Antimicrobial resistance in companion animal pathogens in Australia and assessment of pradofloxacin on the gut microbiota

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A thesis submitted in fulfilment of the requirements of
the degree of Doctor of Philosophy

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Sugiyono Saputra

February 2017

This thesis is dedicated to my beloved parents,

wife and daughter.

And your Lord has decreed that you not worship except Him, and to parents, good treatment. Whether one or both of them reach old age [while] with you, say not to them [so much as], "uff," and do not repel them but speak to them a noble word. And lower to them the wing of humility out of mercy and say, "My Lord, have mercy upon them as they brought me up [when I was] small."

(Al-Qur'an, Surah Al Isra' [17:23-24], Sahih International)

And those who say, "Our Lord, grant us from among our wives and offspring comfort to our eyes and make us an example for the righteous."

(Al Qur'an, Surah Al Furqon [25:74], Shahih International)

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Preamble

I enrolled as a Master of Philosophy (MPhil) as a mixed research and coursework student on 3 March 2014, with an Australian Award Scholarship, sponsored by the Australian Department Foreign Affairs and Trade. As part of my research component, I was involved in the first nation-wide survey of antimicrobial resistance in Australian animals, focusing on phenotypic characterisation of antimicrobial susceptibility patterns in companion animal pathogens. In the second year of my candidature, I applied for a scholarship extension, proposing a program upgrade to a Doctorate (PhD). Considering the satisfactory results of my coursework component and the progress of my research, Australia Awards agreed to extend my scholarship and I was successfully upgraded to a PhD program in Veterinary Science on 9 November 2015. Following my new candidature as a PhD student, my research has expanded to cover the microbial ecology of the rabbit gastrointestinal tract in response to antimicrobial treatment. In addition to my four first author publications, I have also been involved in several other collaborative projects under the over-arching theme of resistance, as indicated by the list of published works produced during my candidature which are not incorporated into this thesis.

This thesis is presented as a series of four research articles and two literature reviews. Chapter 1 is a short general introduction, describing the background and study objectives. The first two published articles (Chapters 2 and 3) are primarily related to my study in antimicrobial resistance in major pathogens, including coagulase-positive staphylococci and *E. coli* isolated from diseased dogs, cats and horses. The literature review presented in Chapter 4 describes a current perspective on antimicrobial resistance in companion animal pathogens in Australia and comparison to similar published work in other countries and includes my work and the work of my colleagues at other Universities who contributed to ARC Linkage project LP130100736 with Zoetis as the major partner. The other three articles (Chapters 5, 6, and 7) are related to my own study on the effects of the new generation veterinary fluoroquinolone on gut microbiota in rabbits. Chapter 5 constitutes a second literature review, describing the effects of antimicrobial administration on the microbial diversity of gut microbiota in humans, food-producing, and laboratory animals. Microbial community changes in faecal samples are presented in Chapter 6, while gastric and caecal microbial community changes are presented in Chapter 7. The major findings and future directions are discussed in Chapter 8.

List of Publications

Research articles by the author incorporated to the thesis:

1. **Saputra S**, Jordan D, Worthing K, Norris, J, Wong HS, Abraham R, Trott DJ, Abraham S. 2017. Antimicrobial Resistance in coagulase-positive staphylococci Isolated from companion animals in Australia: A one year study. PLoS ONE 12(4): e0176379. https://doi.org/10.1371/journal. pone.0176379

2. **Saputra S**, Jordan D, Mitchell T, Wong HS, Abraham RJ, Kidsley A, Turnidge J, Trott DJ, Abraham S. 2017. Antimicrobial resistance in clinical *Escherichia coli* isolated from companion animals in Australia. Vet Microbiol. https://doi.org/10.1016/j.vetmic.2017.09.014

3. **Saputra S**, Mohammadi-Dehcheshmeh M, Woodward N, Page C, Griffiths N, Xie S, Abraham S, Ebrahimie E, Trott DJ. Faecal microbiome profiling in rabbits following single and multiple doses of oral pradofloxacin. Vet J. Accepted 28 Apr 2018.

4. **Saputra S**, Mohammadi-Dehcheshmeh M, Woodward N, Page C, Griffiths N, Xie S, Abraham S, Howarth GS, Ebrahimie E, Trott DJ. Oral pradofloxacin has limited impact on the gastric and caecal microbiota of rabbits. J. Med. Microbiol. Submitted 20 Feb 2018.

Literature reviews by author incorporated to the thesis:

1. **Saputra S**, Jordan D, Worthing KA, Norris JM, Abraham S, Trott DJ. Antimicrobial resistance in companion animal pathogens in Australia: a current perspective. Will be submitted to Aus Vet J.

2. **Saputra S**, Howarth, GS, Trott DJ. Metagenomic studies of gut microbial diversity and structure changes after antibiotic treatment. Will be submitted to Microbiol Ecol.

Additional published research articles as a co-author but not incorporated into the thesis:

1. Abraham S, Kirkwood RN, Laird T, **Saputra S**, Mitchell T, Singh M, Linn B, Abraham RJ, Pang S, Gordon DM, Trott DJ, O'Dea M. Dissemination and persistence of extended-spectrum cephalosporin-resistance encoding IncI1-bla CTXM-1 plasmid among *Escherichia coli* in pigs. ISME J. 2018 Jun 13. doi: 10.1038/s41396-018-0200-3.

2. Badger S, Abraham S, **Saputra S**, Trott DJ, Turnidge J, Mitchell T, Caraguel CGB, Jordan D. Relative performance of antimicrobial susceptibility assays on clinical *Escherichia coli* isolates from animals. 2018. Vet Microbiol 214, 56-64. https://doi.org/10.1016/j.vetmic.2017.12.008

3. Worthing KA, Abraham S, Coombs GW, Pang S, **Saputra S**, Jordan D, Trott DJ, Norris JM. Clonal diversity and geographic distribution of methicillin-resistant S*taphylococcus pseudintermedius* from Australian animals: Discovery of novel sequence types. 2018. Vet Microbiol. 213, 58-65. https://doi.org/10.1016/j.vetmic.2017.11.018.

4. Worthing K, Abraham S, Pang S, Coombs GW, **Saputra S**, Jordan D, Wong HS, Abraham R, Trott D, Norris J. 2017. Molecular characterisation of methicillin-resistant *Staphylococcus aureus* isolated from Australian animals and veterinarians. Microb Drug Res. doi: 10.1089/ mdr.2017.0032

5. Worthing K, Coombs G, Pang S, Abraham S, **Saputra S**, Trott D, Jordan D, Wong H, Abraham R, Norris J. Isolation of *mecC* MRSA in Australia. 2016. J Antimicrob Chemother 71(8):2348-2349 (2 pages). doi: 10.1093/jac/dkw13

6. Abraham S, O'Dea M, Trott D, Abraham R, Hughes D, Pang S, McKew G, Cheong E, Merlino J, **Saputra S** *et al.* Isolation and plasmid characterization of carbapenemase (IMP-4) producing *Salmonella enterica* Typhimurium from cats. Scientific Reports 6:35527, 1-7. doi: 10.1038/srep35527.

Other conference presentations relevant to the thesis

1. **Saputra S**, Woodward N, Page C, Griffiths N, Xie S, Ebrahimie E, Trott DJ. Modulation of faecal and caecal microbiome in rabbit after pradofloxacin treatment. The Australian Microbial Ecology (*AusME2017*), Melbourne 13-15 February 2017.

2. **Saputra S**., K. Worthing, D. J. Trott, J. Norris, H. S. Wong, R. Abraham. 2015. Emerging antimicrobial resistance in staphylococcal infections associated with companion animals in Australia. International Conference on the Evolution and Transfer of Antibiotic Resistance 2015, Amsterdam, the Netherlands. 24-26 June 2015.

3. **Saputra S**., S. Abraham, H.S. Wong, D. Jordan, D. J. Trott. 2015. Wild-type minimum inhibitory concentration (MIC) distribution and antimicrobial susceptibility of pathogenic *Escherichia coli* isolated from companion animals in Australia. 16th Annual Scientific Meeting-Antimicrobials, Australian Society for Antimicrobials, Brisbane, Australia. 26-28 February 2015.

4. Houston-Francis M, Speight N, **Saputra S**, Ebrahimie E and Trott DJ. Presence of *Oxalobacter formigenes* in the gastrointestinal tract of koalas and its association with oxalate nephrosis. The Australian Microbial Ecology (AusME2017), Melbourne 13-15 February 2017.

5. Kidsley A, S. Abraham, **S. Saputra,** J. Turnidge, J. Bell, D. J. Trott. 2015. The major fluoroquinolone-resistant extraintestinal pathogenic *Escherichia coli* clone B2:025b:ST131 is not undergoing epidemic spread in companion animals. 16th Annual Scientific Meeting-Antimicrobials, Australian Society for Antimicrobials, Brisbane, Australia. 26-28 February 2015.

6. Worthing K., J. Norris, **S. Saputra**, D. J. Trott, H. S. Wong, R. Abraham, D. Jordan, S. Abraham. 2015. Characterisation of antimicrobial susceptible and resistant staphylococcal infections in Australian dogs and cats. Australian and NZ Veterinary College Science Week, Queensland, Australia. 9-11 July 2015.

Abstract

Antimicrobials are not only considered as beneficial agents to human and animal health, but are also potentially harmful, both in terms of promoting antimicrobial resistance (AMR), and the potentially negative effects they may have on the gut microbiota. Focusing on these two major detrimental effects, this thesis aims to determine the frequency of AMR in companion animal pathogens isolated from Australia, as well as identify risk factors associated with infection my multidrug-resistant strains; and to describe the gut microbial community changes and other adverse effects in rabbits in response to administration of pradofloxacin, a new veterinary antimicrobial belonging to the critically important fluoroquinolone class.

In the first nation-wide survey of AMR in companion animal pathogens, resistance to antimicrobials from more than ten different classes was described for a total 883 clinical *Escherichia coli* isolated from dogs (n=514), cats (n=341) and horses (n=28). Resistance to critically important antimicrobials not registered for use in animals (imipenem) was not detected in all clinical *E. coli* isolates. Resistance to amikacin, another critically important antimicrobial not registered for use in Australian animals, was low in dog and cat isolates (<2%) but moderately high in horse isolates (10.7%), reflecting its increased use in this species. Resistance to other third line antimicrobials such as third generation cephalosporins (3GC) and fluoroquinolones (FQN), was generally lower in cat isolates (3.8%-5%) compared with dog isolates (9.1%-10.9%) and horse isolates (25%-35.7%). A higher proportion of resistance was observed among the first and second line antimicrobials, ranging from 25%-100% for ampicillin and amoxicillin-clavulanic acid, mainly due to the new interpretation by veterinary-specific clinical breakpoints for dogs and cats which are much lower than human clinical breakpoints. The frequency of multidrug-resistant (MDR) *E. coli* was 18.1%, 11.7% and 42.9% in dog, cat and horse isolates respectively, which was positively associated with the chronicity of infection and prior antimicrobial treatment, in particular for urinary tract infections from dogs.

Furthermore, among coagulase-positive staphylococci (CoPS) isolates from companion animals the frequency of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and methicillin-resistant *Staphylococcus aureus* (MRSA) in the isolate collections was 11.8% (74/629) and 12.8% (15/117),respectively. Resistance to FQN in *S. pseudintermedius* ranged from 8.1%-8.8% and was highly associated with MRSP isolates from dogs (OR 287; 95%CI 91.2–1144.8). MRSA isolates were also co-resistant to FQN (OR 5.4, 95%CI 0.6–252.1), with a frequency ranging from 8.5%-11.8% of total *S. aureus* from dogs (n=47) and cats (n=14). By contrast MRSA isolates from horses, were most likely to be co- resistant to amikacin and rifampicin (OR 6.5, 95%CI 0.7–315.2) with a frequency of 9.4% of total *S. aureus* from horses (n=53). A risk factor analysis showed that MRSP isolates from dogs were significantly more likely in surgical site infections (SSI) and skin

and soft tissue infections (SSTI), particularly if the animals had received prior antimicrobial treatment. Compared with other countries where similar studies have been undertaken, rates of AMR in *E. coli*, were generally lower. However, due to the abrupt emergence and spread of MRSP infections in Australia, rates of AMR in *S. aureus* and *S. pseudintermedius* were moderately higher.

Faecal and gut microbiota profiling using V3-V4 16S rRNA gene Illumina MiSeq DNA sequence analysis confirmed that the veterinary fluoroquinolone pradofloxacin (which has a much broader spectrum of activity compared to earlier generations) had quite different effects on the microbial community in the rabbit stomach and caecum; compared to hard faeces, and soft faeces. Microbial richness and diversity decreased significantly in hard faeces at the end of the 3-day treatment but not in the lumen of the stomach and caecum. *Pseudomonas* spp. was depleted significantly in hard faeces while *Anaeroplasma* significantly diminished in the stomach and caecum. The abundance of several bacterial taxa from the Ruminococcaceae and Lachnospiraceae was significantly overrepresented in all samples. However, overproliferation of bacteria causing enteritis such as *Clostridium* spp. and coliforms was not detected in the two gut compartments nor hard and soft faeces. Further, we hypothesized that stomach microbiota would be less diverse compared with caecum microbiota but our data show that the stomach harboured higher levels of microbial diversity compared with the caecum, regardless of pradofloxacin treatment, most likely due to coprophagy. Although the short-term use of pradofloxacin appears safe in rabbits as we observed no clinically adverse effects, these results confirm that pradofloxacin did result in significant disturbance of the faecal microbial community and changes of metabolic functional diversity.

In conclusion, the first nation-wide AMR survey provides an important snapshot of the current situation of AMR in companion animal pathogens in Australia. Further, this thesis underlined the public health (AMR) and individual health-related (dysbiosis) consequences which should increase the awareness of prudent use of antibiotics especially in animals and broadly in human medicine.

Chapter 1

General Introduction

1.1. Antimicrobials and their consequences

Antimicrobials are one of the most successful forms of chemotherapy in the history of medicine. Ever since the first three antimicrobials (salvarsan, prontosil, and penicillin) were introduced between 1910-1940 announcing the golden era of antibiotic discovery which took place between 1950-1970, the morbidity and mortality associated with bacterial infections has decreased significantly (Aminov, 2010). Antimicrobials have played a fundamental role in achieving major advances in medicine and surgery, as well as successfully preventing and treating infections that occur not only in humans but also in animals (Bengtsson and Greko, 2014; McEwen and Fedorka-Cray, 2002; Ventola, 2015). Along with those obvious benefits, however, administration of antimicrobial agents causes adverse effects, which can be broadly characterised as the development of drug hypersensitivity, toxicological hazards, and effect on the autochthonous microbiota (Francino, 2015; Manten, 1981). This thesis focuses on two main aspects of antimicrobial therapy: the development of antimicrobial resistance (AMR) in pathogens and microbial dysbiosis in the gut microbiota.

1.2. The emergence and monitoring of AMR

Resistance to antimicrobials is a natural process, arising when microorganisms are able to survive under exposure to agents that would normally kill them or stop their growth. When the resistance occurs, antibiotics become less effective for the prevention and treatment of bacterial infections. Bacterial resistance mechanisms associated with several antimicrobials, such as sulphonamides and penicillin, were first reported in the late 1930s and early 1940s soon after the clinical introduction of these early classes (Davies and Davies, 2010). Another case of resistance development, but this time in animals, was observed in the 1940s after poultry were fed dried pseudomycelia of *Streptomyces aureofaciens* containing chlortetracycline to promote growth (Castanon, 2007). For many years, the positive effects of antibiotics as a growth promoter were championed until a 1957 study revealed the negative effect of this practice, when low-dose tetracycline in poultry and pig's feed was shown to increase the incidence of drug-resistant commensal bacteria (Marshall and Levy, 2011).

In terms of their purpose, antimicrobials are used in more diverse ways in animals compared to humans, i.e. for prophylaxis, metaphylaxis, therapeutic and sub-therapeutic purposes (McEwen and Fedorka-Cray, 2002). It is also projected that the volume of antimicrobials used in animals exceeds the volume of use in humans worldwide (Wegener, 2012). The rate of AMR has increased in recent years; presumably by over- and mis-use of antimicrobials. This has had a significant impact on their dissemination into the environment. Zoonotic transmission routes of resistant bacteria, as well as resistant genes shared between humans and animals are

very complex and unpredictable. Resistance gene transfer may occur through direct contact with food-producing animals and companion animals, or indirect contact through food, water, and farm waste (Figure 1.2.1.) (Davies and Davies, 2010; Guardabassi et al., 2004; Lloyd, 2007; Marshall and Levy, 2011). Therefore, recognising the continued emergence of bacterial pathogens that are resistant to critically important antimicrobials is crucial for global public health.

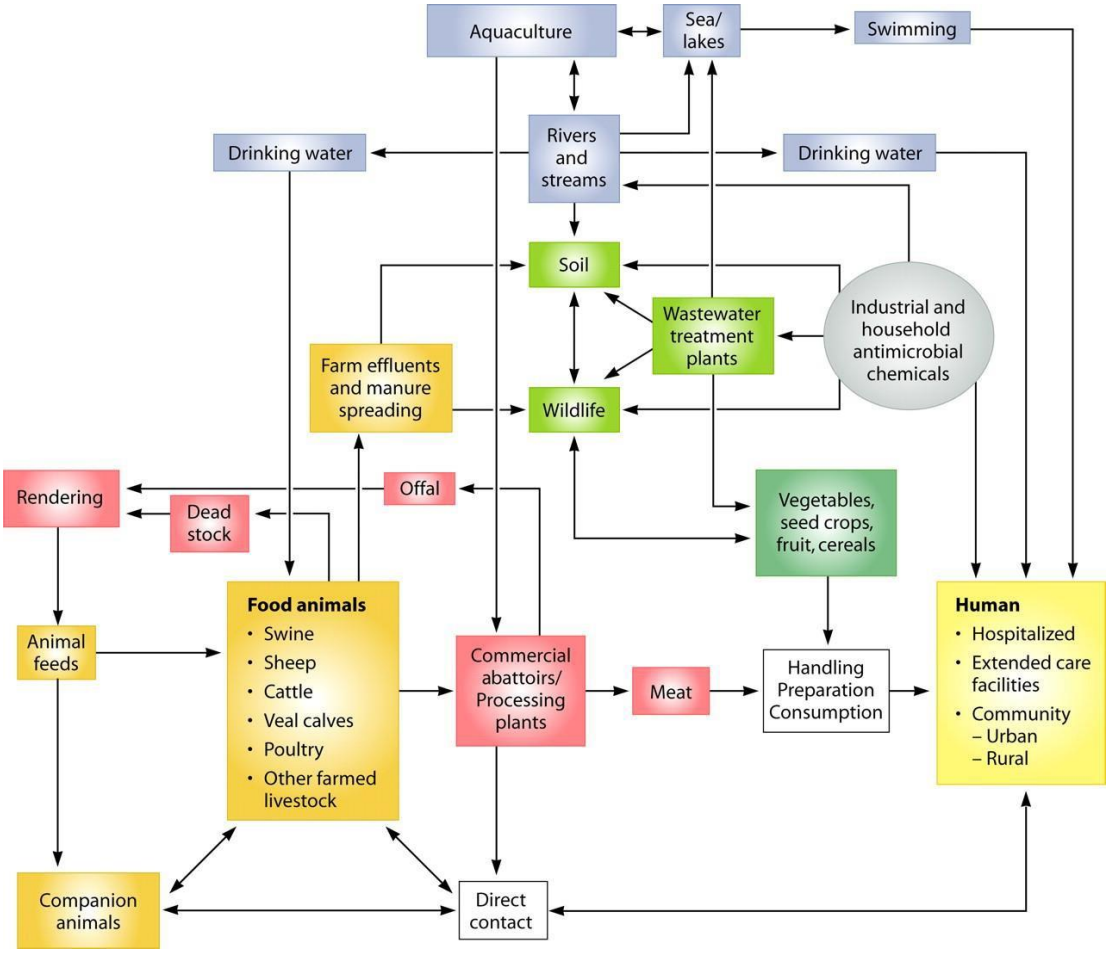

Figure 1.2.1. Dissemination of antimicrobials and AMR in various environmental aspects, including agriculture, the community, hospitals, wastewater treatment, and associated environments (Davies and Davies, 2010).

AMR has been considered a global health problem and is a key element of the One Health and Global Health Security Agendas (GHSA) (Perovic and Schultsz, 2016; Robinson et al., 2016). Several campaigns to prevent and control the emergence of AMR have been developed both nationally and internationally. The World Health Organisation (WHO) has introduced a Global Action Plan on AMR which comprises five objectives: (1) to improve awareness and understanding of AMR; (2) to strengthen knowledge through surveillance and research; (3) to reduce the incidence of infection; (4) to optimize the use of antimicrobial agents; and (5) to develop the economic case for sustainable investment that takes account of the needs of all countries, and increases investment in new medicines, diagnostic tools,

vaccines and other interventions (WHO, 2015a). One of the methods of implementing these global plans is to launch the Global AMR Surveillance System (GLASS), which aims to combine clinical, laboratory and epidemiological data on pathogens that pose the greatest threats to health globally, in particular antibiotic resistant bacteria (WHO, 2015b). Alongside the WHO, the World Animal Health Organization (OIE) and the Food and Agricultural Organization (FAO), as tripartite collaborations, also promote the prudent use of veterinary drugs, help to harmonise national surveillance programs, and raise awareness and increase supporting research on AMR with a "One Health" approach, involving human and animal health alongside agricultural and environmental needs (OIE, 2016).

Historically, the development of an AMR management and surveillance system had already begun in 1995, before the official recommendation by the WHO. Denmark was the first country to establish a continuous monitoring program called the Danish Integrated AMR Monitoring and Research Program (DANMAP). The main objectives of this program are to monitor the consumption of antimicrobial agents and the occurrence of AMR originating in humans and animals, to study the relationship between them and to analyze possible resistance transmission (Hammerum et al., 2007). In 1996, the USA established a similar program, the National AMR Monitoring System (NARMS), collecting resistance data from bacteria isolated from retail meats, human clinical cases and food animals (Gilbert et al., 2007). Subsequently, other countries such as Norway, Sweden and Canada also established similar surveillance programs to monitor AMR both in human and veterinary medicine systematically (NHMRC, 2006).

In Australia, there is no surveillance program on AMR in animals. However, several studies on AMR have been conducted, in particular in food production animals. A study by Murray et al. (1986), undertaken between 1974 and 1982 and initiated by the National Animal Health Committee, was among the first reports describing the frequency of AMR. The survey was conducted by collecting the antimicrobial susceptibility patterns of several pathogenic bacteria from bovine, porcine and avian sources, such as *Salmonella, Staphylococcus aureus* and *E. coli*. In order to monitor the trend of AMR, the Australian Health Ministers' Conference (AHMC) then established the Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) in 1998, although it was disbanded in 2002. This program focused on the spread and transmission of AMR in livestock, using some pathogenic bacteria such as *Salmonella, Campylobacter, E. coli* and *Enterococcus* as examples and explored its potential impact to public health (JETACAR, 1999). To replace this program and deal with the responsibility for monitoring AMR, the Australian Government Department of Agriculture, Fisheries and Forestry finally established the Pilot Surveillance Program for AMR in Bacteria of Animal Origin in 2004. The AMR of more than 1000 strains including *E. coli, Enterococcus* spp. and *Campylobacter* spp. were analyzed from approximately 700 samples of cattle, pig and chicken faeces (DAFF, 2006). No subsequent studies,

however, were undertaken until Meat and Livestock Australia and the Department of Agriculture and Water Resources commissioned further abattoir-based surveys in healthy slaughter age cattle (Barlow et al., 2015, 2017), and pigs and poultry (Schipp, 2018), respectively. However, companion animals (dogs, cats and horses) were not included in the pilot program mentioned above, even though many studies show that they also have a high potential for creating an AMR reservoir. Companion animals have closer contact with humans and are often treated with the same classes of antimicrobial agents used in human medicine, such as fluoroquinolones (e.g. enrofloxacin, marbofloxacin and the newly registered pradofloxacin) (Guardabassi et al., 2004; Lloyd, 2007).

1.3. Effect of pradofloxacin on dysbiosis of normal microbiota in rabbits as an animal model

While penicillin-resistant microorganisms were first reported in 1940, the striking alteration of normal microbiota in various body parts caused by penicillin administration was reported several years later, in 1946 (Keefer, 1951). A sudden change in the bacterial microbiota of the throat from Gram-positive genera to Gram-negative genera was observed. Coliform bacteria also appeared in the throat throughout the course but, subsequent to treatment, coliform organisms gradually disappeared, and the throat became dominated again by Gram-positive cocci. In some cases, new infections appeared after treatment. Bacteria which are present in small numbers and not sensitive to the antibiotic may be proliferating after the susceptible organisms are inhibited. In line with other studies of faecal samples and clinical observation, it was eventually concluded that the disturbance of the gut microbiota caused by the prolonged administration of antibiotics may result in the development of secondary vitamin deficiencies (microbiota alterations of function and immunity) or the evolution of new infectious disease syndromes (such as antibiotic- induced enteritis caused by *Clostridium difficile*) (Keefer, 1951; Smith, 1952; Sullivan et al., 2001). The impact of antimicrobial agents on normal microbiota has been studied widely in skin, genitals and gastrointestinal (GI) tracts using culture-dependant and culture-free methods (Sullivan et al., 2001). Through advances in technology, it is now possible to assess the changes in the gut microbial community in multiple aspects, which include metagenomics (total microbiota), metametabolomics (metabolic function) and metatranscriptomics (function of transcriptomes) as well as through characterization of the development and distribution of resistance mechanisms amongst the microbiota (resistome) (Perez-Cobas et al., 2013).

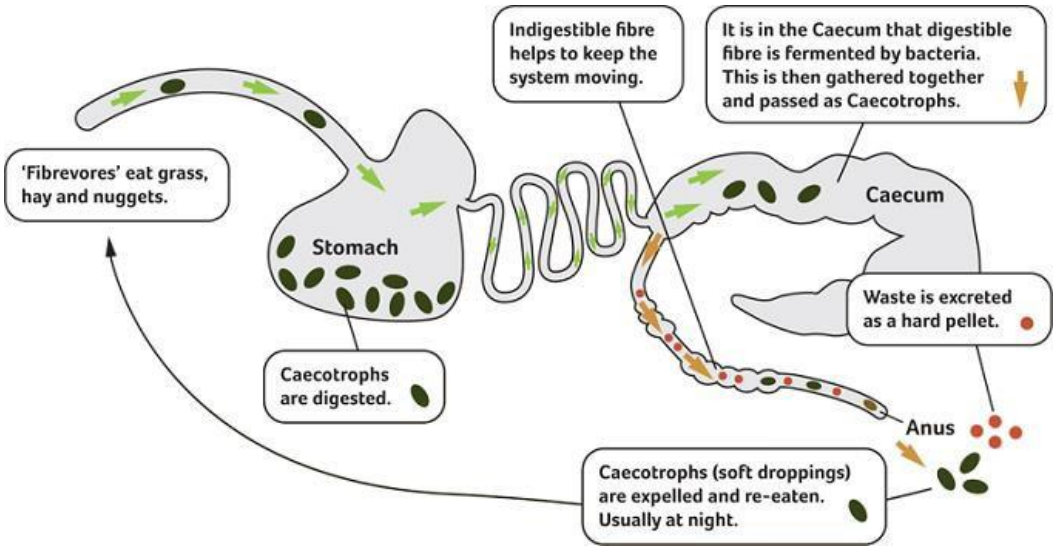

Figure 1.3.1. Description of the rabbit's digestive system (Anonymous, 2014).

The severity of disturbance of the gut microbiota caused by antibiotics may present differently between humans and animal species, depending on their physiological and anatomical properties. Rabbits, as well as hamsters and other rodents, are examples of monogastric animals that have a delicately balanced gastrointestinal tract (Anonymous, 2014; Johnson-Delaney, 2006), that is sensitive to diet changes and certain classes of antibiotics. The rabbit GI tract is a highly complex structure that occupies a large proportion of the body cavity with approximately 88% of all digestible material contained in the stomach and caecum (Halls, 2008). Indigestible fibre is pushed into the colon where it is formed into hard faeces, while the remaining digestible material moves into the caecum and is expelled into the colon to form soft pellets which are reingested, known as caecotrophs (Figure 1.3.1.) (Anonymous, 2014; Halls, 2008). The digestion process is supported by a large and diverse population of microorganisms. The normal functioning and movement of the GI tract and the health of its autochthonous microbial community are vital to the rabbit's overall health, but with such a complex system, any disturbance can tip the balance into a state of disease, which may rapidly become life-threatening (Meredith, 2010).

