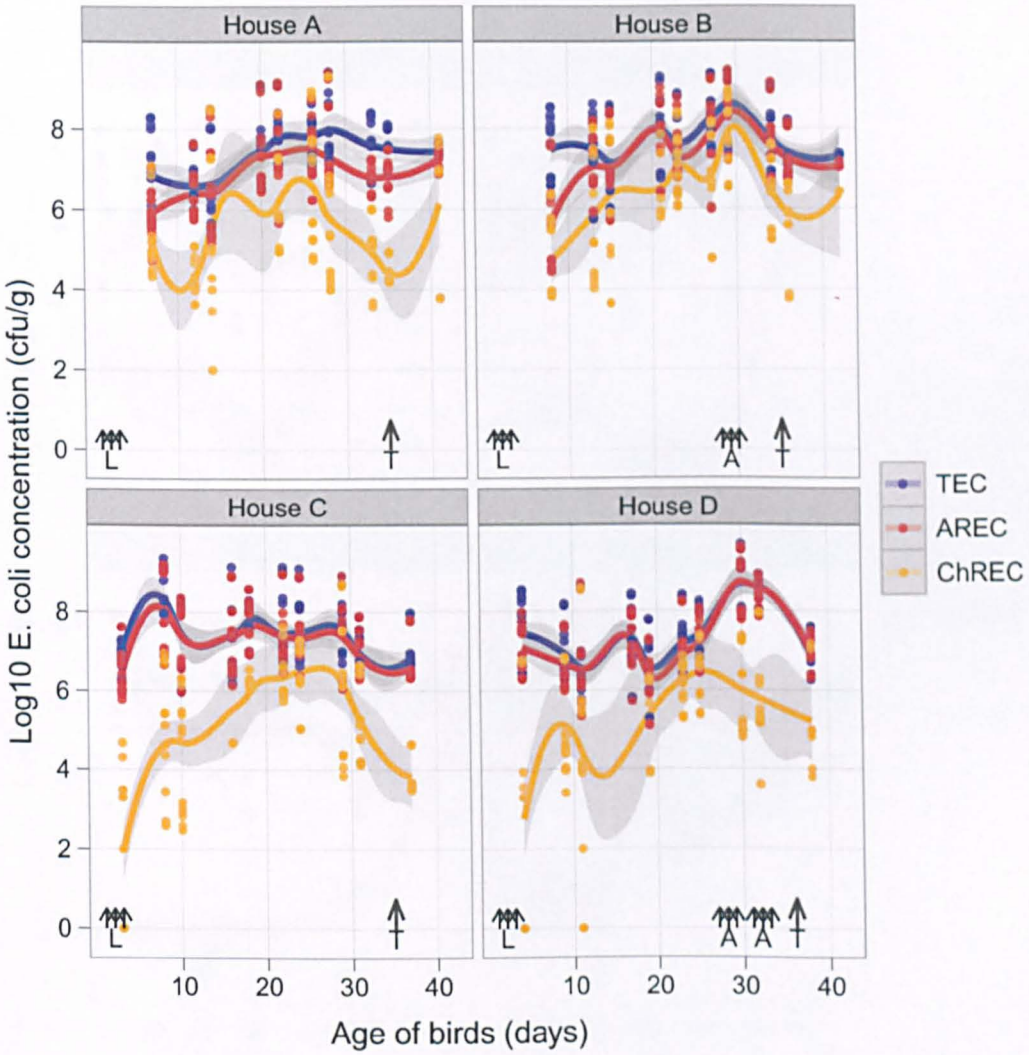

Figure 5.2: Loess smoothed scatter plots showing faecal concentrations of *E. coli* against age for four flocks of birds reared simultaneously on a conventional broiler farm.

Loess smoothing was carried out using first degree polynomial local regression with a span of 0.39. The translucent grey envelopes show the 95% point-wise confidence intervals around the predicted values. The small, black arrows indicate the days upon which the flocks were dosed with antibacterial drugs (L and A) and the large grey arrows indicate the days the flocks were thinned (T).

TEC = total *E. coli*; AREC = ampicillin-resistant *E. coli*; ChREC = chloramphenicol-resistant *E. coli*; L = lincomycin-spectinomycin; A = amoxicillin; T = flock thinned.

Thinning is the removal of a proportion of the male birds in a house at five weeks of age. The rest of the flock are reared in the same house for a further week.

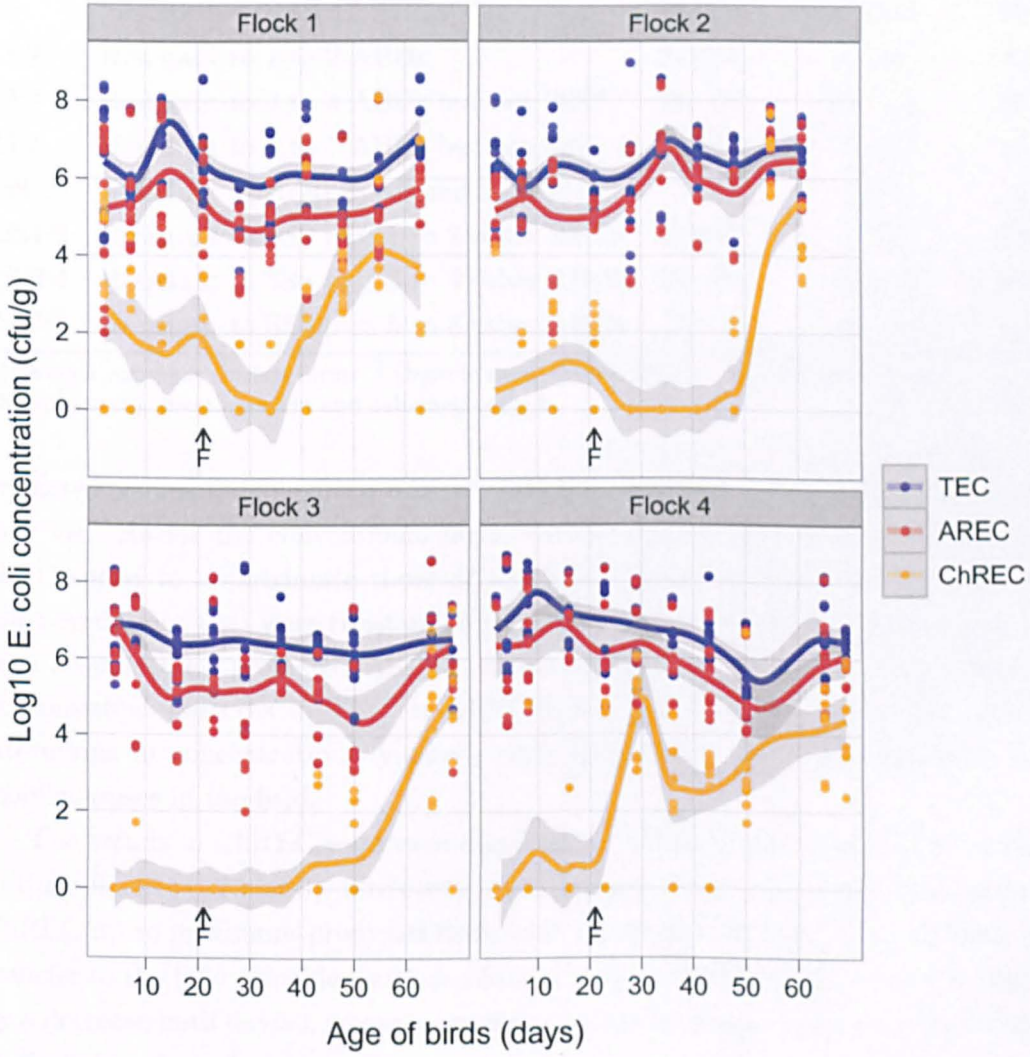

Figure 5.3: Loess smoothed scatter plots showing faecal concentrations of *E. coli* against age for four flocks of birds being reared on an organic meat chicken farm.

Loess smoothing was carried out using first degree polynomial local regression with a span of 0.31. The translucent grey envelopes show the 95% point-wise confidence intervals around the predicted values. The arrows indicate the days upon which the flocks were transferred to the field.

TEC = total *E. coli*; AREC = ampicillin-resistant *E. coli*; ChREC = chloramphenicol-resistant *E. coli*; F = transfer from heated brooder houses to mobile sheds in the field.

Table 5.1: The initial seven defined groups of resistance phenotypes and the distribution of 358 *E. coli* isolates across them.

Group ID	Phenotype category	Number of isolates (%)		
		Conv. fm[a]	Org. fm.[b]	Total
R0	Susceptible to all 17 ABDs[c]	33 (18)	67 (38)	100 (28)
R1:2	Resistant to 1 to 2 ABDs	24 (24)	71 (40)	95 (27)
R3:4	Resistant to 3 to 4 ABDs but not SSS[d]	14 (8)	18 (10)	32 (9)
R5:7	Resistant to 5 to 7 ABDs but not SSS	8 (4)	8 (5)	16 (4)
SSS	Resistant to strep, spect and sulfameth[d]	3 (2)	3 (2)	6 (2)
SSS1:2	Resistant to SSS plus 1 to 2 other ABDs	42 (23)	1 (1)	43 (12)
SSS3:4	Resistant to SSS plus 3 to 4 other ABDs	50 (28)	7 (4)	57 (16)
SSS5:8	Resistant to SSS plus 5 to 8 other ABDs	7 (4)	2 (1)	9 (3)

[a] Conventional meat chicken farm. [b] Organic meat chicken farm. [c] Antibacterial drugs.
[d] Streptomycin, spectinomycin and sulfamethoxazole.

predicted values ranging from 6.26 to 6.68 log; with the raw data ranging from 3.6 to 9 log. As for the conventional farm, within each house the shape of the AREC plots tended to approximate those of the TEC. However, the proportions of the *E. coli* populations that were resistant to ampicillin were lower on the organic farm, with flock-level median predicted AREC concentrations of 5.35 to 6.07 log. There were no consistent patterns in TEC or AREC across the four flocks, nor any consistent alterations in concentration dynamics after transfer of the 21-day old birds to the mobile houses in the field.

The trends in ChREC were even more varied between the organic flocks; however, in three flocks (1, 2 and 3), birds over 30 to 40 days of age shed increasing numbers of ChREC up to maximum predicted flock-level values of 4.72 to 6.71 log. In flock 4, the transfer to the field coincided with a substantial rise in ChREC concentration, followed by a decrease until day 35, whereupon the concentrations rose again in a similar fashion to the other three flocks.

5.3.3 Resistance phenotypes

Descriptive analysis

The overall diversity of *E. coli* resistance phenotypes was high, with 45 different phenotypes present within the full set of 358 isolates. Standard diversity indices showed that phenotypic diversity was higher on the conventional farm compared to the organic, with a probability of 0.92 (SD 0.01) for randomly selecting two isolates of different phenotypes on the conventional farm, and 0.83 (SD 0.04) on the organic. The increased diversity of phenotypes on the conventional farm was also reflected in the mean number of differences in resistance between pairs of isolates, which was 3.13

(+/- 1.64) on the conventional farm and 2.67 (+/- 1.43) on the organic. A comparison of the categories of resistance phenotypes identified within the *E. coli* isolated from the two farms is shown in Table 5.1, which shows that the multidrug resistant phenotypes that included resistance to streptomycin, spectinomycin and sulfamethoxazole (SSS), were generally isolated from the conventional farm.

Table 5.2: Results of an analysis of molecular variance (AMOVA) model investigating the potential clustering of phenotypes at the different levels of the flock-farm hierarchy.

Source of phenotypic variation	d.f.[a]	Sum of squares	Variance components	% Variation
Between-farms	1	82.2	0.98	39.7
Between-flocks[b] within-farms	6	21.2	0.11	4.4
Within-flocks	153	210.4	1.38	55.9
Total	160	313.9	2.46	

[a] Degrees of freedom. [b] A flock is defined as a group of birds that are simultaneously reared in the same house.

Table 5.2 shows the results of the hierarchical AMOVA model that assessed the phenotypic structure of the *E. coli* populations. The variation in phenotypes within a flock was high, with 55.9% of the total variance occurring at this level. In contrast, the variation between flocks within a farm only accounted for a further 4.4% of the total variance, implying that the phenotypes themselves and phenotypic diversity was similar for all flocks on a given farm. However, 39.7% of the total variance occurred at the farm-level: indicating that there were substantial differences between the two farms.

Spatial and temporal dynamics

A visual comparison of the multiple resistance phenotypes of isolates originating from plain ChromagarECC plates (those without ABDs), further demonstrated that there were marked differences in the *E. coli* populations between the two farms (Figure 5.4 on page 132). Of the 51 isolates collected from the paper lining the chick-boxes from both farms, 28 (55%) were fully susceptible, 10 (20%) were resistant to one drug, and 6 (12%) to two drugs. However, *E. coli* isolates originating from conventional birds in the first week after placement (days three to seven) were predominantly expressing resistance to streptomycin, spectinomycin and sulfamethoxazole (the SSS phenotype) with an additional one to four other drug resistances detected per isolate (11 of 13 isolates). Furthermore, this pattern was maintained throughout the growing cycle of the conventional birds (72 of 84 isolates, 86%), with resistance being expressed to a median number of five ABDs per faecal *E. coli* isolate. Although a greater variety of resistance phenotypes were seen after thinning had occurred when the birds reached

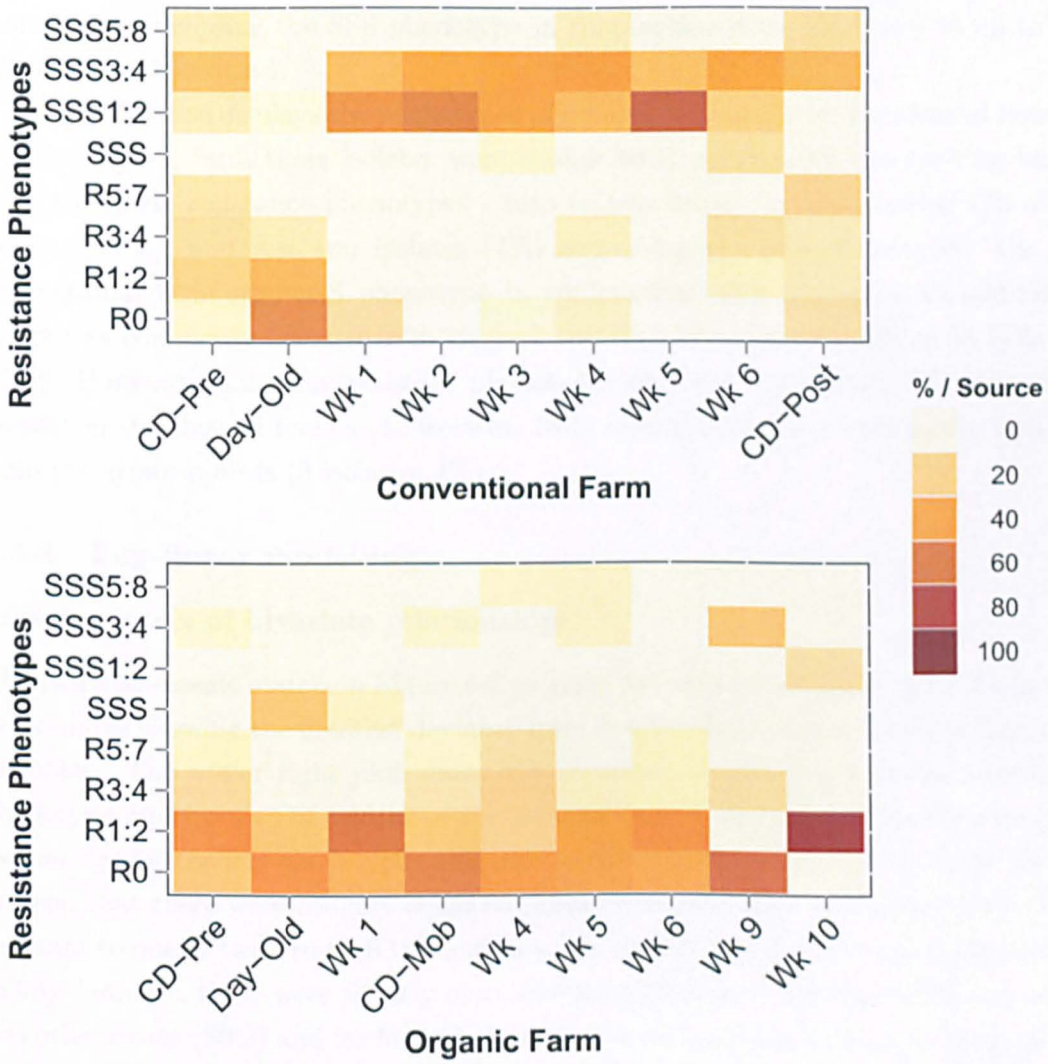

Figure 5.4: Tile plots showing the antibacterial drug resistance phenotypes of *E. coli* isolated from two meat chicken farms at different sampling points (sources) during the flock cycles.

The percentages of isolates within each phenotype category at each sampling point (source) are displayed.

CD-Pre = isolates taken from cleaned sheds prior to the arrival of the monitored flocks; CD-Post = isolates taken from cleaned sheds after the departure of the monitored flocks; CD-Mob = isolates taken from cleaned mobile sheds prior to the transfer of birds from the brooder houses; Day-Old = isolates from the paper liners of the boxes the chicks were delivered in; Wk 1 = isolates from birds up to seven days of age. See Table 5.1 (page 130) for the key to the nomenclature used to identify the categories of ABD resistance phenotypes.

35 days of age. In contrast, the majority of the faecal isolates obtained from growing birds on the organic farm remained in the resistant to zero to two drug categories (62 of 78 isolates, 79%), with resistance shown to a median of one drug per faecal *E. coli*. Nonetheless, 21% of isolates from the organic birds did display resistance to three or more drugs, including the SSS phenotype in conjunction with resistance to up to six other drugs (7 isolates).

Figure 5.4 also displays the phenotypes of isolates collected from the cleaned houses. On the organic farm these isolates were similar to those shed by the growing birds, with low-grade resistance phenotypes - zero to two drugs - predominating (70 of 91 isolates, 77%), and just two isolates (4%) expressing the SSS phenotype. On the conventional farm, the SSS phenotype in conjunction with resistance to additional drugs was commonly isolated from cleaned and disinfected houses (25 of 54 isolates, 46%). However, multidrug resistant phenotypes that did not include SSS were also present in the cleaned houses (15 isolates, 28%) even though they were rarely isolated from the growing birds (3 isolates, 4%).

5.3.4 Log-linear modelling

Mosaic matrix of bivariate relationships

The pairwise mosaic matrix in Figure 5.5 on page 134 highlights that farm of origin was the variable showing the greatest deviance from independence against each of the other variables. The upper right plot shows the bivariate relationship between resistance phenotype and farm. The widths of the vertical bars in this plot reflect the marginal frequencies for the five phenotype categories in the underlying two-by-two table. It can be seen that there were roughly equal numbers of isolates that were susceptible (R0), resistant to one or two drugs (R1:2) and resistant to SSS plus three to eight other drugs (S3:8); however, there were slightly more isolates that were resistant to SSS and up to two other drugs (S0:2) and far fewer isolates that were resistant to three to seven drugs but that did not contain the SSS phenotype (R3:7).

The bars in this plot have then been subdivided horizontally, and the tiles above the horizontal divisions represent the data from the conventional farm, and the tiles below from the organic. There was a very evident dichotomy of phenotypes between the two farms, with significantly more isolates showing R0 or R1:2 phenotypes on the organic farm than would be expected under a hypothesis of independence (as depicted by the two large cells shaded in light blue). Conversely, there are significantly more isolates showing S0:2 and S3:8 phenotypes than expected on the conventional farm. The two intensely shaded small red tiles showed the greatest deviations from independence, i.e. the frequencies of R1:2 phenotypes on the conventional farm and S0:2 phenotypes on the organic, were both far lower than would be expected if phenotype and farm of origin were not associated. The plot in the lower left corner also shows the relationship between phenotype and farm of origin, the corresponding tiles are of the same area

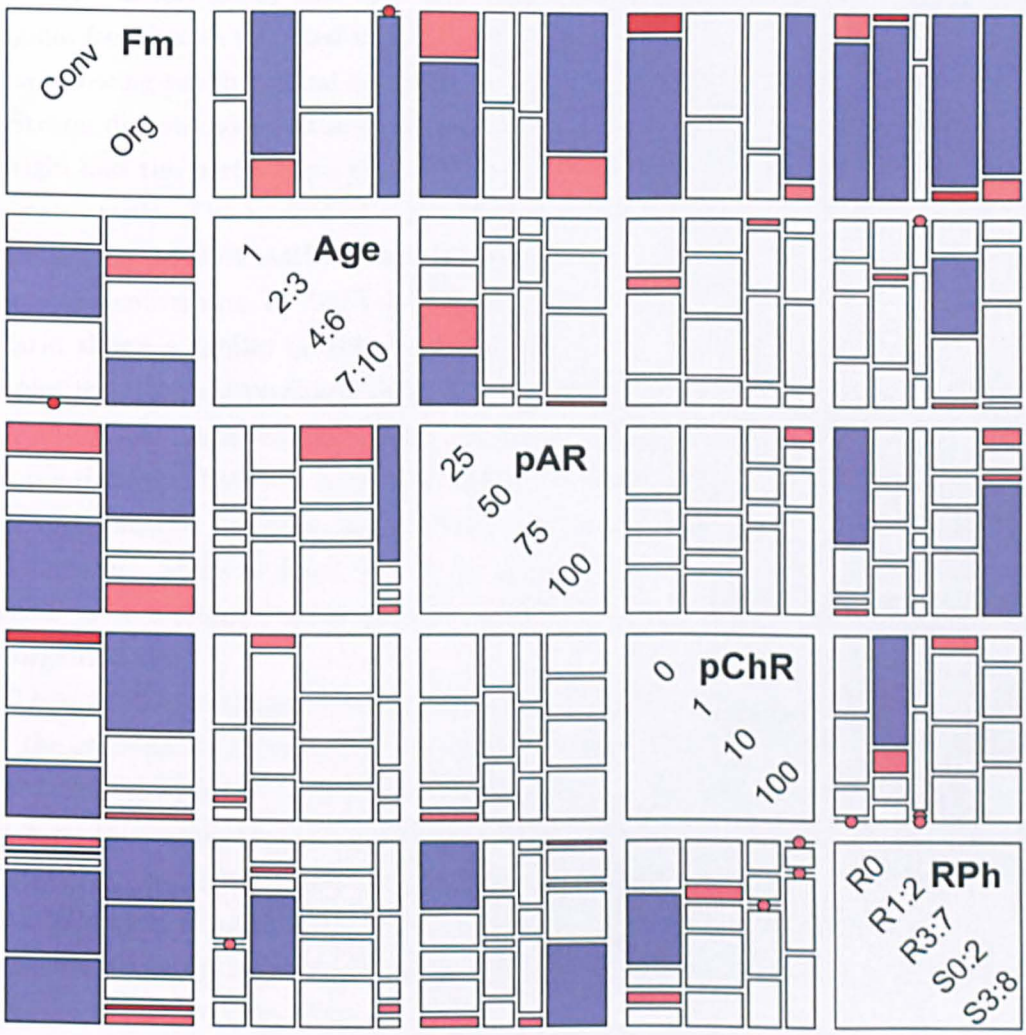

Figure 5.5: Pairwise mosaic matrix displaying the bivariate associations between five categorical variables related to 162 *E. coli* isolates originating from faecal samples collected on two meat chicken farms: one conventional (Conv) and one organic (Org).