Disturbances in the gut microbiota caused by antibiotics depend on the properties of the agents, absorption levels, route of administration and elimination, and possible enzymatic inactivation and/or binding to the faecal material by the agents. Generally, the most common clinical signs of disturbances in the intestinal microbiota are temporary diarrhoea and fungal overgrowth (Sullivan et al., 2001). In rabbits, antibiotic-induced diarrhoea (enteritis), is mainly caused by over-proliferation of enterotoxin-producing *E. coli, Clostridium perfringens* and *Clostridium spiroforme* (Halls, 2005; Prescott, 1978). Enterotoxaemia caused by *C. spiroforme* is

commonly found in recently-weaned fryers (4 to 8 weeks of age) and is thought to be associated with high carbohydrate/high protein/low fibre diets; whilst in adults, administration of antibiotics is usually the main cause (Halls, 2005). Many antibiotics have been associated with enterotoxaemia, including lincomycin, clindamycin, amoxicillin, penicillin, ampicillin, amoxicillin-clavulanic acid, erythromycin, and cephalosporins. However, fluoroquinolone antimicrobials, such as enrofloxacin are better tolerated by rabbits, presumably due to their limited effect on strictly anaerobic bacteria (Kelly and O'Rourke, 2001).

The fluoroquinolones are synthetic bactericidal agents that are broadly used in both human and veterinary medicine for treating a variety of bacterial infections. These agents work through the inhibition of DNA gyrase by interfering with the supercoiling and decatenation of bacterial chromosomal DNA which are primarily active against Gram-negative bacteria, *Mycoplasma,* and some Gram-positive bacteria (Brown, 1996; Hooper, 2001). The first and second-generation fluoroquinolones (nalidixic acid, ciprofloxacin, levofloxacin, enrofloxacin, marbofloxacin) have little or no activity against group D streptococci and obligate anaerobic bacteria but most of the third and fourth generation fluoroquinolones (moxifloxacin and pradofloxacin) have a broader spectrum of activity which includes anaerobes and streptococci (Pallo-Zimmerman et al., 2010; Stein, 1996). Fluoroquinolone resistance develops rapidly, varies greatly among bacterial species, and can be both chromosomal and plasmid-mediated in origin, leading to major public health issues in clinical settings since they are listed as critically important to human health (Redgrave et al., 2014).

Fluoroquinolones can be evaluated by their ability to minimize or limit the clonal expansion of resistant mutants present in large populations of bacteria on the basis of their mutant prevention concentration (MPC), which has been defined as the minimum inhibitory concentration (MIC) of the least susceptible single-step mutant within the population (Blondeau et al., 2001; Smith et al., 2003). Compared with other fluoroquinolones such as marbofloxacin, enrofloxacin, danofloxacin, sarafloxacin, orbifloxacin, and difloxacin; pradofloxacin has the lowest MPCs for both Gram-negative and Gram-positive bacteria; up to 7-fold and 31-fold lower for *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 (Wetzstein, 2005). *In vitro* MPC studies indicate the high potential of pradofloxacin for restricting the selection and amplification of 1st and even 2nd-step fluoroquinolone-resistant mutants naturally present in the population (Schink et al., 2013; Wetzstein, 2005).

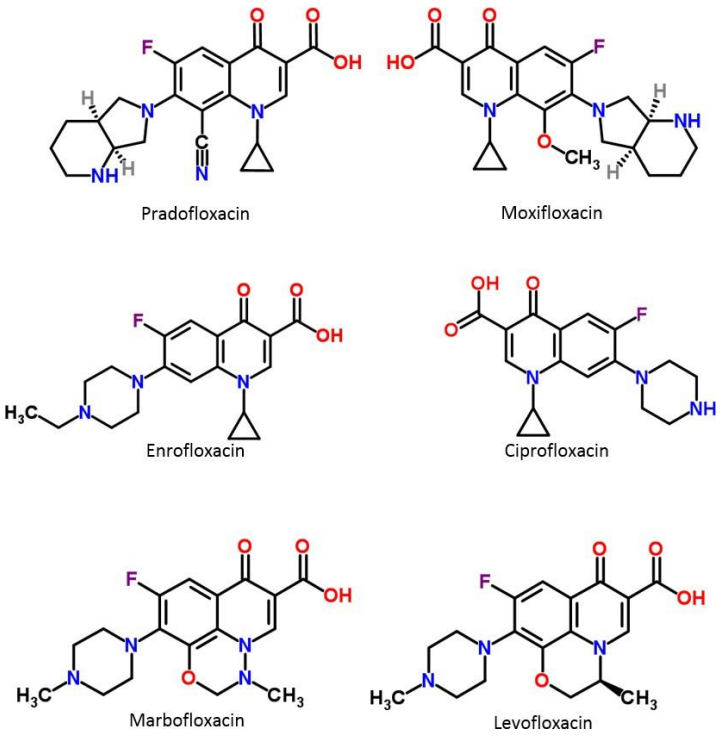

Figure 1.3.2. Chemical structure of fluoroquinolones for animals (left side) and the analogue in human medicine (Anonymous, 2015).

Several veterinary fluoroquinolones have been approved for use in dogs and cats in Australia, including difloxacin (2001), enrofloxacin (2004), orbifloxacin (1999), ibafloxacin (2007) (Cheng et al., 2012) and more recently pradofloxacin (2014). However, of these five, only enrofloxacin, marbofloxacin and pradofloxacin have remained in clinical use. Fluoroquinolones have not been approved for use in food-producing animals in Australia but are frequently used off-label in non-food-producing species. Pradofloxacin, under the name Veraflox®, has been evaluated widely in both *in vitro* and clinical studies. It is intended to be used for dogs suffering from skin infections, periodontal disease, urinary tract infections and cats with wound infections, abscesses, and upper respiratory tract infections (Bayer, 2017).

1.4. Aims and objectives of the present study

The pilot monitoring programs for AMR in Australian food-producing animals were successful in attaining the appropriate samples of bacteria from healthy livestock and provide scientific evidence of low risk to human health posed by AMR in locally produced meat and eggs in this country (APVMA, 2017; JETACAR, 1999). However, this program was predominantly focused on food-producing animals while AMR in companion animals has never been investigated in Australia. As a result, Australia has no national data on antimicrobial-resistant companion animal pathogens, especially *E. coli* and *Staphylococcus* spp. which are the predominant pathogens in human medicine that can acquire AMR. Therefore, the first aim of this thesis was to

determine the frequency of AMR in a national collection of companion animal pathogens in Australia as well as the risk factors for infection by MDR strains. Chapter 2 focuses on the frequency of resistance to critically important antimicrobials in clinical *E. coli*, isolated from dogs, cats and horses in Australia. Risk factors for MDR *E. coli* urinary tract infections are also determined. Chapter 3 reports AMR in staphylococcal species causing infections in dogs, cats and horses; the frequency of methicillin-resistant *S. pseudintermedius* (MRSP) and methicillin-resistant *S. aureus* (MRSA) strains in the collection and risk factors for MRSP infection in dogs. In Chapter 4, a review of AMR studies related to companion animal pathogens in Australia is presented and compared with similar studies in other countries.

In Chapter 5, antimicrobial effects on faecal microbial diversity and structure are investigated and discussed from various metagenomics studies in humans, laboratory animals and food production animals. Despite pradofloxacin's importance as a top shelf reserve antimicrobial agent with a wide spectrum of activity and the lowest MPC values of all the fluoroquinolones, neither the effect of pradofloxacin on the gut microbial community nor its safety in rabbits, rodents and guinea pigs has ever been reported. Thus, the second aim of this thesis is to determine whether pradofloxacin is safe for use in rabbits by analysing the changes in microbial communities in faecal samples (Chapter 6) and stomach and caecum samples (Chapter 7). The gross and histological anatomy of rabbits' gastrointestinal tracts and clinical signs following up to 3 days of treatment were also assessed. This second part of the thesis will also provide valuable information on the in vivo safety of pradofloxacin, using the relatively new technique of microbiota profiling of the gastrontestinal tract.

1.5. References

Aminov, R.I., 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. Front. Microbiol. 1, 134.

Anonymous 2014. Importance of high fibre diet for rabbits, guinea pigs & chinchillas. In Pet Habitat (South Africa).

Anonymous 2015. ChemSpider: search and share chemistry (United Kingdom, The Royal Society of Chemistry).

APVMA 2017. Antibiotic resistance in animals (Kingston, Australia, Australian Pesticides and Veterinary Medicines Authority).

Barlow, R.S., McMillan, K.E., Duffy, L.L., Fegan, N., Jordan, D., Mellor, G.E., 2015. Prevalence and antimicrobial resistance of *Salmonella* and *Escherichia coli* from Australian cattle populations at slaughter. J. Food. Prot. 78, 912-920.

Barlow, R.S., McMillan, K.E., Duffy, L.L., Fegan, N., Jordan, D., Mellor, G.E., 2017. Antimicrobial resistance status of *Enterococcus* from Australian cattle populations at slaughter. PLoS One 12, e0177728.

Bayer 2017. Veraflox Product Manual (Leverkusen, Germany, Bayer).

Bengtsson, B., Greko, C., 2014. Antibiotic resistance-consequences for animal health, welfare, and food production. Ups. J. Med. Sci. 119, 96-102.

Blondeau, J.M., Zhao, X., Hansen, G., Drlica, K., 2001. Mutant prevention concentrations of fluoroquinolones for clinical isolates of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 45, 433-438.

Brown, S.A., 1996. Fluoroquinolones in animal health. J. Vet. Pharmacol. Ther. 19, 1-14.

Castanon, J.I., 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86, 2466-2471.

Cheng, A.C., Turnidge, J., Collignon, P., Looke, D., Barton, M., Gottlieb, T., 2012. Control of fluoroquinolone resistance through successful regulation, Australia. Emerg. Infect. Dis. 18, 1453-1460.

DAFF 2006. Antimicrobial resistance in bacteria of animal origin: pilot surveillance program (Canberra, Australia, Australian Government Department of Agriculture, Fisheries and Forestry).

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74, 417-433.

Francino, M.P., 2015. Antibiotics and the human gut microbiome: dysbioses and accumulation of resistances. Front. Microbiol. 6, 1543.

Gilbert, J.M., White, D.G., McDermott, P.F., 2007. The US national antimicrobial resistance monitoring system. Future Microbiol 2, 493-500.

Guardabassi, L., Schwarz, S., Lloyd, D.H., 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria. J. Antimicrob. Chemother. 54, 321-332.

Halls, A.E. 2005. Enteric diseases in rabbits. In Nutrifax (Canada, Nutreco Canada Inc.).

Halls, A.E. 2008. Caecotrophy in rabbits. In Nutrifax (Canada, Nutreco Canada Inc.).

Hammerum, A.M., Heuer, O.E., Emborg, H.-D., Bagger-Skjøt, L., Jensen, V.F., Rogues, A.-M., Skov, R.L., Agersø, Y., Brandt, C.T., Seyfarth, A.M., Muller, A., Karin Hovgaard, Ajufo, J., Bager, F., Aarestrup, F.M., Frimodt-Møller, N., Wegener, H.C., Monnet, D.L., 2007. Danish integrated antimicrobial resistance monitoring and research program. Emerg. Infect. Dis. 13, 1632-1639.

Hooper, D., 2001. Mechanisms of action of antimicrobials: focus on fluoroquinolones. Clin. Infect. Dis. 32, S9-S15.

JETACAR 1999. The use of antibiotics in food-producing animals: antibiotic resistant bacteria in animals and humans (Canberra, Australia).

Johnson-Delaney, C., 2006. Anatomy and physiology of the rabbit and rodent gastrointestinal system. Proceeding Association of Avian Veterinarians, 9-17.

Keefer, C.S., 1951. Alterations in normal bacterial flora of man and secondary infectionas during antibiotic therapy. Am. J. Med. XI. 11, 665-666.

Kelly, M., O'Rourke, D. 2001. Normal bacterial flora of the rabbit gastrointestinal tract: a clinical approach. In: Seminars in Avian and Exotic Pet Medicine, 45-47.

Lloyd, D.H., 2007. Reservoirs of antimicrobial resistance in pet animals. Clin. Infect. Dis. 45 Suppl 2, S148-152.

Manten, A., 1981. Side effects of antibiotics. Vet. Q. 3, 179-182.

Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24, 718-733.

McEwen, S.A., Fedorka-Cray, P.J., 2002. Antimicrobial use and resistance in animals. Clin. Infect. Dis. 34, S93-106.

Meredith, A. 2010. The rabbit digestive system: a delicate balance. Rabbiting On, Winter 2010, 7-9.

Murray, C., Ratcliff, R., Cameron, P., Dixon, S., 1986. The resistance of antimicrobial agents in *Salmonella* from veterinary sources in Australia from 1975 to 1982. Aus. Vet. J. 63, 286-292.

NHMRC 2006. A comprehensive integrated surveillance program to improve Australia's response to antimicrobial resistance (New South Wales, Australia).

OIE 2016. The OIE strategy on antimicrobial resistance and the prudent use of antimicrobials (Paris, France, The World Organisation for Animal Health).

Pallo-Zimmerman, L.M., Byron, J.K., Graves, T.K., 2010. Fluoroquinolones: then and now. Compendium 32, E1-E9.

Perez-Cobas, A.E., Gosalbes, M.J., Friedrichs, A., Knecht, H., Artacho, A., Eismann, K., Otto, W., Rojo, D., Bargiela, R., von Bergen, M., Neulinger, S.C., Daumer, C., Heinsen, F.A., Latorre, A., Barbas, C., Seifert, J., dos Santos, V.M., Ott, S.J., Ferrer, M., Moya, A., 2013. Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. Gut 62, 1591-1601.

Perovic, O., Schultsz, C., 2016. Stepwise approach for implementation of antimicrobial resistance surveillance in Africa. Afr. J. Lab. Med. 5, 1-7.

Prescott, J.F., 1978. *Escherichia coli* and diarrhoea in the rabbit. Vet. Pathol. 15, 237-248.

Redgrave, L.S., Sutton, S.B., Webber, M.A., Piddock, L.J., 2014. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. Trends Microbiol. 22, 438-445.

Robinson, T.P., Bu, D.P., Carrique-Mas, J., Fevre, E.M., Gilbert, M., Grace, D., Hay, S.I., Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U., Thi Ngoc, P., Van Boeckel, T.P., Woolhouse, M.E., 2016. Antibiotic resistance is the quintessential One Health issue. Trans. R. Soc. Trop. Med. Hyg. 110, 377-380.

Schink, A.K., Kadlec, K., Hauschild, T., Brenner Michael, G., Dorner, J.C., Ludwig, C., Werckenthin, C., Hehnen, H.R., Stephan, B., Schwarz, S., 2013. Susceptibility of canine and feline bacterial pathogens to pradofloxacin and comparison with other fluoroquinolones approved for companion animals. Vet. Microbiol. 162, 119-126.

Schipp, M., 2018. Australian veterinarians - global challenges. Aust. Vet. J. 96, 4-10.

Smith, D.T., 1952. The disturbance of the normal bacterial ecology by the administration of antibiotics with the development of new clinical syndromes. Ann. Intern. Med. 37, 1135.

Smith, H.J., Nichol, K.A., Hoban, D.J., Zhanel, G.G., 2003. Stretching the mutant prevention concentration (MPC) beyond its limits. J. Antimicrob. Chemother 51, 1323–1325.

Stein, G.E., 1996. Pharmacokinetics and pharmacodynamics of newer fluoroquinolones. Clin. Infect. Dis. 23, S19-S24.

Sullivan, Å., Edlund, C., Nord, C.E., 2001. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect. Dis. 1, 101-114.

Ventola, C.L., 2015. The antibiotic resistance crisis. Pharm. Thera. 40, 277-283.

Wegener, H.C. 2012. Antibiotic-resistance-linking human and animal health. In: Improving Food Safety Through a One Health Approach, Washington, 331-349.

Wetzstein, H.G., 2005. Comparative mutant prevention concentrations of pradofloxacin and other veterinary fluoroquinolones indicate differing potentials in preventing selection of resistance. Antimicrob. Agents Chemother. 49, 4166-4173.

WHO 2015a. Global action plan on antimicrobial resistance (Geneva, Switzerland, WHO Press).

WHO 2015b. Global antimicrobial resistance surveillance system manual for early implementation (Geneva, Switzerland, WHO Press).

Chapter 2

Antimicrobial resistance in coagulase-positive staphylococci isolated from companion animals in Australia: A one year study

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Statement of Authorship

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Contribution to the Paper	Performed laboratory work, analysis and interpreted data and wrote manuscript		
Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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PLOS ONE

RESEARCH ARTICLE

Antimicrobial resistance in coagulase-positive staphylococci isolated from companion animals in Australia: A one year study

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Abstract

Methicillin-resistant coagulase-positive staphylococci (CoPS) have become increasingly recognised as opportunistic pathogens that limit therapeutic options in companion animals. The frequency of methicillin resistance amongst clinical isolates on an Australia-wide level is unknown. This study determined antimicrobial susceptibility patterns for CoPS isolated from clinical infections in companion animals (dogs, cats and horses) as part of the first nation-wide survey on antimicrobial resistance in animal pathogens in Australia for a one-year period (January 2013 to January 2014). Clinical *Staphylococcus* spp. isolates (n = 888) obtained from 22 veterinary diagnostic laboratories were identified by MALDI-TOF mass spectrometry and subjected to antimicrobial susceptibility testing for 16 antimicrobials, representing 12 antimicrobial classes. Potential risk factors associated with methicillin resistance in *Staphylococcus pseudintermedius* isolates from dogs were analysed based on demographic factors and clinical history, including gender, age, previous antimicrobial treatment, chronic and/or recurrent diseases and site of infections. The most commonly identified CoPS were *S. pseudintermedius* (70.8%; dogs n = 616, cats n = 13) and *S. aureus* (13.2%, horses n = 53, dogs n = 47 and cats n = 17). Overall, the frequency of methicillin resistance among *S. pseudintermedius* (MRSP) and *S. aureus* (MRSA) was 11.8% and 12.8%, respectively. MRSP isolates were strongly associated with resistance to fluoroquinolones (OR 287; 95%CI 91.2–1144.8) and clindamycin (OR 105.2, 95%CI 48.5–231.9). MRSA isolates from dogs and cats were also more likely to be resistant to fluoroquinolones (OR 5.4, 95%CI 0.6–252.1), whereas MRSA from horses were more likely to be resistant to rifampicin. In multivariate analysis, MRSP-positive status was significantly associated with particular infection sites, including surgical (OR 8.8; 95%CI 3.74–20.7), and skin and soft tissue (OR 3.9; 95%CI 1.97–7.51). *S. pseudintermedius* isolated from dogs with surgical site infections were three times more likely to be methicillin-resistant if cases had received prior antimicrobial treatment. Whilst the survey results indicate the proportion of CoPS obtained from Australian companion animals that are methicillin-resistant is currently moderate, the

collection and analysis, decision to publish, or preparation of the manuscript.

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identified risk factors suggest that it could rapidly increase without adequate biosecurity and infection control procedures in veterinary practice.

Introduction

Coagulase-positive staphylococci (CoPS) cause a range of infections such us bacteraemia, urinary tract infections, pyoderma, abscess and wound infections in both humans and animals [1]. Infections are compounded by the emergence of methicillin-resistant strains that have acquired *mecA* or *mecC* imparting resistance to all the beta-lactams with the exception of a few anti-staphylococci cephalosporins [2]. The major methicillin-resistant CoPS that cause clinical infections are methicillin-resistant *Staphylococcus aureus* (MRSA) in both humans and animals [3], and methicillin-resistant *S. pseudintermedius* (MRSP) in dogs and cats [4]. MRSA and MRSP isolates are often resistant to multiple classes of critically important antimicrobials (CIAs) including fluoroquinolones and aminoglycosides, thereby limiting therapeutic options to treat these infections. In recent years, studies have demonstrated the emergence and clonal spread of MRSA in companion animals (defined here as dogs, cats and horses), and livestock, with potential for bi-directional transmission of these strains between animals and humans [5–7].

In companion animals, distinct MRSA clones appear to colonise specific animal host species. For example, healthcare associated MRSA clone ST22 (EMRSA-15) is most commonly isolated from dogs and cats while community associated MRSA CC 8 (ST8, ST612 and ST254) clones are host-adapted to horses [8]. A recent study by Harrison et al. has also demonstrated that globally disseminated MRSA ST22-IV strains can colonise and cause infection in humans, dogs, and cats without undergoing typical host adaption involving loss or acquisition of antimicrobial resistance and/or prophage genes [9]. These studies demonstrate the need for understanding the ecology and distribution of MRSA clones in companion animals.

Parallel to the emergence of MRSA in companion animals, MRSP has recently emerged in dogs and cats as a cause of skin and soft tissue, post-surgical site and urinary tract infections. Globally, the frequency of MRSP infections in dogs and cats has been increasing and MRSP is now considered to be one of the most important pathogens in small animal medicine [10]. This is attributed to the global spread of MRSP clones and the associated resistance to other CIAs such as fluoroquinolones and aminoglycosides. Unlike MRSA in companion animals, MRSP is not a major zoonotic pathogen and has limited public health impact [10]. However, due to the limited therapeutic options to treat MRSP infections they are now a major animal health issue and require careful monitoring and management [11].

Various studies have demonstrated carriage and zooanthroponotic transmission of MRSA and other multidrug-resistant staphylococci [12] between animals and humans. Consequently, many countries have established surveillance programs to monitor emerging antimicrobial resistance in animals, although companion animals are generally poorly represented in these activities. The frequency and antimicrobial resistance profile of clinical staphylococci in companion animals has been reported in Asia [13, 14], Africa [15], North America and Europe [16]. Sweden and Norway are among the few countries that monitor the occurrence of these resistant strains regularly [17, 18], enabling more accurate estimation of frequency, trends and antimicrobial resistance profiles to be compared on a yearly basis.

In Australia, several one-off studies have documented the recent emergence of MRSA and MRSP as causes of clinical infections as well as carriage by healthy companion animals [11,

19–21]. In addition, carriage of MRSA by Australian veterinarians involved in clinical practice has been well documented with the highest rates of carriage in equine veterinarians (21.4%), mixed-practice veterinarians (11.8%) and those who work exclusively with dogs and cats (4.9%) [7, 19, 22]. However, the frequency of methicillin resistance amongst isolates from infections in companion animals on an Australia-wide level is currently unknown. Therefore, in the present study, we undertook the first national survey of antimicrobial resistance in CoPS isolated from clinical infections in Australian companion animals. The aims were to define the distribution of CoPS species causing clinical infection in companion animals, the frequency of antimicrobial resistance (particularly methicillin resistance and multidrug resistance) and to examine potential risk factors that may contribute to the occurrence of methicillin-resistant strains amongst the most prevalent species.

Materials and methods

Isolate collection and identification

The CoPS isolates were collected during the first national survey of antimicrobial resistance in Australian animal pathogens, which took place over 12 months (January 2013 to January 2014) with the cooperation of all veterinary diagnostic laboratories in each Australian state and territory (n = 22) [23]. Submitting laboratories were instructed to forward coagulase-positive isolates that were considered to be clinically relevant to the presenting condition, as judged by the diagnostic microbiologist. The bacteria were isolated from swabs taken from site of infections or clinical specimens (e.g. urine, biopsies) collected by veterinarians and submitted to veterinary diagnostic laboratories for routine culture and susceptibility testing. All confidential information such as animal name, owner name, address and contract information was removed by the participating veterinary diagnostic laboratories before sending the isolates and clinical information to The University of Adelaide reference laboratory for this study. As a result this study did not require animal ethics approval, as per the Australian National Health and Medical Research Council, Animal Research Ethics code.

Prior to cryopreservation in 20% glycerol broth, isolates were confirmed for purity and haemolysis pattern on Columbia sheep blood agar (SBA; Thermo Fisher Scientific Australia), and identified to genus level using standard phenotypic tests including Gram-stain and the catalase test. A total of 888 isolates were collected in this study, originating from dogs (n = 743, 83.7%), cats, (n = 77, 8.7%) and horses (n = 68, 7.7%). To confirm the identity of staphylococci isolates to species level, all isolates were subjected to MALDI-TOF (Bruker) according to the manufacturer's protocol for bacterial identification.

Antimicrobial susceptibility testing and interpretation

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method of the Clinical Laboratory Standards Institute (CLSI) [24] (Table 1). A total of 16 antimicrobial agents from 12 antimicrobial classes were investigated including aminoglycosides (AMK); ansamycins (RIF); β-lactam/β-lactamase inhibitor combinations (AMC); β-lactams (OXA); fluoroquinolones (CIP, ENR, MRB and PRA); folate-pathway inhibitors (SXT); 1st generation cephalosporins (CEF); 2nd generation cephamycin (FOX); 3rd generation cephalosporins (CVN and CRE); lincosamides (CLI); phenicols (CHL); and tetracyclines (TET). Antimicrobials were obtained from Sigma Aldrich (Australia) and Zoetis (Australia). *Staphylococcus aureus* ATCC 25923 and ATCC 29213 were used as control strains. MIC results were interpreted as resistant (R), susceptible (S) and intermediate (I, if available), according to veterinary specific and human approved interpretative criteria per Clinical and Laboratory Standards Institute (CLSI) VET01S guidelines [25]. When clinical breakpoints were not available in CLSI, MICs

Table 1. Antimicrobial agents and MIC breakpoints (μg/mL) used in this study based on CLSI VET01S and ECOFFs criteria.

Antimicrobial agent	Code	*S. pseudintermedius*	*S. aureus*
Amikacin	AMK	≥16	≥16
Amoxicillin-clavulanate	AMC	≥1/0.5; ≥16/8	≥1/0.5; ≥16/8
Cefovecin	CVN	≥1	≥4
Cefoxitin	FOX	≥1	≥8
Ceftriaxone	CRE	≥4	≥16
Cephalothin	CEF	≥0.5	≥8
Chloramphenicol	CHL	≥32	≥32
Ciprofloxacin	CIP	≥4	≥4
Clindamycin	CLI	≥4	≥4
Enrofloxacin	ENR	≥4	≥4
Marbofloxacin	MRB	≥4	≥4
Oxacillin	OXA	≥0.5	≥4
Pradofloxacin	PRA	≥2	≥2
Rifampicin	RIF	≥4	≥4
Tetracycline	TET	≥1	≥1
Trimethoprim-sulfamethoxazole	SXT	≥4/76	≥4/76

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were interpreted based on epidemiological cut-off values (ECOFFs) as non-wild type (non-WT) organisms derived from assessment of the MIC distribution using ECOFFinder [26, 27] and/or as published by European Committee on Antimicrobial Susceptibility Testing (EUCAST) [28] as presented in Table 1.

For *S. pseudintermedius*, veterinary specific breakpoints were used for AMK, AMC, CLI, ENR, MRB, PRA and TET; human interpretative criteria were used for CHL, CIP, OXA, RIF and SXT and ECOFF criteria as defined by ECOFFinder were used for CVN, FOX, CRE and CEF. For *S. aureus*, veterinary specific breakpoints were used for AMK, AMC, CEF, CLI, ENR, MRB, PRA and TET; human interpretative criteria were used for FOX, CHL, CIP, OXA, RIF and SXT; ECOFF criteria were used for CVN (defined by ECOFFinder) and CRE (defined by EUCAST). In this study, we used breakpoints for CEF of ≥0.5 μg/mL instead of ≥8 μg/mL for *S. pseudintermedius* as stated in CLSI VET01S in order to correspond with ECOFF criteria and presence of *mecA* genes in the isolates. Also, for dog and horse isolates, the veterinary specific breakpoint for AMC of ≥1/0.5 μg/mL was used for isolates from skin and soft tissue infections (SSTIs) and the breakpoint ≥16/8 μg/mL (non-susceptible) was used for isolates from urinary tract infections (UTIs). For cat isolates, a breakpoint for AMC of ≥1/0.5 μg/mL was used for both SSTIs and UTIs.

Isolates showing resistance to three or more antimicrobial classes interpreted by clinical breakpoints were classified as multidrug-resistant (MDR) [29]. The frequency of antimicrobial resistance according to established breakpoints were described as rare: <0.1%; very low: 0.1% to 1.0%; low: >1% to 10.0%; moderate: >10.0% to 20.0%; high: >20.0% to 50.0%; very high: >50.0% to 70.0%; and extremely high: >70.0%; according to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) [30].

Confirmation of methicillin resistance status

Phenotypic confirmation of methicillin resistance status for putative MRSA and MRSP strains was assessed using resistance to cefoxitin and/or oxacillin, as well as colony appearance on Brilliance™ Agar MRSA2 (Thermo Fisher Scientific, Australia). Additionally, *mecA* PCR [31] was undertaken on all *S. aureus* isolates with cefoxitin MICs ≥8 μg/mL and all *S. pseudintermedius*

isolates with oxacillin MICs ≥0.5 μg/mL as recommended by CLSI VET01S [25]. Methicillin-resistant staphylococci were reported as resistant to all penicillins, cephems and β-lactams/β-lactamase inhibitor combinations regardless of *in vitro* test results with those agents [24].