Each bivariate plot is first split vertically and the widths of the vertical bars are proportional to the marginal frequencies of the levels of the variable in the underlying two-way contingency table. Then the vertical bars are split horizontally and the areas of the resultant tiles are proportional to the frequencies of observations in the corresponding cells of the table. Cells deviating from the hypothesis of mutual independence (the hypothesis stating there is no association between the two variables) are indicated by displaying the direction (colour) and size (shading intensity) of the deviance residuals. Positive deviance residuals are denoted by blue-coloured tiles, negative by red. Light blue or red tiles denote > 2 $residual[i, j] < 4$. Dark blue or red tiles denote $residual[i, j] > 4$. There are no dark blue tiles on these plots.

Fm = farm of origin; Age = age of bird when sampled (in weeks); pAR = percentage of *E. coli* in the faecal sample that were resistant to ampicillin; pChR = percentage resistant to chloramphenicol; RPh = resistance phenotype of the isolate; R0 = susceptible; R1:2 = resistant to 1-2 drugs; R3:7 = resistant to 3-7 drugs but not SSS; S0:2 = resistant to SSS and 0-2 other drugs; S3:8 = resistant to SSS and 3:8 other drugs. The labels for the percentage resistant variables denote the upper limit of each category.

and same shade as those in the top right plot, but the shape of the tiles is different. This plot was formed by first splitting upon farm and then upon phenotype and the marginal frequencies reflected in the width of the bars of this plot are those of the two farms, showing roughly equal numbers of isolates from each.

Strong dichotomous patterns of residuals were also seen in the plots between farm of origin and the percentages of *E. coli* resistant to ampicillin and chloramphenicol in a faecal sample. The middle plot on the top row shows an overabundance of samples containing up to 25% AREC from the organic farm compared to an overabundance of samples containing 76–100% AREC from the conventional. The plot of ChREC by farm shows a similar pattern, but in this plot the tile representing the number of samples from the conventional farm from which ChREC were not detected is shaded with an intense, deep red indicating an even stronger departure from independence. The relationships between farm and age of bird reflect the different rearing systems; therefore, because the conventional birds are slaughtered at five to six weeks of age, birds of seven weeks or more are only encountered on the organic farm. There also appears to be a relative under-sampling of birds between two to three weeks of age on the organic farm.

There are suggestions of a relationship between the percentage of AREC in a sample and the resistance phenotype of an isolate cultured from that sample (far right plot on third row), with the most multidrug resistant phenotypes (S3:8) heavily associated with samples containing the highest percentage of AREC (76–100%) and the susceptible isolates (R0) associated with samples containing the lowest percentage of AREC (up to 25%). However, from this simple bivariate plot it is difficult to assess how much that relationship simply reflects the underlying differences in phenotype and percentage AREC between the two farms.

The patterns of residuals in the remaining plots are less straightforward. There is a suggestion that low resistant isolates (R1:2) occurred more frequently in samples from which ChREC were not detected. However, there is little suggestion that samples containing higher percentages of ChREC were more likely to harbour multidrug resistant phenotypes, although it can be seen that no fully susceptible isolates were obtained from samples containing the highest proportions of ChREC (11-100%). There are also few associations between the percentage of AREC and the percentage of ChREC in a faecal sample, with the exception that a sample containing less than 25% AREC was unlikely to contain the highest observed proportions of ChREC (11-100%).

Log-linear models of four-way associations between variables

Figure 5.6 on page 136 consists of four extended mosaic plots derived from a four-way contingency table of: the resistance phenotypes of isolates (RPh), the proportion of faecal *E. coli* that were ampicillin-resistant (pAR), the age of birds (Age), and farm (Fm). Based on the bivariate plots, some of the levels within the phenotype and age

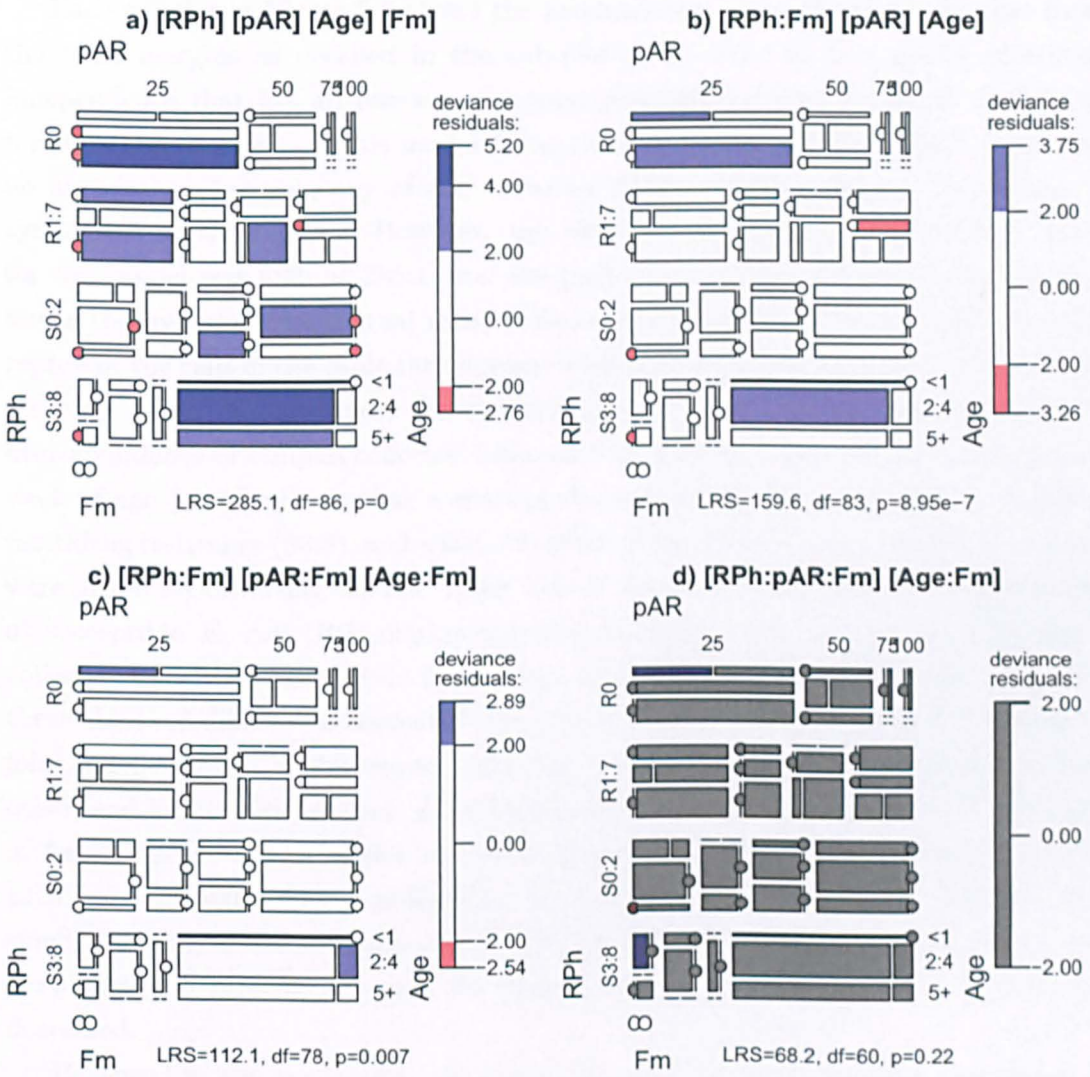

Figure 5.6: Extended mosaic plots showing the results of four sequential log-linear models exploring the relationships between the percentages of *E. coli* in a faecal sample that are resistant to ampicillin (pAR) and three other categorical variables.

The title of each subplot denotes the marginals of the underlying four-way contingency table that have been fitted in each model. The data has been successively divided by each variable, starting on the left with resistance phenotype (RPh) and then moving in a clockwise direction around the plot. The areas of the individual tiles (and groups of tiles) represent the frequencies of the observed data in those cells (or regions) within the contingency table. Individual cells showing significant departures from the hypothesis of independence that has been fitted are highlighted using colour and shading as depicted in the legend to each subplot.

The grey shading in subplot d) signifies that the overall likelihood-ratio chi-square statistic (LRS) for this plot does not indicate a significant departure from the hypothesis of independence that was fitted, and the p-value shows that there is a 22% chance of obtaining the observed frequencies if the true associations between the variables match those that have been fitted in the model.

C = conventional farm, O = organic farm. For other abbreviations see Figure 5.5 on page 134.

variables were collapsed to produce four-level and three-level variables, respectively; this also aided visual clarity within the four-way plots.

Each subplot in Figure 5.6 shows the residuals from a log-linear model that fitted the table margins as detailed in the sub-plot title. Plot 6a is a model of mutual independence that fits all one-way marginal probabilities, but leaves all interaction terms in the residuals. If this model fitted the data it would suggest that there were no associations between any of the variables (Rph $\perp$ pAR $\perp$ Age $\perp$ Fm, where $\perp$ symbolises independence). However, the likelihood-ratio chi-square statistic (LRS) for this model was high at 285.1, and the probability of obtaining the observed data under the hypothesis of mutual independence was zero ($p = 0$). The deep blue tiles represent the cells of the table that deviate most strongly from independence (deviance residuals > 4). For instance, the bottom right of plot 6a shows that there was an over-abundance of samples collected from conventionally-managed birds older than one week of age (category 2:4) that contained the isolates showing the greatest degree of multidrug resistance (S3:8), and where 76–100% of the *E. coli* present within the sample were AREC. Conversely, in the upper left of the plot there was an overabundance of susceptible *E. coli* (R0) originating from samples containing up to 25% AREC collected from the oldest birds (five weeks or more) on the organic farm. Based on these observed differences between farms, the second model fitted (plot 6b) was one of joint independence; in this model, Age and pAR were mutually independent of each other, and jointly independent of the combination of RPh and Fm ([RPh, Fm] $\perp$ pAR $\perp$ Age). The fit of this model improved upon that of mutual independence, but the LRS was still high and the probability of obtaining the observed data was still very small. Looking at the residuals, the two major deviations highlighted in the first plot were both still present, although the magnitudes of the residuals for these cells had decreased.

Because Fm was so strongly associated with all other variables in the bivariate plots (Figure 5.5), the next model fitted was one of conditional mutual independence (plot 6c), where RPh, pAR and Age are all independent of each other given the farm of origin ([RPh, Fm] $\perp$ [pAR, Fm] $\perp$ [Age, Fm]). This model effectively controls for farm effects while any associations between the other three variables remain in the residuals. There were fewer deviant cells in plot 6c, but the LRS was still high and the probability of obtaining the data low ($p = 0.007$), implying that there are other interactions present that have not been fitted. The plot showed an association between R0 phenotypes and faecal samples with $< 25\%$ AREC in conventional birds under one week of age, and another between S3:8 phenotypes and samples with $> 75\%$ AREC in organic birds of two to four weeks. Therefore, the final model fitted (plot 6d) was another conditional independence model that tested the hypothesis that the combination of Rph and pAR was independent of Age given the farm of origin ([RPh, pAR, Fm] $\perp$ [Age, Fm]). This model fitted the data much more closely, the LRS decreased and there was a 22%

chance of obtaining the observed data under this set of associations. In conclusion, although farm of origin was an important factor in both the proportion of faecal *E. coli* populations that were AREC, and the degree of multidrug resistance expressed by *E. coli* isolates, nonetheless when controlling for these farm effects a positive association remained between the percentage of AREC in a faecal sample and the level of multidrug resistance seen in isolates cultured from the sample.

Figure 5.7 (page 139) shows the results of a similar process that examined the associations between the percentage of ChREC in a faecal sample and the other variables. Once again the model of mutual independence (RPh $\perp$ pChR $\perp$ Age $\perp$ Fm) in plot 7a was a very poor fit to the data, and there was evidence of a strong farm effect. The second model followed the pattern of the AREC series by fitting an interaction between RPh and Fm ([RPh, Fm] $\perp$ pChR $\perp$ Age). The residuals from this model (plot 7b) suggested that there was also an association between farm and pChR, at least for samples from which resistant isolates were cultured (R1:7, S0:2 and S3:8). Fitting the model of mutual independence conditional upon farm ([RPh, Fm] $\perp$ [pChR, Fm] $\perp$ [Age, Fm]) does bring the LRS down significantly with a 57% probability of obtaining the data under this hypothesis (plot 7c). There was still one cell with a deviance residual that was just > 2, implying that there could be a weak association between samples for which ChREC were detected in $< 1\%$ of the *E. coli* population and the selection of a susceptible isolate (R0) from that plate in conventional birds of < 1 week of age. Incorporating an interaction between pChR and Age whilst still controlling for farm ([RPh, Fm] $\perp$ [pChR, Age, Fm]) improved the fit still further (plot 7d), resulting in a 95% probability of obtaining the data. Therefore, after controlling for farm of origin, there did not appear to be an interaction between the percentage of ChREC in a sample and the degree of multidrug resistance shown by an isolate selected from the same agar plate. However, there was a suggestion that, over and above the farm-effects, an association may occur between the percentage of ChREC in a sample and the age of bird, in as far as low concentrations of ChREC are present in birds under one week of age; a suggestion that is largely in-line with the population dynamics data shown in Figures 5.2 and 5.3.

5.4 Discussion

This study found salient differences in the dynamics of antibacterial resistant *E. coli* on two meat chicken farms that were operating under contrasting styles of management. The conventional broiler farm was associated with higher overall concentrations of faecal *E. coli* than the organic farm, and the vast majority of *E. coli* shed by the conventional birds were resistant to 8μg/ml ampicillin. Even so, *E. coli* resistant to ampicillin were still routinely shed by birds on the organic farm albeit as a lower proportion of the total *E. coli* population compared to the conventional birds. Differences were also seen

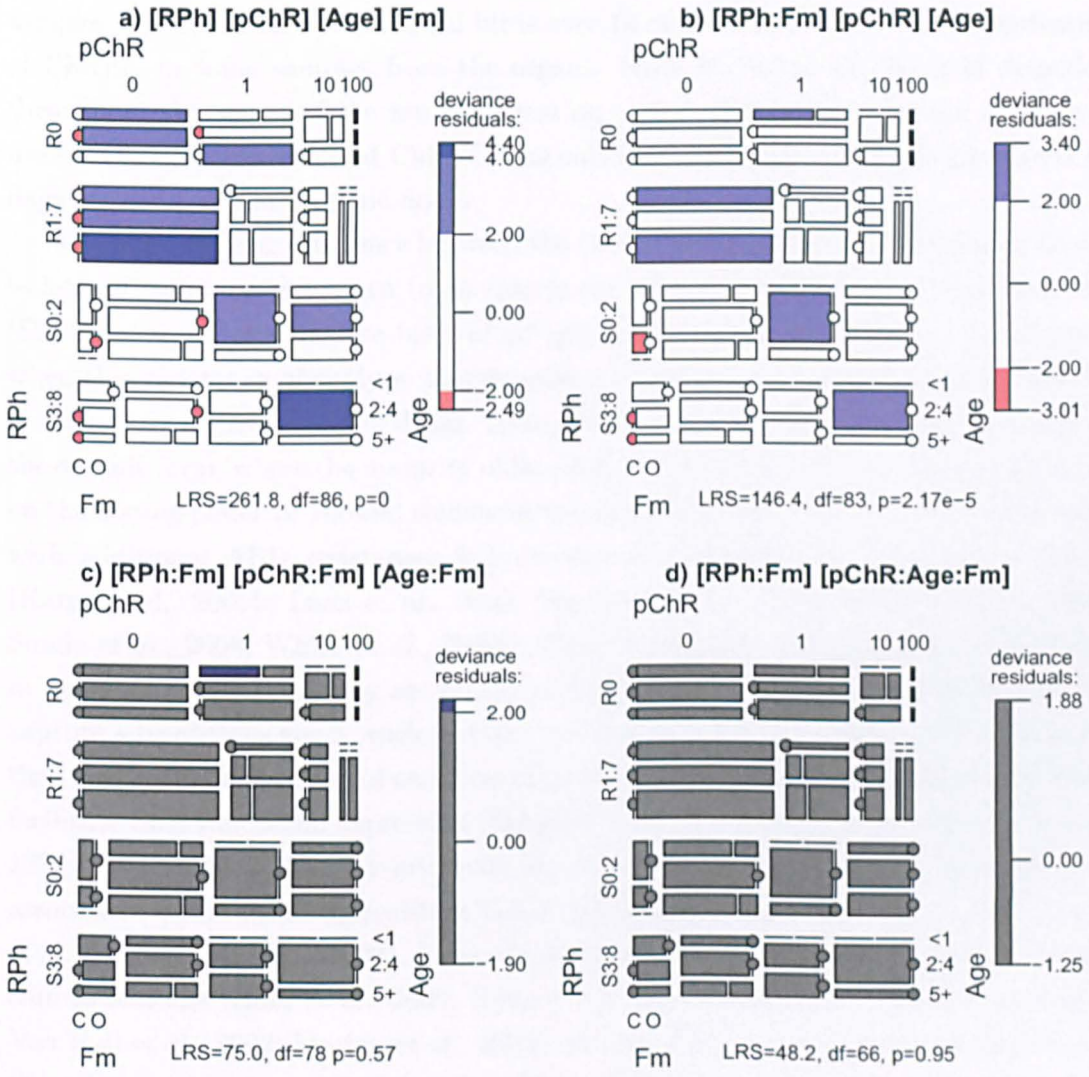

Figure 5.7: Extended mosaic plots showing the results of four sequential log-linear models exploring the relationships between the percentages of *E. coli* in a faecal sample that are resistant to chloramphenicol (pChR) and three other categorical variables.

The title of each subplot denotes the marginals of the underlying four-way contingency table that have been fitted in each model. The data has been successively divided by each variable, starting on the left with resistance phenotype (RPh) and then moving in a clockwise direction around the plot. The areas of the individual tiles (and groups of tiles) represent the frequencies of observed data in those cells (or regions) within the contingency table. Individual cells showing significant departures from the hypothesis of independence that has been fitted are highlighted using colour and shading as depicted in the legend to each subplot.

The grey shading in subplots c) and d) signifies that the overall likelihood-ratio chi-square statistics (LRS) for these plots do not indicate a significant departure from the hypothesis of independence fitted, and the p-values show that there is a 57% and 95% chance, repsectively, of obtaining the observed frequencies if the true associations between the variables match those that have been fitted in these models.

C = conventional farm, O = organic farm. For other abbreviations see Figure 5.5 on page 134.

in the concentrations of faecal chloramphenicol-resistant *E. coli* that were shed by the birds on each farm: measurable concentrations of ChREC were detected in all faecal samples collected from conventional birds over 14 days of age, whilst the concentration of ChREC in some samples from the organic birds fell below the limit of detection throughout the course of the ten week rearing period. Nevertheless, in the absence of use of ABDs, a rise in faecal ChREC concentration was seen in organic birds over 40 days of age in all four organic flocks.

The most striking difference between the two farms was the predominance of *E. coli* isolates showing co-resistance to streptomycin, spectinomycin and sulfamethoxazole (SSS) that were isolated from birds of all ages on the conventional farm. Furthermore, when this resistance phenotype was present it was almost invariably accompanied by resistance to one to four other drugs. In contrast, this phenotype was rarely isolated on the organic farm, where the majority of faecal *E. coli* were either susceptible to all drugs on the testing panel, or showed resistance to just one or two drugs. The SSS phenotype with additional ABD resistances is indicative of the presence of Class 1 integrons (Kang et al., 2005b; Lanz et al., 2003; Maynard et al., 2004; Nogrady et al., 2003; Sunde et al., 2008; White et al., 2002). Class 1 integrons are important mechanisms in bacterial adaptation; they are highly mobilisable genetic elements that function to capture adventitious genes, such as those coding for AB drug resistance. Furthermore, they enable the integration of cassettes of genes into plasmids or chromosomes and also facilitate their functional expression (Boucher et al., 2007; Hall, 1997; Hall and Stokes, 1993; Mazel, 2006). Class 1 integrons are of clinical relevance due to their frequent association with multidrug resistant Gram-negative pathogens (Pai et al., 2003; Singh et al., 2005; Zhao et al., 2003). However, they are also increasingly recognised outside clinical settings (Binh et al., 2009; Kang et al., 2005a; Kim et al., 2005b; Leverstein-Van Hall et al., 2002; Mathai et al., 2004). Several studies have shown the presence of Class 1 integrons, in association with transposon Tn21, in *E. coli* of poultry origin and these isolates characteristically show resistance to sulphonamide drugs, streptomycin and spectinomycin (Bass et al., 1999; Guerra et al., 2003; Nogrady et al., 2006; Yang et al., 2004).

Pulling together the information gathered in this study, a biologically plausible explanation for the predominance of the SSS phenotypes on the conventional farm could be that the prophylactic application of lincomycin-spectinomycin (a broad-spectrum therapeutic ABD) to day-old chicks actively selected for their colonisation with SSS-resistant phenotypes. From this study alone, it is not possible to conclude that the use of lincomycin-spectinomycin (LS) was directly responsible for the predominance of the SSS phenotypes on the conventional farm because the drug was administered to incoming chicks and therefore there were no pre-treatment samples from the 24 birds that were studied. However, on both farms, the majority of *E. coli* associated with incoming, freshly-hatched chicks were either susceptible to all 17 drugs tested,

or showed resistance to one or two drugs. Furthermore, *E. coli* with SSS phenotypes were demonstrated to be present in cleaned houses on the conventional farm, albeit probably in low numbers, and therefore the farm environment would be acting as a source of these strains to the young chicks receiving three days of prophylactic ABDs containing spectinomycin.

Further indirect evidence for the role played by the prophylactic use of LS in selecting for multidrug resistant *E. coli* phenotypes is presented by the predominance of susceptible or low-resistant strains on the organic farm. Even though occasional isolates from the organic birds showed multidrug resistant phenotypes (including the occasional SSS phenotype), the multidrug resistant strains remained in the minority throughout the ten week rearing cycle. This implies that strong selective pressures for these phenotypes were not present on the organic farm. Other studies that have investigated resistance phenotypes in *E. coli* of livestock origin, have also noted that multidrug resistant strains are isolated at lower frequencies both on organic farms and from retail meat originating from organic farms, compared to *E. coli* isolated from conventional farms and farm products (Miranda et al., 2006, 2008; Walk et al., 2007).

Furthermore, having selected for the SSS phenotypes on the conventional farm, these phenotypes then persisted as the majority population until the birds were slaughtered. There are a number of hypotheses that could explain this. Firstly, it seems likely that chicken-adapted strains of *E. coli* have acquired extra-chromosomal DNA and integrons, and therefore resistance is maintained by the natural successions of strains as the birds grow, as previously reported in longitudinal studies of resistant *E. coli* in calves (Khachatryan et al., 2004). Nonetheless, if there were fitness costs associated with carrying extra-chromosomal genes in a non-selective environment one would expect to see a decrease in the SSS phenotype over time. Therefore, the persistence of this phenotype could indicate that mutation-based genetic adaptations have decreased the fitness costs associated with multiple-resistance (Trindade et al., 2009), or that other selective pressures are maintaining resistance. Potential selective pressures could include:

1. changes in feed formulations.
2. the physiological stresses of rapid growth.
3. or the use of in-feed anticoccidial drugs.

On the conventional farm, the birds were fed five different diet formulations during the five to six week rearing period, and for the first four weeks the diets included anticoccidial drugs (initially nicarbazin and narasin, followed by a change to monensin at 14 days).