Risk factor analysis

Of the 794 CoPS isolates analysed in this study, a total of 661 (dogs n = 597, 90.3%; cats n = 16, 2.4%; horses n = 35, 5.3%) were accompanied by a detailed clinical history. However, due to low sample size, only *S. pseudintermedius* isolates from dogs (n = 555) were further interrogated in the risk factors study. *S. pseudintermedius* that were methicillin-resistant (n = 68) were used as the outcome in this analysis. The variables for potential risk factors were gender (male or female), age group (<2 years, 2–10 years or >10 years), previous antimicrobial treatment (yes/ no), chronic and/or recurrent diseases (yes/ no), and site of infection (ear, urinary tract, skin and soft tissue, surgical site or respiratory tract). Initially, univariate analyses were used to assess the effect of various factors on the frequency of methicillin resistance in *S. pseudintermedius* isolates from dogs. This was followed by construction of a multivariate logistic regression model to account for the possible effects of confounding and interaction. Age of animal was forced into the multivariate model as a probable confounder and then each explanatory variable was assessed for its significance on the outcome. The most significant explanatory variables were then added to the model and the process repeated (by adding only significant variables) to obtain a main effects model. Two-way interactions between the main effects variables were then explored and retained when significant at $P<0.05$. Statistical analyses were performed using Stata/MP 14.0 (Stata Corp., College Station, TX, USA).

Results

Distribution of staphylococci species

Of the 888 isolates from companion animals submitted by Australian veterinary diagnostic laboratories for this study, a total of 877 isolates (98.8%) were confirmed to belong to the *Staphylococcus* genus. The most commonly identified CoPS were *S. pseudintermedius* (n = 629) and *S. aureus* (n = 117). Other CoPS identified included *S. schleiferi* (n = 44), *S. intermedius* (n = 2) and *S. delphini* (n = 2). Of the 629 *S. pseudintermedius* isolates, 97.9% were obtained from dogs and 2.1% from cats. Of the 117 *S. aureus* isolates, 45.3% were recovered from horses, 40.1% from dogs, and 14.5% from cats. All *S. schleiferi* isolates originated from dogs while one *S. intermedius* was isolated from a dog and a cat, respectively. Both *S. delphini* isolates came from horses. A small number of coagulase-negative staphylococci were also identified, as either *Staphylococcus felis* (n = 34) isolated from cats, *Staphylococcus epidermidis* (n = 10) from dogs and cats and *Staphylococcus sciuri* (n = 10) from horses and dogs. Coagulase-negative isolates were excluded from further analyses.

Phenotypic antimicrobial resistance among *S. pseudintermedius*

The MIC distribution and frequency of antimicrobial resistance among *S. pseudintermedius* is shown in Table 2. Among 81 isolates with oxacillin MICs ≥0.5 μg/mL, a total of 74 isolates (11.8%, dog n = 72, cat n = 2) were classified as methicillin-resistant *S. pseudintermedius*. The remaining isolates (n = 7) were classified as methicillin-susceptible because of negative results either on the basis of *mecA* PCR and colony appearance on Brilliance™ MRSA 2 Agar (S1 Table).

Among dog isolates (n = 616), resistance to AMC (37.5% of isolates; 45.1% for SSTI, 3.5% for UTI) was most common followed by SXT (37.3%) and TET (22.7%). CLI resistance was

Table 2. MIC distribution and frequency of resistance (%R) among clinical *Staphylococcus pseudintermedius* isolated from dogs (n = 616) and cats (n = 13) in Australia[a].

Antimicrobials	Animals	% R	95% CI	Percentage of isolates with indicated MIC[b]														
				≤0.004	0.008	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	≥64
Amikacin	Dog	1.1	0.5 2.3								1.3	10.7	59.1	22.6	5	0.8		0.3
	Cat	0	0.0 24.7									23.1	53.8	15.4	7.7			
Amoxicillin clavulanate	Dog (SSTI)	45.1	40.7 49.6					3.4	15.3	12.1	24.1	18.7	14.1	1	1.8	1.6	8	
	Dog (UTI)	3.6	1.1 9.4					2.7	8.9	14.2	26.6	30.1	13.3		0.9	0.9	2.7	
	Cat	53.8	26.7 80.9					7.7	23.1	7.7	7.7	23.1	7.7			7.7	15.4	
Cefovecin	Dog	13.1	10.6 16.1					2.8	71.8	11.5	0.6	3.1	0.6	0.3		0.2	0.2	8.8
	Cat	15.4	1.9 45.4						61.5	15.4	7.7							15.4
Cefoxitin	Dog	11.5	9.1 14.3					1.3	36.2	40.1	10.7	3.7	3.6	1.9	1.5	0.2	0.5	0.2
	Cat	23.1	5.0 53.8						46.2	23.1	7.7	7.7	7.7				7.7	
Ceftriaxone	Dog	12.8	10.3 15.7				0.2	0.2	0.5	1	37.2	46.9	1.1	2.1	1.1	0.8	8.8	
	Cat	23.1	5.0 53.8								23.1	53.8		7.7			15.4	
Cephalothin	Dog	13.5	10.9 16.4					28.4	50.5	7.5	2.9	1	0.5	0.6	1.5	0.8	1.9	4.2
	Cat	23.1	5.0 53.8					15.4	61.5			7.7			7.7	7.7		
Chloramphenicol	Dog	5.7	4.0 7.8						0.2				0.5	54.1	39	0.6	0.2	5.5
	Cat	7.7	0.2 36.0											38.5	53.8			7.7
Ciprofloxacin	Dog	8.1	6.1 10.6			0.2	0.5	12	61.5	12.7	2.9	1.3	0.6	1	7.1			
	Cat	0.0	0.2 36.0					23.1	53.8	15.4		7.7						
Clindamycin	Dog	12.7	10.1 15.5				0.2	10.7	69	6.3	0.5	0.3	0.2	0.5	0.6		11.5	
	Cat	7.7	0.2 36.0					7.7	84.6								7.7	
Enrofloxacin	Dog	8.1	6.1 10.6			0.2	0.6	15.9	56.3	12.3	3.6	2.1	0.6	0.8	7.3			
	Cat	0	0.0 24.7					15.4	69.2	15.4								
Marbofloxacin	Dog	8.8	6.7 11.3				0.5	0.2	5.4	61	19.2	4.5	0.3	1.3	7.5			
	Cat	0	0.0 24.7							84.6	15.4							
Oxacillin	Dog	12.7	10.1 15.5				0.2	2.1	61.2	23.7	1.5	0.8	0.8	0.8	0.3	0.3	1.3	6.8
	Cat	23.1	5.0 53.8						76.9		7.7							15.4
Pradofloxacin	Dog	6.5	4.7 8.7			5.7	38.8	42	2.9	1.5	1.3	1.1	5.7	0.8	0.2			
	Cat	0	0.0 24.7				46.2	38.5	7.7	7.7								
Rifampicin	Dog	1	0.1 2.1	37	58.9	2.9			0.2					1				
	Cat	0	0.0 24.7	38.5	61.5													
Tetracycline	Dog	22.7	19.5 26.2					18.7	52.1	6.2	0.3	0.2		0.2			11	11
	Cat	15.4	1.9 45.4					38.5	30.8	15.4								15.4
Trimethoprim sulfamethoxazole	Dog	37.3	33.5 41.3					0.3	0.6	4.7	33	8.1	15.7	26.9	1.1	9.3		
	Cat	30.8	9.1 61.4							30.8	38.5			23.1		7.7		

[a] Among dog isolates, SSTI n = 503, UTI n = 113, cat isolates SSTI n = 10, UTI n = 3.

[b] Unshaded areas show the dilution range for each drug. Vertical solid lines indicate veterinary specific breakpoints.

Double vertical solid lines indicate human interpretative criteria. ECOFFs are indicated as vertical dotted lines. Resistance to CVN, FOX, CRE, CEF and OXA after confirmation of methicillin resistance status is presented in S2 Table.

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observed at a moderate level (12.7%). Similarly, a moderate level of resistance was observed to fluoroquinolones, ranging from 6.5%-8.8% for the four compounds tested in this study (CIP, ENR, MRB, PRA). Resistance to CHL was observed in 5.7% of isolates. A very low number of isolates were resistant to AMK (1.1%; n = 7) and RIF (1%, n = 6).

Among cat isolates (n = 13), the most common resistance found was to AMC (53.8% of isolates) and SXT (30.8%). CHL and CLI resistance was detected in 7.7% isolates. Resistance to AMK, RIF and fluoroquinolones was not detected.

Phenotypic antimicrobial resistance among S. *aureus*

The MIC distribution and frequency of antimicrobial resistance among *S. aureus* is shown in Table 3. Overall, 12.8% of the *S. aureus* isolates were methicillin-resistant, including six isolates from horses (11.3%), six isolates from dogs (12.8%), and three isolates from cats (17.6%). Among methicillin-susceptible *S. aureus*, resistance to at least one or more β-lactam antimicrobials was observed in three isolates (2.6%).

Among isolates from horses (n = 53), resistance to AMC and TET was high (47.1% and 32.1%, respectively). Resistance to SXT was observed in 13.2% of isolates and resistance to AMK and RIF in 9.4% of isolates. Resistance to CHL and fluoroquinolones was observed at a low level (1.9%). Resistance to CLI was not observed.

Among dog isolates (n = 47), AMC (57.4% of isolates; 59.5% for SSTI, 0% for UTI) and CVN (14.9%) had the highest rates of resistance. Resistance to fluoroquinolones was observed in 8.5% of isolates. A low frequency of resistance (2.1%) was observed for AMK and CHL.

Among cat isolates (n = 17), resistance to AMC was the most common (58.8%), followed by resistance to fluoroquinolones (11.8%). Resistance to five antimicrobials (AMK, CHL, CLI, RIF and SXT) was not detected.

Resistance profiles of *S. pseudintermedius* isolates

The resistance profiles of the *S. pseudintermedius* isolates are presented in Table 4. In total, 51.2% of *S. pseudintermedius* isolates were fully susceptible to eight antimicrobial classes. The proportion of single drug resistance in *S. pseudintermedius* was 38.1%, with single SXT resistance the most common pattern (18.8%). MDR was observed in 83 isolates (13.2%) including 74 isolates that were regarded as MRSP based on phenotypic characteristics and *mecA* PCR and nine *S. pseudintermedius* isolates that were methicillin-susceptible. The most common MDR pattern was resistance to phenicols, lincosamides, fluoroquinolones (FQN), β-lactams, tetracycline and folate-pathway inhibitors (CHL-CLI-FQN-OXA-TET-SXT) in 23 canine MRSP isolates. Methicillin resistance was significantly associated with resistance to CLI (OR 105.2, 95%CI 48.5–231.9), FQN (OR 287; 95%CI 91.2–1144.8), TET (OR 7.5, 95%CI 4.4–13.1) and SXT (OR 8.5, 95%CI 4.6–16.6).

Resistance profiles of *S. aureus* isolates

The resistance profiles of *S. aureus* isolates are shown in Table 5. In total, 68.4% of *S. aureus* isolates were fully susceptible to eight antimicrobial classes. MDR was detected with a frequency of 12.8%, including six MRSA isolates from horses, six MRSA isolates from dogs and three MRSA isolates from cats. Resistance to TET was the most common pattern observed in horse isolates (17%) and dog isolates (6.4%). Dog and cat isolates were more likely to be resistant to fluoroquinolones (OR 5.4, 95%CI 0.6–252.1), which was also always associated with methicillin resistance, compared to horse isolates. Horse isolates were more likely to be amikacin-resistant (OR 6.5, 95%CI 0.7–315.2) compared to dog and cat isolates. All rifampicin-resistant *S. aureus* isolates from horses (n = 5) were methicillin-resistant.

Risk factors for MRSP in dogs

In univariate analysis, there was no significant difference in the proportion of MRSP isolates between female versus male dogs; chronic versus non-chronically diseased dogs; or the various age groups (Table 6). Site of infection and prior antimicrobial treatment were significantly associated with MRSP isolation and were retained in the multivariate model. In multivariate analysis, after controlling for the confounding effect of age, isolates from particular infection

Table 3. MIC distribution and frequency of resistance (%R) among clinical *Staphylococcus aureus* isolated from horses (n = 53), dogs (n = 47), and cats (n = 17) in Australia[b].

Antimicrobials	Animals	%R	95% CI	Percentage of isolates with indicated MIC[a]														
				≤0.004	0.008	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	≥64
Amikacin	Horse	9 4	3 1–20 7							1 9	1 9	3 8	43 4	24 5	15 1	7 5	1 9	
	Dog	2 1	0 1–11 3							2 1	2 1	6 4	36 2	46 8	4 3	2 1		
	Cat	0	0 0–19 5										58 8	41 2				
Amoxicillin-clavulanate	Horse (SST)	47 2	33 7–60 5						9 4	22 6	20 8	17	3 8	9 4	3 8	1 9	11 3	
	Dog (SST)	59 5	43 3–74					7 1		14 3	19 1	9 5	14 3	19 1	2 4		14 3	
	Dog (UT)	0	0–0 5							60		40						
	Cat	58 8	35 4–82 2					11 8		5 9	23 5	29 4	17 6				11 8	
Cefovecin	Horse	13 2	5 5–25 3						3 8	3 8	7 5	66	5 7	1 9				11 3
	Dog	14 9	6 2–28 3					2 1	12 8	6 4	12 8	51 1			2 1			12 8
	Cat	17 6	3 8–43 4							5 9	35 3	41 2						17 6
Cefoxitin	Horse	11 3	4 3–23 0						3 8	3 8	1 9	3 8	43 4	32 1			5 7	5 7
	Dog	12 8	4 8–25 7						10 6	10 6	2 1	4 3	38 3	21 3		2 1	2 1	8 5
	Cat	17 6	3 8–43 4										29 4	52 9			5 9	11 8
Ceftriaxone	Horse	13 2	5 5–25 3							1 9	1 9	9 4	15 1	58 5		1 9	11 3	
	Dog	12 8	4 8–25 7							2 1	8 5	12 8	17	46 8			12 8	
	Cat	17 6	3 8–43 4									5 9	52 9	23 5			17 6	
Cephalothin	Horse	11 3	4 3–23 0					5 7	3 8	35 8	32 1	9 4		1 9			5 7	5 7
	Dog	12 8	4 8–25 7					12 8	12 8	8 5	38 3	14 9			4 3			8 5
	Cat	11 8	1 5–36 4						5 9	17 6	41 2	17 6	5 9					11 8
Chloramphenicol	Horse	1 9	0 0–10 1											15 1	83			1 9
	Dog	2 1	0 1–11 3									2 1		25 5	70 2			2 1
	Cat	0	0 0–19 5											5 9	94 1			
Ciprofloxacin	Horse	1 9	0 0–10 1					15 1	37 7	39 6	5 7				1 9			
	Dog	8 5	2 4–20 4					4 3	27 7	29 8	27 7	2 1			8 5			
	Cat	11 8	1 5–36 4							64 7	23 5				11 8			
Clindamycin	Horse	0	0 0–10 1					20 8	77 4		1 9							
	Dog	2 1	0 5–14 5				2 1	34	59 6		2 1						2 1	
	Cat	0	0 0–19 5					47 1	52 9									
Enrofloxacin	Horse	1 9	0 0–10 1				1 9	32 1	54 7	9 4					1 9			
	Dog	8 5	2 4–20 4				2 1	8 5	53 2	23 4	2 1	2 1			8 5			
	Cat	11 8	1 5–36 4					11 8	52 9	23 5					11 8			
Marbofloxacin	Horse	1 9	0 0–10 1					1 9	1 9	75 5	18 9				1 9			
	Dog	8 5	2 4–20 4					2 1	4 3	40 4	40 4	4 3			8 5			
	Cat	11 8	1 5–36 4							29 4	58 8				11 8			
Oxacillin	Horse	11 3	4 3–23 0						17	37 7	30 2	3 8					1 9	9 4
	Dog	12 8	4 8–25 7					2 1	21 3	14 9	44 7	2 1	2 1				4 3	8 5
	Cat	17 6	3 8–43 4						11 8	23 5	47 1			5 9				11 8
Pradofloxacin	Horse	1 9	2 4–20 4			13 2	52 8	30 2			1 9		1 9					
	Dog	8 5	2 4–20 4			4 3	17	46 8	23 4					6 4	2 1			
	Cat	11 8	1 5–36 4			5 9	17 6	64 7					5 9	5 9				
Rifampicin	Horse	9 4	3 1–20 7	45 3	43 4	1 9								9 4				
	Dog	0	0 0–7 5	53 2	44 7			2 1										
	Cat	0	0 0–19 5	35 3	64 7													
Tetracycline	Horse	32 1	19 9–46 3						30 2	28 3	9 4		1 9		1 9		5 7	22 6
	Dog	10 6	3 5–23 1					6 4	38 3	34	10 6		2 1				4 3	4 3
	Cat	5 9	0 1–28 7						47 1	47 1								5 9

(*Continued*)

Table 3. (*Continued*)

Antimicrobials	Animals	% R	95% CI	Percentage of isolates with indicated MIC[a]													
				≤0.004	0.008	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	≥64
Trimethoprim-sulfamethoxazole	Horse	13 2	5 5–25 3							50 9	30 2	3 8	1 9	1 9	3 8	7 5		
	Dog	6 4	1 3–17 5						2 1	44 7	38 3	4 3	4 3	4 3	2 1			
	Cat	0	0 0–19 5					5 9	5 9	52 9	35 3							

[a] Among horse so ates, SSTI n = 53; dog so ates SSTI n = 42, UTI = 5; cat so ates SSTI n = 15, UTI n = 2.

[b] Unshaded areas show the d ut on range for each drug.

Vert ca so d nes nd cate veter nary spec fic breakpo nts.

Doub e vert ca so d nes nd cate human nterpretat ve cr ter a.

ECOFFs are nd cated as vert ca dotted nes.

Res stance to CVN, FOX, CRE, CEF and OXA after confirmat on of meth c n res stance status s presented n S2 Tab e.

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Table 4. Resistance profile per antimicrobial class found in clinical *Staphylococcus pseudintermedius* isolates in Australia (2013–2014)

Resistance profile[a]	No. (%) of isolates	
	Dog (n = 616)	Cat (n = 13)
0: NIL	316 (51.3)	6 (46.2)
1: CLI	6 (1)	-
1: FQN	1 (0.2)	-
1: OXA	7 (1.1)	2 (15.4)
1: TET	45 (7.3)	-
1: SXT	113 (18.8)	3 (23.1)
2: CHL-CLI	1 (0.2)	-
2: CLI-SXT	4 (6.5)	-
2: OXA-CLI	3 (0.5)	-
2: OXA-TET	1 (0.2)	-
2: OXA-SXT	7 (1.1)	-
2: FQN-OXA	1 (0.2)	
2: TET-SXT	46 (7.5)	1 (7.7)
3: CHL-TET-SXT	1 (0.2)	-
3: CLI-TET-SXT	3 (0.5)	-
3: OXA-CLI-SXT	3 (0.5)	-
3: OXA-TET-SXT	2 (0.3)	-
4: AMK-CLI-OXA-RIF	1 (0.2)	-
4: CLI-CHL-TET-SXT	1 (0.2)	-
4: CLI-OXA-RIF-TET	1 (0.2)	-
4: OXA-FQN-CLI-SXT	9 (1.5)	-
4: OXA-CLI-CHL-TET	4 (0.6)	-
5: AMK-CLI-FQN-OXA-SXT	5 (0.8)	-
5: CHL-CLI-FQN-OXA-TET	2 (0.3)	1 (7.7)
5: CHL-CLI-OXA-FQN-SXT	1 (0.2)	-
5: CHL-CLI-FQN-TET-SXT	1 (0.2)	-
5: CLI-FQN-OXA-TET-SXT	7 (1.1)	-
6: CHL-CLI-FQN-OXA-TET-SXT	23 (3.7)	-
6: OXA-FQN-CLI-RIF-TET-SXT	1 (0.2)	-
7: AMK-CLI-OXA-FQN-RIF-TET-SXT	1 (0.2)	-
7: CHL-CLI-OXA-FQN-RIF-TET-SXT	2 (0.3)	-
Total MRSP	72 (11.7)	2 (15.4)
Total MDR but not MRSP	9 (1.5)	-
Total MDR	81 (13.1)	2 (15.4)

[a] Antimicrobial classes included: aminoglycosides (AMK); lincosamides (CLI), phenicols (CHL), fluoroquinolones (FQN, including CIP, ENR, MRB and PRA); β-lactams (OXA, representing methicillin resistance); ansamycin (RIF); tetracyclines (TET); and folate-pathway inhibitors (SXT). NIL, none.

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sites, including surgical sites (OR 8.8; 95%CI 3.74–20.7), and skin and soft tissue (OR 3.9; 95% CI 1.97–7.51) continued to have a strong association with MRSP isolation. In the main effects model, prior antimicrobial treatment was not a significant factor contributing to the isolation of methicillin-resistant strains (OR 1.63; 95%CI 0.86–2.8). However, after inclusion of interaction terms, surgical site infections (OR 15.7; 95%CI 5.37–46.19) and skin and soft tissue infections (OR 6.1, 95%CI 2.52–14.84) were significantly more likely to be methicillin-resistant in dogs who had received prior antimicrobial treatment compared to dogs who had not received prior antimicrobial treatment (Table 7).

Table 5. Resistance profile per antimicrobial class found in clinical *Staphylococcus aureus* isolates from horses, dogs and cats in Australia (2013–2014)

Resistance profile[a]	No. of isolates (%)		
	Horse (n = 53)	Dog (n = 47)	Cat (n = 17)
0: NIL	33 (62.3)	33 (70.2)	14 (82.3)
1: FOX	-	2 (4.2)	-
1: CHL	-	1 (2.1)	-
1: TET	9 (17)	3 (6.4)	-
1: SXT	2 (3.8)	2 (4.2)	-
2: AMK-TET	3 (5.7)	1 (2.1)	-
2: FOX-FQN	1 (1.9)	3 (6.4)	2 (11.8)
2: FOX-TET	-		1 (5.9)
2: TET-SXT	-	1 (2.1)	-
3: FOX-FQN-LNC	-	1 (2.1)	-
4: FOX-RIF-TET-SXT	2 (3.8)	-	-
5: AMK-FOX-RIF-TET-SXT	2 (3.8)	-	-
5: FOX-CHL-RIF-TET-SXT	1 (1.9)	-	-
Total MRSA	6 (11.3)	6 (12.8)	3 (17.6)
Total MDR	6 (11.3)	6 (12.8)	3 (17.6)

[a] Antimicrobial classes included: aminoglycosides (AMK); 2nd cephemycins (FOX, representing methicillin resistance); lincosamides (CLI), phenicols (CHL), fluoroquinolones (FQN, including CIP, ENR, MRB, PRA); ansamycin (RIF); tetracyclines (TET); and folate-pathway inhibitors (SXT). NIL, none.

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Table 6. Univariate analysis of risk-factor variables from *Staphylococcus pseudintermedius* isolates from dogs in Australia (n = 555). Odds ratios define the risk of isolates being classified as methicillin-resistant strains.

Risk factor	n	%MRSP	OR	P value	95% CI
Age in years					
<2	51	5.9	Ref		
2–10	391	13.3	2.45	0.143	0.74–8.17
<10	113	8.8	1.56	0.518	0.41–5.9
Chronic and recurrent disease					
No	492	12	Ref		
Yes	63	9.5	0.77	0.567	0.32–1.87
Prior antimicrobial treatment					
No	419	9.3	Ref		
Yes	136	19.1	2.3	0.002	1.34–3.95
Sex					
Male	247	11.3	Ref		
Female	308	12	1.07	0.805	0.563–1.8
Site of infection					
Ear	255	6.3	Ref		
Skin and soft tissue	138	19.6	3.63	<0.000	1.88–7.01
Urinary tract	104	5.7	0.91	0.865	0.34–2.4
Surgical site	42	35.7	8.3	<0.000	3.7–18.63
Respiratory tract	16	6.2	1	0.997	0.12–8.02

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Table 7. Odds ratios showing the likelihood of isolates being methicillin-resistant in *Staphylococcus pseudintermedius* isolates from dogs in Australia for different combinations of site of infection in the host and exposure of the host to prior antimicrobial treatment.

Prior antimicrobial treatment[a]	Surgical site		Skin and soft tissue	
	n	OR; 95%CI	n	OR; 95%CI
No	19	5.4; 1.65–17.39	96	2.9; 1.32–6.45
Yes	23	15.7; 5.37-46-19	42	6.1; 2.51–14.84

[a] Reference value isolates obtained from dogs with ear infections that did not receive prior antimicrobial treatment.

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Discussion

This is the first comprehensive study describing the distribution of antimicrobial susceptibility profiles in CoPS isolated from clinical infections in companion animals in Australia. This study generated three major findings: 1) The frequency of MRSP and MRSA isolation from clinical infections in companion animals in Australia was estimated as moderate (11.8% and 12.8% of total isolates for each species, respectively); 2) Resistance to critically important antimicrobials used in human medicine (fluoroquinolones, amikacin) remains very low to low among Australian companion animal CoPS; and 3) Prior antimicrobial treatment was identified as a significant risk factor for isolation of MRSP from dogs with surgical site, skin and soft tissue infections.

MRSP infections are increasingly reported in veterinary practice, spreading among companion animals and to a lesser extent among veterinarians [10]. The increased frequency of MRSP that are MDR poses a serious concern for biosecurity and infection control in veterinary practices, due to limited therapeutic options and the ease of transmission between animals. In parallel to other noteworthy studies from Australia [11], MRSP isolates were resistant to more antimicrobial classes than MRSA isolates, exemplified by the high proportion of MRSP isolates showing resistance to more than six antimicrobial classes (n = 29, 4.8%), while this level of multidrug resistance was not identified in any *S. aureus* isolates. Compared to similar surveys in other countries conducted over the same time period, the frequency of methicillin-resistant strains among canine *S. pseudintermedius* in Australia (11.8%) was significantly higher ($P<0.0001$) than that reported in Sweden (0.4%) [17] and Norway (0.5%) [18]. However, resistance to clindamycin in *S. pseudintermedius* in Australia (12.7%) was significantly lower ($P = 0.0001$) than in Sweden (21.6%). While a high level of amikacin resistance in *S. pseudintermedius* isolates has been demonstrated in some studies [32], we found that only a very low proportion of companion animal *S. pseudintermedius* isolates from Australia were resistant to this critically important human drug (n = 7; 1.1%). It is therefore recommended that use of amikacin in veterinary medicine continues to be reserved for MDR infections identified on the basis of culture and susceptibility testing when no other drug class is available [33].

In the only other comparable study conducted in 2006 in two regions of Australia, involving both clinical and non-clinical (i.e. carriage) of S*taphylococcus* spp. isolates from dogs and cats (n = 331), the frequency of methicillin-resistant (*mecA)* and β-lactam-resistant (*blaZ)* strains was only 3% and 6.9%, respectively [20, 34]. Although methodologies for sampling, testing and data interpretation were somewhat different to this study, it might indicate that methicillin resistance amongst Australian companion animal staphylococci has substantially increased in less than a decade.

In a recent Australian study, colonisation of veterinarians by MRSA was dominated by strains belonging to CC8 MRSA (ST8-IV [2B], *spa* t064; and ST612-IV [2B], *spa* variable). These were strongly associated with equine practice veterinarians and were often resistant to

rifampicin and gentamicin [19]. MRSA CC8 (ST8 and ST612) is the most commonly identified clone among both Australian veterinarians and clinical equine samples [35]. Similarly, in the present study, a high proportion of MRSA isolates from cases of infection in Australian horses were also resistant to rifampicin (9.4%) but rarely resistant to fluoroquinolones. Rifampicin is almost exclusively used in equine practice, where it is combined with a macrolide for the oral treatment of *Rhodococcus equi* infections in foals [35, 36]. The equine MRSA isolates identified in the present study were sensitive to range of additional antimicrobial classes including chloramphenicol and fluoroquinolones, demonstrating that additional therapeutic options were still available for treating MRSA infections in horses.

The significant association between methicillin resistance and fluoroquinolone resistance in *Staphylococcus* spp. isolates from dogs and cats in this study reflects the observation that a high proportion of Australian MRSP isolates may belong to internationally disseminated fluoroquinolone-resistant clones such as ST71 and ST45 [11, 37], whereas MRSA isolates are likely to belong to ST22-IV [2B], *spa* variable, commonly found in small animal practice veterinarians in Australia [19, 38] and community-acquired infections [39]. A comparative genomics study is currently underway to determine genetic similarity of methicillin-resistant isolates in this study. Comparative genomics represents the most rapid, cost effective and accurate technique for molecular typing including determination of sequence type.