It is also possible that persistence of the SSS strains may have been enhanced by the 100% enclosed and controlled environment that the birds were reared in on the conventional farm and the biosecurity practices in place. By design, biosecurity

practices are implemented to limit the entry of microbes into the sheds, and this could include potential replacement strains of lower resistance. In line with this hypothesis, there appeared to be a possible increase in phenotype diversity after 12,000 male birds were removed from each shed at five weeks. This practice of thinning the flock involves off-farm catching teams entering the sheds and placing birds into crates. Thinning has previously been implicated in the introduction of *Campylobacter* species into broiler houses (Allen et al., 2008), and it could also facilitate the introduction of *E. coli* of novel resistance phenotypes.

The prophylactic use of LS in day-old chicks on the conventional farm had been implemented after the operating company had withdrawn the sub-therapeutic growth promoter avilamycin from the birds rations in preparation for the European ban on the use of such drugs in 2006 (Regulation (EC) No 1831/2003). After avilamycin had been withdrawn, there was an increase in enteritis of non-specific aetiology (dysbacteriosis), which had initially responded to the administration of amoxicillin. However, over the period of three to four rearing cycles there had been a drop-off in efficacy of the amoxicillin resulting in the instigation of LS prophylaxis. Other countries, that have monitored farming practices following the ban of in-feed, growth promoting antibacterial drugs, have documented similar increases in the use of therapeutic antibacterials in the period immediately after the ban (Arnold et al., 2004; Casewell et al., 2003). However, in some countries farming practices adjusted over time such that only a small percentage of farms continued to rely on in-feed antibacterial drugs to prevent outbreaks of clinical disease (Bengtsson and Wierup, 2006; Grave et al., 2004; Wierup, 2001).

Despite the use of LS, dysbacteriosis still affected two of the four conventional flocks that were studied, and the birds in the affected flocks also received three to six days of amoxicillin during the rearing period. The effects of this additional, clinical use of an ABD upon resistant *E. coli* were harder to ascertain. In both treated flocks, there was an immediate spike in the faecal concentration of AREC, which was particularly pronounced in flock D during their six days of treatment. This spike was short-lived, however, and in both treated flocks faecal concentrations dropped back to pre-treatment values very quickly after the cessation of treatment. Furthermore, due to the general multidrug resistant nature of the *E. coli* on the conventional farm, and the common occurrence of ampicillin-resistant phenotypes (65% of characterised faecal isolates), it was not possible see direct changes in phenotypes during or after amoxicillin treatment.

Whilst the higher degree of ABD resistance observed on the conventional farm could feasibly be related to the use of ABDs, ABD resistant bacteria were still present (albeit at generally lower concentrations) on the organic farm where no ABDs were being administered. Furthermore, chloramphenicol-resistance was seen to increase to over 4 log cfu/g faeces in birds over 40 days of age even though chloramphenicol has not been licensed for use in livestock in the EU since 1994. Similar increases in the

faecal concentrations of ampicillin-resistant *E. coli* in growing birds on an organic farm are reported in chapter 4.

This study has highlighted the complexity of the dynamics of ABD resistant bacteria on meat chicken farms. Single measures of resistance considered in isolation, such as the presence or absence of a particular resistance, or the resistance phenotypes of a set of isolates, may not provide a clear picture of the overall patterns of resistance on a farm. This may be one reason why some studies investigating agricultural ABD use and ABD resistant bacteria fail to find direct links between the two, particularly at the animal level (Jackson et al., 2006; Pol and Ruegg, 2007; Tragesser et al., 2006). The use of log-linear models and mosaic plots in this work allowed for the simultaneous assessment of different measures of resistance (faecal concentrations of resistant *E. coli* and the phenotypes of isolates originating from the same faecal samples) in relation to farm type. The mosaic plots provided clear visual evidence of a strong farm effect on resistance, and the log-linear models then allowed for the assessment of other interactions between the variables whilst controlling for those farm effects. This work showed that some chickens were acting as super-shedders of resistant *E. coli*. These super-shedder birds were shedding not only the highest concentrations of ampicillin-resistant *E. coli* (over 75% of faecal *E. coli* population), but also the isolates characterised from these birds showed the highest degree of multidrug resistance (typically resistant to SSS plus three to eight other drugs). The majority of these birds were on the conventional farm, but after controlling for farm of origin, a significantly higher number than expected were present on the organic farm, suggesting this was not just a farm-related phenomenon.

One of the limitations of this work is that data were collected from just two sites and therefore it is not possible to directly extrapolate these results to all meat chicken farms. However, the main conclusion of this work, that the contrasting farming practices were heavily influencing the dynamics of ABD resistant bacteria on the farms, is not contrary to other studies that have sampled greater numbers of farms but in less detail (Walk et al., 2007; Young et al., 2009).

The advantage of the detailed approach taken here is that data were collected regarding the resistance dynamics of *E. coli* throughout the rearing cycle of meat chickens. This level of detail has shown that the conventional birds were shedding multidrug resistant *E. coli* right up until slaughter, long after receiving prophylactic ABDs as day-old chicks. Furthermore, the close observation of ABD resistance dynamics on an organic farm has shown that the faecal shedding of ABD resistant bacteria can fluctuate even in the absence of ABD use, including an unexplained rise in the faecal concentrations of chloramphenicol-resistant *E. coli* after approximately 35 days of age. Furthermore, the organic birds were evidently encountering multi-resistant *E. coli*, but these strains did not come to predominate within the enteric *E. coli* populations as they did on the conventional farm. Therefore, in conclusion, the use of antibacterial drugs, particularly the prophylactic use in young chicks, clearly aligned

with patterns of resistance in generic *E. coli* present on a conventional broiler farm, but factors other than antibacterial drug use were operating to maintain fewer ABD resistant strains of *E. coli* on the organic farm.

Acknowledgements

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"Speak what you think now in hard words and to-morrow speak what to-morrow thinks in hard words again, though it contradict every thing you said to-day." *Ralph Waldo Emerson*

Chapter 6

Low prevalences of antibacterial drug resistance in Gram-negative bacteria isolated from poultry carcasses in New Zealand

Abstract

In 2005, a panel of experts convened by the New Zealand Food Safety Authority identified that a lack of data on antibacterial drug resistance in livestock was hindering the development of public health risk assessments in this field. The aim of this study was to provide baseline data on the prevalences and patterns of antibacterial drug resistance expressed by Gram-negative bacteria isolated from poultry carcasses in New Zealand. Isolates of *Escherichia coli* (n = 407) originating from carcass rinse samples were submitted by the testing laboratories affiliated to five major poultry processing plants from July to December in 2006. Isolates of *Campylobacter jejuni* (n = 193) originating from retail poultry carcasses in 2005 and 2006 were retrieved from the Massey University archives. Disc diffusion tests were used to ascertain the resistance phenotypes of the isolates. The majority of isolates (71.5% *E. coli* and 99% *C. jejuni*) were fully susceptible to the drugs that were tested, whilst 1% (n = 4) *E. coli* isolates expressed resistance to three or more drugs. The drugs to which resistance was detected in *E. coli* were: cephalothin (18.2% of isolates), ampicillin (4.4%), tetracycline (4.4%) and gentamicin (1.5%). Using ETests to ascertain the minimum inhibitory concentrations of the purportedly cephalothin-resistant *E. coli* isolates gave inconsistent results. This work has demonstrated that the prevalences of resistance shown by Gram-negative bacteria isolated from chicken carcasses in New Zealand are among the lowest reported around the world, and that the use of cephalothin as a marker of resistance to first-generation cephalosporins may not be appropriate for generic *E. coli* of animal origin.

6.1 Introduction

In 2005, an expert panel was convened by the New Zealand Food Safety Authority (NZFSA) to review the impact of the use of antibacterial drugs (ABDs) in agricultural settings on the development of ABD resistant human pathogens. One of the conclusions of the panel was that the development of accurate within-country risk assessments was being impeded by a lack of data regarding resistance in animal-associated bacteria in New Zealand (Expert Panel on Antibiotic Resistance, 2005).

The rate of notifications of campylobacteriosis in New Zealand had been on an upward trajectory since the early 1980s. By 2006, the annual notification rate had reached a peak of 383.5 per 100,000 population, the highest of any developed country in the world (Baker et al., 2007). Poultry meat was accountable for an estimated 80% of infections (Mullner et al., 2009a) and, with 969 people requiring hospitalisation for the disease in 2006, high prevalences of ABD resistance within *Campylobacter* associated with pountry would be of direct public health concern.

The incidence of salmonellosis was comparatively lower at an average of 46.1 notifications per 100,000 population per year between 1995 and 2001 (Thornley et al., 2003). However, this figure is still relatively high for a developed country, and source attribution modelling of data from 2003 estimated that 20% of human cases may originated from poultry (Mullner et al., 2009a).

Many ABD resistance mechanisms are encoded by genes carried on extrachromosomal DNA such as plasmids and transposons, and horizontal gene transfer between bacteria of the same and different species can act to spread these genes through bacterial populations (Salyers and Amabile-Cuevas, 1997; Sunde and Sorum, 2001). The conditions present within the intestinal tracts of mammals and birds are highly conducive for horizontal gene transfer (Blake et al., 2003), and enteric *Escherichia coli* have been shown to be adept at carrying and transferring plasmid-borne resistance genes Sunde and Sorum (2001). For these reasons, non-type-specific *E. coli* are commonly included within resistance surveillance programmes as markers of resistance within livestock populations, but also for their role as potential donors of resistance genes to more pathogenic bacteria (de Jong et al., 2009; Hammerum et al., 2007).

In light of the prominence of foodborne zoonoses in New Zealand and the recommendations of the NZFSA expert panel, a study was undertaken to collect baseline data on the occurrence of antibacterial drug resistant bacteria within the New Zealand broiler industry. The aim of this study was to survey poultry-associated ABD resistance in two zoonotic bacteria of direct impact on human health: *Salmonella enterica* and *Campylobacter jejuni*; as well as non-type-specific *E. coli*.

6.2 Materials and methods

6.2.1 Sampling strategy

A panel of *E. coli* isolates was assembled from carcass rinse samples collected in poultry processing plants in New Zealand between 17 July and 11 December 2006. These samples were routinely collected using a randomised sampling protocol as part of the National Microbiological Database (NMD) surveillance programme administered by the NZFSA (NMD Schedule 1). The aim of this study was to collect approximately 400 *E. coli* isolates across the five main processing plants in New Zealand. These five plants account for approximately 90% of the domestic broiler meat market. The sample size of 400 was chosen to provide 95% confidence limits of +/- 5% around a prevalence estimate of 50%, and acceptable precision around estimates of lower and higher prevalence. The number of samples that was requested from each processing plant was proportional to that plant's share of the domestic market.

Salmonella are only isolated in low numbers from all livestock species within the NMD programme, therefore all *Salmonella* isolated from broiler carcasses at the NMD laboratories during the sampling period were requested for inclusion within this study.

The panel of 193 *C. jejuni* was assembled from the isolate archive at Massey University where they had been stored at −80 °C. The archive had been assembled from *Campylobacter* isolated from retail chickens purchased in the Manawatu region on a monthly basis since March 2005. *Campylobacter jejuni* had been identified using PCR. The sampling strategy relating to that work has been published in detail by Mullner et al. (2009b). Using the criteria of only testing a single isolate from each carcass, a panel of 193 *C. jejuni* isolates were available for testing at the time of this study. The 95% confidence limits around an estimated prevalence of 50% using a sample size of 200 are +/- 7%.

6.2.2 Laboratory methods

The Enterobacteriaceae were submitted to the Massey University laboratory on agar slopes or Petrifilm plates by the NMD laboratories designated to each processing plant. A single isolate of typical morphology was selected from each plate and confirmation of identity for *E. coli* was undertaken using the indole test. The selected isolates were subcultured onto non-selective media and stored at −80 °C using a glycerol stabiliser.

Susceptibility testing of the Enterobacteriaceae isolates was carried out for 12 ABDs using disc diffusion assays in accordance with the guidelines published by the Clinical and Laboratories Standards Institute (CLSI), Baltimore, USA (CLSI document M31-A2). The details of the ABDs tested and the concentrations of discs used are shown in Table 6.1 on page 151, and the control strain used was *E. coli* ATCC 25922.

Forty four *E. coli* isolates showed unusual results to the 30 μg cephalothin disc with large, isolated colonies growing within an obviously demarcated clear zone.

In accordance with CLSI guidelines, colonies growing within the clear zones were subcultured onto plain media, their identity was confirmed using biochemical test strips (API 20E, bioMérieux, France) and they were tested using disc diffusion. Having confirmed that the colonies within the main zone were *E. coli* and upon obtaining similar disc diffusion results to the original isolates, the minimum inhibitory concentrations (MICs) of a randomly selected sub-set of 27 of these nonconforming *E. coli* isolates were ascertained using cephalothin ETest strips (AB Biodisk, Solna, Sweden). The ETests were run in duplicate for each tested isolate.

The *C. jejuni* isolates were tested against six ABDs using disc diffusion tests on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. The ABDs tested are shown in Table 6.4 on page 156, and the control strain used was *C. jejuni* ATCC 35360. The inoculated plates were incubated at 42 °C for 48 hours in a micro-aerobic atmosphere.

Although a number of papers have reported good correlation between disc diffusion and agar dilution assays for *Campylobacter* (Gaudreau and Gilbert, 1997; Gaudreau et al., 2008; Luangtongkum et al., 2007), due to difficulties in standardising the interpretation of results there is no standard CLSI protocol for disc diffusion methods for *Campylobacter* isolates (Fritsche et al., 2007). Full validation of the disc diffusion results in this study was not possible within the project budget, but a subset of 21 randomly selected isolates, together with the isolate identified as resistant to erythromycin using disc diffusion, were retested using broth microdilution as endorsed by CLSI. The subset was tested in triplicate using the EUCAMP microbroth dilution assay test (Trek Diagnostic Systems, East Grinstead, England).

6.2.3 Statistical analysis

Hierarchical clustering of the resistance phenotypes of the 407 *E. coli* isolates was undertaken by designating isolates as resistant or susceptible according to the CLSI breakpoints (shown in Table 6.1, page 151). Isolates of intermediate susceptibility were grouped with the fully susceptible ones. The distance matrix was calculated using a simple matching algorithm, the clustering algorithm used was the unweighted pair group method with arithmetic mean (UPGMA), and the results were displayed as a dendrogram. These methods were chosen as they produced the clearest visual summary of the phenotype data.

Logistic regression modelling was used to estimate the odds ratios of selecting a fully susceptible *E. coli* from each of the participating poultry processing plants in comparison to the plant with the highest percentage of fully susceptible *E. coli*.

Due to the apparent discordance between the *E. coli* disc diffusion results for ampicillin and cephalothin, unordered multinomial logistic regression models (Hosmer and Lemeshow, 2000) were fitted to ascertain whether cephalothin-resistance, as identified using disc diffusion, was associated with consistent shifts in the zone size

measurements for other drugs. In other words, did these variably cephalothin-resistant *E. coli* show similarities in terms of their susceptibility to other drugs that demarcated them from the other isolates? Each *E. coli* isolate was allocated to one of four groups to form a discrete, nominally-scaled outcome variable corresponding to: cephalothin-susceptible/ampicillin-susceptible (CSAS), cephalothin-susceptible/ampicillin-resistant (CSAR), cephalothin-resistant/ampicillin-susceptible (CRAS), cephalothin-resistant /ampicillin-resistant (CRAR). The multinomial model can be expressed as:

$$P(Y = c \mid \mathbf{x}) = \frac{e^{g_c(\mathbf{x})}}{\sum_{s=0}^{3} e^{g_s(\mathbf{x})}} \tag{6.1}$$

$$g_c(\mathbf{x}) = \beta_{c0} + \sum_{k=1}^{4} \beta_{ck} x_k \tag{6.2}$$

Here $P(Y = c \mid \mathbf{x})$ is the conditional probability of outcome category c given $\mathbf{x}$; $\mathbf{x}$ is a vector of the k covariates and the constant term; $g_c(\mathbf{x})$ is the logit of $\mathbf{x}$ for outcome category c; β_{c0} is the intercept and β_{ck} are the regression coefficients for the covariates x_k for the c^{th} outcome category. Outcome categories c and s are coded as zero for the reference category CSAS and one, two and three for CSAR, CRAS and CRAR respectively. The covariates $k = 1, \ldots, 4$ correspond to the zone size measurements for tetracycline, streptomycin, gentamicin and furazolidone, which were centred at their mean value. Drugs to which resistance was shown by only one isolate were excluded from the model.

The dendrogram was produced using BioNumerics 5.1 software (Applied Maths NV, Sint-Martens-Latem, Belgium). The multinomial and logistic regression models were fitted in R Version 2.9.2 (R Development Core Team, Vienna, Austria) (R Development Core Team, 2009).

6.3 Results

6.3.1 Enterobacteriaceae

The disc diffusion test results for the panel of 407 *E. coli* are displayed in Table 6.1 on page 151; the CLSI breakpoints for interpreting the zone sizes have been superimposed upon the table in varying shades of grey. The dendrogram in Figure 6.1, page 152, displays the resistance phenotypes that were present within the *E. coli* panel along with the number of isolates that showed each phenotype and the processing plants from which they originated. The most commonly occurring phenotype was full susceptibility to all drugs tested, and 291 (71.5%) of isolates fell into this category. Sixteen resistance-phenotypes were elucidated among the 116 resistant isolates with 95 (23.3%) of isolates showing resistance to one drug, 17 (4.2%) to two drugs, three (2.6%) to three drugs and one (0.9%) showed resistance to five of the drugs on the panel which included all three

Table 6.1: The disc diffusion zone sizes shown by a panel number of 407 *Escherichia coli* to each of 12 antibacterial drugs. The *E. coli* were isolated from five major poultry processing plants in New Zealand. The table displays the number of isolates that showed each zone size.

Drug	Disc[a] (μg)	%R[b]	95% CI	Zone size (mm)														
				≤ 6	7–11	12	13	14	15	16	17	18	19	20	21	22	23	≥ 24
Ampicillin[c]	10	4.4	2.4–6.4	17	1			1	2	5	24	39	49	50	54	51	44	70
Cephalothin[c]	30	18.2	14.1–22.3	6	9	12	20	27	29	33	43	44	36	39	29	28	17	35
Cefoxitin[c]	30	0	0–0.9												2	27	39	338
Cefotaxime[c]	30	0	0–0.9															407
Streptomycin[c]	10	1.7	0.5–2.9	5	2	9	20	71	81	102	65	35	14	2	1			
Gentamicin[c]	10	1.5	0.4–2.6		4	2		1		5	16	46	65	91	69	58	32	18
Kanamycin[c]	30	0.2	0.2–0.2	1						3	8	27	74	74	76	62	41	41
Tetracycline[c]	30	4.4	2.4–6.4	17	1					1				1	5	7	22	352
Sulfasoxazole[c]	300	0.7	0.1–1.4	3					1		2	2	4	5	11	11	21	347
Ciprofloxacin[c]	5	0	0–0.9															407
Chloramphenicol[c]	30	0	0–0.9									1	4	9	21	30	56	286
Furazolidone[d]	100				6	7	3	9	14	27	27	33	37	32	36	38	34	103
				Resistant zones					Intermediate zones						Susceptible zones			

[a] The concentration of drug in the disc.
[b] The percentage of isolates that were classified as resistant to that drug.
[c] Published CLSI breakpoint concentrations were used; the zone sizes falling within the resistant, intermediate and susceptible categories are highlighted in shades of grey.
[d] No CLSI breakpoint zone sizes were available.

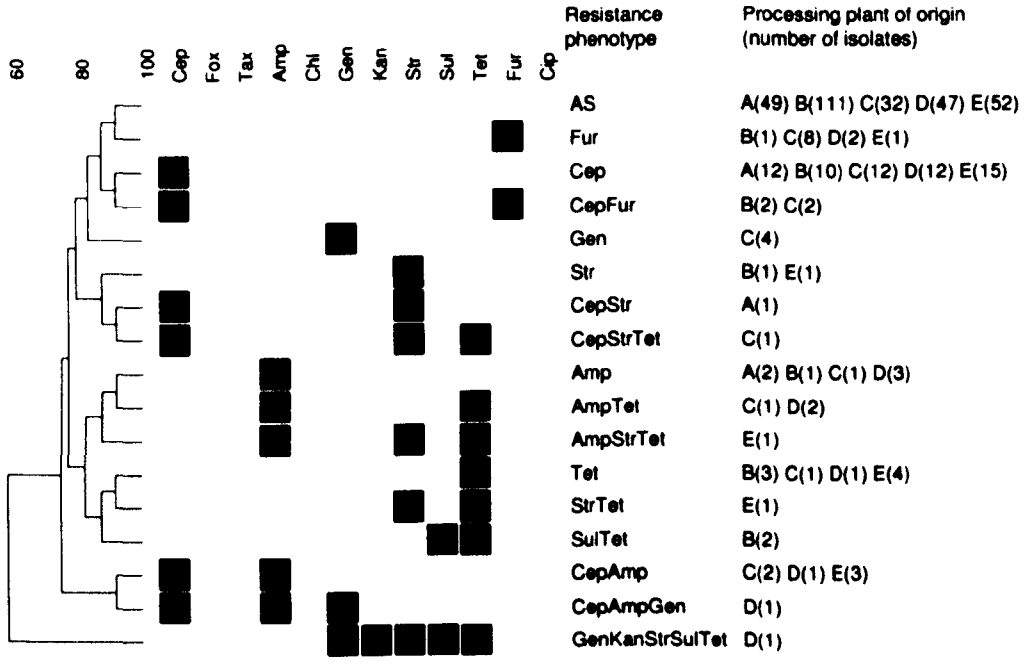

Figure 6.1: UPGMA dendrogram of disc-diffusion-derived antibacterial drug resistance phenotypes displayed by 407 *E. coli* isolates originating from five poultry processing plants (A-E)

Amp = ampicillin; Cep = cephalothin; Chl = chloramphenicol; Cip = ciprofloxacin; Fox = cefoxitin; Fur = furazolidone; Gen = gentamicin; Kan = Kanamycin; Str = streptomycin; Sul = sulfasoxazole; Tax = cefotaxime; Tet = tetracycline.

UPGMA = unweighted pair group method using arithmetic means.

Table 6.2: The results of a logistic regression model of the probability of picking an *E. coli* isolate that was susceptible to each of 12 drugs tested from the samples submitted from each of the five participating poultry processing plants (A-E).

Processing plant	%S[a]	Log-odds (β)	Std. error[b]	Z statistic	p value	Odds ratio (exp(β))	95% CI
B	84.7	1.71	0.24	-	-	-	-
A	76.6	-0.53	0.38	-1.39	0.17	0.59	0.28-1.26
D	67.1	-1.00	0.35	-2.84	0.005	0.37	0.18-0.73
E	66.7	-1.02	0.34	-2.99	0.003	0.36	0.18-0.70
C	50.0	-1.71	0.35	-4.92	0.001	0.18	0.09-0.35

[a] The percentage of isolates from that plant that were fully susceptible to all 12 ABDs.
[b] The standard error of the log-odds.

aminoglycoside drugs tested (gentamicin, kanamycin and streptomycin) in addition to sulfasoxazole and tetracycline.

Using the CLSI guidelines for interpretation of the zone size measurements, 74 (18.2%) of the *E. coli* isolates were designated as resistant to cephalothin and these isolates originated from all five of the participating processing plants (see Figure 6.1). Concurrent cephalothin-ampicillin resistance was seen in just seven isolates originating from plants C, D and E. Ampicillin-resistance was expressed by 18 isolates in total (4.4%), and these originated from all five plants. There were also 18 tetracycline-resistant isolates, originating from all plants except plant A. Resistance to gentamicin was seen in six (1.5%) isolates: four mono-resistant isolates from plant C and two multidrug resistant isolates from plant D, where multidrug resistance refers to an isolate expressing resistance to three or more drugs. None of the *E. coli* isolates were resistant to ciprofloxacin, cefoxitin, cefotaxime or chloramphenicol.