The most important finding from the risk factors study was that particular sites are associated with the risk of a *S. pseudintermedius* infection being methicillin-resistant. In agreement with the present study, other studies have also found that isolates from surgical site infections were at higher risk of being resistant to methicillin when compared to other sites [10, 40]. In parallel to the work here, animals that were hospitalised, visited veterinary clinics frequently or had previous antimicrobial treatment were at higher risk for MRSP infections [41]. Compared to studies from 2006, [20, 34] it appears that MRSP infections are becoming increasingly common in veterinary companion animal practice in Australia. The results strongly reinforce the need for veterinarians to place a high priority on implementing infection control procedures, biosecurity and antimicrobial stewardship such as those recommended by the Australian Veterinary Association [42]. Understanding potential factors that lead to emerging resistance may aid in the development of strategies that could curtail the ongoing spread of MRSP within veterinary hospitals.

This study has some limitations. Inclusion of isolates was performed at the convenience and discretion of the animal owners (who would be expected to pay for tests at the primary laboratory), the consulting veterinarian (who may or may not favour sensitivity testing), and the primary laboratory (who may or may not be interested in the study). Consequently, the resulting size and direction of bias in estimates of resistance frequency is difficult to define. Further, the small sample size of methicillin-resistant feline isolates led to wide confidence intervals, limiting our ability to draw statistically significant conclusions on feline isolates. Future studies should therefore focus on achieving a sufficiently large collection of isolates from cats to increase the accuracy of these estimates. Despite these shortfalls, we are unaware of any collection of isolates that is as representative of the Australian population of companion animals, both in terms of size and geographic diversity. Certainly the data presented here surpasses what is currently available elsewhere in the literature [20, 43] and is therefore a useful basis for reviewing prescribing practices for staphylococcal infections in companion animals both in Australia, and more broadly.

Conclusions

This study shows that antimicrobial resistance is commonly present in the coagulase-positive staphylococci cultured from animal infections in companion animals in Australia. Of greatest

concern is the occurrence of moderate levels of MRSP and MRSA, some of which are also resistant to fluoroquinolones. The data provides important baseline measurements for future surveillance and international benchmarking. A strong association of MRSP with surgical site infections in dogs suggests that there could be shortfalls in infection control in animal hospitals. Periodically repeated surveys of this type are crucial for understanding the trends in emergence and dissemination of antimicrobial resistance in companion animals.

Supporting information

S1 Table. Determination of methicillin resistance in *S. pseudintermedius* and *S. aureus* isolates from dogs, cats and horses in Australia based on phenotypic characteristic and *mecA* PCR.
(DOCX)

S2 Table. Percentage of resistance to penicillins, cephems and β-lactams/β-lactamase inhibitor combinations before and after confirmation of methicillin resistance status.
(DOCX)

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References

1. Savini V, Passeri C, Mancini G, Iuliani O, Marrollo R, Argentieri AV, et al. Coagulase-positive staphylococci: my pet's two faces. Res Microbiol. 2013; 164(5):371–4. https://doi.org/10.1016/j.resmic.2013.02.004 PMID: 23481095
2. Ballhausen B, Kriegeskorte A, Schleimer N, Peters G, Becker K. The *mecA* homolog *mecC* confers resistance against beta-lactams in *Staphylococcus aureus* irrespective of the genetic strain

background. Antimicrob Agents Chemother. 2014; 58(7):3791–8. PubMed Central PMCID: PMC4068569. https://doi.org/10.1128/AAC.02731-13 PMID: 24752255

3. Johnson AP. Methicillin-resistant *Staphylococcus aureus*: the European landscape. J Antimicrob Chemother. 2011; 66 Suppl 4:iv43–iv8. Epub 2011/05/06.
4. Ruscher C, Lubke-Becker A, Semmler T, Wleklinski CG, Paasch A, Soba A, et al. Widespread rapid emergence of a distinct methicillin- and multidrug-resistant *Staphylococcus pseudintermedius* (MRSP) genetic lineage in Europe. Veterinary Microbio. 2010; 144(3–4):340–6. Epub 2010/02/26.
5. Smith TC. Livestock-associated *Staphylococcus aureus*: the United States experience. PLoS Pathog. 2015; 11(2):e1004564. PubMed Central PMCID: PMC4412291. https://doi.org/10.1371/journal.ppat.1004564 PMID: 25654425
6. Gingrich EN, Kurt T, Hyatt DR, Lappin MR, Ruch-Gallie R. Prevalence of methicillin-resistant staphylococci in Northern Colorado shelter animals. J Vet Diagn Invest. 2011; 23(5):947–50. https://doi.org/10.1177/1040638711407301 PMID: 21908352
7. Jordan D, Simon J, Fury S, Moss S, Giffard P, Maiwald M, et al. Carriage of methicillin-resistant *Staphylococcus aureus* by veterinarians in Australia. Aust Vet Jo. 2011; 89(5):152–9. Epub 2011/04/19.
8. Walther B, Monecke S, Ruscher C, Friedrich AW, Ehricht R, Slickers P, et al. Comparative molecular analysis substantiates zoonotic potential of equine methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol. 2009; 47(3):704–10. PubMed Central PMCID: PMC2650932. https://doi.org/10.1128/JCM.01626-08 PMID: 19109463
9. Harrison EM, Weinert LA, Holden MT, Welch JJ, Wilson K, Morgan FJ, et al. A shared population of epidemic methicillin-resistant *Staphylococcus aureus* 15 circulates in humans and companion animals. mBio. 2014; 5(3):e00985–13. Epub 2014/05/16. PubMed Central PMCID: PMC4030480.
10. van Duijkeren E, Kamphuis M, van der Mije IC, Laarhoven LM, Duim B, Wagenaar JA, et al. Transmission of methicillin-resistant *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics. Vet Microbiol. 2011; 150(3–4):338–43. https://doi.org/10.1016/j.vetmic.2011.02.012 PMID: 21420256
11. Siak M, Burrows AK, Coombs GW, Khazandi M, Abraham S, Norris JM, et al. Characterization of methicillin-resistant and meticillin-susceptible isolates of *Staphylococcus pseudintermedius* from cases of canine pyoderma in Australia. J Med Microbiol. 2014; 63(Pt 9):1228–33. https://doi.org/10.1099/jmm.0.076117-0 PMID: 24994658
12. Guardabassi L, Schwarz S, Lloyd DH. Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother. 2004; 54(2):321–32. Epub 2004/07/16. https://doi.org/10.1093/jac/dkh332 PMID: 15254022
13. Youn J-H, Yoon JW, Koo Hye Cheong, Lim S-K, Park YH. Prevalence and antimicrogram of *Staphylococcus intermedius* group isolates from veterinary staff, companion animals, and the environment in veterinary hospitals in Korea. J Vet Diagn Invest. 2011; 23:268–74. https://doi.org/10.1177/104063871102300211 PMID: 21398446
14. Feng Y, Tian W, Lin D, Luo Q, Zhou Y, Yang T, et al. Prevalence and characterization of methicillin-resistant *Staphylococcus pseudintermedius* in pets from South China. Vet Microbiol. 2012; 160(3–4):517–24. Epub 2012/07/10. https://doi.org/10.1016/j.vetmic.2012.06.015 PMID: 22770517
15. Youn JH, Park YH, Hang'ombe B, Sugimoto C. Prevalence and characterization of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from companion animals and environment in the veterinary teaching hospital in Zambia, Africa. Comp Immunol Microbiol Infect Dis. 2014; 37(2):123–30. Epub 2014/02/01. https://doi.org/10.1016/j.cimid.2014.01.003 PMID: 24480623
16. Perreten V, Kadlec K, Schwarz S, Gronlund Andersson U, Finn M, Greko C, et al. Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study. J Antimicrob Chemother. 2010; 65(6):1145–54. Epub 2010/03/30. https://doi.org/10.1093/jac/dkq078 PMID: 20348087
17. SWEDRES/SVARM. Use of antimicrobials and occurrence of antimicrobial resistance in Sweden 2014. Upsala: 2015.
18. NORM/NORM-VET. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Oslo, Norway: 2013.
19. Groves MD, Crouch B, Coombs GW, Jordan D, Pang S, Barton MD, et al. Molecular Epidemiology of methicillin-resistant *Staphylococcus aureus* isolated from Australian veterinarians. PloSOne. 2016; 11(1):e0146034. PubMed Central PMCID: PMC4703204.
20. Malik S, Coombs GW, O'Brien FG, Peng H, Barton MD. Molecular typing of methicillin-resistant staphylococci isolated from cats and dogs. J Antimicrob Chemother. 2006; 58(2):428–31. Epub 2006/06/20. https://doi.org/10.1093/jac/dkl253 PMID: 16782740

21. Worthing KA, Coombs GW, Pang S, Abraham S, Saputra S, Trott DJ, et al. Isolation of mecC MRSA in Australia. J Antimicrob Chemother. 2016:dkw138.
22. Trott D, Jordan D, Barton M, Abraham S, Groves M. Vets versus pets: methicillin-resistant Staphylococcus aureus in Australian animals and their doctors. Microbiol Aust. 2013; 10:25–7.
23. Abraham S, Jordan D, Wong HS, Johnson JR, Toleman MA, Wakeham DL, et al. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals. Journal of Global Antimicrobial Resistance. 2015; 3(4):273–7. https://doi.org/10.1016/j.jgar.2015.08.002 PMID: 27842872
24. CLSI. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved Standard-Fourth Edition. CLSI document VET01-A4. Wayne, PA, USA: CLSI; 2013.
25. CLSI. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals- Third Edition. CLSI document VET01S. Wayne, PA, USA: CLSI; 2015.
26. Turnidge J, Kahlmeter G, Kronvall G. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clin Microbiol Infect. 2006; 12(5):418–25. https://doi.org/10.1111/j.1469-0691.2006.01377.x PMID: 16643517
27. CLSI. ECOFFinder [Internet]. 2016 [cited 10 June 2016]. Available from: http://clsi.org/standards/micro/ecoffinder/.
28. EUCAST. Antimicrobial wild type distributions of microorganisms 2016 [cited 10 June 2016]. Available from: http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=init.
29. Magiorakos A, Srinivasan A, Carey R, Carmeli Y, Falagas M, Giske C, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012; 18:268–81. https://doi.org/10.1111/j.1469-0691.2011.03570.x PMID: 21793988
30. EFSA ECDC. EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. EFSA Journal 2015. 2015; 13(2):1–178.
31. Merlino J, Watson J, Rose B, Beard-Pegler M, Gottlieb T, Bradbury R, et al. Detection and expression of methicillin/oxacillin resistance in multidrug-resistant and non-multidrug-resistant *Staphylococcus aureus* in Central Sydney, Australia. J Antimicrob Chemother. 2002; 49(5):793–801. PMID: 12003973
32. Gold RM, Cohen ND, Lawhon SD. Amikacin resistance in *Staphylococcus pseudintermedius* isolated from dogs. J Clin Microbiol. 2014; 52(10):3641–6. Epub 2014/08/01. PubMed Central PMCID: PMC4187778. https://doi.org/10.1128/JCM.01253-14 PMID: 25078911
33. Shaban R, Simon G, Trott D, Turnidge J, Jordan D. Surveillance and reporting of antimicrobial resistance and antibiotic usage in animals and agriculture in Australia. Canberra, Australia: Department of Agriculture, The Australian Government, 2014.
34. Malik S, Peng H, Barton MD. Partial nucleotide sequencing of the *mecA* genes of *Staphylococcus aureus* isolates from cats and dogs. J Clin Microbiol. 2006; 44(2):413–6. PubMed Central PMCID: PMC1392638. https://doi.org/10.1128/JCM.44.2.413-416.2006 PMID: 16455893
35. Axon JE, Carrick JB, Barton MD, Collins NM, Russell CM, Kiehne J, et al. Methicillin-resistant *Staphylococcus aureus* in a population of horses in Australia. Aust Vet J. 2011; 89(6):221–5. https://doi.org/10.1111/j.1751-0813.2011.00711.x PMID: 21595643
36. Crowley J, Po E, Celi P, Muscatello G. Systemic and respiratory oxidative stress in the pathogenesis and diagnosis of *Rhodococcus equi* pneumonia. Equine Vet J Suppl. 2013;(45):20–5. https://doi.org/10.1111/evj.12166 PMID: 24304399
37. Solyman SM, Black CC, Duim B, Perreten V, van Duijkeren E, Wagenaar JA, et al. Multilocus sequence typing for characterization of *Staphylococcus pseudintermedius*. J Clin Microbiol. 2013; 51(1):306–10. PubMed Central PMCID: PMC3536184. https://doi.org/10.1128/JCM.02421-12 PMID: 23115265
38. Paul NC, Moodley A, Ghibaudo G, Guardabassi L. Carriage of methicillin-resistant *Staphylococcus pseudintermedius* in small animal veterinarians: indirect evidence of zoonotic transmission. Zoonoses Public Health. 2011; 58(8):533–9. https://doi.org/10.1111/j.1863-2378.2011.01398.x PMID: 21824350
39. Enrright MC, Day NPJ, Davies CE, Peacock SJ, Sprati BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol. 2000; 38(3):1008–15. PMID: 10698988
40. Soares Magalhaes RJ, Loeffler A, Lindsay J, Rich M, Roberts L, Smith H, et al. Risk factors for methicillin-resistant *Staphylococcus aureus* (MRSA) infection in dogs and cats: a case-control study. Vet Res. 2010; 41(5):55. PubMed Central PMCID: PMC2879574. https://doi.org/10.1051/vetres/2010028 PMID: 20423695
41. Lehner G, Linek M, Bond R, Lloyd DH, Prenger-Berninghoff E, Thom N, et al. Case-control risk factor study of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) infection in dogs and cats in

Germany. Vet Microbiol. 2014; 168(1):154–60. https://doi.org/10.1016/j.vetmic.2013.10.023 PMID: 24290489

42. Developing a national antimicrobial resistance strategy for Australia [Internet]. Australian Veterinary Association; 2014 [Cited 10 June 2016]; [1–5]. Available from: http://www.ava.com.au/sites/default/files/AVA_website/pdfs/AVA%20AMR%20strategy%20discussion%20paper%20submission%20FINAL.pdf

43. Gandolfi-Decristophoris P, Regula G, Petrini O, Zinsstag J, Schelling E. Prevalence and risk factors for carriage of multi-drug resistant Staphylococci in healthy cats and dogs. J Vet Sci. 2013; 14(4):449. https://doi.org/10.4142/jvs.2013.14.4.449 PMID: 23820161

Supplementary information

S1 Table. Determination of methicillin resistance in *S. pseudintermedius* and *S. aureus* isolates from dogs, cats and horses in Australia based on phenotypic characteristic and *mecA* PCR.

Species	Lab ID	Animal origin	IZD (mm)		MIC (µg/mL)		Colony appearance in Brilliance^{TM} MRSA2	*mecA* PCR detection
			FOX	OXA	FOX	OXA		
S. pseudintermedius	N13/1/238	Dog	24	0	1	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/359	Dog	25	0	1	≥64	Pale blue- slow growth	Yes
S. pseudintermedius	N13/1/373	Dog	21	0	4	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/438	Dog	28	0	1	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/480	Dog	20	0	16	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/634	Dog	26	0	2	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/649	Dog	23	0	2	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/651	Dog	24	0	2	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/704	Dog	25	0	2	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/748	Dog	22	0	4	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/839	Dog	23	0	2	≥64	Pale blue	Yes
S. pseudintermedius	N13/4/115	Dog	24	0	8	≥64	Pale blue	Yes
S. pseudintermedius	N13/4/121	Dog	25	10	2	≥64	Pale blue	Yes
S. pseudintermedius	N13/4/123	Dog	24	0	4	≥64	Pale blue	Yes
S. pseudintermedius	N13/4/25	Dog	33	0	2	≥64	Pale blue- slow growth	Yes
S. pseudintermedius	N13/4/39	Dog	31	15	1	≥64	Pale blue- slow growth	Yes
S. pseudintermedius	N13/4/52	Dog	32	0	2	≥64	Pale blue	Yes
S. pseudintermedius	N13/4/75	Dog	30	10	1	≥64	Pale blue- slow growth	Yes
S. pseudintermedius	N13/4/94	Dog	23	0	8	≥64	Pale blue	Yes
S. pseudintermedius	Q13/1/190	Dog	20	0	8	≥64	Pale blue	Yes
S. pseudintermedius	Q13/1/243	Dog	26	0	2	≥64	Pale blue	Yes
S. pseudintermedius	Q13/1/294	Cat	14	0	32	≥64	Pale blue	Yes
S. pseudintermedius	Q13/1/311	Dog	29	0	0.5	≥64	Pale blue	Yes
S. pseudintermedius	Q13/1/317	Dog	23	0	8	≥64	Pale blue	Yes
S. pseudintermedius	Q13/1/326	Dog	28	0	8	≥64	Pale blue	Yes
S. pseudintermedius	Q13/3/18	Dog	26	0	4	≥64	Pale blue	Yes
S. pseudintermedius	Q13/3/24	Dog	26	0	2	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/18	Dog	22	0	4	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/125	Dog	33	16	4	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/133	Dog	29	0	2	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/140	Dog	32	0	2	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/152	Dog	26	0	8	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/191	Dog	28	0	1	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/393	Dog	25	0	8	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/407	Dog	28	0	1	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/413	Dog	21	0	1	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/440	Dog	29	0	0.5	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/441	Dog	30	0	1	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/475	Dog	30	0	1	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/488	Dog	30	0	1	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/52	Dog	18	0	32	≥64	Pale blue	Yes
S. pseudintermedius	V13/2/92	Cat	30	12	2	≥64	Pale blue	Yes
S. pseudintermedius	W13/1/11	Dog	24	0	4	≥64	Pale blue	Yes
S. pseudintermedius	N13/1/103	Dog	23	0	8	32	Pale blue	Yes
S. pseudintermedius	N13/1/304	Dog	12	0	32	32	White blue	Yes
S. pseudintermedius	N13/1/580	Dog	28	10	4	32	Pale blue	Yes
S. pseudintermedius	N13/1/77	Dog	27	17	2	32	Pale blue	Yes
S. pseudintermedius	Q13/1/35	Dog	25	0	2	32	Pale blue	Yes

S. pseudintermedius	V13/2/193	Dog	25	0	2	32	Pale blue	Yes
S. pseudintermedius	V13/6/5	Dog	30	0	0.25	32	Pale blue	Yes
S. pseudintermedius	W13/1/12	Dog	23	12	4	32	Pale blue	Yes
S. pseudintermedius	V13/2/173	Dog	30	0	2	16	Pale blue	Yes
S. pseudintermedius	V13/2/83	Dog	29	0	2	16	Pale blue	Yes
S. pseudintermedius	V13/2/220	Dog	33	0	1	8	Pale blue- slow growth	Yes
S. pseudintermedius	N13/4/21	Dog	29	16	0.5	4	Pale blue	Yes
S. pseudintermedius	Q13/1/21	Dog	26	0	1	4	Pale blue	Yes
S. pseudintermedius	Q13/1/219	Dog	30	13	1	4	Pale blue	Yes
S. pseudintermedius	V13/2/227	Dog	26	10	1	4	Pale blue- slow growth	Yes
S. pseudintermedius	V13/2/242	Dog	30	0	1	4	Pale blue	Yes
S. pseudintermedius	V13/2/194	Dog	30	0	1	2	Pale blue	Yes
S. pseudintermedius	V13/2/229	Dog	31	20	32	2	Pale blue	Yes
S. pseudintermedius	V13/6/4	Dog	25	0	8	2	Pale blue	Yes
S. pseudintermedius	V13/6/7	Dog	27	0	1	2	Pale blue	Yes
S. pseudintermedius	N13/1/307	Dog	27	14	0.5	1	Pale blue- slow growth	Yes
S. pseudintermedius	N13/1/386	Dog	26	0	0.5	1	Pale blue- slow growth	Yes
S. pseudintermedius	N13/1/421	Dog	28	10	0.5	1	Pale blue	Yes
S. pseudintermedius	N13/1/446	Dog	25	0	0.5	1	Pale blue	Yes
S. pseudintermedius	N13/1/616	Dog	27	20	0.5	0.5	Pale blue	Yes
S. pseudintermedius	N13/1/700	Dog	28	15	0.5	0.5	Pale blue- slow growth	Yes
S. pseudintermedius	N13/4/19	Dog	32	20	0.5	0.5	Pale blue- slow growth	Yes
S. pseudintermedius	V13/2/16	Dog	32	21	1	0.5	Pale blue- slow growth	Yes
S. pseudintermedius	V13/2/379	Dog	0	0	0.25	0.5	Pale blue- slow growth	Yes
S. pseudintermedius	W13/1/4	Dog	27	20	1	0.5	Pale blue	Yes
S. pseudintermedius	W13/1/5	Dog	12	14	0.5	0.5	Pale blue- slow growth	Yes
S. pseudintermedius	N13/1/317	Dog	25	0	2	≥64	Pale blue	No
S. pseudintermedius	V13/2/42	Dog	0	0	≥64	8	No growth	No
S. pseudintermedius	S13/1/74	Dog	34	27	4	2	No growth	No
S. pseudintermedius	N13/4/59	Dog	32	19	0.5	1	Pale blue- slow growth	No
S. pseudintermedius	N13/1/466	Cat	25	17	1	0.5	No growth	No
S. pseudintermedius	N13/1/627	Dog	33	19	0.25	0.5	Pale blue	No
S. pseudintermedius	N13/1/677	Dog	30	19	2	0.5	No growth	No
S. aureus	N13/1/17	Cat	8	0	≥64	≥64	Blue	Yes
S. aureus	N13/1/382	Cat	10	0	≥64	≥64	Blue	Yes
S. aureus	N13/1/648	Horse	12	0	≥64	≥64	Blue	Yes
S. aureus	N13/1/715	Horse	7	0	≥64	≥64	Blue	Yes
S. aureus	Q13/1/141	Dog	0	0	≥64	≥64	Blue	Yes
S. aureus	Q13/1/145	Dog	0	0	≥64	≥64	Blue	Yes
S. aureus	Q13/1/305	Dog	12	0	≥64	32	Blue	Yes
S. aureus	V13/2/426	Dog	11	0	≥64	≥64	Blue	Yes
S. aureus	V13/2/458	Horse	12	0	≥64	≥64	Blue	Yes
S. aureus	N13/1/396	Horse	12	0	32	≥64	Blue	Yes
S. aureus	N13/1/408	Horse	13	0	32	≥64	Blue	Yes
S. aureus	N13/4/96	Horse	16	0	32	32	Blue	Yes
S. aureus	V13/2/439	Dog	16	18	32	≥64	Blue	Yes
S. aureus	Q13/1/325	Dog	16	0	16	32	Blue	Yes
S. aureus	V13/6/23	Cat	17	10	32	4	Blue- small colonies	No

*IZD- inhibition zone diameter; MIC- minimum inhibitory concentration; FOX- cefoxitin; OXA- oxacillin. Colony appearance on Brilliance™ Agar was described as slow growth if colonies of typical blue or pale blue appearance were observed after 48 hrs instead of the typical 24hrs incubation. Isolates shaded in grey were negative for the *mecA* PCR and for the purposes of this paper- classified as methicillin-susceptible *S. pseudintermedius*. A single *S. aureus* isolate from a cat (V12/6/23) that was negative for *mecA* PCR- was subsequently shown to contain a *mecC* element [21].

S2 Table. Percentage of resistance to penicillins, cephems and β-lactams/β-lactamase inhibitor combinations before and after confirmation methicillin resistance status.

Antimicrobial agent	No. of resistant isolates determined by MIC breakpoints	No. of isolates reported as resistant determined by MIC breakpoints and methicillin resistance status
S. pseudintermedius (dogs n=616)		
Amoxicillin-clavulanate	231 (37.5 %)	239 (38.8 %)
Cefovecin	81 (13.1 %)	86 (14 %)
Cefoxitin	71 (11.5 %)	84 (13.6%)
Ceftriaxone	79 (12.8 %)	85 (13.8 %)
Cephalothin	83 (13.5 %)	93 (15.1 %)
Oxacillin	78 (12.7 %)	78 (12.7%)
S. pseudintermedius (cats n=13)		
Amoxicillin-clavulanate	7 (53.8 %)	7 (53.8 %)
Cefovecin	2 (15.4%)	2 (15.4%)
Cefoxitin	3 (23.1 %)	3 (23.1 %)
Ceftriaxone	3 (23.1 %)	3 (23.1 %)
Cephalothin	3 (23.1 %)	3 (23.1 %)
Oxacillin	3 (23.1 %)	3 (23.1 %)
S. aureus (horses n=53)		
Amoxicillin-clavulanate	25 (47.2 %)	25 (47.2 %)
Cefovecin	7 (13.2 %)	7 (13.2 %)
Cefoxitin	6 (11.3 %)	6 (11.3 %)
Ceftriaxone	7 (13.2 %)	7 (13.2 %)
Cephalothin	6 (11.3 %)	7 (13.2 %)
Oxacillin	6 (11.3 %)	6 (11.3 %)
S. aureus (dogs n=47)		
Amoxicillin-clavulanate	27 (54.7 %)	27 (54.7 %)
Cefovecin	7 (14.9 %)	7 (14.9 %)
Cefoxitin	6 (12.8 %)	6 (12.8 %)
Ceftriaxone	6 (12.8 %)	6 (12.8 %)
Cephalothin	6 (12.8 %)	6 (12.8 %)
Oxacillin	6 (12.8 %)	6 (12.8 %)
S. aureus (cats n=17)		
Amoxicillin-clavulanate	10 (58.8 %)	10 (58.8 %)
Cefovecin	3 (17.6%)	3 (17.6%)
Cefoxitin	3 (17.6%)	3 (17.6%)
Ceftriaxone	3 (17.6%)	3 (17.6%)
Cephalothin	2 (11.8%)	3 (17.6%)
Oxacillin	3 (17.6%)	3 (17.6%)

*Shaded areas show changes of percentage after methicillin resistance status.

Chapter 3

Antimicrobial resistance in clinical *Escherichia coli* isolated from companion animals in Australia

Statement of Authorship

Title of Paper	Antimicrobial resistance in clinical *Escherichia coli* isolated from companion animals in Australia
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Principal Author

Name of Principal Author (Candidate)	Sugiyono Saputra		
Contribution to the Paper	Performed laboratory work, analysis and interpreted data and wrote manuscript		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19/2/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Antimicrobial resistance in clinical *Escherichia coli* isolated from companion animals in Australia

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ABSTRACT

Multidrug-resistant (MDR) *Escherichia coli* have become a major public health concern to both humans and animal health. While the frequency of antimicrobial resistance (AMR) in clinical *E. coli* is monitored regularly in human medicine, current frequency of AMR in companion animals remains unknown in Australia. In this study we conducted antimicrobial susceptibility testing (AST) and where possible, determined potential risk factors for MDR infection among 883 clinical *Escherichia coli* isolated from dogs (n = 514), cats (n = 341) and horses (n = 28). AST was undertaken for 15 antimicrobial agents according to the Clinical Laboratory Standards Institute (CLSI) guidelines and interpreted using epidemiological cut-off values (ECOFFs) as well as CLSI veterinary and human clinical breakpoints. The AST revealed complete absence of resistance to carbapenems while resistance to amikacin was observed at a low level in isolates from dogs (1.6%) and cats (1.5%) compared to horses (10.7%). Among dog isolates, resistance to fluoroquinolones ranged from 9.1%–9.3% whereas among cat isolates, it ranged from 3.2%–5%. Among dog isolates, the proportion showing a 3rd generation cephalosporin (3GC) non-wild type phenotype was significantly higher ($P < 0.05$) in skin and soft tissue infection (SSTI, n = 122) isolates (17.2%–20.5%) compared to urinary tract infection (UTI, n = 392) isolates (9.9%–10.2%). The frequency of multidrug resistance was 18.1%, 11.7% and 42.9% in dog, cat and horse isolates, respectively. Risk factor analysis revealed that MDR *E. coli* isolated from UTI were positively associated with chronicity of infection and previous antimicrobial treatment. Dogs and cats with chronic UTI that had been previously treated with antimicrobials were eight times and six times more likely to be infected with MDR *E. coli* compared to dogs and cats with non-chronic UTI, and no history of antimicrobial treatment, respectively. This study revealed that pre-existing disease condition and prior antimicrobial use were the major risks associated with UTI with MDR *E. coli* in companion animals.

1. Introduction

Escherichia coli, a Gram negative bacteria normally residing in the intestinal tract, is among the most common pathogenic agents in hu mans and animals. It is classified into various pathotypes, causing in testinal and extra intestinal infections, including gastroenteritis, ur inary tract infections (UTI), skin and soft tissue infections (SSTI), and septicaemia (Hammerum and Heuer, 2009). Infections are usually less responsive to treatment when multidrug resistant (MDR) *E. coli* are encountered, especially when they are resistant to critically important antimicrobials (CIA), including extended spectrum β lactams (e.g. 3rd and 4th generation cephalosporins and carbapenems) and fluor oquinolones (FQN) (Abraham et al. (2014b). Therefore, effectiveness of treatment of bacterial disease is becoming a challenge because of emerging MDR strains contributing to more costly and protracted in fections.

Antimicrobial resistance (AMR) in bacteria that are well adapted for colonisation of both humans and animals is also a significant concern as shared environments provide the opportunity for rapid dissemination of resistant strains from one host to the other (Guardabassi et al., 2004).