The percentage of *E. coli* isolates that were susceptible to all 12 antibacterial drugs varied between the individual processing plants with the range extending from 50 to 84.7%. Logistic regression modelling showed that the odds of picking an isolate from a chicken carcass that was susceptible to all 12 drugs were significantly lower for plants C, D and E compared to plant B (Table 6.2 on page 153).

Of the 74 cephalothin-resistant *E. coli* detected by disc diffusion, six (1.5% of the total 407 isolates) grew right up to the edge of the disc (zone size $\leq$6 mm) while the rest showed zone sizes of 10 to 14 mm (median 13 mm). However, scattered individual colonies grew within a mainly clear zone around the cephalothin disc for 44 (59.5%) of these 74 purportedly cephalothin-resistant isolates. Repeating the tests using the original stored isolates returned the same result, and the results of API20E tests confirmed that the scattered resistant colonies were *E. coli*. Disc diffusion tests of purified cultures of colonies within the main clear zone also produced clearly demarcated zones containing individual scattered colonies, implying that this result was not due

to a mix of two different strains within the original stored cultures. The duplicate ETests determined that only three of a subset of 21 of these non-conforming isolates showed a conclusive MIC $\geq$32 μg/ml in both tests. However, for six isolates, scattered colonies within a main zone of no-growth were also seen using Etest, and for three of these there was discordance between the duplicate test results, with a clear zone of no-growth corresponding to a MIC of 32 μg/ml for one of the two tests. Two of the jointly cephalothin and ampicillin-resistant isolates were also retested using ETest and the MICs of cephalothin for both isolates were $\geq$32 μg/ml.

Multinomial modelling highlighted a number of associations between the cephalothin and ampicillin resistance status of the *E. coli* isolates and the mean-centred zone size measurements obtained for other drugs (Table 6.3 on page 155). Compared to isolates that were susceptible to both cephalothin and ampicillin (CSAS), the parameter for the intercept (β_0) was negative for all three of the resistant categories: CSAR, CRAS, and CRAR. The mean zone size diameters for tetracycline, streptomycin, gentamicin and furazolidone were 25.7, 15.5, 20.2 and 21.0 mm, respectively, which were all larger than the breakpoint zone sizes for susceptibility for these drugs. Therefore, the negative intercept parameters show that isolates with susceptibility to all four of these drugs were less likely to show resistance to ampicillin, or cephalothin, or both. Furthermore, a 1 mm increase in zone size for tetracycline (i.e. an increase in susceptibility), was associated with a further decrease in the odds of an isolate being ampicillin-resistant but cephalothin susceptible (CSAR) compared to the CSAS baseline category. In other words, ampicillin-resistant isolates also showed smaller zone size diameters (a relative decrease in susceptibility) to tetracycline. In contrast, the isolates that were designated as cephalothin resistant (both CRAS and CRAR) were more likely to show reductions in susceptibility to gentamicin, but not tetracycline.

A total of three Salmonella were submitted during the sampling period. All three originated from a single processing plant (plant C) and each isolate was fully susceptible to all of the drugs on the Enterobacteriaceae panel.

6.3.2 *Campylobacter jejuni*

For all drugs except nalidixic acid, over 94% of isolates showed zone diameters of 24 mm or wider, indicating that the isolates were likely to be susceptible to these drugs (Table 6.4 on page 156). The median zone size for nalidixic acid was 24 mm with the full range of zone sizes being 16 to 34 mm. A single isolate showed total resistance to 15 μg erythromycin with growth extending to the edge of the disc ($\leq$ 6 mm).

Ascertaining the minimum inhibitory concentrations (MICs) for a subset of 22 *C. jejuni* isolates using microbroth dilution confirmed that these isolates were susceptible to nalidixic acid, ciprofloxacin, tetracycline and chloramphenicol (Table 6.5 on page 157). The MIC of the isolate that grew to the edges of the erythromycin disc was $\geq$ 32 μg/ml, therefore confirming the resistance of this isolate. Based on microbroth

Table 6.3: The estimated β coefficients, standard errors and odds ratios obtained from an unordered multinomial logistic regression model of cephalothin and ampicillin resistance status of 407 *E. coli* isolates against the mean-centred disc diffusion zone sizes (in mm) of four other antibacterial drugs.

Ceph/Amp resistance status	Other ABD covariates	Log-odds (β)	Std. error[a]	Odds-ratio (Exp(β))	95% CI
CSAS[b]		Ref[c]	-	-	-
CSAR[d]	Intercept	-3.68	0.37	-	-
	Tetracycline[e]	**-0.12**	**0.04**	**0.88**	**0.83–0.94**
	Streptomycin	-0.06	0.13	0.94	0.77 1.16
	Gentamicin	0.08	0.18	1.08	0.80 1.46
	Furazolidone	-0.004	0.08	1.00	0.87 1.14
CRAS[f]	Intercept	-1.74	0.15	-	-
	Tetracycline	-0.01	0.03	0.99	0.94 1.05
	Streptomycin	-0.04	0.08	0.96	0.84 1.08
	Gentamicin	**-0.26**	**0.07**	**0.77**	**0.69–0.87**
	Furazolidone	**-0.07**	**0.03**	**0.93**	**0.88–0.98**
CRAR[g]	Intercept	-4.30	0.50	-	-
	Tetracycline	0.01	0.10	1.01	0.86 1.19
	Streptomycin	0.18	0.23	1.20	0.83 1.75
	Gentamicin	**-0.43**	**0.12**	**0.65**	**0.54–0.79**
	Furazolidone	0.12	0.09	1.13	0.96 1.31

[a] The standard error of the log-odds.
[b] CSAS = Cephalothin-susceptible/Ampicillin-susceptible.
[c] Reference category of the outcome variable.
[d] CSAR = Cephalothin-susceptible/Ampicillin-resistant.
[e] The ABD covariates showing statistically significant associations with cephalothin/ampicillin resistance status were deemed to be those for which the log-odds were at least twice the standard error and the 95% CI around the odds ratios did not include 1. These significant associations have been highlighted in bold.
[f] CRAS = Cephalothin-resistant/Ampicillin-susceptible.
[g] CRAR = Cephalothin-resistant/Ampicillin-resistant.

Table 6.4: The disc diffusion zone sizes shown by 193 *Campylobacter jejuni* isolates to each of six antibacterial drugs. The isolates were obtained from retail chicken meat purchased in the Manawatu region of New Zealand. The table displays the number of isolates that showed each zone size.

Drug	Disc[a] (μg)	%R[b]	Zone size (mm)															
			≤ 6	7–14	15	16	17	18	19	20	21	22	23	24	25	26	27	≥ 28
Erythromycin	15	0.5	1[c]					1			2	3	4	8	19	12	21	122
Ciprofloxacin	5	0												1	1	1	2	188
Enrofloxacin	5	0												1		3	4	185
Nalidixic acid	30	0				2	2	3	8	12	13	19	24	22	33	17	12	26
Chloramphenicol	30	0									1	1				9	10	172
Tetracycline	30	0														1		192

[a] The concentration of drug in the disc.

[b] The percentage of isolates that were classified as resistant to that drug.

[c] No CLSI breakpoint zone sizes are available for *Campylobacter* species; however, CLSI recognise that isolates for which there is no clear zone of no growth ($\leq$6 mm) are resistant to that strength of drug and this zone is highlighted in grey.

Table 6.5: A comparison of the minimum inhibitory concentrations obtained using microbroth dilution with the zone sizes obtained using disc diffusion for 22 *Campylobacter jejuni* isolates.

Antibacterial drug	MIC-derived resistance category	Isolates (n)	Mean zone size (mm)[a]	Std. error[b]	95% CI[c]
Erythromycin	Resistant	1	≤ 6	-	-
	Intermediate	2	19.5	-	-
	Susceptible	19	29.3	0.63	27.9–30.6
Nalidixic acid	Susceptible	22	23.6	0.82	21.9–25.3
Ciprofloxacin	Susceptible	22	33.9	0.96	31.9–35.9
Tetracycline	Susceptible	22	37.7	0.93	35.8–39.7
Chloramphenicol	Susceptible	22	30.2	0.74	28.6–31.7

[a] The size of the zone of no growth around an ABD disc obtained using disc diffusion.
[b] The standard error for zone size.
[c] The 95% CI around the mean disc diffusion zone size.

dilution, two of the subset of 22 isolates demonstrated intermediate susceptibility to erythromycin. The disc diffusion zone sizes for these two isolates (18 and 21 mm) were smaller than the mean zone size of the isolates designated as susceptible to erythromycin (29.3 mm, 95% CI 27.9–30.6). Using the commercial microbroth dilution plates, one of the 22 isolates tested was also found to show resistance to streptomycin with an MIC of $> 16\,\mu g/ml$, but this drug had not been included in the original disc diffusion panel.

6.4 Discussion

Comparing the results of this study (Tables 6.1 and 6.4) with data from other countries reveals that the prevalences of ABD resistance in Gram-negative bacteria recovered from broiler chickens at slaughter in New Zealand are among the lowest reported in the world.

6.4.1 Enterobacteriaceae

For instance, 4.4% of *E. coli* isolates in this study were resistant to ampicillin, with an equal number (although mainly different isolates) showing resistance to tetracycline. These two drugs have been used in human and veterinary medicine for many years and bacteria carrying resistance to them are commonly isolated from both diseased and healthy people (Dominguez et al., 2002; Nys et al., 2004) and animals (Dai et al., 2008; Hendriksen et al., 2008), and even from free-living wildlife species (Livermore et al., 2001; Nascimento et al., 2003). Data collected from five central European countries in

2002 and 2003 found between country differences in the proportion of *E. coli* collected from chickens at slaughter that were resistant to ampicillin with a range from 38.6% to 71.1% (de Jong et al., 2009). Likewise tetracycline resistance ranged from 49% to 81.2%. Figures from Canada in 2005 were of similar magnitudes with 38.5% and 57.3% of isolates expressing ampicillin and tetracycline resistance, respectively (CIPARS 2005). Data from the Scandinavian countries were more similar to the New Zealand data with ampicillin resistance ranging between 4% and 17.1%, and tetracycline 3.7% and 6.5% (DANMAP 2006; NORM/NORM-VET 2006; SVARM 2004). Furthermore, the *E. coli* isolated from retail chicken meat samples across Scandinavia showed similar prevalences of resistance to those obtained from caecal samples from slaughtered birds with 0.7% to 7.6%, and 5% to 14.4% of isolates showing ampicillin and tetracycline resistance, respectively.

Although resistance was generally uncommon, there was a significantly lower probability of obtaining a susceptible isolate from three plants (C, D and E) compared to plant B, the plant with the highest percentage of fully-susceptible isolates. All the farms supplying an individual plant belonged to the same poultry company and therefore were using the same husbandry protocols. At the time of the study, all the farms supplying each of the five plants were administering sub-therapeutic doses of zinc bacitracin as an in-feed prophylactic measure against necrotic enteritis, and the use of other ABDs in the face of clinical disease on any farm was rare (see Chapter 7 and Pleydell et al. (2010a)). However, the farms supplying plants C, D and E had also used two other drugs (tylosin and avilamycin) as routine feed additives at various times over the six years preceding the study; whereas the farms supplying plants A and B had used zinc bacitracin exclusively over the same time period. It is possible that these company-level differences in in-feed ABD use may be the cause of the differences seen between-plants in the patterns of *E. coli* resistance. In a similar manner, between-plant differences in the serotypes and resistance phenotypes of *Salmonella* on chicken carcasses have been previously reported in the USA (Logue et al., 2003).

None of the *E. coli* isolates in this study were resistant to cefotaxime or cefoxitin. Sampling from the probability mass function of the binomial distribution, it can be estimated that if the true prevalence of cefotaxime or cefoxitin resistance within *E. coli* isolated from poultry carcasses was either 1% or 0.1%, then the probabilities of zero of 407 isolates showing resistance to that drug would be 0.02 or 0.67 respectively. Therefore, although this study has found no phenotypic evidence of CTX-M or AmpC extended spectrum beta-lactamases (ESBLs) among *E. coli* of poultry origin in New Zealand, this sample size cannot exclude the possibility that CTX-M or AmpC could be present at very low levels. This situation is worthy of future monitoring, due to the recent detection of CTX-M-15 enzymes in *E. coli* causing human urinary tract infections in New Zealand (Moor et al., 2008), and because other countries are starting to report the presence of genes encoding for the CTX-M family within *E. coli* isolated

from poultry (Costa et al., 2009; Dai et al., 2008; Smet et al., 2008).

Despite a low prevalence of ampicillin resistance and no evidence of the presence of ESBLs, evidence of cephalothin resistance was seen for 18.2% of *E. coli* isolates. This was surprising, because beta-lactamase enzymes with a spectrum of activity that includes cephalothin also degrade ampicillin (Li et al., 2007; Livermore, 1995). However, these observations are not unprecedented. One study of *E. coli* isolated from human sewage, animals and the environment found that, using disc diffusion, 20–30% of isolates originating from animal faeces and farm environments were resistant to cephalothin with just 4–6% of the same isolates showing ampicillin resistance (Sayah et al., 2005). In the work reported here, ETest results were also indeterminate for these atypical isolates, and stable cephalothin-resistance could only be confirmed in three of 21 isolates. Other researchers have also found disparities in the assessment of the susceptibility to cephalothin in *E. coli* isolates using different testing methods (Zhang et al., 2007).

One potential explanation for the appearance of a low number of resistant colonies on a plate of pure growth of a single strain could be that the strain was hypermutable in nature. However, whilst hypermutator strains due to defects within the methyl-directed mismatch repair system have been well described in *E. coli* (LeClerc et al., 1996), running disc diffusion tests on purified cultures of the potentially mutant colonies produced the same result of a clearly demarcated zone of no growth with scattered individual colonies growing within it. An alternative hypothesis could be that these strains are demonstrating epigenetic phase regulation, whereby the expression of a gene is switched on and off under differing environmental conditions (Deitsch et al., 2009), which has been documented for the expression of surface pili by uropathogenic *E. coli* in the presence of urine (Holden et al., 2007). Such systems have been most extensively studied in pathogenic species, and particularly *Neisseria* where it has been demonstrated that a single DNA methylation event can simultaneously switch multiple genes on and off, including genes responsible for efflux pump activity and those encoding resistance to specific antibacterial agents (Srikhanta et al., 2009). The results of the multinomial model indicating that these purportedly cephalothin-resistant strains, regardless of their ampicillin-status, were associated with a concurrent decrease in susceptibility to gentamicin (Table 6.3) implies that these difficult-to-classify strains do share common characteristics that differ from those of the more definitively cephalothin-susceptible strains.

A further hypothesis is suggested by previous observations that some strains of *E. coli* can produce acylesterase enzymes that are narrow-spectrum cephalosporinases that act on the side chain of the cephalosporin ring (Nishida et al., 1968). These strains do not produce beta-lactamases, but with increasing incubation periods they are able to grow in broths containing cephalothin in concentrations of up to four times the MIC (Nishiura et al., 1978). Growth curves from Nishiura's work showed

that an acylesterase producing strain of beta-lactamase-negative *E. coli* was in the exponential stage of growth in a broth containing cephalosporin at the MIC after 16 to 18 hours incubation: the incubation period for disc diffusion assays. As the acylesterase enzymes have not been considered to be a clinically important resistance mechanism in pathogenic bacteria (Ogawara, 1981) there is no recent literature in this area; however, it is possible that such strains could be represented within the diversity of *E. coli* populations residing in the intestinal tracts of mammals and birds.

However, regardless of the actual mechanism behind these observations, it would appear that cephalothin is not suitable for use as a marker of resistance to first-generation cephalosporins in generic *E. coli* of animal origin.

With respect to *Salmonella*, this work confirms that *Salmonella* are rarely isolated from freshly dressed broilers in New Zealand within the NMD programme, and the three isolates that were tested were not expressing ABD resistance.

In this study, the Enterobacteriaceae isolates were obtained from carcass rinse samples that had been collected after the carcasses had passed through an immersion chiller. This sample type was chosen for economic and logistic reasons as these samples were already being routinely collected for the NZFSA's National Microbiological Database. However, many of the surveillance programmes in other countries utilise caecal contents or cloacal swabs taken from broiler chickens at slaughter (CIPARS 2005; DANMAP 2006; SVARM 2004). Therefore, potential problems with direct comparison of results could arise if the population of *E. coli* present in a carcass rinse is not representative of the population within the lower intestinal tract of the bird or, alternatively, if immersion chilling exerts a selective pressure upon the resistance phenotypes of *E. coli* on the carcass.

There is a scarcity of data assessing the genotypes and phenotypes of *E. coli* at different stages within a processing plant, however there are some published papers relating to *Campylobacter*. One study demonstrated that the subtypes of *Campylobacter* present in the live birds immediately prior to slaughter did correspond to those present on the carcasses (Simmons et al., 2008). However, others have shown that populations of *Campylobacter* on post-chill carcasses were less genetically diverse than those on pre-chilled (Hunter et al., 2009), and that certain subtypes were more adept at surviving within the processing plant and therefore more likely to contaminate carcasses of subsequent flocks (Allen et al., 2007; Newell et al., 2001). Whether there are similar changes in the genetic subtypes of *E. coli* present along the processing chain, and whether such changes in subtypes present on a carcass would bias the patterns of ABD resistance elucidated by a surveillance project remains to be tested. However, from a public health point of view, isolates taken from the carcasses of chickens may be preferable, in terms of being potentially more representative of the possible risks to consumers compared to isolates from the caeca.

6.4.2 *Campylobacter jejuni*

Disc diffusion methods found that 192 of 193 *C. jejuni* isolates were fully susceptible to the six drugs tested, with the exception being a single isolate that grew to the edge of the erythromycin disc (Table 6.4). The use of microbroth dilution assays for a subset of isolates confirmed that isolates with wide zone sizes were susceptible to the drugs on the panel and that one isolate (0.5% of the total tested) was resistant to erythromycin. In comparison, 11% of *C. jejuni* isolated from retail poultry meat reared in Denmark in 2007 were resistant to nalixidic acid and ciprofloxacin (DANMAP 2007). Whilst, 42% of *C. jejuni* isolated from chicken imported into Denmark were resistant to those two quinolone drugs in that year. In Sweden, 5-7% of *C. jejuni* isolated from chickens at slaughter showed resistance to nalidixic acid, enrofloxacin or ciprofloxacin (SVARM 2004). In central Europe, 10.6–83.3% of *C. jejuni* isolated from slaughtered poultry were resistant to ciprofloxacin, and 23.5–58.3% to tetracycline, but no resistance was seen in any of five countries to erythromycin (de Jong et al., 2009). In contrast to the European trends, the *C. jejuni* isolated from retail chicken meat in Canada were fully susceptible to the quinolone drugs, but 5.7% were resistant to erythromycin (CIPARS 2005). Elsewhere in the world resistance in *Campylobacter* can be even more prominent, in a Korean study, for instance, 87.9% of 594 isolates of *Campylobacter* spp. isolated from raw retail chicken meat were resistant to ciprofloxacin, 87.2% to tetracycline and 19.4% to erythromycin (Kang et al., 2006).

6.4.3 Concluding summary

This study has provided base-line data on ABD resistance in Gram-negative bacteria present on post-chill chicken carcasses in poultry processing plants in New Zealand. It demonstrated that the majority of *Campylobacter jejuni* and non-type-specific *Escherichia coli* isolates were fully susceptible to first-line and second-line ABDs. However, there are between-plant differences with increased prevalences of resistance being detected in *E. coli* obtained from some plants. The study has also provided evidence that the use of cephalothin as a marker of resistance to first-generation cephalosporins may produce inconsistent results if applied to generic *E. coli* of animal-origin. The data provided by this study has contributed to the scope and design of a national surveillance programme of ABD resistance in bacteria of animal origin in New Zealand, and provides a statistically valid set of results for comparison with data collected in the future.

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"Science is the tool of the Western mind and with it more doors can be opened than with bare hands. It is part and parcel of our knowledge and obscures our insight only when it holds that the understanding given by it is the only kind there is." *Carl Jung*

Chapter 7

Evidence for the clustering of antibacterial drug resistance phenotypes of enterococci within integrated poultry companies[1]

Abstract

From July-December 2006, a panel of 401 enterococci was isolated from carcass rinse samples collected in five poultry processing plants in New Zealand. Agar diffusion assays for nine antibacterial drugs were used to obtain a resistance phenotype for each isolate. Hierarchical clustering techniques and diversity indices showed a high diversity of resistance phenotypes within each plant, with populations of *Enterococcus faecalis* showing greater heterogeneity than *Enterococcus faecium*. Bayesian modelling identified three clusters of phenotype patterns within the panel: the *E. faecium* isolates showed a high probability of containing two distinct clusters, whilst the *E. faecalis* isolates all grouped together to form the third cluster. The validity of these three clusters was examined using pairwise fixation indices and analysis of variance. Comparing the three clusters to the structure of the participating companies showed that, resistance phenotypes for *E. faecium* isolated from processing plants that were geographically separated but were operated by the same integrated poultry company were more similar than *E. faecium* isolated from unconnected companies. Company-level management factors, such as the routine use of antibacterial drugs and the genetic line of birds reared, mirrored the structure of these clusters; thus indicating that company-level factors were the dominant selective pressures upon resistance phenotypes across all operating units within these integrated poultry companies.

[1] A manuscript based on this chapter has been accepted for publication and is available as: E. Pleydell, L. Rogers, E. Kwan, and N. French. Evidence for the clustering of antibacterial resistance phenotypes of enterococci within integrated poultry companies. *Microbial Ecology*, 59(4):678–688, May 2010a. doi: 10.1007/s00248-009-9625-6

7.1 Introduction

Antibacterial drug resistance phenotypes are a well established clinical tool for determining the appropriate chemotherapeutic treatment for a bacterial infection (CLSI document M31-A3). Resistance phenotypes are also frequently used to assess the probable source of faecal contamination within an environment (Ebdon and Taylor, 2006; Wiggins et al., 2003). However the interpretation of resistance phenotypes in epidemiological studies is often limited to determining the percentage of resistance shown to each of an array of drugs tested.

Many epidemiological studies and surveillance programmes use indicator organisms such as *Escherichia coli* or *Enterococcus* species as a means of assessing the drugs to which bacteria are expressing resistance in a given environment (DANMAP 2006, 2007; Hershberger et al., 2005). These bacterial species are readily isolated from the intestinal tracts of mammals and birds, and from environments that have been contaminated by faeces (Graves et al., 2002). They are also adept at acquiring and transferring resistance genes, and therefore are used as markers of the antibacterial resistance genes that are present within that environment (Rizzotti et al., 2005). Investigating patterns within the resistance phenotypes of these indicator organisms may offer a means for identifying the major selective pressures influencing ABD resistance present in an environment and this, in turn, could be useful for developing evidence-based resistance control programmes. However, studies that have assessed the resistance phenotypes within populations of indicator bacteria on livestock farms tend to report a high degree of heterogeneity with little immediate evidence of links between the phenotypes present and factors such as species of origin, antibacterial drug use or farm management practices (Aarestrup et al., 2000a; Garcia-Migura et al., 2005; Klein et al., 1998). Nonetheless, it is now well established that the frequent use of antibacterial drugs within a given environment, such as a hospital or a livestock farm, will select for increased ABD resistance in the bacterial populations residing within that environment (Bantar et al., 2003; Emborg et al., 2004; Jensen et al., 2006; Polk, 2003). This being the case, one would also expect to be able to distinguish between the resistance phenotypes of bacteria replicating in environments of differing selective pressures such as antibacterial drug use.

In New Zealand, poultry farms within a livestock company will be operating under standardised husbandry procedures that will include protocols relating to the use of antibacterial drugs on those farms. The feed mills and poultry processing plants are also company-owned, thus allowing for potential common sources of bacteria within a company, as well as possible cross contamination of carcasses. Furthermore, the larger livestock companies operate their own transport systems, thereby offering potential transmission routes for spreading bacteria and resistance mechanisms throughout the company network.

No routine surveillance of antibacterial resistance in livestock has previously been

undertaken in New Zealand, and there is little published data available (Nulsen et al., 2008). This lack of data impedes the assessment of the possible risks to human health posed by resistant bacteria carried by livestock (Expert Panel on Antibiotic Resistance, 2005). Therefore, the aims of this study were to assess the prevalences of resistance to nine antibacterial drugs displayed by the Gram-positive indicator organisms *Enterococcus faecium* and *Enterococcus faecalis* originating from freshly dressed, post-chill chicken carcasses in five poultry processing plants. Statistical methods commonly used in population genetics studies were then applied to the dataset of resistance phenotypes to look for evidence of biologically plausible clustering of antibacterial resistance phenotypes at the processing plant or company level.

7.2 Methods

7.2.1 Sampling protocol

Between 17 July and 11 December 2006, 401 enterococci, *E. faecium* and *E. faecalis*, were isolated from freshly dressed poultry carcasses at five major poultry processing plants in New Zealand. These five plants account for over 90% of the domestic market. Four plants were located across the North Island and one on the South Island. The carcass rinse samples were being routinely collected at each plant on a daily basis as part of the National Microbiological Database (NMD) surveillance programme operated by the New Zealand Food Safety Authority (NZFSA) (NMD Schedule 1). Trained plant workers randomly select three post-chill carcasses per day for whole carcass rinse sampling in accordance with a standard protocol. The samples are submitted to NMD-registered testing laboratories for the ongoing surveillance of food-borne bacteria. For this study the NMD-registered laboratories sent 1 ml of rinse fluid in 9 ml of azide broth by courier-post to the Massey University laboratory; the samples were held at 4 °C during transportation.

A minimum sample size of 400 was chosen to provide 95% confidence of estimating the prevalence of resistance to a given antibacterial drug with a precision of +/-5% around a true prevalence of 50% and with acceptable precision at lower and higher prevalences. The number of samples requested from each processing plant was proportional to that plant's share of the domestic market and the NMD-laboratories were asked to supply a specified number of samples on a weekly basis.

Subsequent to the statistical and cluster analyses, data were collected concerning the drug use patterns and the genetic line of birds used by the companies operating the five plants.

7.2.2 Laboratory methods

The azide broths were incubated for 24 to 48 hours at 42 °C. Incubated broths in which turbidity was seen were then subcultured onto Slanetz and Bartley chromogenic

agar (Fort Richard Laboratories, Auckland, New Zealand) and incubated at 42 °C for 48 hours. Isolates were selected upon the basis of their colour and morphology, and were presumptively confirmed as enterococci using the Remel^{TM} PYR test kit (Thermo Fisher Scientific, Lenexa, Kansas) to detect pyrrolidonyl arylamidase activity. Definitive confirmation of identity and the simultaneous differentiation of *E. faecium* and *E. faecalis* was performed using real-time PCR utilising previously published primers (Depardieu et al., 2004) and thermal melt curve analysis. Of the genetically confirmed isolates, the first 401 to arrive at the laboratory underwent susceptibility testing.

Susceptibility testing was performed using Kirby-Bauer disc diffusion in accordance with the guidelines published by the Clinical and Laboratories Standards Institute (CLSI), Baltimore, USA (CLSI document M31-A3). A panel of 9 drugs were tested: ampicillin, chloramphenicol, gentamicin, tetracycline, erythromycin, vancomycin, quinupristin-dalfopristin, zinc bacitracin, and furazolidone. The control organism used was *E. faecalis* ATCC 29212 and, wherever possible, CLSI criteria were used for the interpretation of the measured zone sizes. In the absence of CLSI breakpoints for zinc bacitracin and furazolidone, isolates which produced no detectable zone (zone size $\leq$ 6 mm) were classified as 'resistant'; those with zone diameters of $\geq$ 18 mm or more were classified as 'susceptible'; and the rest were classified as 'intermediate'. In fact, the majority of isolates (95% and 80% respectively) produced zone sizes of $\leq$ 6 mm for zinc bacitracin and furazolidone.

Isolates with zone diameters within the 'resistant' category for vancomycin were sent to the Communicable Disease Group within the Environmental Science and Research Institute Ltd (ESR), New Zealand, for further characterization. Minimum Inhibitory Concentrations (MICs) to vancomycin and teicoplanin were determined using ETest®(AB bioMérieux, Solna, Sweden) on Mueller-Hinton agar (Difco/Becton Dickinson, Sparks, Maryland, USA) and interpreted in accordance with CLSI standards (CLSI document M31-A2). For isolates with a vancomycin MIC <4 mg/L, the presence and type of van gene was investigated using PCR as per published methods (Clark et al., 1993). To allow for comparison with vancomycin-resistant clones of *E. faecalis* previously isolated in New Zealand (Manson et al., 2003), DNA analysis was undertaken using Pulsed Field Gel Electrophoresis (PFGE) after restriction digestion with *Sma*I.

7.2.3 Analytical methods

Initially, the resistance phenotypes were coded in three ways:

- A string of nine binary elements where "1" corresponds to resistant and "0" to susceptible or intermediate.
- A string of nine letters "R","I" and "S" (RIS) determined using the breakpoints displayed in Table 7.2 on page 171.

- A one-dimensional vector of nine zone size measurements in millimetres.

Hierarchical clustering of resistance phenotypes was undertaken to provide an initial visual assessment of phenotypes against bacterial species and processing plant of origin. In order to produce a succinct dendrogram, a simple matching coefficient algorithm was utilised to produce a similarity matrix from the binary-coded phenotypes. The linkage rule utilised was the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

The RIS coding provided a good compromise between the more restrictive binary and the over-diverse numeric phenotype codes. Therefore, for the rest of the analyses the tertiary-coded (RIS) resistance phenotypes were used as analogues for standard haplotypic data. The diversity of phenotypes was measured using two indices: the standard diversity and mean number of pairwise differences. The standard diversity (D) of resistant phenotypes from a given processing plant is the probability that two randomly chosen isolates from that plant will show different phenotypes (Nei, 1987). The estimated pairwise differences ($\hat{\pi}$) is the mean number of differences in RIS categories shown by all pairs of isolates obtained from the same plant (Tajima, 1993).

In order to estimate the number of clusters of RIS phenotypes within the set of 401 isolates, a Markov chain Monte Carlo (MCMC) model-based clustering approach was taken using the methods of Pritchard et al. 2000. This model implements frequency-based clustering, whereby each isolate is probabilistically assigned to each of a pre-specified number of clusters in dependence upon the frequencies of RIS categories for each of the nine antibacterial drugs within each cluster. The model assumes that the frequencies of resistance categories within each model-identified cluster are at a steady state and that the frequency of an RIS category for one drug is not dependent upon that of another drug. Due to the high diversity of resistance phenotypes within each processing plant, the model settings implemented were those recommended for dealing with subtle population structures (Falush et al., 2003). An admixture model was chosen, meaning that the model allowed for the possibility that the bacterial populations within each of the five processing plants may not be in total isolation from each other. The model inferred the degree of admixture (alpha) from the data itself with the initial value of alpha being set to 1, i.e. a high degree of admixture. Ten prior groupings of isolates were used to seed the model corresponding to two separate populations of *E. faecium* and *E. faecalis* within each of the five processing plants. The model allowed for the possibility that the frequencies of resistance categories were similar between different clusters by using correlated frequencies. The model was run using different values (1 to 10) for the number of clusters (K) within the data: $K = 1$ would imply that there were no clusters of phenotypes and the isolates from each plant were equally heterogeneous; whereas $K = 10$ would imply that the 10 groups used to initialise the model were all phenotypically distinct from each other. For each value of K the model was run 100 times. Each run consisted of a burn-in period of 50,000

iterations and thereafter sampling of the chain occurred over a further 50,000 iterations.

The MCMC models return an estimate of the log of the posterior probabilities of obtaining the observed data (D) given the K-value stipulated for that model ($\ln P(D)|K$) (Pritchard et al., 2000). In order to determine the most likely value of K, the mean values and standard deviations of $\ln P(D)$ were calculated for each batch of 100 runs for K = 1–10. The q-matrix for the individual isolates, derived from the K value returning the highest posterior probabilities, was visualised as a confluent, stacked bar plot using the *distruct* program, available from the University of Michigan (Rosenberg, 2004). The run of the model chosen to draw the plot was the run with the $\ln P(D)$ value closest to the mean value of all 100 runs for that value of K.

The validity of the three MCMC-derived clusters was assessed by calculating the pairwise fixation indices (F_{ST}) between all possible pairings of the ten prior groupings of isolates (Reynolds et al., 1983). In these analyses, the F_{ST} values represent a measure of the degree of variation in resistance phenotypes between each pair of groups: such that a value of 0 would indicate that the two groups were indistinguishable, whilst the theoretical value of 1 would indicate that the two groups were totally distinct from each other.

An analysis of molecular variance model (AMOVA) was used to partition the total variance within the 401 RIS phenotypes into the covariance components of the imposed hierarchy, i.e. between the model-derived clusters, between processing plants within the clusters, and within the individual plants. AMOVA was calculated using a non-parametric permutation approach (Excoffier et al., 1992).

The dendrogram was constructed using Bionumerics® Version 5 (Applied Maths Inc., Austin, Texas). The MCMC clustering model was run using *structure* Version 2.2 (Falush et al., 2003, 2007). The diversity indices, pairwise fixation indices and AMOVA were estimated using Arlequin Version 3.1 (Excoffier et al., 2005).

7.3 Results

7.3.1 Descriptive analysis

Enterococcus faecium or *E. faecalis* were recovered from 68% of the samples submitted. Differences in recovery were seen between processing plants: four plants yielded either species from 68-78% of samples, but enterococci were only recovered from 45% of samples submitted from plant C (Table 7.1 on page 170). Genetic confirmation of identity was obtained for 425/459 presumptive *E. faecium* or *E. faecalis*. Of these, 362 (80%) were identified as *E. faecium*, and differences in the proportions of the two species were seen between plants with *E. faecium* ranging between 60% to 91% of isolates (Table 7.1).

Table 7.2 on page 171 shows the agar diffusion results for the full panel of 401 enterococci that were tested. Bacitracin (Bac) resistance was high in both species with

Table 7.1: A comparison of the isolation frequencies and diversity of antibacterial resistance phenotypes of *E. faecium* and *E. faecalis* isolated from carcass rinse samples collected at five poultry processing plants (A–E)

	Processing plant of origin				
	A	B	C	D	E
% samples from which enterococci grew	78	74	45	71	68
Proportion of isolates identified as *E. faecium*	0.91	0.84	0.60	0.64	0.85
Enterococcus faecium					
Number of isolates tested	77	109	27	47	58
Standard diversity[a]	0.83	0.91	0.92	0.94	0.96
(SD sampling variance)	(0.03)	(0.02)	(0.03)	(0.02)	(0.01)
Pairwise differences[b]	1.82	2.02	2.03	2.39	2.40
(PD sampling variance)	(1.06)	(1.15)	(1.47)	(1.32)	(1.32)
Enterococcus faecalis					
Number of isolates tested	8	20	18	25	12
Standard diversity	1.00	0.98	0.97	0.97	0.98
(SD sampling variance)	(0.06)	(0.02)	(0.03)	(0.02)	(0.04)
Pairwise differences	3.21	3.13	2.8	3.02	2.72
(PD sampling variance)	(1.83)	(1.69)	(1.54)	(1.62)	(1.55)

[a] Standard diversity is the probability that two randomly chosen isolates from the same plant are of different resistance phenotypes.

[b] Pairwise differences are the mean number of differences in resistance to each of the nine drugs shown by all pairs of isolates collected from the same plant

Table 7.2: The distributions of disc diffusion zone sizes for 318 *E. faecium* and 83 *E. faecalis* isolates against nine antibacterial drugs

Drug (disc)[a]	Spp.[b]	%R[c]	[95% CI]	Zone size (mm)															
				≤ 6	7–9	10	11	12	13	14	15	16	17	18	19	20	21	22	≥ 23
Amp[d]	1	12.3	[8.9–16.4]						3	9	13	14	14	17	18	15	18	27	170
(10 μg)	2	0	[0–0.04]											1	3	4	7	5	53
Chl[d]	1	1.9	[0.7–4.0]	2				4		4	3	4	10	20	24	41	38	32	136
(300 μg)	2	0	[0–0.04]							3	3	7	9	3	12	12	9	10	15
Gen[d]	1	0.3	[0.01–1.7]	1								1	6	15	16	23	36	42	177
(200 μg)	2	2.4	[0.29–8.4]	2				1		3	3	13	12	16	13	9	2	6	3
Tet[d]	1	32.1	[27.0–37.5]	48	4	9	15	15	3	8	8	3	4	4	11	7	2	12	165
(30 μg)	2	48.2	[37.1–59.4]	12	4	13	6	1	2	2	3		1		2	3	7	3	24
Ery[d]	1	36.5	[31.1–42.0]	109		2		3	2	2	3	9	8	18	25	15	8	9	105
(15 μg)	2	42.2	[31.4–53.5]	10	1	3	7	7	7	10	4	2	4	3	7	8	4		8
Van[d]	1	0.3	[0.01–1.7]							1		2	3	4	7	8	26	50	217
(30 μg)	2	3.6	[0.8–10.2]	1						2	4	11	29	14	7	7	1	2	5
QDn[d]	1	13.8	[10.2–18.1]	1	2	4	7	6	6	6	12	23	27	36	46	32	42	22	46
(15 μg)	2	83.1	[73.3–90.5]	1	1	9	20	26	9	1	2	2	1	2	1	2	2		4
Bac[e]	1	97.2	[94.7–98.7]	309	2	1	2								2				2
(10 iu)	2	88.0	[79.0–94.1]	73			1				1	1		2		1	2	1	1
Fur[e]	1	95.6	[92.7–97.6]	304				2		1	2	3	2	2	1	1			
(100 μg)	2	20.5	[12.4–30.8]	17		2	3	5	12	8	9	7	6	8	4	1		1	
				Resistant zones					Intermediate zones						Susceptible zones				

[a] Concentration of drug in the disc.
[b] Species of *Enterococcus*: 1 = *E. faecium*; 2 = *E. faecalis*.
[c] Percentage of isolates of that species that were classified as resistant to that drug.
[d] CLSI breakpoint zone sizes were used.
[e] No CLSI breakpoint zone sizes were available.

97% *E. faecium* and 88% *E. faecalis* producing zone sizes $\leq$ 6 mm. Furazolidone (Fur) resistance was also very common in *E. faecium* with 96% isolates with zone sizes $\leq$ 6 mm, but was considerably lower in *E. faecalis* at 21%. The prevalences of resistance to tetracycline (Tet) and erythromycin (Ery) were similar for both species ranging from 32% to 48%. No *E. faecalis* isolates, but 12% of *E. faecium* isolates were resistant to ampicillin (Amp). Quinupristin-dalfopristin (QDn) resistance, which is commonly associated with *E. faecalis*, was significantly more frequent within the *E. faecalis* isolates (83%) compared with the *E. faecium* (14%) (χ^2 = 156.7; 1 df; n = 402; $P \leq 0.001$). Furthermore, three isolates showed resistance to high-levels (200 μg disc) of gentamicin (Gen) and four isolates were found to have disc zones that fell within the resistant category (6 to 14 mm) for vancomycin (Van).

Further characterisation work showed that the *E. faecalis* isolate with a zone size for vancomycin of $\leq$ 6 mm had an MIC of $\geq$ 256μg/ml for both vancomycin and teicoplanin, whilst all three isolates that had shown zone sizes of 14 mm to vancomycin had MICs of $\leq$ 4μg/ml for both drugs, thereby identifying them as susceptible to vancomycin. The *vanA* vancomycin-resistance gene was detected in the vancomycin-resistant *E. faecalis*, and the PFGE profile of this isolate was indistinguishable from the profiles of a clone of vancomycin-resistant *E. faecalis* previously described within the New Zealand broiler industry (Manson et al., 2003).

7.3.2 Statistical analysis

The dendrogram in Figure 7.1 (page 173) shows the full array of resistance phenotypes that were elucidated using the breakpoints for resistance that are shown in Table 7.2. The dendrogram shows that whilst some resistance patterns were only detected in one or other of the *Enterococcus* species, many were displayed by isolates of both species. The dendrogram also provides an easy visual assessment of the degree of multidrug resistance seen in the isolates. Of the 401 isolates tested, only one *E. faecalis* was fully susceptible to all nine drugs. At the other end of the spectrum, two *E. faecium* isolates (from plants A and D) were resistant to six of the nine drugs, and these two isolates displayed similar phenotypes: 'AmpTetEryQDnBacFur' and 'AmpChlTetEryBacFur'. The most commonly occurring phenotype for the *E. faecium* isolates was the 'BacFur' pattern and this was detected in a high proportion of isolates from plants A and B. The *E. faecalis* isolate that was confirmed to be carrying the *vanA* vancomycin-resistance gene had a unique, bi-resistant phenotype of 'EryVan', and showed zone sizes of 21 mm and 16 mm to bacitracin and furazolidone, respectively.

The calculated diversity indices showed that phenotype diversity was high across the plants and species (Table 7.1). The majority of *E. faecalis* isolates within a given plant displayed a unique phenotype and the median number of differences in RIS categories between isolates within a plant was 3.02. However, for the *E. faecium* isolates, the standard diversity values (range 0.83 to 0.96) and the median number of differences

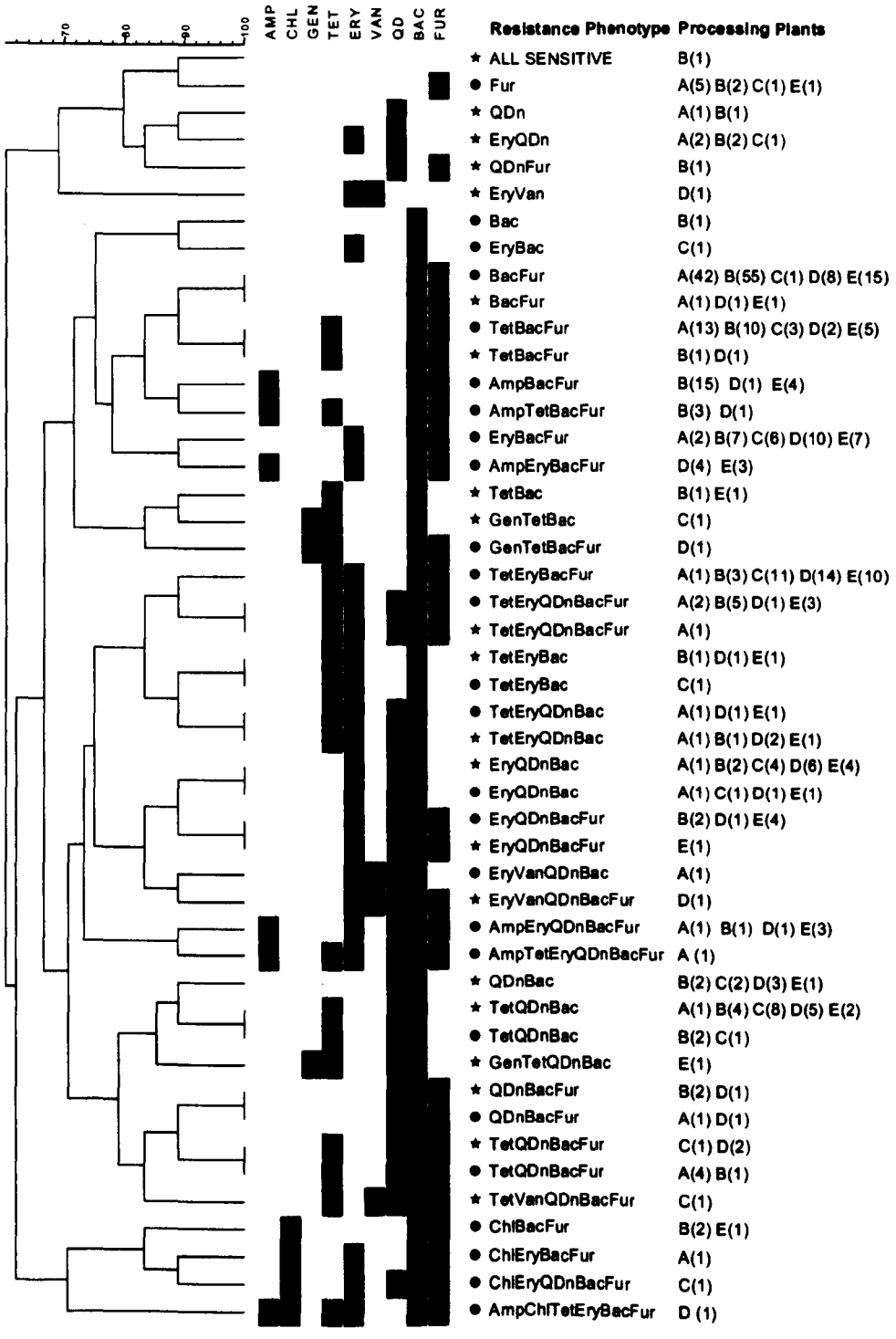

Figure 7.1: UPGMA dendrogram of disc-diffusion-derived antibacterial resistance phenotypes displayed by 401 *Enterococcus isolates*

Solid circles = *E. faecium*; Solid stars = *E. faecalis.*

Amp = ampicillin; Bac = zinc bacitracin; Chl = chloramphenicol; Ery = erythromycin; Fur = Furazolidone; Gen = high-level gentamicin; QDn = quinupristin-dalfopristin; Tet = tetracycline; Van = vancomycin.

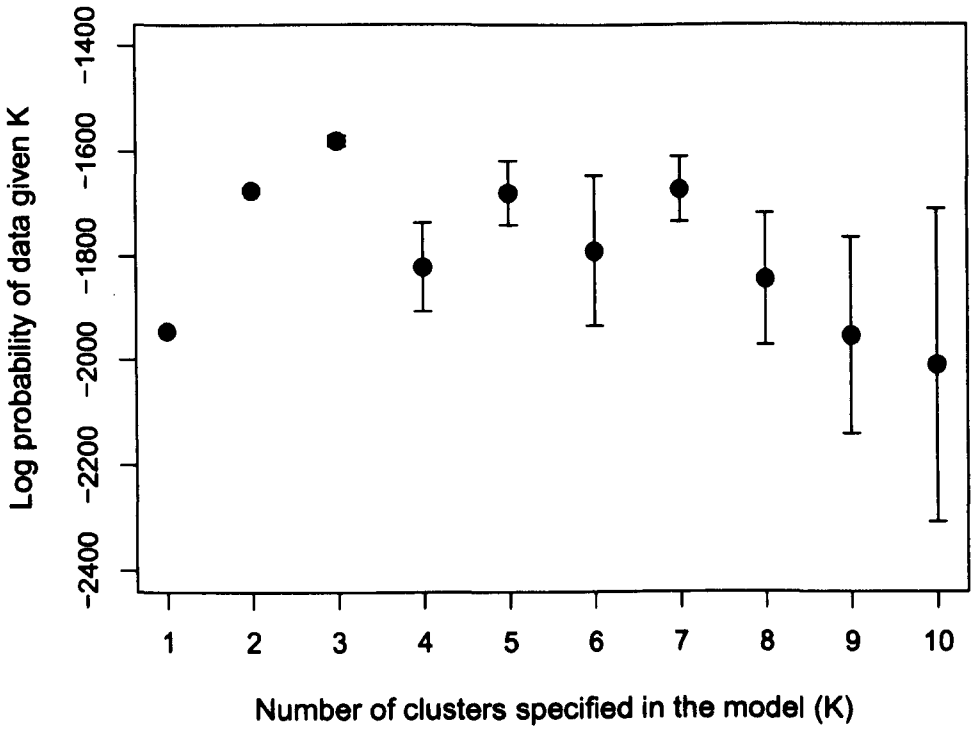

Figure 7.2: Error bar plot showing the mean values and standard deviations of the posterior probabilities of the data as a function of the number of clusters (K) specified in a series of MCMC models that probabilistically assigned 401 *Enterococcus* isolates into K clusters that were characterised by the patterns and frequencies of resistance to each of nine antibacterial drugs

Points represent the mean values of 100 runs of the model for each value of K.
Bars represent two standard deviations of the mean.