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Environmental exposure and direct exposure to companion animals play important roles in transmission of resistant bacteria (Abraham et al., 2014b; Groves et al., 2016; Guardabassi et al., 2004). Frequent or intimate contact between companion animals and humans increases potential for transmission of resistant bacteria to humans, as readily transferable strains have been documented in several studies (Johnson et al., 2009).

MDR *E. coli* is increasing in frequency in both human and compa nion animal settings, including strains possessing extended spectrum β lactamases (ESBLs). Public health implications of ESBLs in animal iso lates have mainly been considered in food producing animals (Abraham et al., 2014a; Jahanbakhsh et al., 2016), but should also be extended to companion animals. Similarly, human to animal transmission is just as significant an issue for strains that predominately infect humans, such as 025b:ST131 and O75:ST1193 (Platell et al., 2011, 2012).

Monitoring of ongoing emergence and dissemination of resistance is a critical component of management systems that aim to keep re sistance at low levels (WHO, 2014). Many countries have established surveillance programmes to understand the emergence and severity of resistance in major pathogenic bacteria in both and humans and ani mals (EFSA and ECDC, 2015). These activities enable the early detec tion of the acquisition and spread of antimicrobial resistance. In Aus tralia, surveillance of human pathogens conducted by the Australian Group of Antimicrobial Resistance (AGAR) shows an increasing trend in the frequency of resistance amongst *E. coli*, especially involving the major classes of antibiotics used for treatment in humans (Turnidge et al., 2014). Recently, AMR in clinical *E. coli* isolated from food pro ducing animals in Australia has been reported (Abraham et al., 2015). However, current frequency of AMR in companion animals (defined here as dogs, cats and horses) remains unknown although the presence of multidrug resistance in pathogenic *E. coli* from dogs has been re ported in some localised studies (Guo et al., 2013, 2015). Therefore, as part of the first Australia wide survey of antimicrobial resistance in pathogens from animals, we generated antimicrobial susceptibility data for putative pathogenic *E. coli* isolated from companion animals with clinical illness. We used epidemiological cut off values (ECOFFs) re commended by the European Committee on Antimicrobial Suscept ibility Testing (EUCAST) to define wild type populations and the Clin ical Laboratory Standards Institute (CLSI) veterinary and human breakpoints to define the frequency of AMR from both a veterinary and public health standpoint. We then used these data to determine mul tiple resistance patterns and identify potential risk factors for UTI by strains defined as MDR *E. coli* in dogs and cats.

2. Material and methods

2.1. Isolates collections and identifications

Clinical *E. coli* isolates were obtained from 22 government, private and university veterinary diagnostic laboratories throughout Australia from January 2013 January 2014. Isolates were collected as part of the first nation wide survey of antimicrobial resistance in animals in Australia (Abraham et al., 2015; Saputra et al., 2017). These isolates were accompanied by clinical history and a laboratory submission re port with the details of the client having been de identified. Attributes of each case (age, gender, infection site, prior antimicrobial treatment) were extracted from clinical histories to use in the study of risk factors for MDR *E. coli* infection. Although all isolates had been identified as *E. coli* by submitting laboratories, this was repeated by detection of the *E. coli* specific universal stress protein A (*uspA*) gene (Chen and Driffiths, 1998), performing the indole spot test (BactiDrop™ Spot Indole, Ther mofisher Scientific) as well as observing colony morphology pattern on Sheep Blood Agar (SBA) prior to cryopreservation of isolates in 20% glycerol broth.

2.2. Antimicrobial susceptibility testing (AST)

Minimum inhibitory concentrations (MICs) were determined using broth microdilution performed in 96 well plates by the method of CLSI (CLSI, 2013). Susceptibility to a total of 15 antimicrobial agents from 9 antimicrobial categories was assessed, including: amikacin (AMK) and gentamicin (GNT), from the aminoglycosides (AMG); amoxicillin cla vulanic acid (AMC) from β lactam/β lactamase inhibitor combinations (BLI); ampicillin (AMP) from the β lactam (BLA) group; imipenem (IMP) from carbapenems (CRB), cephalothin (CEF), from the 1st gen eration cephalosporins (1GC); cefoxitin (FOX) a 2nd generation ce phamycin (2GC); cefovecin (CVN), ceftiofur (CTR) and ceftriaxone (CRO), representing 3rd generation cephalosporins (3GC); ciprofloxacin (CIP), enrofloxacin (ENR), marbofloxacin (MRB) and orbifloxacin (ORB), representing fluoroquinolones (FQN); and tetracycline (TET). The antimicrobials were all obtained from Sigma Aldrich except for CVN and CTR, which were obtained from Zoetis (Australia). Quality controls were monitored on every MIC testing by using *E. coli* ATCC 25922 as a control strain.

2.3. Interpretation of antimicrobial susceptibility profiles

Each isolate was designated as non wild type (non WT) to each antimicrobial based on ECOFFs published by the EUCAST or assessment using actual MIC distribution with ECOFFinder (Turnidge et al., 2006). This interpretation aimed to assess “microbiological resistance” for detection emerging resistance in the community. Further, to assess “clinical resistance” (to advise on therapy in the patients) and public health significance, MIC results were also interpreted based on both veterinary and human clinical breakpoints according to CLSI VET01S (CLSI, 2015a) and CLSI M100 S25 (CLSI, 2015b) as listed in Table 1. Additionally, AGAR have used the term “non susceptibility” to include both intermediate and resistance isolates interpreted based on CLSI clinical breakpoints (Turnidge et al., 2014). Note that non WT isolates

Table 1
Determination of non-wild type (non-WT) organisms based on epidemiological cut-off values (ECOFFs) and resistant (R) organisms based on clinical breakpoints according to CLSI documents.

Antimicrobials	Code	Non-WT	R[1]	R[2]
Amikacin	AMK	≥16	≥64	≥16
Ampicillin	AMP	≥16	≥32	≥1 and ≥16[a]; ≥32[b,c]
Amoxicillin-clavulanate	AMC	≥32	≥32	≥1/0.5 and ≥16/8[a,b]; ≥32/16[c]
Cefoxitin	FOX	≥16	≥32	–
Ceftiofur	CTR	≥2	–	≥8
Ceftriaxone	CRO	≥0.25	≥4	–
Cefovecin	CVN	≥4	–	≥4
Cephalothin	CEF	≥32	≥32	≥8[a]: ≥32[b,c]
Ciprofloxacin	CIP	≥0.12	≥4	–
Enrofloxacin	ENR	≥0.25	–	≥4
Gentamicin	GEN	≥4	≥16	≥8[a,c]; ≥16[b]
Imipenem	IPM	≥2	≥4	≥4
Marbofloxacin	MRB	≥0.25	–	≥4
Orbifloxacin	ORB	≥1	–	≥8
Tetracycline	TET	≥4	≥16	≥16

For dog isolates, an AMP breakpoint ≥1 μg/mL was used for SSTI and ≥16 μg/mL for UTI, an AMC breakpoint ≥1/0.5 μg/mL was used for SSTI and ≥16/8 μg/mL for UTI. An AMC breakpoint ≥1/0.5 μg/mL was used for both UTI and SSTI isolates from cats. A CEF resistance breakpoint ≥8 μg/mL was used for isolates from dogs and was adapted from veterinary breakpoints established for bovine mastitis. Proposed breakpoints for cefovecin were established in this study.

[1] Clinical breakpoints were adapted from human interpretative criteria according to CLSI M100 S25.

[2] Veterinary clinical breakpoints were adapted according to CLSI VET01S.

[a] Breakpoints used for dog isolates.

[b] Breakpoints used for cat isolates.

[c] Breakpoints used for horse isolates.

as defined by ECOFFs may or may not respond clinically to antimicrobial treatment while resistant isolates defined by CLSI clinical breakpoints are not likely to respond clinically to the usually achievable concentrations of the agent with normal dosage regiments (CLSI, 2015a; Silley, 2012).

The frequency of non WT, non susceptibility or resistance for each antimicrobial was described as rare: < 0.1%; very low: 0.1% to 1.0%; low: > 1% to 10.0%; moderate: > 10.0% to 20.0%; high: > 20.0% to 50.0%; very high: > 50.0% to 70.0%; and extremely high: > 70.0%; according to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) (EFSA and ECDC, 2015). Resistance profiles were generated using CLSI clinical breakpoints which include intermediately resistant and resistant isolates (i.e. non susceptible isolates), and this interpretation was retained in order to classify isolates as MDR for the risk factors study. Given the differences in ampicillin, amoxicillin clavulanate and 1st generation cephalosporin clinical breakpoints established on the basis of pharmacokinetics for skin and soft tissue vs urinary tract infection sites in companion animals, strains were defined as MDR if they showed resistance to at least one drug in three or more antimicrobial classes using human clinical breakpoints, as previously described (Magiorakos et al., 2012).

2.4. Detection of β lactamase genes

Following phenotypic detection of non WT isolates to either carbapenems or 3rd cephalosporins/cephamycins (MICs for imipenem ≥1 μg/mL; ceftriaxone ≥0.25 μg/mL; ceftiofur ≥2 μg/mL; cefovecin ≥4 μg/mL and/or cefoxitin ≥16 μg/mL), isolates were screened by PCR for the major groups of genes encoding β lactamases including bla_{IMP}, $bla_{CTX\ M}$, $bla_{CMY\ 2}$ and bla_{TEM} (Abraham et al., 2015; Dallenne et al., 2010).

2.5. Statistical analysis and risk factors study for multidrug resistant E. coli

All statistical analyses were performed using Stata 14.0 (Stata Corp., College Station, TX, USA) (Barlow et al., 2015). MIC distributions were produced per animal category with corresponding 95% confidence intervals (CIs) for the proportion of isolates showing non susceptibility to each antimicrobial. For the assessment of risk factors, only *E. coli* isolates originating from UTI in dogs (n = 366) and cats (n = 306) were analysed for factors contributing to isolation of a MDR *E. coli* strain since there were too few isolates from SSTI and from horses to be included. AMC was excluded during risk factor analysis for the cat isolates because the veterinary clinical breakpoint is actually lower than the wild type ECOFFs. The proportion of *E. coli* isolates expressing multidrug resistance was derived for combinations of possible risk factors, including animal (dogs and cats), age group (< 2, 2 10 years and > 10 years), sex (male and female), chronic and recurrent diseases (yes/no), concurrent diseases, such as diabetes, kidney and immune disease (yes/no), and the source case having received prior antimicrobial treatment (yes/no). Univariate and multivariate analyses based on logistic regression were used to assess the effect of various factors on the proportion of resistant vs susceptible isolates. MDR *E. coli* isolates (interpreted by human clinical breakpoints) were used as the outcome. In multivariate logistic regression, variables with a significant result from univariate analysis (P < 0.2) were included in the model to obtain a main effects model. Two way interactions arising from the main effects were retained where significant (p < 0.05).

3. Results

3.1. Isolate MIC distributions according to host species

Overall, 883 isolates were confirmed as *E. coli* using the *uspA* PCR and spot indole test. Among these clinical *E. coli*, 514 isolates (58.2%) originated from dogs, 341 isolates (38.6%) from cats, and only 28 isolates (3.2%) from horses. Isolates were obtained from all mainland states of Australia, with over 45% of the isolates originating from New South Wales (NSW) (Table 2). The distribution of MIC values and overall frequency of non WT and resistance based on ECOFFs, veterinary and human clinical breakpoints are presented for all antimicrobials in Table 3.

Table 2
Number of total *E. coli* isolated from clinical infections in dogs, cats and horses from five regions in Australia.

Region	No. of isolates	%	Animal origin		
			Dog	Cat	Horse
New South Wales (NSW)	403	45.6	233	157	13
Queensland (QLD)	236	26.7	130	100	6
Victoria (VIC)	212	24.0	124	82	6
South Australia (SA)	25	2.8	21	2	2
Western Australia (WA)	7	0.8	6	0	1
Total	883	100	514	341	28

3.2. Frequency of resistance among clinical E. coli according to CLSI breakpoints

Most notably, resistance to carbapenems (imipenem) was not detected in any isolates (0/883, 0%). Among the dog isolates (n = 514), the highest frequency of resistance based on veterinary breakpoints was to CEF (n = 486, 94.6%) followed by AMC (n = 234, 45.5%) and AMP (n = 227, 44.2%). However, if human clinical breakpoints were used, the percentage of isolates exhibiting resistance to CEF, AMC and AMP was reduced to 21.6% (n = 111), 27.8% (n = 143) and 11.3% (n = 58), respectively. The frequency of resistance to 3GC used in veterinary (CTR and CVN) and human medicine (CRO) was moderate with frequencies of 10.1% (n = 52), 10.9% (n = 56) and 10.3% (n = 53), respectively. Resistance frequency to FQN was slightly lower at 9.1% (n = 47) for CIP and ENR and 9.3% (n = 51) for MRB and ORB. Low levels of resistance to AMK (n = 8, 1.6%) and GEN (n = 25, 4.9%) were also recorded.

Among cat isolates (n = 341), all isolates (100%) would be classified as resistant to AMC based on recently approved veterinary specific breakpoints, but if human clinical breakpoints were used, the frequency of resistance was markedly lower (n = 26, 7.6%). The frequency of resistance to 3GC (n = 29, 5.6% for CTR and CRO; n = 33, 6.5% for CVN), FQN (n = 11, 3.2%) and GEN (n = 6, 1.8%) was significantly lower among cat isolates compared to dog isolates (P < 0.05).

With such a low number of isolates obtained from horses in the nation wide study (n = 28), confidence intervals were large, however, the frequency of resistance to CIA was generally higher than observed for cat and dog isolates including aminoglycosides (AMK n = 3, 10.7%; GEN n = 11, 39.3%), 3GC (n = 9, 32.1%), and FQN (n = 7, 25%) based on veterinary specific breakpoints.

For the remainder of the antimicrobials, interpretation of MIC results using either ECOFFs, or human and veterinary specific breakpoints did not differ significantly.

3.3. Frequency of resistance and non WT among clinical E. coli according to site of infection

Generally, the proportion of isolates showing resistance to each antimicrobial was lower among *E. coli* obtained from canine UTI (n = 392, 76.3%) compared to SSTI (n = 122, 23.7%) but did not reach significance. However, based on EUCAST ECOFFs, among dog isolates, a significantly higher proportion of non WT isolates from SSTI was observed for CEF, 3GC and TET (P < 0.05) compared to UTI. Among cat isolates, the proportion of non WT isolates from SSTI

Table 3
MIC distribution and frequency of non-wild type (non-WT, based on ECOFFs) and resistant isolates (R, based on CLSI breakpoints) among *E. coli* obtained from dogs (n = 514), cats (n = 341) and horses (n = 28) in Australia.

Antimicrobials	Animal	% non-WT	% R[1]	% R[2]	Percentage of isolates with indicate d MIC (μg/mL)														
					≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	≥128
Amikacin	Dog	1.6	0.2	1.6							0.2	7	42.4	40.1	8.8	1.4		0.2	
	Cat	1.5	0	1.5							0.3	5	45.2	41.1	7	1.5			
	Horse	10.7	0	10.7								3.6	25	46.4	14.3	10.7			
Ampicillin	Dog	28.8	27.8	44.2								1.4	15.6	46.7	7.6	1	0.2	0.4	27.2
	Cat	26.7	26.7	26.7								1.8	29	37	5.6			0.9	25.8
	Horse	50	50	50									7.1	42.9				3.6	46.4
Amoxicillin-clavulanate	Dog	11.3	11.3	45.5								0.4	1.6	14.6	53.1	19.1	2.7	8.6	
	Cat	7.6	7.6	100									2.6	25.5	47.8	16.4	2.3	5.3	
	Horse	17.9	17.9	7.8									3.6	10.7	50	17.9		17.9	
Cefoxitin	Dog	13	10.5	-								1	16.9	54.1	15	2.5	1.2	1.8	7.6
	Cat	9.1	5.9	-								2.1	32.8	44	12	3.2	1.2	1.8	2.9
	Horse	10.7	7.1	-								3.6	7.1	46.4	32.1	3.6			7.1
Ceftiofur	Dog	12.3	-	10.1				0.4	3.5	24.7	48.8	10.3	1.9	0.2		1.6	2.1	6.4	
	Cat	6.7	-	5.6				0.6	7.6	40.8	36.7	7.6	1.2			0.9	0.6	4.1	
	Horse	32.1	-	32.1				3.6		35.7	25	3.6						32.1	
Ceftriaxone	Dog	12.6	10.3	-			26.7	48.1	12.6	1.4	0.4	0.2	0.4		0.2	0.6	9.5		
	Cat	8.2	5.6	-			43.4	41.1	7.3	2.1		0.3	0.3			0.3	5.3		
	Horse	35.7	32.1	-			28.6	32.1	3.6	3.6							32.1		
Cefovecin	Dog	11.5	-	10.9					0.8	10.1	41.8	31.3	4.5	0.6	0.6		0.4	0.2	9.7
	Cat	7.3	-	6.5					3.8	19.4	46.6	19.1	3.8	0.9	0.6	0.3	0.3	0.6	4.7
	Horse	28.6	-	28.6					3.6	10.7	32.1	25						3.6	25
Cephalothin	Dog	21.6	21.6	94.6									1.2	4.3	34.2	38.7	8.9	0.2	12.5
	Cat	15	15	15									0.9	11.7	43.4	29	6.5	0.3	8.2
	Horse	39.3	39.3	39.3									7.1	3.6	32.1	17.9			39.3
Ciprofloxacin	Dog	10.5	9.1	-	30.7	49.6	6.2	0.6	2.3	0.8	0.2	0.4		1	8.2				
	Cat	3.8	3.2	-	44.9	44.6	4.7	0.9	1.2		0.6				3.2				
	Horse	25	25	-	35.7	35.7		3.6							25				
Enrofloxacin	Dog	12.8	-	9.1	0.2	10.5	55.1	20	1.4	1.2	2.1	0.2	0.2		9.1				
	Cat	5	-	3.2	0.9	24	56.9	11.7	1.5	0.3	1.2	0.3			3.2				
	Horse	28.6	-	25		7.1	46.4	17.9		3.6					25				
Gentamicin	Dog	4.9	4.5	4.9						5.3	50.6	35.2	4.1		0.4	0.6	3.5	0.4	
	Cat	2.6	1.8	1.8					0.6	2.6	49.3	39.3	5.6	0.9			1.5	0.3	
	Horse	39.3	39.3	39.3						3.6	17.9	39.3					25	14.3	
Imipenem	Dog	0	0	0				3.1	31.3	44.6	16.1	4.9							
	Cat	0	0	0				4.4	29.6	44.3	18.8	2.9							
	Horse	0	0	0				10.7	42.9	28.6	10.7	7.1							
Marbofloxacin	Dog	12.8	-	9.3	0.2	9.9	62.3	14.2	0.6	1.8	1.4		0.4	0.8	8.6				
	Cat	5	-	3.2		20.8	61.6	11.4	1.2	0.9	0.9				3.2				
	Horse	28.6	-	25		14.3	46.4	10.7		3.6					25				
Orbifloxacin	Dog	12.5	-	9.3		0.2	1	25.3	49.8	9.9	1.4	1.4	1.4	0.4	0.2	9.1			
	Cat	4.4	-	3.2		0.3	3.8	37.8	42.5	10	1.2	0.3	0.6	0.3		3.2			
	Horse	25	-	25				21.4	46.4	3.6	3.6					25			
Tetracycline	Dog	17.1	16.7	16.7					0.2	0.6	8.9	57.6	15.6	0.2	0.2	0.2	1	2.9	12.6
	Cat	11.7	10	10						0.6	21.1	60.7	5.9	1.5	0.3			1.5	8.5
	Horse	50	50	50						0	7.1	35.7	7.1				3.6	10.7	35.7

*Unshaded areas show the dilution range for each drug. Epidemiological cut-off values (ECOFFs) are indicated as vertical dotted lines. [1]Frequency of resistance according to human clinical breakpoints (CLSI M100 S25), indicated by double vertical solid lines. [2]Frequency of resistance based on veterinary clinical breakpoints (CLSI VET01S), indicated by vertical solid lines.

(n = 21, 6.2%) was significantly higher compared to UTI (n = 320, 93.8%) in FQN and TET (P < 0.05) (Table 4).

3.4. Phenotypic resistance patterns derived from veterinary and human clinical breakpoints

Overall, 541 isolates (61.3%) were susceptible to all antimicrobial agents, interpreted according to CLSI clinical breakpoints. A moderate proportion of dog isolates (n = 93, 18.1%) and cat isolates (n = 40, 11.7%) and a high proportion of horse isolates (n = 12, 42.9%) were classified as MDR (Table 5). The most common resistance profile was BLA BLI detected in 71 isolates while the most common MDR profile was BLA BLI 2GC 3GC observed in 20 dog isolates and 8 cat isolates. A total 13 isolates, including 10 isolates from dogs, two isolates from cats and one isolate from a horse shared a MDR profile to seven anti microbial classes (AMG BLA BLI 2GC 3GC FQN TET).

3.5. Presence of β lactamase genes

Corresponding with the interpretation of imipenem susceptibilities, bla_{IMP} was not detected in any isolate with an MIC > 0.5 μg/mL. Among 112 isolates that satisfied the selection criteria and were screened for β lactamase genes, $bla_{CMY\ 2}$ was dominant, having been detected in 58 isolates (51.8%), followed by bla_{TEM} (n = 48; 42.9%) and $bla_{CTX\ M}$ (n = 23; 20.5%). A total of 28 isolates (25%) contained both $bla_{CMY\ 2}$ and bla_{TEM}; 13 isolates (11.6%) contained $bla_{CTX\ M}$ and bla_{TEM} and four isolates (3.6%) contained $bla_{CMY\ 2}$ and $bla_{CTX\ M}$. A total of 27 isolates that satisfied the β lactamase screening criteria were negative for all resistance genes tested.

3.6. Risk factors associated with MDR E. coli in urinary tract infections

The epidemiological data and univariate analysis for the risk factors study are shown in Table 6. *E. coli* urinary tract infections were more common in female dogs (n = 264, 72.1%) and cats (n = 222, 72.5%). There was a significant difference in the proportion of *E. coli* isolates expressing multidrug resistance in two potential risk factor groups, those reported to have chronic infection and prior antimicrobial treat ment. A significant association of MDR *E. coli* with age groups including 2 10 years and > 10 years, concurrent disease and prior antimicrobial treatment was observed in dog isolates only. In multivariate analysis, chronicity of UTI was the factor that remained significantly associated with MDR *E. coli* isolates in dog (OR 4.3; 95%CI 2.1 9; P < 0.0001) and cat isolates (OR 3; 95%CI 1.2 7.4; P < 0.02). However, after in clusion of interaction terms, dogs and cats with chronic UTI that had been previously treated with antimicrobials were eight times and six

Table 4
Percentage of non-WT (based on ECOFFs) and resistance (based on CLSI clinical breakpoints) in *E. coli* obtained from dogs (n = 514), cats (n = 341) and horses (n = 28) by site of infection.

Antimicrobial agents	Dogs				Cats				Horses			
	ECOFF		CLSI		ECOFF		CLSI		ECOFF		CLSI	
	UTI n = 392	SSTI n = 122	UTI n = 392	SSTI n = 122	UTI n = 320	SSTI n = 21	UTI n = 320	SSTI n = 21	UTI n = 4	SSTI n = 24	UTI n = 4	SSTI n = 24
Amikacin	1.8	0.8	1.8	0.8	1.2	4.8	1.2	4.8	0	12.5	0	12.5
Ampicillin	26.7	35.2	26.8	100	26.3	31.8	26.3	33.3	0	58.3	0	58.3
Amoxicillin-clavulanate	10.2	14.6	28.6	100	7.8	4.8	100	100	0	20.8	0	20.8
Cefoxitin	12.2	15.6	9.2	14.8	9	9.5	5.6	9.5	0	41.7	0	41.7
Ceftiofur	10.2[a]	18.9	8.7	14.8	6.6	9.5	5.3	9.5	0	37.5	0	37.5
Ceftriaxone	10.2[a]	20.5	8.9	14.8	8.1	9.5	5.3	9.5	0	41.6	0	41.6
Cefovecin	9.9[a]	17.2	9.2	16.4	7.2	9.5	6.3	9.5	0	33.3	0	33.3
Cephalothin	11[a]	18	95.4	92.6	8.6	9.5	15	14.3	0	45.8	0	45.8
Ciprofloxacin	9.4	13.9	8.2	12.3	3.1[b]	14.3	2.8	9.5	0	29.2	0	29.2
Enrofloxacin	12	15.6	8.2	12.3	4.7[b]	14.3	2.8	9.5	0	33.3	0	33.3
Gentamicin	4.3	6.6	4.3	6.6	2.5	4.8	1.6	4.8	0	45.8	0	45.8
Imipenem	0	0	0	0	0	0	0	0	0	0	0	0
Marbofloxacin	11.7	16.4	8.4	12.3	4.4[b]	14.3	2.8	9.5	0	33.3	0	33.3
Orbifloxacin	11.7	14.6	8.4	12.3	3.8[b]	14.3	2.8	9.5	0	29.2	0	29.2
Tetracycline	14.3[a]	26.2	16.3	25.4	10.9[b]	23.8	9.1	23.8	25	54.2	25	54.2

*Clinical breakpoints for FOX, CRO and CIP were adapted from CLSI M100 S25.
[a] A significantly lower proportion ($P < 0.05$) of non-WT was observed in dog isolates from UTI compared to SSTI (CTR, CRO, CVN, CEF and TET).
[b] A significantly lower proportion ($P < 0.05$) of non-WT was observed in cat isolates from UTI compared to SSTI (CIP, ENR, MRB, ORB and TET).

times more likely to be infected with MDR *E. coli* compared to dogs and cats with acute UTI and/or no history of prior antimicrobial treatment, respectively (Table 7).

4. Discussion

In this study, we report the findings of the first nation wide survey of antimicrobial resistance in clinical *E. coli* isolated from dogs, cats and horses in Australia. Major findings from this study are: 1) the overall frequency of resistance to CIA registered for veterinary use in dog isolates was classified as low for FQN (9.1% 9.3%) and moderate for 3GC (10.1% 10.9%), whilst it was low among cat isolates (FQN 3.2% 5%; 3GC 5.6% 6.5%); (2) among dog and cat isolates, resistance to CIA registered in human medicine was either not reported (carbapenems) or low (amikacin; 1.5% 1.6%); and 3) chronic and/or recurrent disease and prior antimicrobial treatments were the main risk factors for the isolation of MDR *E. coli* from UTI in both dogs and cats.

To date, information related to the frequency of resistance among companion animal clinical *E. coli* to CIA classes in Australia is very limited. Since large scale surveys of companion animals for AMR have not been performed previously, it is uncertain over what timescale resistance has evolved. Ideally, resistance to CIA would be detected early and this may only happen if such surveys are performed at regular time intervals. In contrast to pathogenic *E. coli* in companion animals, more information is available for commensal *E. coli* (Barlow et al., 2015) and for some pathogenic *E. coli* (Abraham et al., 2015) from Australian livestock, but once again, these studies are very recent and require ongoing surveillance to determine trends. For example, the frequency of resistance to CIA in both commensal and pathogenic *E. coli* from livestock is negligible in Australia, possibly because of strict regulation of antimicrobials in food animals and/or animal management systems that do not favour bacterial disease (Barlow et al., 2015; Cheng et al., 2012). By comparison, treatment of infections in companion animals closely mirrors human medicine, with veterinarians able to prescribe 3GC and FQN registered for use in dogs and cats, largely without any restrictions other than the availability of prudent use guidelines (AIDAP, 2016). Furthermore, companion animal veterinarians in Australia do have access to off label use of human formulations (e.g. amikacin and carbapenems), and although the numbers of animals actually treated with these drug classes is thought to be very limited (Gibson et al., 2008),

Table 5
The most prevalent resistance profile per antimicrobial category found in clinical *E. coli* isolated from dogs (n = 514), cats (n = 341) and horses (n = 28) in Australia based on CLSI human clinical breakpoint data.

No. antimicrobial category	No. of isolates (%)	Resistance pattern (no. of isolates)		
		Dogs	Cats	Horses
All susceptible	541 (61.3)	302	227	12
1	83 (9.4)	BLI (29)	BLA (15)	TET (2)
2	95 (10.8)	BLA-BLI (38)	BLA-BLI (32)	BLA-BLI (1)
3	42 (4.8)	BLA-BLI-TET (14)	BLA-BLI-TET (9)	BLA-BLI-2GC (1)
4	43 (4.9)	BLA-BLI-2GC-3GC (20)	BLA-BLI-2GC-3GC (8)	AMG-BLA-BLI-TET (1)
5	26 (2.9)	AMG-BLA-BLI-FQN-TET (5)	AMG-BLA-BLI-FQN-TET (5)	AMG-BLA-BLI-FQN-TET (2)
6	21 (2.4)	AMG-BLA-BLI-3GC-FQN-TET (4)	AMG-BLA-BLI-2GC-3GC-FQN (2)	AMG-BLA-BLI-3GC-FQN-TET (4)
7	13 (1.5)	AMG-BLA-BLI-2GC-3GC-FQN-TET (10)	AMG-BLA-BLI-2GC-3GC-FQN-TET (2)	AMG-BLA-BLI-2GC-3GC-FQN-TET (1)
Non-MDR	738 (83.6)	420	301	16
MDR	145 (16.4)	93	40	12

*Antimicrobial categories included: aminoglycosides, AMG (AMK and GEN); penicillin, BLA (AMP), beta-lactam/inhibitors, BLI (AMC), 2nd generation cephalosporins, 2GC (FOX); 3rd generation cephalosporins, 3GC (CVN, CTR, CRO), fluoroquinolones, FQN (CIP, ENR, MRB and ORB); and tetracycline, TET.

Table 6
Univariate analysis of risk-factor variables from MDR *E. coli* isolated from UTI in dogs (n = 366) and cats (n = 306). Odds ratios define the risk of isolates being classified as MDR.

Risk factor	Dogs					Cats				
	n	%MDR	OR	P value	95% CI	n	%MDR	OR	P value	95% CI
Age in years										
< 2	45	4.4	Ref			9	11.1	Ref		
2–10	154	18.9	5	0.033	1.1–21.8	78	9	0.8	0.834	0.1–7.3
< 10	167	17.4	4.5	0.045	1–19.7	219	12.3	1.1	0.913	1.1–9.3
Sex										
Male	102	16.7	Ref			84	13.1	Ref		
Female	264	16.3	1	0.93	0.5–1.8	222	10.8	0.8	0.576	0.4–1.7
Chronic and recurrent diseases										
No	317	12.6	Ref			276	10.5	Ref		
Yes	49	40.8	4.8	< 0.0001	2.5–9.2	30	20	3.2	0.011	0.8–5.6
Concurrent diseases										
No	323	14.6	Ref			268	11.6	Ref		
Yes	43	30.2	2.5	0.011	1.3–5.2	38	10.5	0.9	0.85	0.3–2.7
Prior antimicrobial treatment										
No	301	13.6	Ref			275	10.5	Ref		
Yes	65	29.2	2.6	0.003	1.4–5	31	19.4	2	0.151	0.8–5.4

Table 7
Odds ratios showing the likelihood of *E. coli* isolates obtained from UTI in dogs and cats being MDR using chronicity of infection and exposure of the host to prior antimicrobial treatment as the variables.

Prior antimicrobial treatment	Dogs				Cats			
	Chronic		Non chronic		Chronic		Non chronic	
	n	OR; 95%CI	n	OR; 95%CI	n	OR; 95%CI	n	OR; 95%CI
Yes	22	8.3; 3.2–21.3	43	1.8; 0.7–4.3	6	4.8; 0.8–28	25	2; 0.6–6.5
No	27	4.2; 1.7–10.5	274	Ref.	25	23.1; 1.1–8.7	250	Ref.

antimicrobial stewardship programmes governing use are still in their infancy (Abraham et al., 2014b).

International comparison of the results obtained in this study are difficult to interpret due to differences in study design, drugs tested, breakpoint determination, and temporal or geographic variation. In comparison with an analogous study in the USA conducted over a five year period (2008 2013; dog isolates n = 2390; cat isolates n = 780) using CLSI breakpoints (Thungrat et al., 2015), a moderately high level of resistance was observed among dog isolates to FQN (CIP 10.7%; ENR 11.7%) and a low level among cat isolates (CIP 5.3%; ENR 5.9%). In our Australian study, when we applied the same clinical breakpoints to our data, we observed a similar though slightly lower level of resistance to CIP and ENR among dog (both 9.1%) and cat isolates (both 3.2%). Resistance to GEN in the Australian study was also lower (dog isolates 4.9%, cat isolates 1.8%) compared to the US study (dog isolates 8.5%; cat isolates 5.9%).

Additionally, using cefotaxime and cefpodoxime as representatives of the 3GC class, the proportion of resistant isolates in the US study ranged from 13.4% 13.9% among dog isolates and 7.6% 9.5% among cat isolates, which is analogous to our Australian study (dog isolates 11.5% 12.6%, cat isolates 6.7% 8.2%). However, in contrast to these two studies, a surveillance study conducted in Sweden among clinical *E. coli* isolated from UTI in dogs (n = 943) and cats (n = 461) in 2014, showed a very low to low frequency of non WT isolates for the critically important antimicrobials such as 3GC (cefotaxime: dogs 0.7%, cats 1%) and FQN (dogs 7%; cats 7%) (Swedres Svarm, 2015) when ECOFFs were used. In our study, low and moderately high proportions of isolates from UTI cases were classified as non WT for both 3GC (CRO: dogs 10.2%, cats 8.1%) and FQN (dogs 12%, cats 4.7%) using the same breakpoints.

Comparison of AMR among human isolates from Australia obtained over a similar time scale is difficult, as in 2013, AGAR switched to surveying AMR in blood sepsis isolates only, whereas in previous surveys they alternated each year between hospital and community sourced isolates. Nevertheless, comparison with data from the AGAR 2012 report, confirmed that the proportion of non susceptibility for some CIA was generally higher among dog isolates and lower among cat isolates. The non susceptibility rate of CRO and CIP in *E. coli* isolates causing UTI in humans (n = 2025) was 4.2% and 6.9%, respectively (Turnidge et al., 2014) while in our study, the proportion of non susceptibility among dog isolates was 8.9% and 8.2%, respectively. Resistance to CRO in cat isolates was also slightly higher (5.3%) but much lower for CIP (2.8%) when compared to human isolates. By using the same definition, the proportion of multidrug resistance (non susceptibility to three or more antimicrobial classes) among human isolates (n = 1871) was 13.8%, while among dog isolates it was much higher (18.1%) and slightly lower among cat isolates (11.7%).

Although the frequency of multidrug resistance among horse *E. coli* isolates was much higher compared to dog and cat isolates, the sample size was extremely small compared to the total population of horses in Australia. Nevertheless, the fact that a number of equine isolates were resistant to amikacin, a critically important drug only registered for use in humans in Australia, raises some concerns that should be followed up with a further survey on larger numbers of horses. However, a study in the UK recommended that WHO designated and prioritised critically important antimicrobials should be discouraged from use as first line therapies in horses, especially with the significant increase in resistance among clinical *E.coli* to these drug classes and reports of extended spectrum β lactamase (ESBL) genes being identified in equine isolates (Johns and Adams, 2015).

As reported previously, $bla_{\text{CMY 2}}$ and $bla_{\text{CTX M}}$ genes are the most frequent AmpC β lactamase and ESBL encoding genes identified in both human and veterinary medicine (Abraham et al., 2015; Sidjabat et al., 2014), and are often associated with bla_{TEM} on MDR plasmids (Hordijk

et al., 2013). In this study, $bla_{CMY\ 2}$ and $bla_{CTX\ M}$ were collectively identified in 68.7% (77/112) of *E. coli* isolates with a 3GC and 38.4% (43/94) of isolates with FQN non WT phenotype. A comparative genomics study is currently underway to confirm phylogenetic groups, multilocus sequence types, plasmids, and resistance genes in selected MDR *E. coli* isolates from this study.

Owing to the much larger number of dog and cat isolates, it was possible to identify potential risk factors for isolation of MDR *E. coli* from UTI, with the most significant factors being the presence of chronic and/or recurrent disease and prior use of antimicrobial agents. Although UTIs are positively associated with older aged and/or female dogs and cats (Thompson et al., 2011), we found that there was no significant correlation between these two variables and frequency of antimicrobial resistance. Another study identified that the proportion of MDR *E. coli* from dogs with complicated UTI was significantly higher (36%) compared to isolates from dogs with uncomplicated infections (21%) (Wong et al., 2015). Previous antimicrobial treatments also significantly influenced the likelihood of the *E. coli* isolate to exhibit a MDR phenotype, in particular resistance to 3GC and FQN (Leite Martins et al., 2014). A study in cats also showed that prior antimicrobial treatment significantly influenced the risk of AMR while the type of infection did not reach significance (Hernandez et al., 2014). Other factors in addition to predisposing disease condition and prior treatment with antimicrobials that could not be examined in the present study include number of previous hospitalizations and length of hospitalization which have both been associated with carriage of MDR extraintestinal pathogenic *E. coli* in dogs (Gibson et al., 2008).

This study has some limitations. One major factor is the current differences in interpretative criteria applied to AST results. Much higher rates of resistance were observed for some antimicrobials (AMP, AMC and CEF), according to the latest veterinary specific breakpoints (CLSI), particularly according to body site specific breakpoints (SSTI compared to UTI breakpoints), which is in contrast with an interpretation based on EUCAST ECOFFs or human specific CLSI clinical breakpoints (Table 3). If only veterinary specific clinical breakpoints were applied, many SSTI isolates would be classified as resistant even though their ECOFFs indicate that they are wild type *E. coli*. This is important from a PK/PD and antibiotic stewardship perspective, but it gives neither an indication of the isolate's propensity to carry β lactamase genes nor its public health significance. Further, the estimates of frequency of resistance amongst horse isolates had very wide confidence limits owing to the small sample size. Future studies should therefore focus on achieving a sufficiently large collection of isolates from horses to increase the accuracy of frequency estimates beyond that obtained here. Caution must therefore be exercised in making any public health recommendations based on the resistance frequencies generated in this study using veterinary specific breakpoints. Despite these shortfalls, we are unaware of any collection of *E. coli* isolates that is as representative of the Australian population of dogs and cats, both in terms of size and geographic source.

5. Conclusion

In conclusion, using a combination of ECOFFs and CLSI clinical breakpoints, the first Australia wide survey of antimicrobial resistance in pathogenic *E. coli* originating from companion animals identified no resistance to carbepenems and low to moderate levels of resistance to other CIA (amikacin, 3GC and FQN) in cat and dog isolates. Pathogenic *E. coli* isolates from horses represented only a small fraction of the total and it is recommended this group of animals is more appropriately targeted in future surveys. Dogs and cats with chronic and/or recurrent UTI or that had previous antimicrobial treatment/s were at increased risk of yielding a MDR *E. coli* isolate on a urine culture and susceptibility test. To maintain or even lower these levels of resistance, it is crucial for all veterinarians and pet owners to reduce inappropriate antimicrobial use by following prudent use guidelines and reduce the potential for transmission by applying biosecurity, infection control and antimicrobial stewardship strategies in companion animal practice.

Ethical approval

Not required.

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References

AIDAP, 2016. Antibiotic Prescribing Detailed Guidelines. (Vets Australia).
Abraham, S., Trott, D.J., Jordan, D., Gordon, D.M., Groves, M.D., Fairbrother, J.M., Smith, M.G., Zhang, R., Chapman, T.A., 2014a. Phylogenetic and molecular insights into the evolution of multidrug-resistant porcine enterotoxigenic *Escherichia coli* in Australia. Int. J. Antimicrob. Agents 44, 105–111.
Abraham, S., Wong, H.S., Turnidge, J., Johnson, J.R., Trott, D.J., 2014b. Carbapenemase-producing bacteria in companion animals: a public health concern on the horizon. J. Antimicrob. Chemother. 69, 1155–1157.
Abraham, S., Jordan, D., Wong, H.S., Johnson, J.R., Toleman, M.A., Wakeham, D.L., Gordon, D.M., Turnidge, J.D., Mollinger, J.L., Gibson, J.S., Trott, D.J., 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals. J. Glob. Antimicrob. Res. 3, 273–277.
Barlow, R.S., McMillan, K.E., Duffy, L.L., Fegan, N., Jordan, D., Mellor, G.E., 2015. Prevalence and antimicrobial resistance of Salmonella and *Escherichia coli* from Australian cattle populations at slaughter. J. Food Prot. 78, 912–920.
CLSI, 2013. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, approved standard-fourth edition. CLSI document VET01-A4 (Wayne, PA, USA).
CLSI, 2015a. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, third edition. CLSI document VET01S (Wayne, PA, USA).
CLSI, 2015b. Performance Standards for Antimicrobial Susceptibility Testing, twenty-fifth informational supplement. CLSI document M100-S25 (Wayne, PA, USA).
Chen, J., Driffiths, M.W., 1998. PCR differentiation of *Escherichia coli* from other Gram-negative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein. Lett. Appl. Microbiol. 27, 369–371.
Cheng, A.C., Turnidge, J., Collignon, P., Looke, D., Barton, M., Gottlieb, T., 2012. Control of fluoroquinolone resistance through successful regulation. Australia Emerg. Infect. Dis. 18, 1453–1460.
Dallenne, C., Da Costa, A., Decre, D., Favier, C., Arlet, G., 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. J. Antimicrob. Chemother. 65, 490–495.
EFSA, ECDC, 2015. EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. EFSA J. 2015 (13), 1–178.
Gibson, J., Morton, J., Cobbold, R., Sidjabat, H., Filippich, L., Trott, D., 2008. Multidrug-resistant *E. coli* and *Enterobacter* extraintestinal infection in 37 dogs. J. Vet. Intern. Med. 22, 844–850.
Groves, M.D., Crouch, B., Coombs, G.W., Jordan, D., Pang, S., Barton, M.D., Giffard, P., Abraham, S., Trott, D.J., 2016. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* isolated from Australian veterinarians. PLoS One 11, e0146034.
Guardabassi, L., Schwarz, S., Lloyd, D.H., 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria. J. Antimicrob. Chemother. 54, 321–332.
Guo, S., Brouwers, H.J., Cobbold, R.N., Platell, J.L., Chapman, T.A., Barrs, V.R., Johnson, J.R., Trott, D.J., 2013. Fluoroquinolone-resistant extraintestinal pathogenic *Escherichia coli*, including O25b-ST131, isolated from faeces of hospitalized dogs in an Australian veterinary referral centre. J. Antimicrob. Chemother. 68, 1025–1031.
Guo, S., Wakeham, D., Brouwers, H.J., Cobbold, R.N., Abraham, S., Mollinger, J.L., Johnson, J.R., Chapman, T.A., Gordon, D.M., Barrs, V.R., Trott, D.J., 2015. Human-associated fluoroquinolone-resistant *Escherichia coli* clonal lineages, including ST354, isolated from canine feces and extraintestinal infections in Australia. Microbes Infect. 17, 266–274.
Hammerum, A.M., Heuer, O.E., 2009. Human health hazards from antimicrobial-resistant Escherichia coli of animal origin. Clin. Infect. Dis. 48, 916–921.
Hernandez, J., Bota, D., Farbos, M., Bernardin, F., Ragetly, G., Medaille, C., 2014. Risk factors for urinary tract infection with multiple drug-resistant *Escherichia coli* in cats. J. Feline Med. Surg. 16, 75–81.
Hordijk, J., Schoormans, A., Kwakernaak, M., Duim, B., Broens, E., Dierikx, C., Mevius, D., Wagenaar, J.A., 2013. High prevalence of fecal carriage of extended spectrum beta-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs. Front.

Microbiol. 4, 242.
Jahanbakhsh, S., Smith, M.G., Kohan-Ghadr, H.R., Letellier, A., Abraham, S., Trott, D.J., Fairbrother, J.M., 2016. Dynamics of extended-spectrum cephalosporin resistance in pathogenic *Escherichia coli* isolated from diseased pigs in Quebec. Can. Int. J. Antimicrob. Agents 48, 194–202.
Johns, I.C., Adams, E.L., 2015. Trends in antimicrobial resistance in equine bacterial isolates: 1999–2012. Vet. Rec. 176, 334.
Johnson, J.R., Miller, S., Johnston, B., Clabots, C., DebRoy, C., 2009. Sharing of *Escherichia coli* sequence type ST131 and other multidrug-resistant and urovirulent *E. coli* strains among dogs and cats within a household. J. Clin. Microbiol. 47, 3721–3725.
Leite-Martins, L.R., Mahu, M.I., Costa, A.L., Mendes, A., Lopes, E., Mendonca, D.M., Niza-Ribeiro, J.J., de Matos, A.J., da Costa, P.M., 2014. Prevalence of antimicrobial resistance in enteric *Escherichia coli* from domestic pets and assessment of associated risk markers using a generalized linear mixed model. Prev. Vet. Med. 117, 28–39.
Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18, 268–281.
Platell, J.L., Cobbold, R.N., Johnson, J.R., Heisig, A., Heisig, P., Clabots, C., Kuskowski, M.A., Trott, D.J., 2011. Commonality among fluoroquinolone-resistant sequence type ST131 extraintestinal *Escherichia coli* isolates from humans and companion animals in Australia. Antimicrob. Agents Chemother. 55, 3782–3787.
Platell, J.L., Trott, D.J., Johnson, J.R., Heisig, P., Heisig, A., Clabots, C.R., Johnston, B., Cobbold, R.N., 2012. Prominence of an O75 clonal group (clonal complex 14) among non-ST131 fluoroquinolone-resistant *Escherichia coli* causing extraintestinal infections in humans and dogs in Australia. Antimicrob. Agents Chemother. 56, 3898–3904.
Saputra, S., Jordan, D., Worthing, K.A., Norris, J.M., Wong, H.S., Abraham, R., Trott, D.J., Abraham, S., 2017. Antimicrobial resistance in coagulase-positive staphylococci isolated from companion animals in Australia: a one year study. PLoS One 12, e0176379.
Sidjabat, H.E., Seah, K.Y., Coleman, L., Sartor, A., Derrington, P., Heney, C., Faoagali, J., Nimmo, G.R., Paterson, D.L., 2014. Expansive spread of IncI1 plasmids carrying blaCMY-2 amongst *Escherichia coli*. Int. J. Antimicrob. Agents 44, 203–208.
Silley, P., 2012. Susceptibility testing methods, resistance and breakpoints: what do these terms really mean? Rev. Sci. Tech. Off. Int. Epiz 31, 33–41.
Swedres-Svarm, 2015. In: Hellman, J., Aspevall, O., Bengtsson, B., Pringle, M. (Eds.), A Report on Swedish Antibiotic Utilisation and Resistance in Human Medicine (Swedres) and Swedish Veterinary Antibiotic Resistance Monitoring (Svarm). Public Health Agency of Sweden and National Veterinary Institute, Uppsala.
Thompson, M.F., Litster, A.L., Platell, J.L., Trott, D.J., 2011. Canine bacterial urinary tract infections: new developments in old pathogens. Vet. J. 190, 22–27.
Thungrat, K., Price, S.B., Carpenter, D.M., Boothe, D.M., 2015. Antimicrobial susceptibility patterns of clinical *Escherichia coli* isolates from dogs and cats in the United States: January 2008 through January 2013. Vet. Microbiol. 179, 287–295.
Turnidge, J., Kahlmeter, G., Kronvall, G., 2006. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clin. Microbiol. Infect. 12, 418–425.
Turnidge, J.D., Gottlieb, T., Mitchell, D.H., Coombs, G.W., Daley, D.A., Bell, J.M., 2014. Community-onset Gram-negative surveillance program annual report, 2012. Commun. Dis. Intell. 38, E54–E58.
WHO, 2014. Antimicrobial Resistance: Global Report on Surveillance. World Health Organization, France.
Wong, C., Epstein, S.E., Westropp, J.L., 2015. Antimicrobial susceptibility patterns in urinary tract infections in dogs (2010–2013). J. Vet. Intern. Med. 29, 1045–1052.

Chapter 4

Antimicrobial resistance in companion animal pathogens in Australia: a current perspective

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Contribution to the Paper	Collected all relevant data for review, analysis and interpretation and wrote manuscript, acted as corresponding author		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Antimicrobial resistance in companion animal pathogens in Australia: a current perspective

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Abstract

Globally, antimicrobial resistance (AMR) is considered to be a dire threat to the health and well-being of humans and animals. A key step in controlling the dissemination of antimicrobial-resistant bacteria is to monitor their occurrence in hosts. Historically, the role of companion animals in the ecology of AMR has received comparatively little attention and thus very few countries include companion animals in their AMR surveillance efforts. In this review we summarize recent AMR studies attempting to quantify the presence of AMR in pathogens isolated from companion animals in Australia and compare them to other countries. In Australia, a nationwide survey of pathogenic coagulase-positive staphylococci (CoPS, including *Staphylococcus pseudintermedius* and *Staphlylococcus aureus*) and *Escherichia coli* isolated from companion animals has been recently conducted. The findings provide evidence of emerging antimicrobial resistance issues and the potential for bi-directional transmission of antimicrobial-resistant bacteria between companion animals and humans. Such studies are also invaluable for informing treatment choices for common bacterial infections encountered in veterinary practice. Further, in order to maximise the gain from such work in the future, a harmonised and co-ordinated process for collection and laboratory-evaluation of the AMR status of pathogens from companion animals needs to be established.

Keywords: antimicrobial resistance, companion animals, *S. pseudintermedius, S. aureus, E. coli*

Background

Companion animals (dogs, cats and horses) have become an integral part of modern society. According to a recent survey by Animal Medicines Australia (AMA, 2016) almost two in five Australian households have dogs (38%) while nearly three in ten households have cats

(29%), with an average of 1.3 dogs and 1.4 cats per household. The proportion of households owning horses is much lower (less than 3%). The relationship between these animals and humans has drastically changed through the years, in particular dogs and cats. More than 65% households viewed dogs and cats as part of the family while less than 25% viewed them as a companion. The term "fur baby" is a colloquial expression now used to describe this phenomenon (AMA, 2016) indicating that humans are having more personal and closer physical contact with companion animals. Consequently, the possibility for bi-directional transmission of zoonotic pathogens has increased. This can occur by direct contact or indirectly through environmental contamination. Additionally, the demand for antibiotics for companion animals in Australia has increased by nearly 20%, although it comprises only 5% of market share of pet healthcare products (AMA, 2016). This high demand reflects incremental increasing antibiotic consumption that may increase selective pressures for AMR among companion animal bacterial pathogens. Over 80% of Australian veterinary graduates seek employment in the companion animal sector (Heath, 2002). Therefore, two issues need to be considered in companion animal veterinary medicine as it is currently practised in Australia:

1) Development of antimicrobial resistance among commensal and pathogenic bacteria isolated from companion animals.
2) The risk of zoonotic transmission of antimicrobial-resistant bacteria to in contact humans, in companion animal hospitals, the family home and community settings.

Antimicrobial use and antimicrobial resistance in companion animal pathogens

There is an increasing body of evidence supporting the direct association between antimicrobial use and emergence of antimicrobial resistance in companion animal pathogens. A study conducted in Canada showed increased resistance to cephalothin, enrofloxacin and gentamicin in staphylococci isolates from dogs related to increased use of those antibiotics during the period 1984-1998 (Prescott et al., 2002). A Swedish study also demonstrated increased lincosamide resistance in staphylococci isolates from canine pyoderma in parallel with increased used of clindamycin during the period 1990-1998. The higher level of co-resistance to other antibiotics such as tetracycline and streptomycin in recurrent cases may also have been influenced by the use of lincosamides (Holm et al., 2002). Another study in the USA in 1997 showed an increase in fluoroquinolone resistance amongst *E. coli* isolates from canine urinary tract infections (UTIs) following increased enrofloxacin use in the previous year (Cooke et al., 2002).

Zoonotic and nosocomial transmission in veterinary hospital settings

Zoonotic transfer of pathogens between humans and animals has also been reported to occur in veterinary hospitals. In most cases, colonisation of cats and dogs by zoonotic pathogens usually occurs without the presence of overt clinical signs (van Duijkeren et al., 2011a). More physical contact and interaction with animals has been shown to result in higher probably of colonisation by zoonotic pathogens such as coagulase-positive staphylococci (*S. pseudintermedius* and *S. aureus*) and extraintestinal pathogenic *E. coli*. In support of this, the prevalence of methicillin-resistant *S. aureus* (MRSA) colonisation among Australian veterinarians was higher among equine vets (11.9%) and small animals practitioners (4.9%), compared to veterinarians who have had less interaction with animals (industry and government veterinarians) with only 0.9% of a total of 771 participants (Jordan et al., 2011). A more recent Australian study also found 8% of 46 veterinary personnel carried MRSA, while none carried MRSP (Worthing *et al.,* 2018: under review). Molecular typing suggests that particular MRSA clones are found in specific animal groups and the veterinarians that treat these animals (Worthing et al., 2017). For instance, in a follow up to the MRSA nasal colonisation study among Australian veterinarians, ST22-IV MRSA were the most common MRSA clones identified in cats and dogs as well as veterinarians treating cats and dogs (Loeffler et al., 2005; Worthing et al., 2017) while ST8-IV MRSA was common amongst both horses and equine veterinarians (Groves et al., 2016; Worthing et al., 2017). These results were similar to those obtained in studies undertaken in the UK and Ireland (Moodley et al., 2006).

While MRSA carriage is frequently reported among veterinarians, MRSP carriage is a more recent observation. A Danish study demonstrated that MRSP colonisation occurred among 4% of veterinarians (5/128 participants) (Paul et al., 2011). Whilst MRSP transmission between animals and humans is quite rare, transmission between animal species (i.e. dogs and cats) has also been reported; and usually results from a single resistant clone circulating within a pet population (van Duijkeren et al., 2011a; van Duijkeren et al., 2011b; Zubeir et al., 2007). MRSP zoonotic infections have also been reported in dog owners who are immunosuppressed (Stegmann et al., 2010) and direct link between MRSP carriage in a dog and MRSP infection in its owner with skin and soft tissue infections has been recently found (Somayaji et al., 2016).

Evidence of transmission of Gram-negative pathogens between animals and humans, has also been found in both the family home (Johnson et al., 2009) and veterinary hospital settings (Sidjabat et al., 2006a). This is particularly the case for multidrug-resistant extraintestinal pathogenic *Escherichia coli* (ExPEC) expressing AmpC and CTX-M β-lactamases and/or

fluoroquinolone resistance (Guo et al., 2015; Sidjabat et al., 2006a). It is presumed that the faecal–oral route is the most likely mode of transmission (Sidjabat et al., 2006a). Unexpectedly, carbapenem-resistant Enterobacteriaceae (CRE) were also recently isolated from a clinically unwell cat and several in contact healthy cats in an animal shelter in Australia, raising the concern that shelters and veterinary hospitals could potentially play a role in the dissemination of MDR pathogens and their genes (Abraham et al., 2016). Genome sequencing of the isolates which belong to ST19 revealed that the plasmid pIMP4-SEM1 carrying blaIMP-4 showed greatest similarity to two blaIMP-8 carrying IncHI2 plasmids from *Enterobacter* spp. isolated from humans (Abraham et al., 2016).

AMR in clinical isolates of coagulase positive staphylococci from companion animals in Australia

In Australia, the issues associated with antimicrobial resistance among bacteria infecting companion animals were first raised by Love (1989) who highlighted the susceptibility profiles of staphylococci isolates submitted through the Veterinary Pathology Diagnostic Service at the University of Sydney from 1982-1988. A further survey was conducted in the following years until July 1993 (Barrs et al., 1996) Among a total of 485 isolates, cloxacillin/oxacillin resistance in *S. aureus* and *S. intermedius* (since reclassified as *S. pseudintermedius*) (Sasaki et al., 2007) was not detected. The authors highlighted the importance of monitoring susceptibility patterns in staphylococci isolates, as they may change over time due to evolving prescribing practices in the hospital. Since then, several one-off surveys and case reports describing clinical cases of antimicrobial resistance in major pathogens (coagulase-positive staphylococci and *E. coli*) in companion animals have been reported in Australia, as presented in Table 1.

A study from veterinary clinics in Adelaide was among the first reports describing MRSA in dogs in Australia (Malik et al., 2006) In total, the proportion of methicillin-resistant staphylococci was 4% of 252 samples, covering healthy and diseased dogs and cats. The MRSA strains, confirmed by detection of the *mecA* gene, were successfully isolated from dogs with clinical disease only (n=2), and showed resistance to all antimicrobials tested except for rifampicin and vancomycin. Demonstrating how antimicrobial-resistant strains can appear in unexpected ways, a clone of MRSA typically found among livestock-associated strains was recently reported in a cat in Australia (Worthing et al., 2016). The MRSA isolate harboured a *mecC* gene and was clonal type ST425, a type which had hitherto been restricted to cattle in Europe (Worthing et al., 2016) Despite the occasional occurrence of 'livestock-associated'

MRSA (Abraham et al., 2017) clones in companion animals, screening companion animal-derived collections of clinical strains for genetic host adaptation markers reveals that most are 'human-adapted' MRSA clones, suggesting that the rare cases of MRSA infection and/or carriage in companion animals usually originate from humans (Worthing et al., 2017).