(2.03) were comparatively lower than the equivalent values for *E. faecalis*.

Using the MCMC model to assess the number of clusters (K) of phenotypes within the data showed that the mean log of the posterior probabilities rose to a maximum value at $K = 3$ before decreasing again with a corresponding increase in the variance (Figure 7.2 on page 174). Running the model using different sets of initial parameters continued to return the highest log probability when $K = 3$ and, in some cases, the model became less stable with some runs failing to converge.

The individual q-matrices from the model of $K = 3$ are presented as a confluent stacked bar plot in Figure 7.3. The predominantly blue region on the right hand side of the plot corresponds to the *E. faecalis* isolates that, due to the high diversity of phenotypes in this species, showed similar probability profiles regardless of the processing plant of origin. The *E. faecium* isolates, however, were split into two clusters: the isolates from plants A and B showed the highest probability of being in cluster 1 (yellow), and the isolates from plants C and D showed a higher probability of being in cluster 2 (red-brown). The isolates from plant E were more mixed with some showing

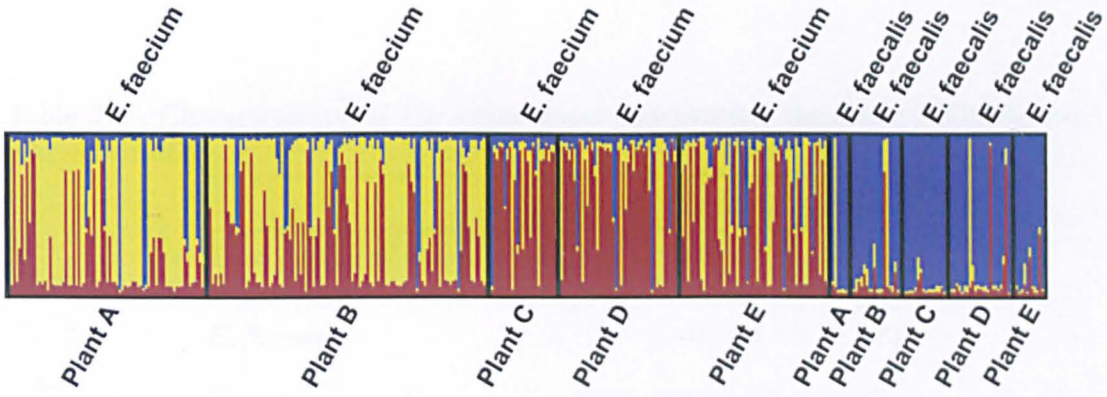

Figure 7.3: Confluent stacked bar plot showing the probabilities (y axis) that each of the individual 401 *Enterococcus* isolates (x axis) belonged to each of three MCMC-model-derived clusters

The *vertical black lines* demarcate the ten prior groupings of the isolates along the x axis into processing plant of origin and species of *Enterococcus*. The width of each group is proportional to the number of isolates within it.

The three MCMC-derived clusters are depicted in *different colours*: Cluster 1, *yellow*; Cluster 2, *red-brown*; Cluster 3, *blue*.

profiles resembling those in cluster 2, whilst others showed a closer resemblance to cluster 1.

Using pairwise F_{ST} values to check the validity of the three MCMC derived clusters found that the median F_{ST} value between pairs of isolates taken from plants within the same cluster was low at 0.02. This was significantly lower than that between pairs of isolates from plants in different clusters at 0.29 (Mann-Whitney $U = 433$, $n_1 = 14, n_2 = 31, P < 0.0001$ two-tailed); therefore, confirming that the within-cluster pairings showed more similar phenotypes than those between clusters.

The AMOVA model confirmed a high overall diversity of resistance phenotypes with 76% of the variance within the data being found within the groups of isolates from individual processing plants. Nonetheless, 22% of the variance was found between the three model-derived clusters, with only 2% of the variance occurring between the plants within the same cluster.

Assigning the individual isolates to the cluster to which they showed the greatest posterior probability allowed for an exploration of the most commonly occurring phenotypes within each cluster as shown in Table 7.3 (page 176). It can be seen that cluster 1 contains mainly *E. faecium* isolates with a predominance of 'BacFur' phenotypes (71%), and lower numbers of the same phenotype but with an additional Amp or Tet resistance. The isolates in cluster 2, the other *E. faecium* dominated cluster, showed a wider distribution of phenotypes; as did cluster 3 which contained the majority of the *E. faecalis* isolates.

Table 7.4 (page 176) shows a comparison of variables related to the farms supplying the five processing plants against the two *E. faecium*-dominated clusters. Cluster

Table 7.3: Characteristics of the enterococci constituting the three MCMC-model-derived clusters

	Cluster 1	Cluster 2	Cluster 3
Total number of isolates	161	126	114
E. faecium	158 (98)[a]	123 (98)	37 (32)
E. faecalis	3 (2)	3 (3)	77 (68)
Phenotype	% isolates within each cluster[b]		
AmpBacFur[c]	8	6	-
BacFur	71	7	-
EryBacFur	-	25	-
EryQDnBac	-	-	18
TetBacFur	14	10	-
TetEryBacFur	-	32	-
TetEryQDnBacFur	-	-	11
TetQDnBac	-	-	20

[a] The figures in brackets are the percentage of isolates of that species within that cluster.
[b] The three most common phenotypes in each cluster are shown; when a phenotype is present in two clusters the percentages within both clusters are shown for comparative purposes; the symbol (-) denotes that a phenotype is not present within that cluster.
[c] For a guide to the nomenclature used to denote phenotypes see Figure 7.1 on page 173.

Table 7.4: Comparing the two Bayesian model-derived clusters that contained predominantly *E. faecium* isolates (clusters 1 and 2) with management factors related to the farms supplying each of the five poultry processing plants (A–E)

	Processing plant				
	A	B	C	D	E
Assigned cluster of highest probability for an *E. faecium*	1	1	2	2	2
Median Rs[a] per *E. faecium* isolate	2.5	2.6	3.3	3.4	3.2
Operating company	a	b	c	c	c
Genetic line of birds	A	A	B	B	B
Number of sub-therapeutic ABDs[b] used in six years	1	1	3	3	3
Number of therapeutic ABDs used in six years	0	3	1	0	0
Therapeutic-drug-days[c] in six years	0	121	693	0	0

[a] Number of drugs to which resistance was expressed.
[b] Antibacterial drugs.
[c] Therapeutic-drug-days are the product of the number of farms that administered an antibacterial drug at therapeutic doses and the number of days for which that drug was administered on a farm.

2 is associated with *E. faecium* from plants C, D and E. These three plants are geographically separated across the North and South Islands of New Zealand, but they operate within the same integrated livestock system. Plants A and B, associated with cluster 1, are operated by two different livestock companies, however there are links between the two companies and similar management protocols were used on the farms supplying these plants. Table 7.4 also shows that the two *E. faecium* clusters have mirrored the differences in the genetic lines of birds used on the farms supplying the processing plants and also the patterns of use of in-feed sub-therapeutic drugs over the six years preceding the study. In contrast, the two clusters do not align with the sporadic use of drugs at therapeutic doses over the same time period on the farms supplying plants B and C.

7.4 Discussion

This study identified three clusters of resistance phenotypes within populations of *E. faecium* and *E. faecalis* isolated from post-chill chicken carcasses within five poultry processing plants. The *E. faecalis* isolates from all five plants showed a uniformly high degree of diversity and largely clustered together as a single group. However, the two predominantly *E. faecium* clusters corresponded to the structure of the integrated poultry industry in New Zealand, indicating that specific patterns of resistance can occur across multiple, geographically separated units within a livestock company. This is likely to be due to standardised operating procedures exerting uniform selective pressures across the individual farms and processing plants. Two plausibly influential farm-level variables did align with the *E. faecium* clusters, and these were the patterns of use of in-feed drugs at sub-therapeutic doses, and the genetic line of birds being reared. Between-company differences in the genetic lines of birds reared may result in the selection and persistence of different resistance phenotypes due to underlying differences in the population structures of the birds' enteric flora. Furthermore, differences in medication policies relating to the parent and grandparent flocks could also result in the vertical transmission of resistant bacteria through an integrated livestock system, as has been previously reported for fluoroquinolone-resistant *E. coli* (Petersen et al., 2006).

In terms of the antibacterial drugs being used on the rearing farms, all farms supplying plants A and B were administering sub-therapeutic doses of zinc bacitracin as a prophylactic measure against necrotic enteritis throughout the rearing period. The farms supplying plants C, D and E were also using zinc bacitracin in the same manner, but in addition to this sub-therapeutic doses of tylosin and avilamycin had also been administered across all the company-operated farms for extended periods of time during the six years preceding the study.

Interestingly, the occasional use of therapeutic antibacterial drugs on some of the

actual rearing farms supplying plants B and C did not appear to strongly influence the clusters seen; thus suggesting that the occasional, clinically orientated use of antibacterial drugs exerts less of a long-term selective-pressure than the routine use of drugs at sub-therapeutic doses. In general, the administration of therapeutic antibacterial drugs occurs infrequently on New Zealand broiler farms with zero use being recorded on all farms supplying plants A, D and E over the six years preceding this study.

In accordance with the administration of zinc bacitracin on 100% of the farms supplying the processing plants in this study, 95% of the enterococci isolated showed total resistance to 10 IU of this drug. Work undertaken in New Zealand into the genetic basis of bacitracin-resistance in *E. faecalis* demonstrated that high level bacitracin resistance ($\geq 256\mu g/l$) was mediated by an ATP-activated efflux pump in the bacterial membrane (Manson et al., 2004a). The genes encoding for this pump and its expression were carried on a plasmid that was shown to be transferable at high frequency to other strains of *E. faecalis*. Therefore, the administration of zinc bacitracin to groups of animals could be expected to select rapidly for the transfer of resistance between populations of *E. faecalis* present within that environment.

Quinupristin-dalfopristin resistance occurred significantly more frequently within the *E. faecalis* isolates (84%) compared with the *E. faecium* (14%). *Enterococcus faecalis* is said to be intrinsically resistant to quinupristin-dalfopristin (QD) as it carries a species-specific resistance gene, *lsa* (Singh et al., 2002). In contrast, QD-resistance in *E. faecium* is regarded as acquired and is commonly coded for by the plasmid borne *vatD* and *vatE* genes often carried in combination with the macrolide resistance gene *ermB* (DANMAP 2006, 2007). However, isolates of *E. faecalis* have also been recovered from poultry meat carrying *vatE* genes (Jones and Deshpande, 2004) and these genes have been shown to be transferable to *E. faecium* (Simjee et al., 2002). Quinupristin-dalfopristin is not licensed for use in animals and the related compound virginiamycin has not been widely used within the New Zealand broiler industry. However, there have previously been some short-term trials using virginiamycin as a growth-promoter on a limited number of broiler farms (D. Marks, personal comment). Further genetic analysis of isolates would elucidate whether the QD-resistant enterococci identified in this study are carrying mobile *vatD* or *vatE* genes.

One of the 401 isolates in this study was identified as carrying the *vanA* vancomycin-resistance gene, and PFGE analysis showed that this *E. faecalis* isolate was genetically indistinguishable to the clone that was identified within the New Zealand broiler industry in 2001 (Manson et al., 2003, 2004b). Thus, in 2004, the clone was still persisting within the industry five years after the use of avoparcin as a growth promoter had been discontinued in New Zealand, which is in-line with reports from various European countries (Borgen et al., 2000a; Garcia-Migura et al., 2007a; Heuer et al., 2002a). However, in 2006 only one isolate was identified as the previously recognised

vancomycin-resistant clone corresponding to 1.2% of the *E. faecalis* population with a 95% exact binomial confidence interval of 0.03-6.5%. The relative scarcity of this clone may be related to the fact that the resistance phenotype of this *vanA E. faecalis* was unique (see Figure 7.1) and it was susceptible to zinc bacitracin (zone size 21 mm) which was the predominant drug being used within the industry at the time the isolates were collected.

In this work, cluster analysis techniques have been used as exploratory methods to look for patterns within a dataset in line with the approach taken by one of the original proponents of cluster analysis, the psychologist J. H. Ward (Blashfield, 1980). Hierarchical clustering has previously been used to assess clusters of antibacterial resistance patterns in panels of *Salmonella* (Berge et al., 2006; Tatavarthy et al., 2006) and *E. coli* (Moreno et al., 2007). Previous work has also demonstrated that the algorithm used to produce the clusters has a strong influence on the within-cluster diversity and, therefore, the numbers of clusters produced (Berge et al., 2003). The authors of that work concluded that several hierarchical clustering algorithms should be compared in order to ascertain the clustering within a particular dataset. In the present study, hierarchical clustering has been used as a way of providing a visual summary of the relationships between the resistance phenotypes, species of enterococci and processing plants of origin. To this end the form of the data analysed and the clustering algorithm selected were those that provided a clear and succinct, summary dendrogram.

If hierarchical clustering is used as a means of determining the number of probable clusters within a dataset, then the investigator has to specify a cut-off value that denotes the numbers of nodes or branches of the hierarchy that are deemed to define separate clusters. An alternative approach was taken in the study reported here, whereby a Bayesian model designed for multi-locus genotype data (Pritchard et al., 2000) was adapted to look for clusters of resistance phenotypes which, being strings of discrete data, resemble the structure of multi-locus genotypes. A series of models were run that showed that altering the values of the prior parameters either caused the model to fail to converge or produced very similar q-matrices with the highest probability that there were three clusters within the dataset. An assumption of the model is that the frequencies of R, I and S categories within each cluster are at a steady-state; therefore, this technique may not be applicable to situations where there are likely to be frequent changes in the antibacterial drugs being administered during the sampling period. The model also assumes that the frequency of R, I and S categories for a drug are independent to those of other drugs. This assumption would be violated if two resistance genes were consistently linked on the same genetic vehicle such as a plasmid or transposon. Therefore, in order to check the validity of the three MCMC-derived clusters, two further population genetics techniques were employed which demonstrated that these three clusters were ascertainable within the data using other methods,

namely the fixation index (F_{ST}) and analysis of molecular variance (AMOVA).

In summary, this study has demonstrated that the resistance phenotypes of indicator bacterial species can be conserved within integrated poultry networks. Furthermore, the findings of this study support the hypothesis that, in a country where there is minimal use of therapeutic agents within the broiler industry, the long-term prophylactic use of sub-therapeutic drugs presents a measurable selective force upon the resistance phenotypes of the bacterial populations associated with broiler chickens.

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"Our lives begin to end the day we become silent about things that matter." *Martin Luther-King*

Chapter 8

Concluding discussion

This thesis set out to study patterns of antibacterial drug (ABD) resistant bacteria on pig and poultry meat farms, and to look for associations between farm management practices and resistance. During the course of this work ABD resistance within bacterial populations was measured in a variety of ways. Initially, farm-level estimates of resistance were obtained using direct plating techniques to detect ABD resistant bacteria in pooled faecal samples. This method revealed that vancomycin-resistant *Enterococcus faecium* (VREF) were detectable on some conventionally managed meat chicken farms, but were rarely isolated from organic chickens and neither conventional nor organic pigs. Similarly, gentamicin-resistant *Escherichia coli* (GREC) were more commonly isolated from conventional pigs than the other three categories of livestock, and ciprofloxacin-resistant *E. coli* (CREC) were uncommonly detected on any type of farm, but when they did occur they were more likely to be found on the conventional farms. However, some categories of resistant bacteria, namely ampicillin-resistant *E. coli* (AREC) and erythromycin-resistant *E. faecium* (EREF), were commonly detected on the majority of farms, and often within a high proportion of samples collected. Thus, seeking to detect resistant bacteria within faecal samples proved to be useful for comparing the prevalences of certain resistant bacteria, but was not discriminatory enough to readily distinguish farm-level differences for the most commonly occurring ones.

To this end, protocols were developed to assess the actual concentrations of resistant bacteria within individual faecal samples. These methods allowed for a closer study of resistance dynamics within individual birds and flocks over relatively short periods of time. In one study on a single farm, an increase in the proportions of the faecal *E. coli* that were resistant to ampicillin were seen in the older and heavier birds, and in a second study on the same farm, a similar increase in the proportion of *E. coli* that were resistant to chloramphenicol was elucidated as organic birds increased in age. That these increases in resistance were seen in the absence of use of ABDs indicates that factors other than drug use are involved in increasing numbers of some resistant bacteria occurring on farms.

In comparison to the organic birds, the median faecal concentration of non-specific *E. coli* was one log higher on the conventional farm. Such differences in the structure of the underlying enteric microbial populations may be related to factors such as the genetic line of bird used, but could also be a consequence of the use of prophylactic ABDs in young chicks. This prophylactic application of ABDs for the first three days after the arrival of the chicks on the conventional farm may also have contributed to the less distinct temporal patterns of resistance that were seen compared to the organic data, not least because it prevented the collection of pre-dosage data. Nonetheless, increases in the concentration of *E. coli*, both the total populations and the ampicillin-resistant subsections, did occur when some of the conventional flocks were dosed with amoxicillin at an older age, due to outbreaks of enteritis of uncertain origin. This work also highlighted that the relative concentrations of the three types of *E. coli* studied changed markedly after the flocks had been thinned at 35 days, which could be due to the introduction of alternative strains of *E. coli* by the catching teams as well as possible stress related changes in enteric bacterial population dynamics.

Whilst both methods (detection and measuring faecal concentrations) produced interesting data, both also involved considerable laboratory work, which placed logistical limits on the number of different resistant bacteria that could be analysed at once. The alternative approach of assessing the resistance phenotypes of purified bacterial isolates allowed for a broader assessment to be made of concurrently expressed resistance mechanisms. This method revealed more conspicuous contrasts between the *E. coli* present on the conventional and organic farms, with multidrug-resistant (MDR) bacteria (with resistance expressed to a median number of five drugs per isolate) predominating within the isolates obtained from the conventional birds by the end of the first week. Thereafter, these MDR *E. coli* persisted as the most common phenotypes right up until slaughter, suggesting that they were either biologically fitter than other bacteria encountered by the birds, or that the birds were not being exposed to susceptible strains. In contrast, on the organic farm the relatively low level of MDR seen in isolates from the incoming chicks (resistance expressed to 0 to 2 drugs) persisted as the most common phenotypes throughout the rearing cycle. Therefore, although the sporadic detection of highly MDR isolates (resistance expressed to up to ten drugs) indicated that the organic birds were encountering resistant *E. coli*, these MDR phenotypes did not come to predominate within the enteric *E. coli* populations on this farm.

Looking for associations between the various measures of resistance and the use of ABDs required estimates to be made of the quantities of ABDs used on the farms studied. However, this was not an easy task due to the lack of a standardised recording system for drug use on UK farms, resulting in a variety of types and quality of information being available on different farms. Collecting the drug data via face-to-face interviews with the farm managers allowed for the consultation of a variety

of records, such as: the medicines record book, veterinary invoices, feed labels and, in some circumstances, visits to the farm pharmaceutical store and farmer recall. Personal collection of data in this way undoubtedly resulted in more accurate estimations of drug use than may have been obtained using other techniques such as a mailed questionnaire, but this accuracy came at the expense of time and could be difficult to implement in larger studies.

Besides the lack of readily available, standardised, drug use data, the methods of estimating drug use also imparted a degree of uncertainty in the estimates. In order to control for differences in farm size and drug potencies, species-specific standardised measures of animal daily doses were calculated per 1000 head of livestock finished. Due to the retrospective nature of the data, the exact weights of the animals or birds at the time of dosing were not available and therefore the calculation of animal daily doses administered included a standard value for weight, set at 50% of the average finishing weight for that species, thus inevitably underestimating some animals and over estimating others.

Other challenges were encountered when trying to estimate the quantities of in-feed oral ABDs that were consumed on the farms, which required farm-specific estimations to be made of the feed intake of growing animals during the periods of ABD administration. Furthermore, many of the drugs that are used as sub-therapeutic growth promoting drugs are not licensed for use at therapeutic doses, and alternative methods were sought in order to try to standardise this data. In the final analysis, however, the much simpler measure of number of days of routine oral ABD administration (growth promoters and therapeutic drugs) provided nearly as adequate a fit to the ampicillin-resistant *E. coli* data as the more complicated standardised measures. Thus suggesting, that in countries without centralised drug recording systems, recording the periods of time for which routine oral drugs are administered on a farm may be an adequate measure of the strength of ABD selective pressures on that farm. This method in isolation, however, could under estimate the ABD selective pressures on farms that were not administering prophylactic drugs, but were regularly having to use high levels of therapeutic drugs in the face of frank clinical disease. Although a careful definition of routinely administered drugs could also help to capture information on frequently used therapeutic ABDs.

Having quantified ABD use, it was seen that, although ampicillin-resistant *E. coli* (AREC) was commonly detected on the majority of UK pig and poultry farms that were studied, nonetheless, there was an association between farms using the highest quantities of ABDs, and upon which the highest proportion of faecal samples were positive for AREC. However, regression modelling demonstrated that the measure of the quantity of actual beta-lactam drugs that were administered on a farm did not fit the data as well, particularly on the pig farms where there was little evidence for any link at all. In fact, the only bacterium studied for which a direct link was seen

between its isolation and the use of a specific and related ABD was ciprofloxacin-resistant *E. coli* (CREC), which was detected in a significantly greater proportion of samples collected on pig farms administering the related drug enrofloxacin than those that were not. There was also evidence of co-selection occurring on some of the conventional poultry farms where a strong relationship was seen between the use of the combination agent lincomycin-spectinomycin and the isolation of vancomycin-resistant *E. faecium* (VREF).

For the other resistant bacterium-livestock combinations studied, however, increases in isolation frequency were associated with increases in more generalised ABD use variables, particularly the routine administration of oral ABDs: both as prophylactic therapeutics and/or sub-therapeutic growth promoters. For the more commonly occurring resistant bacteria, the age and production status of the animals also appeared to be influential, with the lowest frequencies of detection being seen in the growing and finishing pigs, compared to the breeding adults and weaners. Furthermore, the multivariate analysis also showed that other factors, such as: the type of feed, diseases present on a farm, and mortality rate, also appeared to be associated with higher frequencies of detection of AREC and EREF. Thus suggesting that host physiology and factors at the host-bacterium interface may significantly influence the frequency of detection of resistant bacteria on a farm.

However, many of the farm-level variables were associated with each other, and this collinearity within the data meant that regression modelling alone could not provide an all encompassing view of the farm practices associated with resistance, because the models struggled to converge and the standard errors of the coefficients were inflated. For this reason, the multiple correspondence analyses (MCA) added greater breadth to the analytical process by enabling the visualisation of groups of associated farm and resistance covariates. For instance, MCA revealed that the detection of VREF was not only associated with broiler farms using lincomycin-spectinomycin, but the frequencies of detection of VREF were highest on farms administering this drug to young chicks as a prophylactic measure against necrotic enteritis. Furthermore, the poultry MCA model also demonstrated that these were also the farms on which the lowest rates of flock mortality were recorded. This suggests that, in this instance, the use of prophylactic drugs was efficacious in enhancing bird health and welfare, and this poses an interesting ethical dilemma between the desire to minimise resistant bacteria on farms for possible public health reasons, and the wish to protect animal health.

On a wider scale, the frequent occurrence of certain ABD resistant bacteria on organic farms, could also support the theory that, to a degree, ABD resistance expressed by faecal bacteria in a region can be due to other human activities in the area that are outside the control of the farmer. The much lower prevalences of resistance expressed by *E. coli* isolated from slaughtered chickens in New Zealand, compared to the UK data, could also support a link between human population density and resistance

levels. Whilst the primary reason for the low prevalence of ABD resistance in bacteria associated with New Zealand broilers was likely to be the relative lack of therapeutic ABD use on the majority of New Zealand broiler farms, a stark contrast to the situation in the UK, the human population density in New Zealand is much lower than the UK and resistance in human pathogens is also, currently, lower than that seen in the UK (Health Protection Agency Centre for Infections, 2008; Public Health Surveillance, 2008). These lower levels of ABD resistance in general may also reflect the geographical isolation of New Zealand from more heavily populated countries.

The New Zealand data also included food borne pathogens: the three *Salmonella* isolates were fully susceptible to all ABDs tested, and very little resistance to ABDs was detected within the panel of *Campylobacter jejuni* isolates tested. The lack of resistance to erythromycin and ciprofloxacin shown by the New Zealand *C. jejuni* panel was particularly striking within a global context, with many countries reporting much higher frequencies of resistance. Furthermore, in the mid 2000s, New Zealand had some of the highest case reporting rates for human campylobacteriosis in the world, including some of the highest rates of hospitalisation due to the disease. Therefore, a lack of resistance to the two commonly used ABDs within the *C. jejuni* isolates from this country strongly suggests that the use of ABDs within the broiler industries in other countries is the major selective pressure for ABD resistance amongst the circulating strains of this bacterium.

A large part of the work detailed in this thesis has been the adaptation and application of analytical techniques that are not yet in common use in veterinary epidemiology, such as: MCA, methods for visualising and exploring categorical data (mosaic plots and log-linear models), and a variety of cluster analysis techniques. These methods were found to be particularly well suited to analysing the resistance phenotypes of bacterial isolates, demonstrating that more information can be contained within such data than is commonly extracted and published. Furthermore, the addition of other covariates, such as farm management practices, can greatly enhance the interpretation of the patterns of ABD resistance that have been elucidated. For example, the use of a Bayesian model designed for genomic cluster analysis, revealed that the resistance phenotypes of *E. faecium* isolated from freshly slaughtered chickens in New Zealand fell into two clusters that aligned with the structure of the broiler industry in that country. This observation demonstrated that phenotypes of bacterial isolates from spatially separated processing plants within the same company were more similar than those of isolates from different companies - suggesting that company-level management factors applied across all farms operated by that company were strongly influential. The post-analytical collection of management data showed that these two clusters aligned with the genetic lines of birds used by a company, and also the number of different sub-therapeutic agents that had been administered prophylactically within the companies over the six years preceding data collection. Furthermore, the highest degree

of MDR was associated with the company that had used the greatest number of different drugs. Interestingly, the occasional use of full-dose therapeutic agents within farms supplying two of the five processing plants did not appear to have greatly influenced the predominant resistance phenotypes within the two clusters of *E. faecium*.

With respect to other methods used, the mosaic plots were found to be excellent visual aids for exploring the data from the two longitudinal studies on a conventional and an organic chicken farm. In particular, the use of a pairwise mosaic matrix highlighted the dichotomous nature of several resistance covariates; showing clearly, that the proportions of faecal *E. coli* that were resistant to ampicillin and chloramphenicol were very different between the two farms. The plot matrix also confirmed that the degree of multidrug resistance shown by individual *E. coli* isolates also differed markedly between the two farms. Log-linear modelling then went on to suggest that even when farm of origin was controlled for, there was an association between the highest proportions of ampicillin-resistant *E. coli* within a faecal sample and the highest degree of multidrug resistance for individual isolates from the same faecal samples, This work again suggests that bird-level factors may also be acting to increase the shedding of ABD resistant bacteria.

Although many associations have been revealed by these studies, due to the relatively small number of farms that have been studied and the clustering of samples within farms, these results should not be directly extrapolated to all UK farms. However, the strongest associations seen were between the prophylactic use of lincomycin-spectinomycin and the detection of VREF on poultry farms, and the use of enrofloxacin on pig farms and the detection of fluoroquinolone-resistant *E. coli*, and these results are both biologically plausible and supported by the results of other studies in the UK (Garcia-Migura et al., 2007b; Taylor et al., 2009).

Regarding the other associations that have been seen in this work, at all times the statistical techniques used were chosen to be those appropriate to the structure of the underlying data: regression models included nested hierarchies of random effects to control for the clustered nature of the data, and multiple correspondence analysis was developed within the social sciences in order to explore data comprised of large numbers of covariates and smaller numbers of observations. However, many of the novel techniques utilised were exploratory in nature, and further work would be useful to investigate more closely some of the hypotheses raised by the work.

For instance, a larger cross-sectional study collecting faecal samples from a greater number of farms utilising a wide variety of ABD use practices (perhaps across multiple countries), would be useful to ascertain whether it is indeed the routine and prophylactic use of oral ABDs that is providing the greatest selection pressures for increasing numbers of ABD resistant bacteria on farms. This work would also allow for the examination of the suggestion that simply recording the number of days for which these oral drugs are routinely administered on a farm is a possible alternative to laborious

calculations aiming to accurately estimate the actual quantity of drugs used. One could envisage this study using direct-plating techniques to obtain prevalence estimates at the farm, company, region or country level. Bacterial isolates collected from the samples could also be assessed for multidrug resistance using phenotyping as described in this work, but the accessibility of genomic techniques such as microarrays now also open up the possibilities of directly assessing the resistance genes carried by a bacterium, with or without prior bacteriological culture. Furthermore, some of the techniques used in this work, such as correspondence analysis and cluster analysis, are also being applied to analyse genomics data (Fellenberg et al., 2001).

With respect to the longitudinal studies of faecal concentrations of resistant *E. coli*, it would be interesting to undertake a third study in order to follow flocks on a conventional broiler farm that was not administering ABDs to day-old chicks. These results would make for interesting comparisons with those obtained from the two farms that were studied, and could shed some light upon the extent to which the prophylactic administration of a broad-spectrum ABD affects the dynamics of faecal *E. coli* populations throughout the rearing cycle of a flock.

In relation to the further examination of the effects of prophylactic ABD use, an observation from the phenotype studies worthy of follow-up, is the persistence of MDR strains of *E. coli* within conventional broiler sheds after dosing the birds as day-old chicks. Furthermore, the persistence of MDR *E. coli* that were likely to be carrying Class I integrons, together with the alteration of *E. coli* dynamics after the flocks had been thinned, suggests that there could be a possible role for probiotics in rearing conventional broiler birds. Probiotics could be a means by which susceptible strains of bacteria could be re-introduced into conventional broiler sheds. Although the possibility that alternative resistance genes could be taken on to farms by the bacteria present within the probiotics themselves would need consideration, as would the possibility that the susceptible probiotic strains would themselves develop the typical MDR phenotype due to gene transfer events within the intestinal tracts of the birds.

In summary, the application of a variety of novel techniques to data regarding ABD resistant bacteria has clearly shown that the use of ABDs on livestock farms does select for increased ABD resistance within the bacterial populations on those farms, both in terms of the numbers of resistant bacteria shed by the animals, and the degree of multidrug resistance shown by individual isolates. The strongest selective pressures were associated with the prophylactic use of oral therapeutic drugs and the long-term administration of sub-therapeutic growth promoters. However, there was also evidence that other farm-level factors were influential as well, and there were insights into the potential influences of regional effects and factors at the level of the bacterium.

Whilst, the extent of the risks posed to public health by the presence of resistant commensal bacteria on farms is still not clear, nonetheless, the enhancement and maintenance of vast reservoirs of resistance genes on livestock farms should not be

dismissed as negligible. Bearing in mind that the majority of resistance genes present in pathogenic bacteria occur on transferable genetic vehicles, and that the original source of these genes is likely to be the reservoir of genes present within populations of soil bacteria, there are no grounds to suppose that other reservoirs of resistance, such as livestock farms that are routinely using ABDs, are of no potential consequence for human, animal and environmental health.

However, multivariate analysis also revealed that whilst farms using prophylactic ABDs and growth promoting drugs were indeed likely to be those on which ABD resistant bacteria were more frequently detected, these were also the farms on which there were the lowest livestock mortality rates, which is of course why the farms were using ABDs in this manner. Nonetheless, there are countries that have managed to dramatically cut the use of ABDs on pig and poultry farms without those industries as a whole becoming economically nonviable. Therefore, in order to preserve the long-term efficacy of antibacterial drugs in human and veterinary medicine, scientists, veterinarians and livestock industries should work closely together to find ways to maintain animal health, welfare and productivity, whilst minimising the numbers of ABD resistant bacteria on livestock farms. A crucial aspect of this approach must include working to raise the general knowledge of the mechanisms and potential consequences of bacterial resistance to antibacterial drugs amongst practising and training veterinarians.

"A magician creates various things
Such as horses, elephants and so forth;
His creations do not actually exist.
You should know all things in the same way."
King of Concentration Sutra, Buddha Shakyamuni

Appendix A

Supplementary information relating to Chapter 3

Details of the resistances studied and control strains used

For *E. coli* see Table A.1 on page 192. For *E. faecium* see Table A.2 on page 193.

Using randomly generated data to assess the number of dimensions to retain from the MCA models

See Figure A.1 on page 194.

Cluster validation using silhouette coefficients

Silhouette coefficients were used to assess the most probable number of true clusters within the two datasets (Rousseeuw, 1987). The silhouette coefficient (s) of element i is:

$$s_i = \frac{b_i - a_i}{\max(a_i, b_i)} \tag{A.1}$$

where a_i is the average distance between element i and all other elements in that cluster, and b_i is the minimum average distance between element i and all elements in another cluster. Elements with silhouette coefficients close to one are well assigned ($a_i << b_i$); values close to zero indicate that an element is split between two clusters ($a_i \approx b_i$); and negative values imply that the element has been assigned to an inappropriate cluster ($a_i > b_i$).

The silhouette plots obtained for the pig data are displayed in Figure A.2 on page 195, and the contents of the clusters resulting from two candidates for optimal cluster number (six and eleven) are shown in Table A.3 on page 196. The corresponding plots and tables for the poultry data are on pages 197 and 198, respectively.

Table A.1: Details of the resistances studied for *E. coli* and the control strains used to check the drug concentrations in the CHROMagarECC plates.

Resistance	Bkpt[a]	Control strains	Relevance	Basis of resistance mechanisms
Ampicillin	8 μg/ml	NCTC 10418 ATCC 25922 LR22[b] S28/99[b]	Used frequently in human and veterinary medicine.	Beta-lactamase genes can be chromosomal and/or present on mobile genetic elements.
Gentamicin	4 μg/ml	NCTC 10418 G33/G36[b]	Used more frequently in human medicine. Cross-resistance seen with apramycin: a veterinary therapeutic ABD.	Genes encoding for drug inactivation enzymes are carried by plasmids and transposons.
Ciprofloxacin	1 μg/ml	NCTC 10418 F3/F13/F15[b]	Used exclusively in human medicine. Cross-resistance seen with veterinary therapeutic fluoroquinolones.	Chromosomal mutations in conjunction with efflux pumps, but plasmid-borne resistance genes increasingly recognised.

[a] Breakpoint concentrations of ABDs incorporated into the CHROMagar plates. Based on the Health Protection Agency breakpoints used at that time.

[b] Strains sourced from work within the VLA: LR = Dr Luke Randall, S and F = Carol Clouting, G = own work.

Table A.2: Details of the resistances studied for *E. faecium* and the control strains used to check the drug concentrations in the Slanetz and Bartley agar plates.

Resistance	Bkpt[a]	Control strains	Relevance	Basis of resistance mechanisms
Erythromycin	4 μg/ml	NCTC 12697 NCTC 7171 NCTC 775 NCTC 12202	Used more frequently in human medicine. Cross-resistance seen with veterinary drugs such as tylosin.	Modification of drug-target site encoded by genes carried on plasmids and transposons.
Vancomycin	16 μg/ml	NCTC 12697 NCTC 7171 NCTC 775 NCTC 12202	Used exclusively in human medicine. Cross-resistance seen with the growth promoting agent avoparcin.	The *vanA* operon of genes encode for pathways that prevent the disruption of cell wall synthesis by vancomycin, and are carried on transposons and plasmids.

[a] Breakpoint concentrations of ABDs incorporated into the Chromagar plates. Based on the Danish Veterinary Laboratory breakpoints used at that time.

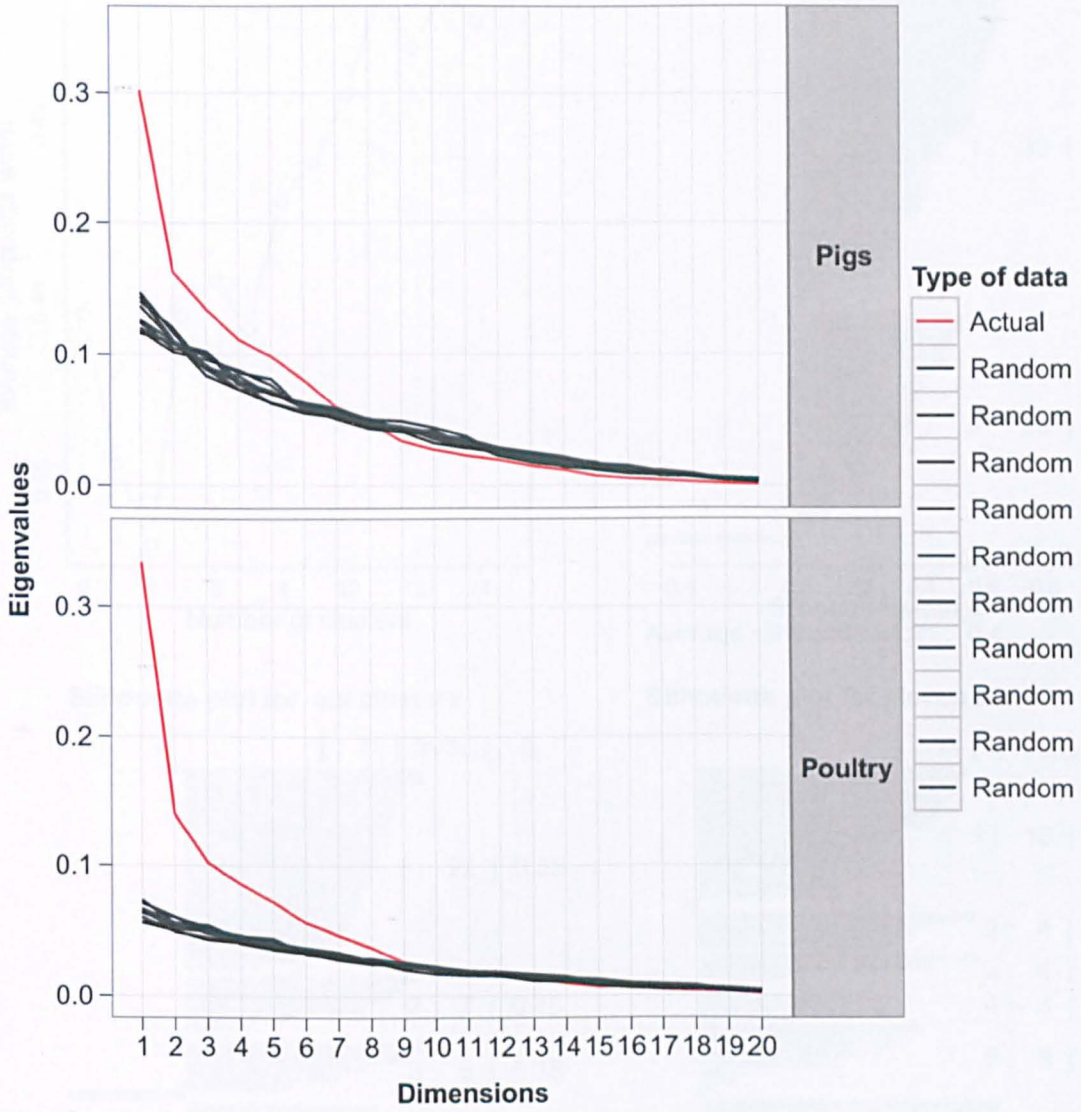

Figure A.1: Two plots of the eigenvalues associated with each of one to twenty dimensions identified by multiple correspondence analyses of the occurrence of ABD resistant bacteria on pig and poultry farms alongside other farm-level covariates.

The red lines depict the eigenvalues obtained from MCA models of the actual data; and the black lines depict the eigenvalues obtained from ten sets of randomly generated data of the same structure as the original.

The last dimension of the models of actual data that returned an eigenvalue above that of the randomly generated data was assumed to represent the last dimension that contained information about meaningful associations between the covariates.

Six dimensions were chosen for the pig data and eight for the poultry.

For more details regarding the implementation of this method see page 73 in Chapter 3

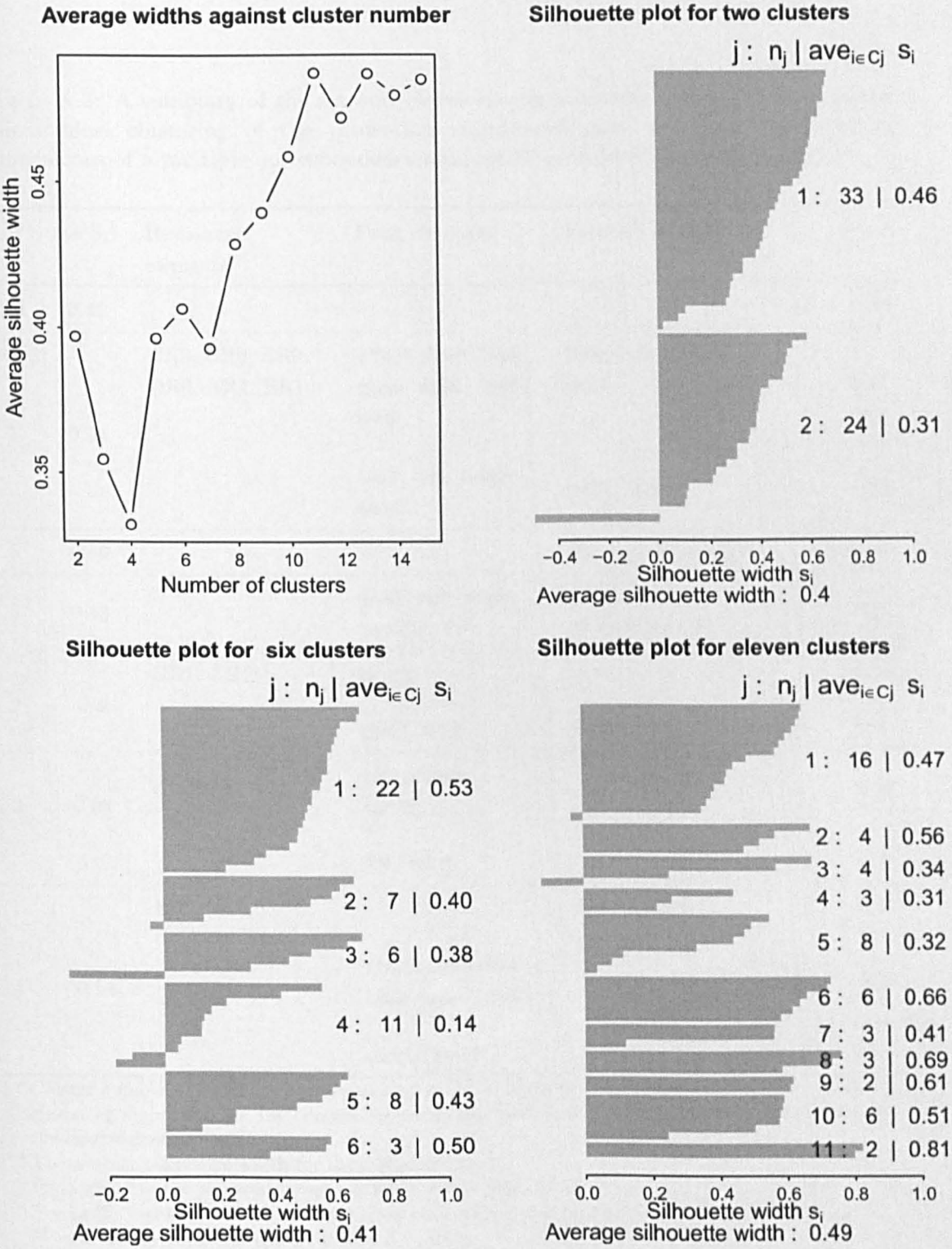

Figure A.2: Using silhouette plots to determine the most appropriate number of clusters within the 57 pig variable elements.

$j : n_j \mid ave_{i \in C_j} S_i$, where j is the cluster number, n_j is the number of variable elements in cluster j (C_j), S_i is the silhouette coefficient (width) for variable element i, and $ave_{i \in C_j}$ is the mean of the silhouette coefficients for all variable elements i in cluster j.

Table A.3: A summary of the six and eleven cluster solutions derived from weighted hierarchical clustering of the projection coordinates from the first six principal dimensions of a multiple correspondence analysis of farm-level elements related to pig farms.

C_j[a]	ave S_i[b]	Resistance elements[c]	Drug elements	Farm elements	C_j	ave S_i
1:6	0.41				1:11	0.49
1	0.54	AR0, CR0, ER0, GR0, AR1, ER1.	amg0, dexr0, fq0, gpd0, mls0, oral0, tet0.	dpop0, feed1**[2],[d] wat1.	1	0.47
			bla1, inj1, ndg1, tadd1.	onhd, fmsz2.	6	0.66
6	0.50			dip1, feed2, wat2.	7	0.41
5	0.43		bla0, inj0, ndg0, tadd0.	cdhd, dip0, pmws0, fmsz1.	5	0.32
2	0.4	GR1, AR2*[1].	dexr1.	pmws1.	2	0.56
			gpd1, tet2.	fmsz3.	8	0.69
3	0.38	CR1[bb11].	tet1, oral2, tadd2[b2].		3	0.34
			fq1, mls2.		11	0.81
4	0.14	ER2.		dip2[b2], dpop1.	4	0.31
			amg1, mls1, oral1, bla2, inj2[b3], ndg2.		10	0.51
			dexr2, gpd2.		9	0.61

[a] Cluster number. The clusters are numbered in the order in which the dendrogram was cut by the clustering algorithm, but the clusters appear in the table in the order in which they appeared in the dendrogram.

[b] The average silhouette width for the stated cluster/s.

[c] For a guide to the element names see Table 3.1 on page 74.

[d] * = in the 6-cluster scheme, element i has silhouette width S_i where $-0.05 < S_i < 0.05$;
** = in the 11-cluster scheme, $-0.05 < S_i < 0.05$;
[b] = in the 6-cluster scheme, $S_i < -0.05$;
[bb] = in the 11-cluster scheme, $S_i < -0.05$;
The number following the superscript symbol of each uncertainly assigned element denotes the nearest alternative cluster.