MRSP in Australia was first reported more than a decade ago (Barton et al., 2003) however, this report which was documented by personal communication could not be verified by testing the isolate. Further cases of MRSP infection were reported in a study conducted in 2011 and 2012 by collecting clinical samples from a veterinary dermatology referral clinic in Australia (Siak et al., 2014). More recently, the whole genome profiling of MRSP isolates has shed more light on its epidemiology in this country (Siak et al., 2014; Worthing et al., 2018). The first comprehensive study describing MRSP from Western Australia examined 171 samples from dogs with *S. pseudintermedius* pyoderma and found twelve isolates (7%) were MRSP (Siak et al., 2014). The MRSP isolates were characterised using *dru* typing, which revealed that most isolates were *dru* type dt11cb. This same *dru* type was found in isolates from Western Australia in a subsequent study (Worthing et al., 2018). Molecular analysis revealed that Australian MRSP isolates from diseased dogs and cats are phylogenetically diverse, demonstrate geographic clustering and exhibit more co-resistance compared to MRSA (Saputra et al., 2017b; Worthing et al., 2018). Genotyping and phylogenetic analysis also revealed that the globally dominant MRSP clone, ST71, is present in Australia and closely related to European strains of ST71, indicating that ST71 MRSP in Australia may have originated in Europe (Worthing et al., 2018).

The first nationwide survey of AMR in companion animal pathogens in Australia has recently provided deeper insights into the frequency of AMR among CoPS and *E. coli*. To some extent, the earlier reported frequencies of methicillin-resistant staphylococci, 4% in a study of *S. aureus* isolates completed in 2006 (Malik et al., 2006) and 7% in *S. pseudintermedius* isolates from 2011-2012 (Siak et al., 2014), were significantly lower compared to the national survey conducted in 2013-2014 which identified MRSP and MRSA frequencies of 12.8% and 12.8%, respectively (Saputra et al., 2017b) (Table 1). This may indicate an increase in the occurrence of resistance in staphylococci associated with companion animals. However, these data should be interpreted with caution because of different sampling methods and antimicrobial susceptibility testing, making direct comparison between studies problematic.

Table 1. Summary of antimicrobial susceptibility attributes of clinical CoPS and *E. coli* isolates in companion animals in Australia

Species	Sampling period	State	AST method	No of AM tested	Findings	Ref.
Staphylococci isolates from dogs (n=190)	1982-1988	NSW	Disc diffusion	11	- resistance to cloxacillin, amoxiclav, neomycin and kanamycin were not detected in S. intermedius[1] and S. aureus	(Love, 1989)
Staphylococci isolates from dogs (n=268)	October 1987-July 1993	NSW	Disc diffusion	15	- resistance to cloxacillin/oxacillin, amoxiclav, norfloxacin and cephalothin were not detected in S. intermedius and S. aureus	(Barrs et al., 1996)
MDR *E. coli* from dogs (n=10)	Clinical cases-18 months period	QLD	Disc diffusion	8	- no imipenem and amikacin resistance - the first report of combined 3GC and FQN resistance in dogs in Australia	(Warren et al., 2001)
Staphylococci from healthy dogs (n=55) and cats (n=51); diseased dogs (n=141; cats (n=5)	unspecified	SA	broth microdilution	13	- MRSA isolates obtained from two infected dogs - no vancomycin and rifampin resistance - identical *mecA* genes to that found in human strains	(Malik et al., 2006)
Extraintestinal pathogenic *E. coli* (ExPEC) from dogs (n=11)	unspecified	QLD	Broth microdilution	20	- first report on the detection of plasmid-mediated blaCMY-7 in animal isolates in Australia	(Sidjabat et al., 2006b)
***E. coli* (dogs n=59 and cats n=17) and *S. intermedius* (dogs n=21)**	October 2004-May 2007	NSW	Agar dilution, E-test	6	- no FQN resistance in all cat isolates and *S. intermedius* - 12 out of 56 *E. coli* isolates are FQN resistant strains	(Gottlieb et al., 2008)
MDR *E. coli* n=76 isolated from 47 dogs, 5 cats, 4 horses, one koala	October 1999-December 2007	QLD	Disc diffusion	5	- all isolates were resistant to FQN except two isolates - high resistance to cefoxitin (84%) and 3GC (50%) in (group A and B1 and chloramphenicol (53%) in group A	(Gibson et al., 2010)
FQN resistant *E. coli* n=125 (canine n=120 and feline n=5)	October 2007-October 2009	NSW, QLD, VIC	Disc diffusion	7	- a strong association of FQN resistance and MDR phenotypes - exhibited co-resistance to tetracycline (75%), cefalotin (69%), SXT (69%), amoxicillin/clavulanic acid (53%)	(Platell et al., 2010)
***S. pseudintermedius* isolated from 19 dogs with pyoderma**	February 2011-November 2012	WA	Disc diffusion	15	- twelve isolates were identified as MRSP by *mecA* PCR - exhibited co-resistance to erythromycin (91.6 %), gentamicin (83.3 %), ciprofloxacin (83.3 %), chloramphenicol (75 %), clindamycin (66 %), oxytetracycline (66%) and tetracycline (50 %)	(Siak et al., 2014)
Clinical *E. coli* (n=148) from dogs and cats	unspecified	NSW	Disc diffusion and e-test	2	-resistance to titarcillin/clavulanic acid was 6% total *E. coli* as determined by disc diffusion	(Bennett et al., 2013)
Clinical *E. coli* (n=29) from dogs and cats with UTI	Clinical cases-6 months period	NSW	Disc diffusion and e-test	2	-resistance to doxycycline and tetracycline was 13.8%, as determined by dick diffusion	(Wilson et al., 2006)
Clinical *E. coli* n=883 (dogs n=514, cats n=341 and horses n=28)	January 2013-January 2014	NSW, QLD, VIC, SA, WA	broth microdilution	15	- no imipenem resistance, amikacin resistance was observed at a low level - resistance to FQN was higher in dog isolates (9.1%–9.3%) compared to cat isolates (3.2%–5%); frequency of MDR was 18.1%, 11.7% and 42.9% in dog, cat and horse isolates, respectively.	(Saputra et al., 2017a)
Clinical staphylococci isolates from dogs, cats and horses (n=877)	January 2013-January 2014	NSW, QLD, VIC, SA, WA	broth microdilution	16	- frequency of MRSP and MRSA in companion animals was 11.8% and 12.8%, respectivelywhich were highly associated with resistance to FQN	(Saputra et al., 2017b)

[1]*Staphylococcus pseudintermedius* is the most common cause of bacterial skin infections in dogs, previously identified as *S. intermedius.* *Note: AST, antimicrobial susceptibility testing; AM, antimicrobial; 3GC, third generation cephalosporins; FQN, fluoroquinolones.

Table 2. Recent trends of antimicrobial resistance in major pathogens companion animals in other countries

Species	Sampling period	Country	AST method	No of AM tested	Findings	Ref.
Clincal E. coli from horses (n=324), dogs (n=1162), cats (n=537); S. aureus from horse (n=399); S. pseudintermedius from dogs (n=376)	2016 (since 2000)	Sweden	Broth microdilution		-in horse isolates MDR E. coli 10% (31/324), MRSA was not detected -in dog isolates MDR E. coli 9% (99/1162), MRSP 0.5% (2/376) -in cat isolates MDR E. coli 3% (16/537) -proportion of resistance relatively stable over the years	(SWEDRES-SVARM, 2017)
Clinical *S. pseudintermedius* from dogs (n=201) in 2013	2004, 2008, 2013	Norway	Disc diffusion	12	-increased proportion of multidrug resistant (>3 class antimicrobials: 10% in 2004 but nearly 25% in 2013)	(NORM/NORM-VET, 2014)
Clinical E. coli accounted for 59.45 % (n =13231/ 22256) of all UTI isolates	2008-2013	14 European countries	Disc diffusion and broth microdilution	16	- the highest 3GC resistance frequencies were found in Portugal (31.25 %), Italy (24.64 %) and Spain (21.15 %) while Sweden, Denmark, Netherlands <5% - Sweden, Denmark, Belgium and the Netherlands had <10 % FQN resistant E. coli; Portugal and Spain nearly 30%	(Marques et al., 2016)
***E. coli* (n=3135) from canine urinary samples**	January 2005 and December 2012	New Zealand	Disc diffusion	6	- increased resistance to amoxiclav (2.3% vs 6.5%), cephalothin (6.7% vs 17.9%) and enrofloxacin (0.3% vs 1.8%) between 2005 and 2012, respectively	(McMeekin et al., 2017)
***E. coli* (n=1746) and *S. aureus* (n=170) from dogs, cats and horses; *S. pseudintermedius* (n=1952*)* from dogs and cats**	June 2011-December 2015	Finland	broth microdilution	6	- proportion of MRSA ranged from 0-9.8%, the highest in 2014 - proportion of MRSP was 18% in 2011 and 14% in 2015 - non-susceptibility to enrofloxacin in E. coli was increasing up to 14% in 2015	(Nykäsenoja et al., 2017)
Staphylococci isolates from dogs (n=537), cats (n=80), horses (n=10) and other (n=5)	1999-2014	Portugal	Disc diffusion	38	- resistance to the majority of antimicrobials and the number of mecA-positive isolates increased significantly over time	(Couto et al., 2016)
***S. intermedius group* (SIG) in NWL lab: (n=13,313) from dogs (n=12,785) and cats (528); RCV lab: n=1242 from dogs (n=11870 and cats (n=55)**	NWL lab: March 2006-August 2012; RVC lab: January 2003-December 2012	UK	Disc diffusion	13	- a significant increasing trend of resistance to ampicillin, amoxicillin, cefovecin and enrofloxacin - resistance to cephalexin, clindamycin and SXT increased over time in referral hospital isolates	(Beever et al., 2015)
***S. aureus* (n=1393) from horses**	January 2007-December 2013	France	Disc diffusion	17	- prevalence of MRSA were ranged from 0% in 2009 to 12.9% in 2012	(Guerin et al., 2017)
Clinical *E. coli* from dogs (n=401) and cats (n=329)	January 2007-December 2013	Poland	Disc diffusion	13	- increasing MDR E. coli and resistance to amoxiclav, amoxicillin, the extended spectrum cephalosporins, SXT and tetracycline	(Rzewuska et al., 2015)
Clinical *S. pseudintermedius* from dogs (n=10281)	2004-2013	Netherlands	unspecified	14	-proportion of MRSP increased from 0.95% in 2014 to 7.38% in 2013	(Duim et al., 2016)

*Note: AST, antimicrobial susceptibility testing; AM, antimicrobial; 3GC, third generation cephalosporins; FQN, fluoroquinolones, SXT, trimethoprim-sulfamethoxazole; NWR, Nationwide Laboratory Service; RCV, Royal Veterinary College

AMR in clinical isolates of extraintestinal pathogenic *E. coli* from companion animals in Australia

AMR in Gram-negative bacteria isolated from companion animals in Australia has mainly focused on the emergence of extended-spectrum β-lactamase (ESBL) and fluoroquinolone resistance in extraintestinal pathogenic *E. coli* and *Enterobacter* spp. isolated from dogs (Sidjabat et al., 2007; Warren et al., 2001). MDR *E. coli* isolates were first reported as a cause of nosocomial infections in a veterinary teaching hospital in 2001. Whilst the isolates were resistant to all classes of antimicrobial agent registered for use in dogs, resistance to critically important human antibiotics such as imipenem and amikacin was not detected (Warren et al., 2001). Subsequently, the study of co-resistance to third generation cephalosporins and fluoroquinolones has been a major priority. Resistance to third generation cephalosporins in extraintestinal *E. coli* (ExPEC) is mainly encoded by plasmid-mediated AmpC (CMY-7) or blaCTX-M-15 beta-lactamases (Platell et al., 2010; Sidjabat et al., 2006b). Further investigation revealed that the clonal groups carrying blaCMY-7 isolated from dogs appear less virulent than a reference human ExPEC strain (Sidjabat et al., 2009). Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) analysis of *E. coli* isolates from human and companion animals indicated high similarity profile (>94%) and confirmed that they belong to the ST131 group (Platell et al., 2011) which also common in North America (Johnson et al., 2009). The clonal group ST131 was previously reported to be more prevalent in the human isolates (35%, n=585) rather than in the companion animal isolates (7.2%, n=125) (Platell et al., 2010).

The study focusing on the monitoring of emerging AMR in Gram-negative isolates was limited. In most cases, the studies were designed to screen and characterise the resistance against particular antibiotics, as well as snapshot and clinical cases studies, without considering continuous monitoring to development of AMR over time. For instance, amikacin resistance was not observed in dog isolates in the previous study (Warren et al., 2001) while in the 2013-2014 study, amikacin resistance was observed at low levels (<2%) in dog and cat isolates (Saputra et al., 2017a). However, again, comparison to other studies in terms of frequency of AMR is difficult due to no standardisation in the methodology so it cannot be deduced whether AMR in *E. coli* is also increasing.

Comparison of data describing AMR in pathogenic isolates from companion animals compared to humans

A great deal of attention has been given to the comparison of AMR detection rates from different species of companion animals and humans. It is important to note that in terms of monitoring of AMR, there are some key differences in the manner that medical services are implemented in the human and companion animal population. In most cases, a smaller proportion of clinical isolates in companion animals are likely to be captured for inclusion in surveys because the laboratory investigations (isolation, culture and antimicrobial susceptibility testing) from diseased animals are performed on a fee basis at the discretion of a paying client, while in human medicine, the clinical services are provided or subsidised by the government-funded health services (Shaban et al., 2014). Additionally, the isolates submitted to veterinary diagnostic laboratories are often derived from recurrent clinical cases which may provide biased information (Shaban et al., 2014). Therefore, direct comparison of AMR in veterinary and human medicine may not be appropriate, particularly with respect to measuring the frequency of particular genotypes and phenotypes.

It is important to note that comparison of companion animal and human AMR surveillance data may not be directly comparable, as there are considerable differences in sampling in methodology (The Australian Group on Antimicrobial Resistance alternated between human hospital and community-acquired isolates between 2008 and 2012 and then in 2013 switched to only consider isolates from cases of sepsis). Additionally, similar time frames or sample time period need to be considered to minimise biased interpretation. Comparison with data from the Community-onset Gram-negative Surveillance Program by the Australian Group on Antimicrobial Resistance (AGAR) 2012 found that the proportion of non-susceptibility for third generation cephalosporins (3GC, ceftriaxone) among *E. coli* isolated from the urine in dogs (8.9%) and cats (8.2%) was higher compared to human isolates (4.2%). Also, the non-susceptibility rate of fluorquinolone (ciprofloxacin) in *E. coli* isolated from dogs (8.2%) was higher than human isolates (ciprofloxacin, 6.9%), although a much lower frequency of resistance was observed among cat isolates (2.8%) (Saputra et al., 2017a; Turnidge et al., 2014). Comparing staphylococci isolates from human skin and soft tissue infection specimens from the Community-onset *Staphylococcus aureus* Surveillance Programme 2012, found the proportion of MRSA among human isolates was 17.9%, slightly higher than the proportion of MRSA among companion animal isolates (12.8%) (Coombs et al., 2014).

Comparison of AMR in companion animal pathogens in Australia to other countries

It is often useful to compare countries on the basis of frequency of specific pathogens to generate insight on the best methods of controlling disease. However, the same technical difficulties occur with comparison between countries as occurs in determining trends in AMR emergence within a country. For this reason, strong inferences should not be made about small differences in frequency of specific phenotypes or genotypes to allow for probable differences in the way isolates were procured and tested. With this limitation in mind, the current study in *E. coli* from urine samples in Australia (Saputra et al., 2017a) can be compared to studies with similar objectives which were all interpreted according to clinical breakpoints as published by The Clinical & Laboratory Standards Institute (CLSI) (Figure 1). Although a recent methodology for antimicrobial susceptibility testing was different among countries (Table 2), the current study (Badger et al., 2018) suggests that disc diffusion is as accurate as minimum inhibitory concentration (MIC) at predicting resistance of clinical *E. coli* from animals and thus studies using the two methods are comparable. Compared to other countries, Australia falls in the range of countries with a relatively low prevalence of AMR in clinical *E. coli* isolated from urinary tract infections (UTI) in all animals, in particular resistance to third generation cephalosporins (3GC) and fluoroquinolones (FQN) in cat isolates. The proportion of 3GC and FQN-resistant *E. coli* in both dog and cat isolates was below 10%.

On the other hand, the proportion of MRSP isolated from skin and soft tissue infections (SSTI) in dogs in Australia was at moderately high level (13%, n=513) (Saputra et al., 2017b), in similar proportion with Finland (11%, n=500) (Nykäsenoja et al., 2017) and Germany (11.6%, n=2130)(Lehner et al., 2014) (Figure 2). Interestingly, a significantly lower proportion of MRSP was observed in UTI samples (5.3%, n=123), compared to SSTI in the Australian study (Saputra et al., 2017b) Sweden showed consistently very low and low levels of MRSP, with 0.9% and 1.15% frequencies reported in SSTI (n=556) and UTI samples (n=174), respectively (Marques et al., 2016; SWEDRES-SVARM, 2013). In the Netherland study, a slightly higher proportion of MRSP was observed in UTI (10.92%, n=174) (Marques et al., 2016) compared to SSTI (7.38%, n=1077) (Duim et al., 2016) The differing frequencies of MRSP amongst infection sites indicates that risk factors for MRSP colonisation and infection cannot be generalised to all countries.

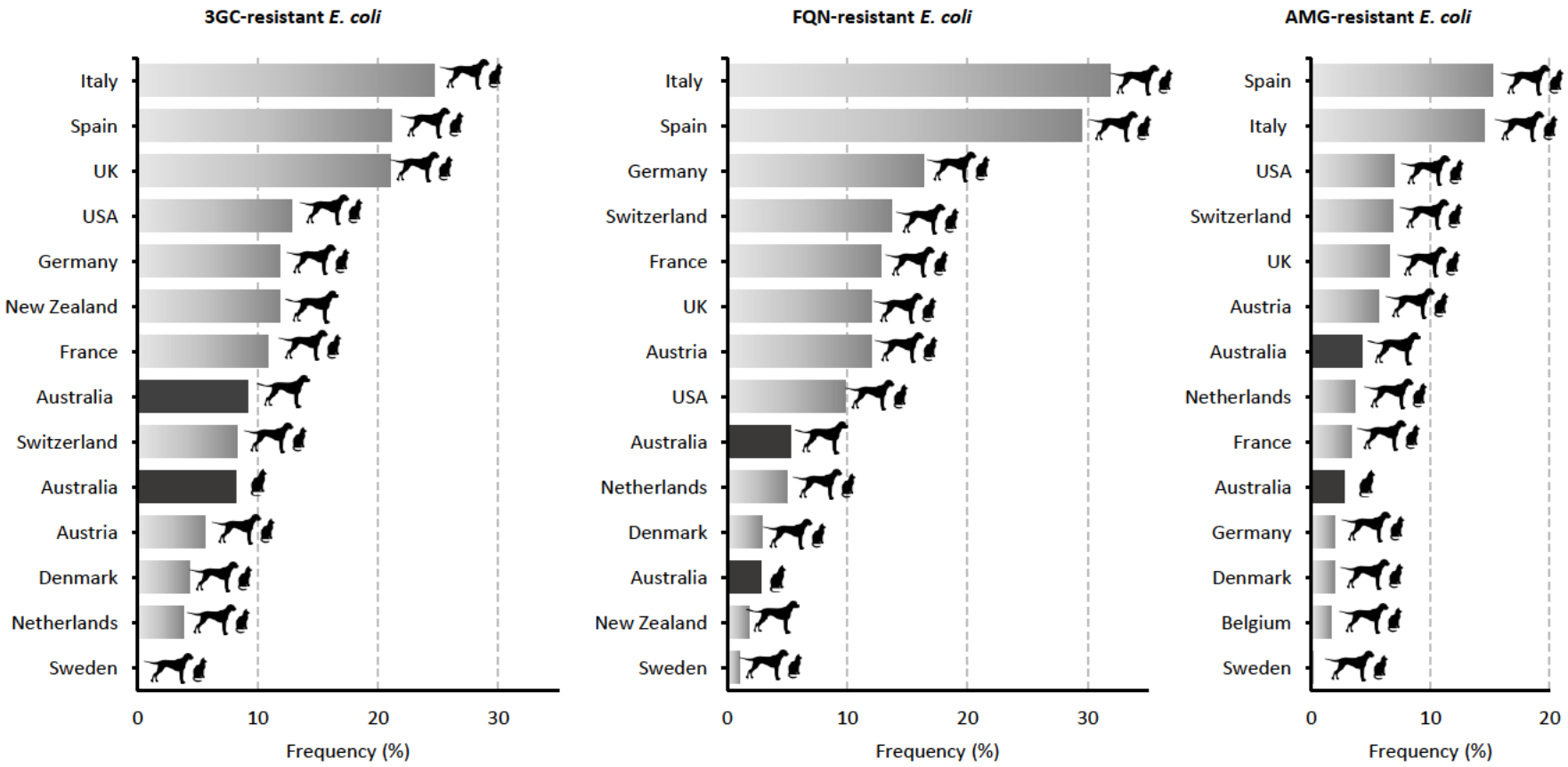

Figure 1. Frequency of resistance to third generation cephalosporins (3GC), fluoroquinolones (FQN) and aminoglycosides (AMG) in *E. coli* isolated from urine samples in dogs and cats. Methodology and interpretation based on CLSI documents. Sampling periods and number of isolates tested were varied: European countries 2012-2013 (Austria n=142, Denmark n=206-208, France n=933-954, Germany n=152-153, Italy n=69, the Netherlands n=81-1461, Spain n=46-61, Sweden n=2082-2091, Switzerland n=132-133, the UK n=143) (Marques et al., 2016); New Zealand 2012 n=1082 (McMeekin et al., 2017); USA 2008-2013 n=3172 (Thungrat et al., 2015); and Australia 2013-2014 dogs n=392 cat n=320 (Saputra et al., 2017a).

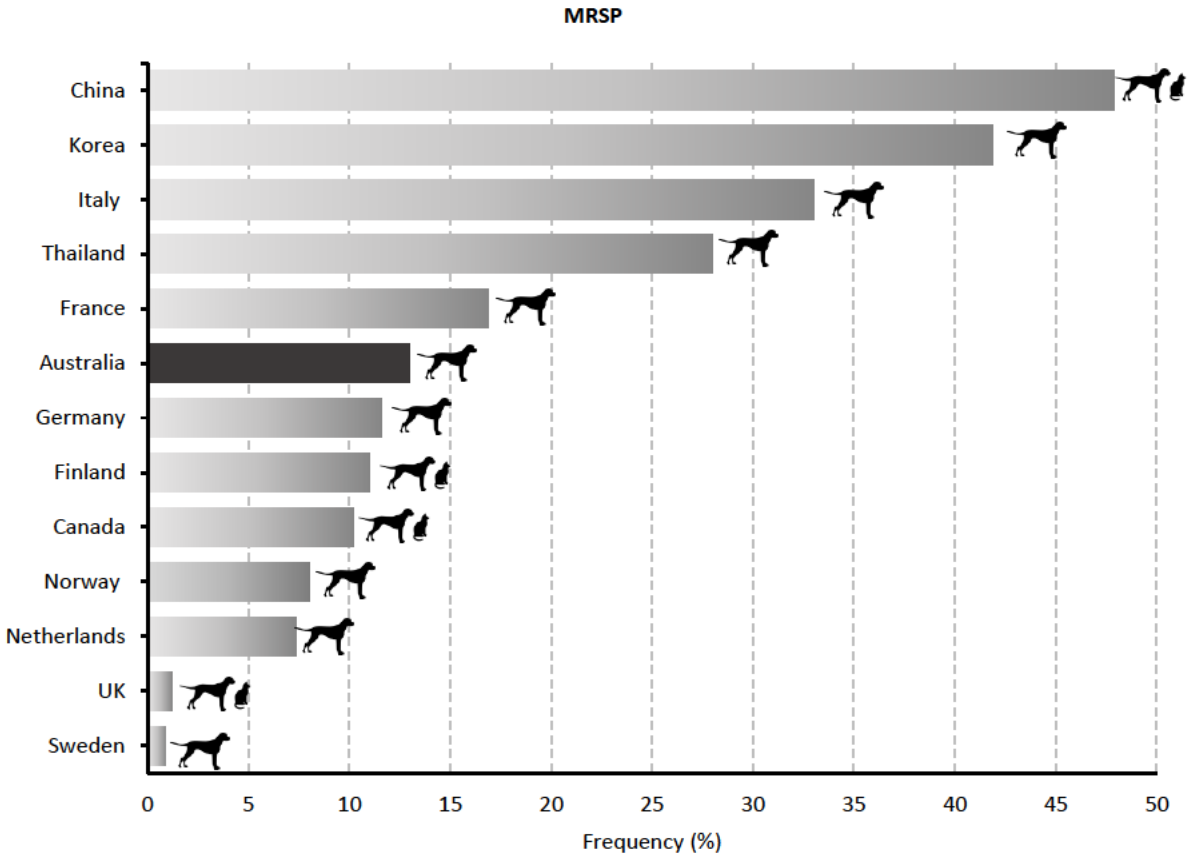

Figure 2. Frequency of MRSP isolated from skin and soft tissue infections in companion animals. MRSP isolates confirmed based on susceptibility testing using oxacillin/cefoxitin as surrogate and *nuc*/*mecA* PCR. Frequency of MRSP calculated based on total *S. pseudintermedius* isolates in each study. Sampling periods and number of isolates tested were varied: Sweden 2013 n=566 (SWEDRES-SVARM, 2013), the Netherlands 2013 n=1163 (Duim et al., 2016); Norway 2013 n=201 (NORM/NORM-VET, 2014); Finland 2013 n=500 (Nykäsenoja et al., 2017); the UK 2012 (n=1273); (Beever et al., 2015) Canada 2014 n=149 (Joffe et al., 2015); Germany 2010-2011 n=2130 (Lehner et al., 2014); Australia 2013-2014 n=513 (Saputra et al., 2017b); France 2010 n=243 (Haenni et al., 2014); Thailand 2006-2013 n=100 (Kadlec et al., 2016); Italy 2011-2014 n=63 (Ventrella et al., 2017); Korea 2011-2015 n=143 (Kang et al., 2017); China 2007-2009 n=144 (Feng et al., 2012).

Trends and surveillance of AMR in companion animal pathogens

One of the notable first studies to report the rising trends of AMR in dogs and cats was the UK study by Normand and colleagues in 2000 (Normand et al., 2000). During 1989-1997, increasing levels of resistance to amoxicillin, amoxicillin-clavulanate and streptomycin were observed in *E coli* isolates while a rise in resistance to erythromycin and cephalexin was observed in staphylococci (Normand et al., 2000). Since then, other studies have also reported a similar trend of AMR in companion animals in Canada (Prescott et al., 2002), Sweden (Holm et al., 2002) and the USA (Cooke et al., 2002). While the evidence of increasing trend of AMR

in companion animals is clear, national surveillance on AMR in animals generally do not include data from companion animals. The only exceptions are the surveillance programmes in Sweden (SVARM), Finland (FINRES-Vet), and France (RESAPATH), which monitor on a yearly basis, while Norway (NORM-VET) monitors AMR in companion animals every 4-5 years. A pan-European study (ComPath) which was part of the CEESA (The European Animal Health Study Centre) monitoring programme also reported susceptibility patterns of representative bacterial pathogens isolated from diseased dogs and cats, in particular samples from urine (Moyaert et al., 2017) dermatological disease (Ludwig et al., 2016) and respiratory tract samples (Morrissey et al., 2016). This program included nine countries, Czech Republic, France, Germany, Hungary, Italy, The Netherlands, Poland, Spain and the United Kingdom, representing a major proportion of the companion animals in the EU.

A recent European study demonstrated that AMR trends vary significantly and are specific to geographic regions (Marques et al., 2016). For instance, the level of fluoroquinolone-resistant *E. coli* isolated in Belgium, Denmark, France and the Netherlands decreased significantly during 2008-2013, but the decrease was not observed in Italy, Spain and Portugal. This may reflect national patterns of antimicrobial use and varying interventions for prevention and control of AMR (Guardabassi, 2017). In a Swedish study (SWEDRES-SVARM 2014), a temporal trend was observed when the frequency of MRSP isolates from dogs reached a peak at 5% (n=381) in 2009, but then dropped in the following years (Figure 3). Since then, infection control programmes were implemented in veterinary hospitals including an antimicrobial usage policy for the treatment in dogs with dermatological disorders. Molecular analysis revealed that the drop was primarily due to a decreasing occurrence of the globally dominant MRSP clone, ST71-J-t02-II-III. Interestingly, an outbreak of MRSP in 2009 was also observed in Finland (Nykäsenoja et al., 2017) and Netherlands (Duim et al., 2016) which also slowly decreased.

In general, most European countries demonstrate an increasing trend of AMR in companion animal pathogens with few exceptions. According to the current SWEDRES-SVARM report, resistance to most antimicrobials in dog, cat and horse isolates fluctuates slightly over the years, but remains at approximately the same level overall (SWEDRES-SVARM, 2017). This may indicate that monitoring programmes are helpful in stabilising AMR levels. The frequency of MRSP in the UK is relatively stable but resistance to enrofloxacin, clindamycin and trimethoprim/sulfamethoxazole increased over time (Beever et al., 2015). Other studies also demonstrate increasing trend of AMR as demonstrated in Norway, Finland, France, Portugal and Poland (Table 2).