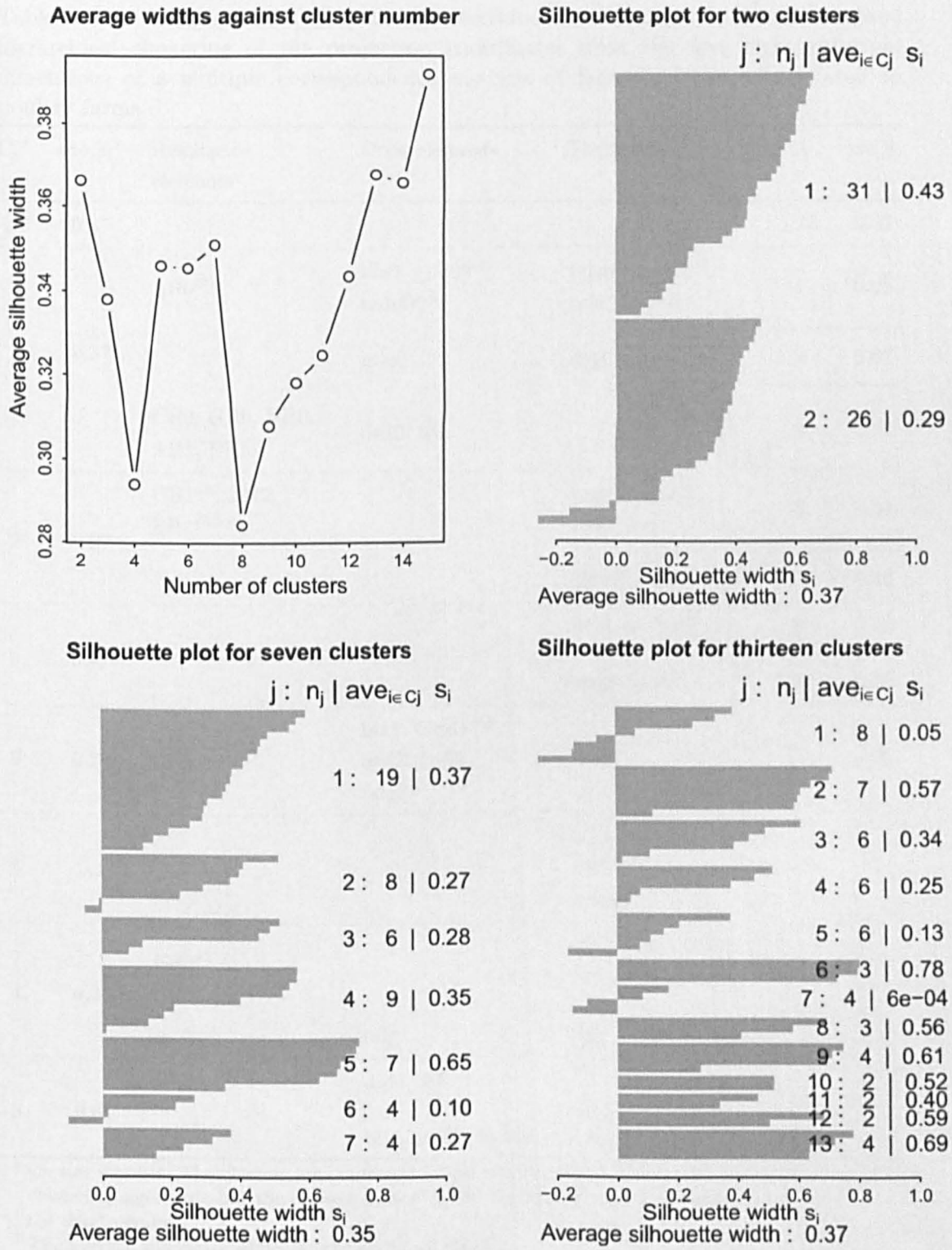

Figure A.3: Using silhouette plots to determine the most appropriate number of clusters within the 57 poultry variable elements.

$j : n_j \mid ave_{i \in C_j} S_i$, where j is the cluster number, n_j is the number of variable elements in cluster j (C_j), S_i is the silhouette coefficient (width) for variable element i, and $ave_{i \in C_j}$ is the mean of the silhouette coefficients for all variable elements i in cluster j.

Table A.4: A summary of the seven and thirteen cluster solutions derived from weighted hierarchical clustering of the projection coordinates from the first eight principal dimensions of a multiple correspondence analysis of farm-level elements related to poultry farms.

C_j[a]	ave S_i[b]	Resistance elements[c]	Drug elements	Farm elements	C_j	ave S_i
1:7	0.37				1:13	0.37
		AR0$^{\flat\flat 2}$.[d]	bla0, gpd0$^{\flat\flat 2}$, tadd0$^{\flat\flat 9}$.	mage, mspp$^{\flat\flat 9}$, scln, hage3.	1	0.05
1	0.37		ndg0.	dp0, fmsz1, rng1.	9	0.61
		CR0, GR0, VR0, AR1, ER1.	dsd0, ls0.		2	0.57
2	0.27	GR1^{*4}, AR2, ER2^{*4,**8}.		hage1, mort1, wat1.	3	0.34
				emp2, flw0.	11	0.40
7	0.27			dft0, mort3.	10	0.52
				emp0, wat2.	12	0.59
3	0.28	CR1.	bla1, tadd1^{**8}, dsd2, gpd2, ndg2^{*5,**13}.		4	0.25
6	0.1			ccln, sage, ssp$^{*4,\flat\flat 8}$, hage2$^{\flat 3,\flat\flat 4}$.	7	0
4	0.35	ER0^{*1}.		dft1, emp1, flw1, fmsz2$^{\flat\flat 8}$, mort2.	5	0.13
			ndg1.	dp1, rng0.	8	0.56
5	0.65	VR1.	dsd1, ls1.		6	0.78
			bla2, gpd1, tadd2.	fmsz3.	13	0.69

[a] Cluster number. The clusters are numbered in the order in which the dendrogram was cut by the clustering algorithm, but the clusters appear in the table in the order in which they appeared in the dendrogram.

[b] The average silhouette width for the stated cluster/s.

[c] For a guide to the element names see Table 3.1 on page 74.

[d] * = in the 6-cluster scheme, element i has silhouette width S_i where $-0.05 < S_i < 0.05$;
** = in the 11-cluster scheme, $-0.05 < S_i < 0.05$;
$^{\flat}$ = in the 6-cluster scheme, $S_i < -0.05$;
$^{\flat\flat}$ = in the 11-cluster scheme, $S_i < -0.05$;
The number following the superscript symbol of each uncertainly assigned element denotes the nearest alternative cluster.

Appendix B

Supplementary information relating to Chapter 5

Details of the sampling strategies and control strains used

For details of the birds sampled during the conventional study see Table B.1 on page 201, and for the organic birds see Table B.2 on page 202. For details regarding the control strains used to check the levels of antibacterial drugs in the CHROMagar plates see Table B.3 on page 203. For a breakdown of the numbers of isolates from each source that were characterised further see Tables B.4 and B.5 on page 203.

Choosing the bandwidth for the smoothing models

The `loess` function in R is uses nearest-neighbour bandwidth adjustments and, therefore, one needs to stipulate what proportion of the total data is fitted within each model: known as the span value. The span values were selected using a leave-one-out cross-validation (LOOCV) technique based on the prediction sum of squares (PRESS) (Allen, 1974). Leave-one-out cross-validation involves removing a single data point from the dataset, fitting a loess model with a given span value, and using the fitted model to predict the value of the data point that has been removed. This process is repeated for every value within the data set, and the predicted sum of squared errors is calculated (Cleveland, 1979). The span value that minimises PRESS is designated the optimal value.

An optimal span was calculated for each of 24 subsets of data: the three categories of *E. coli* within four flocks on each of two farms (see Table B.6 on page 204). An example plot resulting from the LOOCV of faecal ampicillin-resistant *E. coli* concentrations in one of the conventional houses is shown in Figure B.1 on page 200. The mean optimal value of span of all subsets of data for a given farm was the value chosen for the smoothed scatter plots of *E. coli* concentrations on that farm (see Table B.6). These two selected values (0.39 for the conventional data and 0.31 for the organic) were further

assessed using plots of the residuals versus fitted values to check that the resultant loess models provided an adequate fit to the data (see Figure B.2 on page 205).

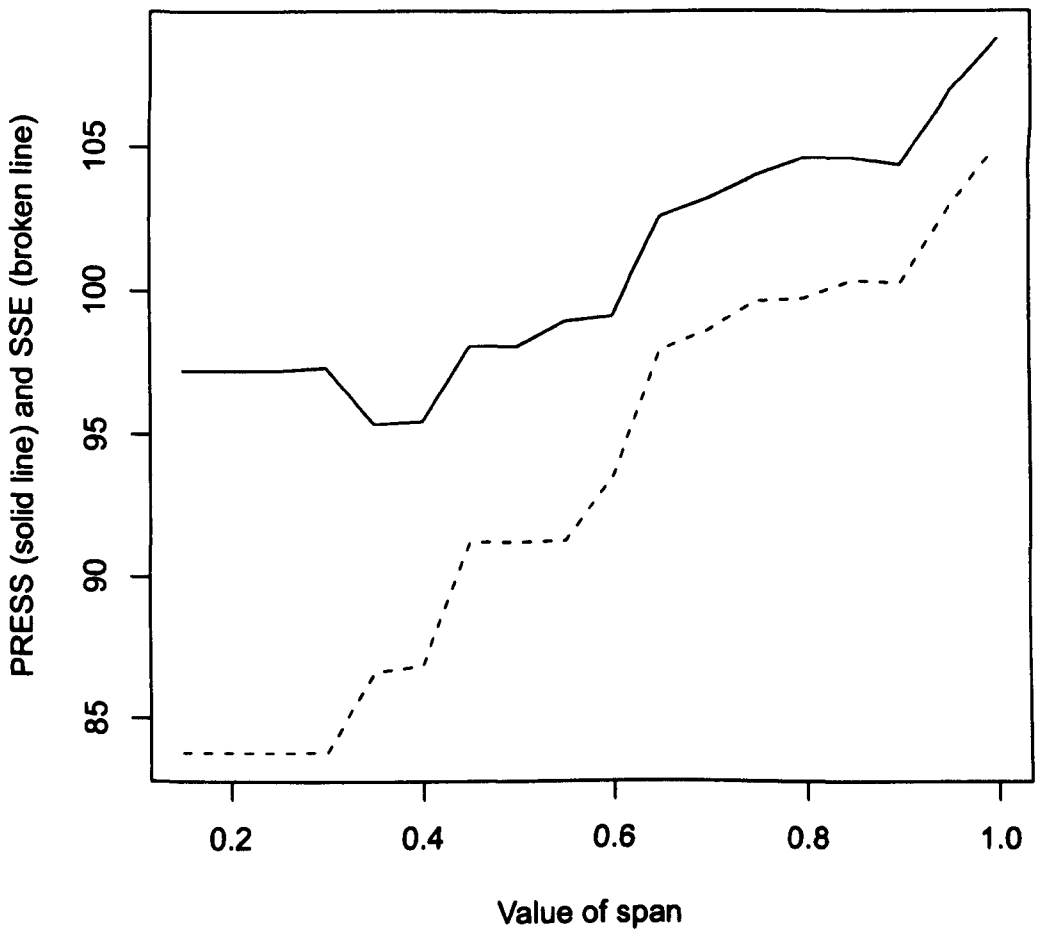

Figure B.1: Plot of predicted sum of squares (PRESS) and sum of the squared error (SSE) against various values of span resulting from leave-one-out cross-validation of a linear loess fit of ampicillin-resistant faecal *E. coli* concentrations against age of bird in a single house on a conventional broiler farm.

Using this technique, the optimal span value (bandwidth) is the value that minimises PRESS, i.e. 0.35 within this subset of data.

Table B.1: A table showing the ages of each of the marked birds on the conventional farm on the days on which they were caught and sampled.

House	Bird	Age of bird on sampling visit (n = 1–10)										Total
	ID	1	2	3	4	5	6	7	8	9	10	samples
A	1	7	12	14	x[a]	22	26	28	33	x	x	7
	2	7	12	14	x	x	x	x	x	x	x	3
	3	7	12	14	x	22	26	28	33	35	41	9
	4	7	12	14	20	x	x	x	x	x	x	4
	5	7	12	14	20	22	x	28	33	35	41	9
	6	7	12	14	x	22	26	x	x	x	x	5
	25	-[b]	-	-	20	22	26	28	33	35	41	7
	26	-	-	-	20	22	26	28	33	x	x	5
	27	-	-	-	20	22	x	x	x	x	x	2
	31	-	-	-	-	-	26	28	33	35	x	4
B	19	8	13	15	21	23	27	29	34	36	42	10
	20	8	13	15	x	x	x	x	x	x	x	3
	21	8	13	15	21	23	27	x	34	36	x	8
	22	8	13	15	21	x	27	29	34	36	x	8
	23	8	13	15	21	23	27	29	x	x	x	7
	24	8	13	15	x	23	x	29	34	36	x	7
	28	-	-	-	21	23	27	29	34	x	x	5
	19	-	-	-	21	23	27	29	34	36	x	6
C	13	3	8	10	16	18	22	24	29	31	x	9
	14	3	8	10	x	x	x	x	x	x	x	3
	15	3	8	10	16	18	22	24	29	31	37	10
	16	3	8	10	16	18	22	24	29	31	37	10
	17	3	8	10	16	18	22	24	29	31	37	10
	18	3	8	10	16	18	22	24	29	31	37	10
	30	-	-	-	16	18	22	24	29	31	37	7
D	7	4	9	11	17	19	23	25	30	32	x	9
	8	4	9	11	17	19	23	25	30	32	x	9
	9	4	9	11	17	19	23	25	30	32	38	10
	10	4	9	11	17	19	23	25	30	32	38	10
	11	4	9	11	17	19	23	25	30	32	38	10
	12	4	9	11	17	19	23	25	30	32	38	10

[a] x = it was not possible to obtain a sample from the bird on that visit.
[b] - = the bird had not been recruited onto the study on that visit.

Table B.2: A table showing the ages of each of the marked birds on the organic farm on the days on which they were caught and sampled.

Flock	Bird	Age of bird on sampling visit (n = 1–11)											Total
	ID	1	2	3	4	5	6	7	8	9	10	11	samples
1	1	2	7	x[a]	13	21	28	34	41	48	55	s[b]	9
	2	2	7	x	13	21	28	34	41	48	55	s	9
	3	2	7	x	13	21	28	34	41	48	55	s	9
	4	2	7	x	13	21	28	34	41	48	55	s	9
	5	2	7	x	13	21	28	34	41	48	55	s	9
	6	2	7	x	13	21	28	34	41	48	55	s	9
2	7	2	7	x	13	21	28	34	41	x	x	s	7
	8	2	7	x	13	21	28	34	41	48	55	s	9
	9	2	7	x	13	21	28	34	41	48	55	s	9
	10	2	7	x	13	21	28	34	41	48	55	s	9
	11	2	7	x	13	21	28	34	41	48	55	s	9
	12	2	7	x	13	21	28	34	41	48	55	s	9
3	13	-[c]	x	4	x	x	x	x	x	x	x	x	1
	14	-	x	4	8	16	23	29	36	43	50	65	9
	15	-	x	4	8	16	23	29	36	43	50	65	9
	16	-	x	4	8	16	23	29	36	43	50	65	9
	17	-	x	4	8	16	23	29	36	43	50	65	9
	18	-	x	4	8	16	23	29	36	43	50	65	9
	25	-	-	-	8	16	23	29	36	43	50	65	9
4	19	-	x	4	8	16	23	29	36	43	50	65	9
	20	-	x	4	8	16	23	29	36	43	50	65	9
	21	-	x	4	8	16	23	29	x	43	50	65	8
	22	-	2	4	8	x	23	29	36	43	50	65	9
	23	-	x	4	8	16	23	29	36	43	50	65	9
	24	-	x	4	8	16	23	29	36	43	50	65	9

[a] x = it was not possible to obtain a sample from the bird on that visit.
[b] s = the bird had been slaughtered.
[c] - = the bird had not yet arrived on the farm.

Table B.3: Details of the control strains used to check the levels of ampicillin and chloramphenicol in the CHROMagar ECC plates.

E. coli strain	Source	MIC of ampicillin (μg/ml)	MIC of chloramphenicol (μg/ml)
NCTC 10418	International control	susceptible	susceptible
ATCC 29522	International control	2–8	-
LR22	VLA archive: Dr Luke Randall	2–4	≥ 8
S28/99	VLA archive: Carol Clouting	> 256	> 64
1232/04	VLA archive: *Salmonella*-serotyping	≥ 2	≥ 8
1250/04	VLA archive: *Salmonella*-serotyping	-	≥ 8
S/502/04	VLA archive: *Salmonella*-serotyping	> 32	≥ 64

Table B.4: A breakdown of the numbers and sources of 181 *E. coli* isolates that had originated from a conventional broiler farm and that were tested for resistance to 17 antibacterial drugs using microbroth dilution methods. pos

Source of isolates	House				Total
	A	B	C	D	
Cleaned houses: pre-flock	-	16	-	17	33
Incoming chicks	13	8	12	10	43
Faecal samples (growing birds)	18	20	22	24	84
Cleaned houses: post-flock	-	4	-	17	21

Table B.5: A breakdown of the numbers and sources of 177 *E. coli* isolates that had originated from an organic meat chicken farm and that were tested for resistance to 17 antibacterial drugs using microbroth dilution methods. pos

Source of isolates	Flock				Total
	1	2	3	4	
Cleaned brooding houses	14	10	15	14	53
Incoming chicks	2	2	2	2	8
Faecal samples (growing birds)	24	16	19	19	78
Cleaned mobile houses	10	10	10	8	38

Table B.6: The optimal smoothing span values obtained for 24 subsets of data (three categories of bacteria from four flocks on each of two meat chicken farms) using a leave-one-out cross-validation method to calculate the prediction sum of squares for a series of spans ranging from 0.05 to 1.

Group of birds	Optimal value for span[a]			Mean
	TEC[b]	AREC[c]	ChREC[d]	
Conventional farm				
House A	0.50	0.40	0.53	0.48
House B	0.35	0.35	0.45	0.38
House C	0.30	0.30	0.15	0.25
House D	0.25	0.30	0.75	0.43
Mean	0.35	0.34	0.47	0.39[e]
Organic farm				
Flock 1	0.20	0.50	0.35	0.35
Flock 2	0.18	0.45	0.20	0.28
Flock 3	0.50	0.20	0.20	0.30
Flock 4	0.30	0.35	0.30	0.32
Mean	0.30	0.38	0.26	0.31[e]

[a] Span refers to the proportion of data that is fitted within each local model.
[b] Total faecal *E. coli* population.
[c] Ampicillin-resistant faecal *E. coli*.
[d] Chloramphenicol-resistant faecal *E. coli*.
[e] These mean span values for the two farms were the values selected for span for the smoothed scatter plots of faecal *E. coli* concentrations against age that were shown in Chapter 5 on pages 129 and 128.

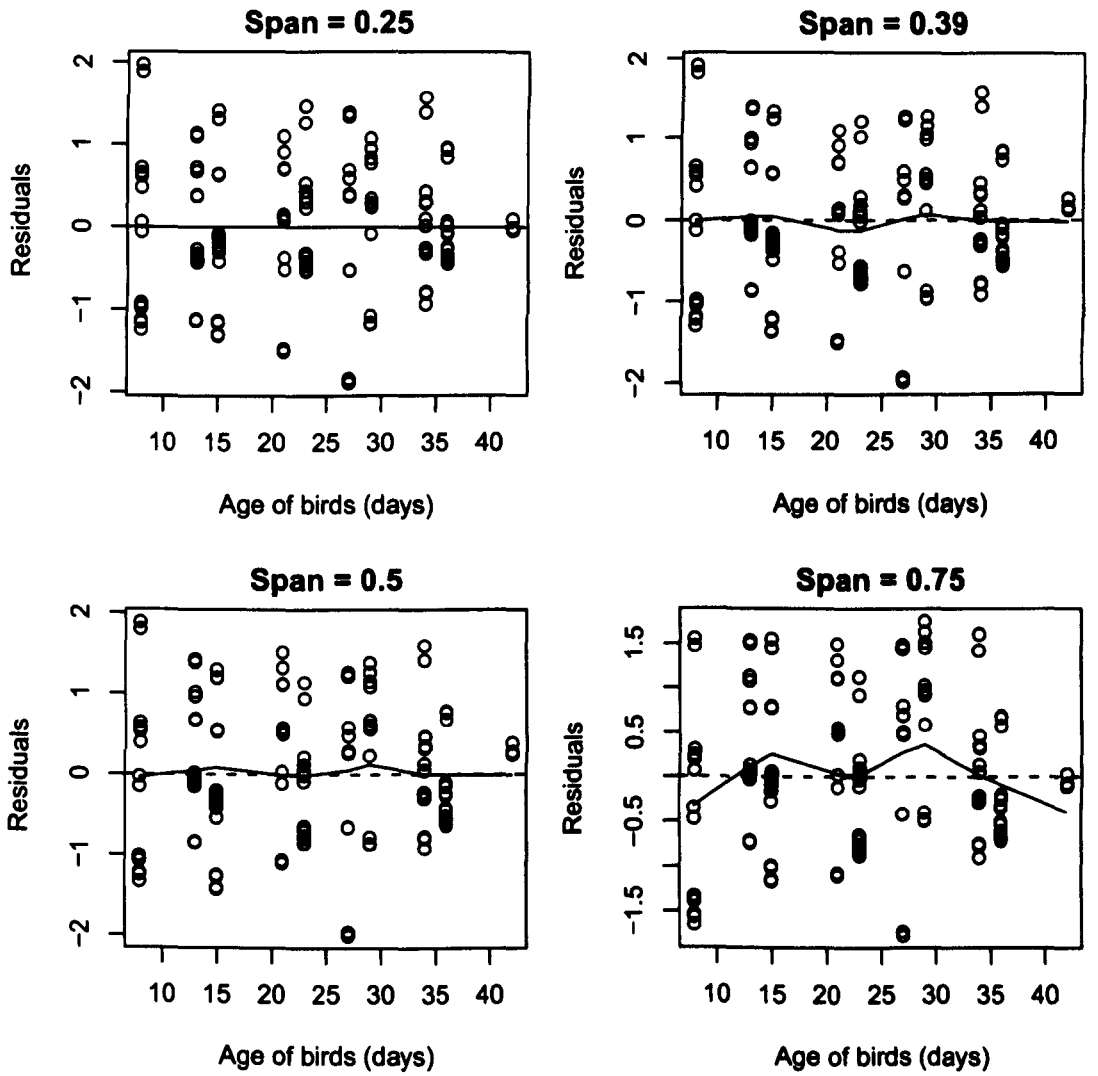

Figure B.2: Plots displaying the residuals from four separate loess fits of faecal concentrations of ampicillin-resistant *E. coli* against age of bird in a single poultry house on a conventional broiler farm. The four models differ only in the value of span that has been used.

The mean of the optimal span values for all subsets of data from the conventional farm was 0.39, slightly higher than the optimal value of 0.35 obtained for this particular subset as shown in Figure B.1. However, this graphic shows that the residuals from a loess fit of the actual data using a span of 0.39 were not substantially deviating from zero. Therefore, using a span of 0.39 provided a reasonable fit to the data, whereas a span of 0.75 was a rather poor fit to the data.

A first degree polynomial loess fit with a span of 0.5 has been used to smooth the residuals in each of these four subplots.

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"Science is a wonderful thing if one does not have to earn one's living by it." *Albert Einstein*