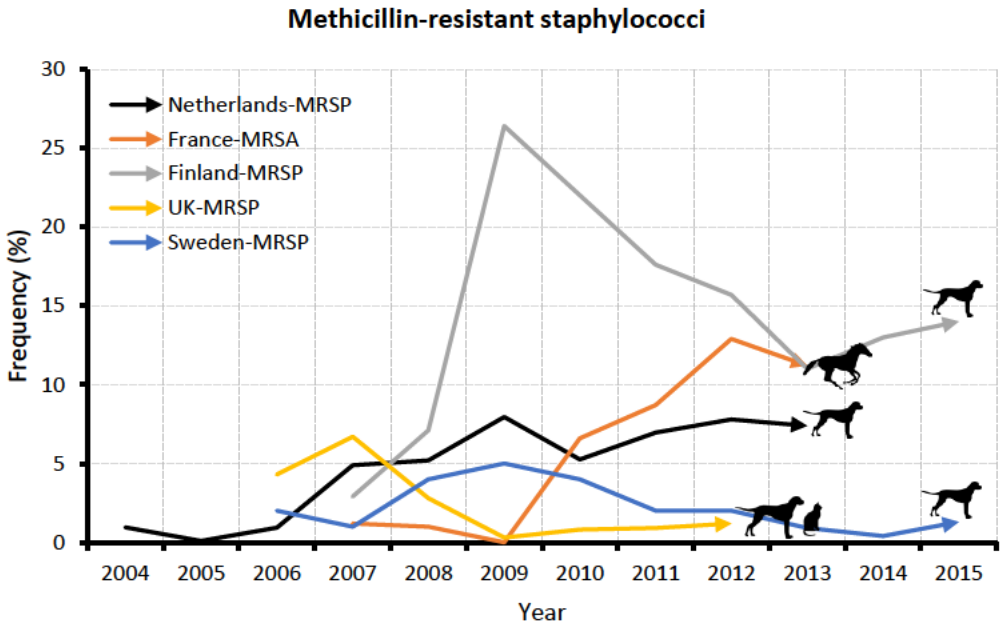

Figure 3. Trends of MRSP isolated from dogs in Sweden n=89-566 (SWEDRES-SVARM report) (SWEDRES-SVARM, 2017); Finland n=210-500 (FINRES-Vet report) (Nykäsenoja et al., 2017); the Netherlands n=969-1163 (Duim et al., 2016) and the UK n=670-2497 (Beever et al., 2015). Trend of MRSA is obtained from France study (n=163-253) isolated from horses (Guerin et al., 2017) The frequency was calculated per total of *S. pseudintermedius* (Sweden, Finland, and the Netherlands) and *S. aureus* isolates (France). The UK study calculated the frequency per total *Staphylococcus intermedius* group (SIG).

Conclusion and future work

Until recently, there was no continuous monitoring programs on AMR in animals in Australia, in particular in companion animals. Although the emergence of antimicrobial resistance associated with companion animals in Australia has been studied more in recent years, it is difficult to define current trends of AMR in companion animal pathogens. The first nation-wide survey of AMR in companion animals which was conducted in 2013-2014 provides an important basis for future studies. Genotyping of isolates provides valuable insights into the epidemiological origin of isolates, and future works should strive to include molecular typing to allow comparison with existing reports. Continuous surveillance of AMR is vital in determining the size of the problem, to see whether resistance is increasing or not, to detect any previously unknown types of resistance and to determine whether any particular type of resistance is spreading or is associated with an outbreak (Huntera and Reeves, 2002; Shaban et al., 2014). Therefore, specific objectives should be defined to control emerging AMR as well as adequate action plans for the next surveillance study. Unlike monitoring programmes in

human and food production animals, there is no harmonisation of antimicrobial resistance surveillance in companion animal pathogens published by an international organisation (i.e. WHO, FAO, OIE). Such harmonisation would include sampling methods, animal and bacterial species, antibiotic tested, and methods used for susceptibility testing, interpretive criteria and reporting. Harmonisation and standardisation are essential, enabling meaningful comparison of AMR data over time and among countries, which is useful for future strategies and recommendations to combat AMR.

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Competing interest

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References

Abraham, S., Jagoe, S., Pang, S., Coombs, G.W., O'Dea, M., Kelly, J., Khazandi, M., Petrovski, K.R., Trott, D.J., 2017. Reverse zoonotic transmission of community-associated MRSA ST1-IV to a dairy cow. Int. J. Antimicrob. Agents 50, 125-126.

Abraham, S., O'Dea, M., Trott, D.J., Abraham, R.J., Hughes, D., Pang, S., McKew, G., Cheong, E.Y., Merlino, J., Saputra, S., Malik, R., Gottlieb, T., 2016. Isolation and plasmid characterization of carbapenemase (IMP-4) producing *Salmonella enterica* Typhimurium from cats. Sci. Rep. 6, 35527.

AMA 2016. Pet Ownership in Australia 2016 (Animal Medicines Australia).

Badger, S., Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. Relative performance of antimicrobial susceptibility assays on clinical *Escherichia coli* isolates from animals. Vet. Microbiol. 214, 56-64.

Barrs, V.R., Malik, R., Love, D.N., 1996. Antimicrobial susceptibility of staphylococci isolated from various disease condition in dogs: a further study. Aus. Vet. Prac. 25, 37-42.

Barton, M.D., Pratt, R., Hart, W.S., 2003. Antibiotic resistance in animals. Commun. Dis. Intell. 27, S121-S126.

Beever, L., Bond, R., Graham, P.A., Jackson, B., Lloyd, D.H., Loeffler, A., 2015. Increasing antimicrobial resistance in clinical isolates of *Staphylococcus intermedius* group bacteria and emergence of MRSP in the UK. Vet. Rec. 176, 172.

Bennett, A.B., Martin, P.A., Gottlieb, S.A., Govendir, M., 2013. In vitro susceptibilities of feline and canine *Escherichia coli* and *Pseudomonas* spp. isolates to ticarcillin and ticarcillin-clavulanic acid. Aust. Vet. J. 91, 171-178.

Cooke, C.L., Singer, R.S., Jang, S.S., Hirsh, D.C., 2002. Enrofloxacin resistance in *Escherichia coli* isolated from dogs with urinary tract infections. J. Am. Vet .Med. Assoc. 220, 190-192.

Coombs, G.W., Daley, D.A., Pearson, J.C., Nimmo, G.R., Collignon, P.J., McLaws, M.-L., Robinson, J.O., Turnidge, J.D., 2014. Community-onset *Staphylococcus aureus*: surveillance programme annual report 2012. Commun. Dis. Intell. 38, E59-E69.

Couto, N., Monchique, C., Belas, A., Marques, C., Gama, L.T., Pomba, C., 2016. Trends and molecular mechanisms of antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16 year period. J. Antimicrob. Chemother. 71, 1479-1487.

Duim, B., Verstappen, K.M., Broens, E.M., Laarhoven, L.M., van Duijkeren, E., Hordijk, J., de Heus, P., Spaninks, M., Timmerman, A.J., Wagenaar, J.A., 2016. Changes in the

population of methicillin-resistant *Staphylococcus pseudintermedius* and dissemination of antimicrobial-resistant phenotypes in the Netherlands. J. Clin. Microbiol. 54, 283-288.

Feng, Y., Tian, W., Lin, D., Luo, Q., Zhou, Y., Yang, T., Deng, Y., Liu, Y.H., Liu, J.H., 2012. Prevalence and characterization of methicillin-resistant *Staphylococcus pseudintermedius* in pets from South China. Vet. Microbiol. 160, 517-524.

Gibson, J.S., Cobbold, R.N., Trott, D.J., 2010. Characterization of multidrug-resistant *Escherichia col*i isolated from extraintestinal clinical infections in animals. J. Med. Microbiol. 59, 592-598.

Gottlieb, S., Wigney, D.I., Martin, P.A., Norris, J.M., Malik, R., Govendir, M., 2008. Susceptibility of canine and feline *Escherichia coli* and canine *Staphylococcus intermedius* isolates to fluoroquinolones. Aus. Vet. J. 86, 147-152.

Groves, M.D., Crouch, B., Coombs, G.W., Jordan, D., Pang, S., Barton, M.D., Giffard, P., Abraham, S., Trott, D.J., 2016. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* isolated from Australian veterinarians. Plos One 11, e0146034.

Guardabassi, L., 2017. Antimicrobial resistance: a global threat with remarkable geographical differences. N. Z. Vet. J. 65, 57-59.

Guerin, F., Fines-Guyon, M., Meignen, P., Delente, G., Fondrinier, C., Bourdon, N., Cattoir, V., Leon, A., 2017. Nationwide molecular epidemiology of methicillin-resistant *Staphylococcus aureus* responsible for horse infections in France. BMC Microbiol. 17, 104.

Guo, S., Wakeham, D., Brouwers, H.J., Cobbold, R.N., Abraham, S., Mollinger, J.L., Johnson, J.R., Chapman, T.A., Gordon, D.M., Barrs, V.R., Trott, D.J., 2015. Human-associated fluoroquinolone-resistant *Escherichia coli* clonal lineages, including ST354, isolated from canine feces and extraintestinal infections in Australia. Microbes Infect. 17, 266-274.

Haenni, M., de Moraes, N.A., Chatre, P., Medaille, C., Moodley, A., Madec, J.Y., 2014. Characterisation of clinical canine meticillin-resistant and meticillin-susceptible *Staphylococcus pseudintermedius* in France. J. Glob. Antimicrob. Resist. 2, 119-123.

Heath, T., 2002. Longitudinal study of veterinarians from entry to the veterinary course to 10 years after graduation: attitudes to work, career and profession. Aust. Vet. J. 80, 474-478.

Holm, B.R., Petersson, U., Morner, A., Bergstrom, K., Franklin, A., Greko, C., 2002. Antimicrobial resistance in staphylococci from canine pyoderma: a prospective study of first-time and recurrent cases in Sweden. Vet. Rec. 151, 600-605.

Huntera, P.A., Reeves, D.S., 2002. The current status of surveillance of resistance to antimicrobial agents: report on a meeting. J. Antimicrob. Chemother. 49, 17-23.

Joffe, D., Goulding, F., Langelier, K., Magyar, G., McCurdy, L., Milstein, M., Nielsen, K., Villemaire, S., 2015. Prevalence of methicillin-resistant staphylococci in canine pyoderma cases in primary care veterinary practices in Canada: A preliminary study. Can. Vet. J. 56, 1084–1086.

Johnson, J.R., Miller, S., Johnston, B., Clabots, C., Debroy, C., 2009. Sharing of *Escherichia coli* sequence type ST131 and other multidrug-resistant and urovirulent *E. coli* strains among dogs and cats within a household. J. Clin. Microbiol. 47, 3721-3725.

Jordan, D., Simon, J., Fury, S., Moss, S., Giffard, P., Maiwald, M., Southwell, P., Barton, M.D., Axon, J.E., Morris, S.G., Trott, D.J., 2011. Carriage of methicillin-resistant *Staphylococcus aureus* by veterinarians in Australia. Aust. Vet. J. 89, 152-159.

Kadlec, K., Weiss, S., Wendlandt, S., Schwarz, S., Tonpitak, W., 2016. Characterization of canine and feline methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) from Thailand. Vet. Microbiol. 194, 93-97.

Kang, J.H., Chung, T.H., Hwang, C.Y., 2017. Clonal distribution of methicillin-resistant *Staphylococcus pseudintermedius* isolates from skin infection of dogs in Korea. Vet. Microbiol. 210, 32-37.

Lehner, G., Linek, M., Bond, R., Lloyd, D.H., Prenger-Berninghoff, E., Thom, N., Straube, I., Verheyen, K., Loeffler, A., 2014. Case-control risk factor study of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) infection in dogs and cats in Germany. Vet. Microbiol. 168, 154-160.

Loeffler, A., Boag, A.K., Sung, J., Lindsay, J.A., Guardabassi, L., Dalsgaard, A., Smith, H., Stevens, K.B., Lloyd, D.H., 2005. Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. J. Antimicrob. Chemother. 56, 692-697.

Love, D.N., 1989. Antimicrobial susceptibility of staphylococci isolated from dogs. Aus. Vet. Prac. 19, 196-200.

Ludwig, C., de Jong, A., Moyaert, H., El Garch, F., Janes, R., Klein, U., Morrissey, I., Thiry, J., Youala, M., 2016. Antimicrobial susceptibility monitoring of dermatological bacterial

pathogens isolated from diseased dogs and cats across Europe (ComPath results). J Appl. Microbiol. 121, 1254-1267.

Malik, S., Peng, H., Barton, M.D., 2006. Partial nucleotide sequencing of the *mecA* genes of *Staphylococcus aureus* isolates from cats and dogs. J Clin Microbiol 44, 413-416.

Marques, C., Gama, L.T., Belas, A., Bergstrom, K., Beurlet, S., Briend-Marchal, A., Broens, E.M., Costa, M., Criel, D., Damborg, P., van Dijk, M.A., van Dongen, A.M., Dorsch, R., Espada, C.M., Gerber, B., Kritsepi-Konstantinou, M., Loncaric, I., Mion, D., Misic, D., Movilla, R., Overesch, G., Perreten, V., Roura, X., Steenbergen, J., Timofte, D., Wolf, G., Zanoni, R.G., Schmitt, S., Guardabassi, L., Pomba, C., 2016. European multicenter study on antimicrobial resistance in bacteria isolated from companion animal urinary tract infections. BMC Vet Res 12, 213.

McMeekin, C.H., Hill, K.E., Gibson, I.R., Bridges, J.P., Benschop, J., 2017. Antimicrobial resistance patterns of bacteria isolated from canine urinary samples submitted to a New Zealand veterinary diagnostic laboratory between 2005-2012. N Z Vet J 65, 99-104.

Moodley, A., Stegger, M., Bagcigil, A.F., Baptiste, K.E., Loeffler, A., Lloyd, D.H., Williams, N.J., Leonard, N., Abbott, Y., Skov, R., Guardabassi, L., 2006. spa typing of methicillin-resistant *Staphylococcus aureus* isolated from domestic animals and veterinary staff in the UK and Ireland. J. Antimicrob. Chemother. 58, 1118-1123.

Morrissey, I., Moyaert, H., de Jong, A., El Garch, F., Klein, U., Ludwig, C., Thiry, J., Youala, M., 2016. Antimicrobial susceptibility monitoring of bacterial pathogens isolated from respiratory tract infections in dogs and cats across Europe: ComPath results. Vet. Microbiol. 191, 44-51.

Moyaert, H., Morrissey, I., de Jong, A., El Garch, F., Klein, U., Ludwig, C., Thiry, J., Youala, M., 2017. Antimicrobial susceptibility monitoring of bacterial pathogens isolated from urinary tract infections in dogs and cats across Europe: ComPath Results. Microb. Drug. Resist. 23, 391-403.

NORM/NORM-VET 2014. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway 2013 (Oslo, NORM/NORM-VET).

Normand, E.H., Gibson, N.R., Carmichael, S., Reid, S.W.J., 2000. Trends of antimicrobial resistance in bacterial isolates from a small animal referral hospital. Vet. Rec. 146, 151-155.

Nykäsenoja, S., Kivilahti-Mäntylä, K., Rantala, M., Thomas Grönthal, Pohjanvirta, T., Olkkola, S., Pekkanen, K., Nummela, M., Helin-Soilevaara, H. 2017. FINRES-Vet 2013–2015: Finnish

Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents (Helsinki, Finland).

Paul, N.C., Moodley, A., Ghibaudo, G., Guardabassi, L., 2011. Carriage of methicillin-resistant *Staphylococcus pseudintermedius* in small animal veterinarians: indirect evidence of zoonotic transmission. Zoonoses Public Health 58, 533-539.

Platell, J.L., Cobbold, R.N., Johnson, J.R., Heisig, A., Heisig, P., Clabots, C., Kuskowski, M.A., Trott, D.J., 2011. Commonality among fluoroquinolone-resistant sequence type ST131 extraintestinal *Escherichia coli* isolates from humans and companion animals in Australia. Antimicrob. Agents Chemother. 55, 3782-3787.

Platell, J.L., Cobbold, R.N., Johnson, J.R., Trott, D.J., 2010. Clonal group distribution of fluoroquinolone-resistant Escherichia coli among humans and companion animals in Australia. J. Antimicrob. Chemother. 65, 1936-1938.

Prescott, J.F., Hanna, W.J.B., Reid-Smith, R., Drost, K., 2002. Antimicrobial drug use and resistance in dogs. Can. Vet. J. 43, 107-116.

Rzewuska, M., Czopowicz, M., Kizerwetter-Swida, M., Chrobak, D., Blaszczak, B., Binek, M., 2015. Multidrug resistance in *Escherichia col*i strains isolated from infections in dogs and cats in Poland (2007-2013). Sci. World. J. 2015, 408205.

Saputra, S., Jordan, D., Mitchell, T., Wong, H.S., Abraham, R.J., Kidsley, A., Turnidge, J., Trott, D.J., Abraham, S., 2017a. Antimicrobial resistance in clinical *Escherichia coli* isolated from companion animals in Australia. Vet. Microbiol. 211, 43-50.

Saputra, S., Jordan, D., Worthing, K.A., Norris, J.M., Wong, H.S., Abraham, R., Trott, D.J., Abraham, S., 2017b. Antimicrobial resistance in coagulase-positive staphylococci isolated from companion animals in Australia: A one year study. PLoS One 12, e0176379.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K., 2007. Reclassification of phenotypically identified *Staphylococcus intermedius* strains. J Clin Microbiol 45, 2770-2778.

Shaban, R.Z., Simon, G.I., Darren J. Trott, Turnidge, J., Jordan, D. 2014. Surveillance and Reporting of Antimicrobial Resistance and Antibiotic Usage in Animals and Agriculture in Australia (Australia, Department of Agriculture, Griffith University and University of Adelaide).

Siak, M., Burrows, A.K., Coombs, G.W., Khazandi, M., Abraham, S., Norris, J.M., Weese, J.S., Trott, D.J., 2014. Characterization of meticillin-resistant and meticillin-susceptible

isolates of *Staphylococcus pseudintermedius* from cases of canine pyoderma in Australia. J. Med. Microbiol. 63, 1228-1233.

Sidjabat, H.E., Chin, J.J., Chapman, T., Wu, K., Ulett, G.C., Ong, C.Y., Schembri, M.A., Johnson, J.R., Trott, D.J., 2009. Colonisation dynamics and virulence of two clonal groups of multidrug-resistant *Escherichia coli* isolated from dogs. Microbes Infect. 11, 100-107.

Sidjabat, H.E., Gobius, K.S., Hanson, N.D., Fegan, N., Townsend, K.M., Bensink, J.C., Lorentzen, M., Chin, J.J.-C., Bettelheink, K.A., Trott, D.J., 2006a. Emergence and spread of two distinct clonal groups of multidrug-resistant *Escherichia coli* in a veterinary teaching hospital in Australia. J. Med. Microbiol. 55, 1125-1134.

Sidjabat, H.E., Hanson, N.D., Smith-Moland, E., Bell, J.M., Gibson, J.S., Filippich, L.J., Trott, D.J., 2007. Identification of plasmid-mediated extended-spectrum and AmpC beta-lactamases in *Enterobacter* spp. isolated from dogs. J. Med. Microbiol. 56, 426-434.

Sidjabat, H.E., Townsend, K.M., Hanson, N.D., Bell, J.M., Stokes, H.W., Gobius, K.S., Moss, S.M., Trott, D.J., 2006b. Identification of bla(CMY-7) and associated plasmid-mediated resistance genes in multidrug-resistant *Escherichia coli* isolated from dogs at a veterinary teaching hospital in Australia. J. Antimicrob. Chemother. 57, 840-848.

Somayaji, R., Priyantha, M.A., Rubin, J.E., Church, D., 2016. Human infections due to *Staphylococcus pseudintermedius,* an emerging zoonosis of canine origin: report of 24 cases. Diagn. Microbiol. Infect. Di.s 85, 471-476.

Stegmann, R., Burnens, A., Maranta, C.A., Perreten, V., 2010. Human infection associated with methicillin-resistant *Staphylococcus pseudintermedius* ST71. J. Antimicrob. Chemother. 65, 2047-2048.

SWEDRES-SVARM 2013. Use of Antimicrobials and Occurrence of Antimicrobial Resistance in Sweden (Solna/Uppsala).

SWEDRES-SVARM 2017. Consumption of Antibiotics and Occurrence of Resistance in Sweden 2016 (Uppsala, Public Health Agency of Sweden and National Veterinary Institute).

Thungrat, K., Price, S.B., Carpenter, D.M., Boothe, D.M., 2015. Antimicrobial susceptibility patterns of clinical *Escherichia col*i isolates from dogs and cats in the United States: January 2008 through January 2013. Vet. Microbiol. 179, 287-295.

Turnidge, J.D., Gottlieb, T., Mitchell, D.H., Coombs, G.W., Daley, D.A., Bell, J.M., 2014. Community-onset Gram-negative surveillance program annual report, 2012. Commun. Dis. Intell. 38, E54-E58.

van Duijkeren, E., Catry, B., Greko, C., Moreno, M.A., Pomba, M.C., Pyorala, S., Ruzauskas, M., Sanders, P., Threlfall, E.J., Torren-Edo, J., Torneke, K., Scientific Advisory Group on, A.,

2011a. Review on methicillin-resistant *Staphylococcus pseudintermedius*. J. Antimicrob. Chemother. 66, 2705-2714.

van Duijkeren, E., Kamphuis, M., van der Mije, I.C., Laarhoven, L.M., Duim, B., Wagenaar, J.A., Houwers, D.J., 2011b. Transmission of methicillin-resistant *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics. Vet. Microbiol. 150, 338-343.

Ventrella, G., Moodley, A., Grandolfo, E., Parisi, A., Corrente, M., Buonavoglia, D., Guardabassi, L., 2017. Frequency, antimicrobial susceptibility and clonal distribution of methicillin-resistant *Staphylococcus pseudintermedius* in canine clinical samples submitted to a veterinary diagnostic laboratory in Italy: A 3-year retrospective investigation. Vet. Microbiol. 211, 103-106.

Warren, A.L., Townsend, K.M., King, T., Moss, S., O'Boyle, D., Yates, R.M., Trott, D.J., 2001. Multi-drug resistant *Escherichia coli* with extended-spectrum β-lactamase activity and fluoroquinolone resistance isolated from clinical infections in dogs. Aus. Vet. J. 79, 621-623.

Wilson, B.J., Norris, J.M., Malik, R., Martin, P.A., Wigney, D.I., Baral, R.M., Govendir, M., 2006. Susceptibility of bacteria from feline and canine urinary tract infections to doxycycline and tetracycline concentrations attained in urine four hours after oral dosage. Aust. Vet. J. 84, 8-11.

Worthing, K.A., Abraham, S., Coombs, G.W., Pang, S., Saputra, S., Jordan, D., Trott, D.J., Norris, J.M., 2018. Clonal diversity and geographic distribution of methicillin-resistant *Staphylococcus pseudintermedius* from Australian animals: Discovery of novel sequence types. Vet. Microbiol. 213, 58-65.

Worthing, K.A., Abraham, S., Pang, S., Coombs, G.W., Saputra, S., Jordan, D., Wong, H.S., Abraham, R.J., Trott, D.J., Norris, J.M., 2017. Molecular characterization of methicillin-resistant *Staphylococcus aureus* isolated from Australian animals and veterinarians. Microb. Drug. Resist. 24, 203-212.

Worthing, K.A., Coombs, G.W., Pang, S., Abraham, S., Saputra, S., Trott, D.J., Jordan, D., Wong, H.S., Abraham, R.J., Norris, J.M., 2016. Isolation of *mecC* MRSA in Australia. J. Antimicrob. Chemother. 71, 2348-2349.

Zubeir, I.E., Kanbar, T., Alber, J., Lammler, C., Akineden, O., Weiss, R., Zschock, M., 2007. Phenotypic and genotypic characteristics of methicillin/oxacillin-resistant *Staphylococcus intermedius* isolated from clinical specimens during routine veterinary microbiological examinations. Vet. Microbiol. 121, 170-176.

Chapter 5

Metagenomic studies of gut microbial diversity and structure changes after antibiotic treatment

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Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Metagenomic studies of gut microbial diversity and structure changes after antibiotic treatment

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Abstract

It is widely known that the use of antibiotics heavily disrupts the ecology of the gut microbiota. As a complex assemblage of microbes, disturbance in some populations may lead to other changes; not only to the community structure, but also there may be functional diversity changes related to nutrient supply and immunity. Over the last decade, metagenomic studies using next-generation sequencing (NGS) have utilized deep analysis to understand the effects of antibiotics on microbial composition and function. With the increasing number of metagenomic studies, we explore current research to summarize the commonalities and differences of antibiotic effects on the gut microbiota in humans, laboratory animals and production animals. Altered microbial phylotypes were detected in most cases. However, only 80% of the total antibiotic studies of humans and laboratory animals, with fewer in food animals (25%), demonstrated a significant decrease in the microbial richness and/or diversity in the gastrointestinal tract, while the others indicated minor changes or, surprisingly, an increased microbial richness and induced proliferation of beneficial bacteria (known as the eubiotic effect). Antibiotic features such as class and spectrum affect the gut microbiota in different ways, with varied recovery times. Additionally, the unique effect in food animals was observed when antibiotics were administered as feed-additives in sub therapeutic doses, resulting in a distinctive microbial composition without reducing microbial diversity. The findings in this field emphasize the variation in gut microbial community changes and its consequences following antibiotic treatment, depending on the dosing and the properties of the antibiotic, and the initial structure and function of the microbial community.

Keywords: antibiotics, gut, microbial community, metagenomics.

Introduction

The gastrointestinal tract (GI) in each individual hosts its own, unique community of immensely diverse microbial species including bacteria, viruses, fungi, archaea and eukaryotic organisms (Fuerst, 2014; Lloyd-Price et al., 2016). This microbial community, which contains a similar number of bacterial cells compared with the number of human body cells (Sender et al., 2016), plays a crucial role in the body's nutrient supply and immunity levels (Morowitz et al., 2011). Several factors influence the microbial members and composition, including diet intake, medicines, genotype of the host, early-life and initial state of the microbiota, age, stress and geographic origin (Backhed et al., 2012; Gibson et al., 2015; Lozupone et al., 2012; Willing et al., 2011). Although there is no standard measurement, a healthy gut microbiota is defined in terms of its ecological stability, ability to resist stress-related change (intestinal homeostasis) and maintenance of an ideal microbial composition with a desirable functional profile (Backhed et al., 2012). The effects of several pharmaceutical agents on gut microbiota have been reported in recent studies, including antibiotics, antidiabetic drugs (Montandon and Jornayvaz, 2017), non-steroidal anti-inflammatory drugs (Rogers and Aronoff, 2016) and other host-targeted drugs (Maurice et al., 2013). Antibiotics are of greatest concern due to their potency to kill a wide range of bacterial species, leading not only to a beneficial clinical outcome by treating pathogenic bacteria but also causing disturbance to the regulation of the host's immunity levels and metabolism, and the development of antimicrobial resistance (Backhed et al., 2012; Francino, 2015; Gasparrini et al., 2016).

Our knowledge of the gut microbiome and the changes caused by administration of antibiotics has been described widely over the last decade due to the development of culture-free methods such as high-throughput sequencing which has become the gold standard technique to assess the microbial community or environment (metagenomics) (Oulas et al., 2015). This method enables a deep analysis of the microbial members and structures of communities and, further, predicts their functional diversity. As a result, metagenomic studies have rapidly expanded, producing a dramatic increase in the scientific findings in the field (Bragg and Tyson, 2014; Oulas et al., 2015). The 454 Life Sciences, Ion Torrent PGM and Illumina systems are the three commonly used NGS technologies (Allali et al., 2017). To date, they have been widely used in metagenomic studies in humans, animals and the environment. By performing amplification of specific genes of interest (16S rRNA or resistance genes) and subjecting them to an NGS platform, metagenomic data can be generated to create community diversity profiles and functional composition analyses (Dhariwal et al., 2017). This

method can be utilised to understand the hidden nature of the environmental community which include the species of microorganism and their role in the community and the in a balanced ecological niche (Oulas et al., 2015). Comparable with NGS, phylogenetic microarray analysis or human intestinal tract chip (HITChip) hybridizations also provide robust results, obtaining equivalent biological conclusions to pyrosequencing of microbial communities, regardless of technology or primer choice (Claesson et al., 2009; van den Bogert et al., 2011).

Specific members of bacterial communities affected by antibiotics have been described previously, primarily using cultivation-based methods (Lagier et al., 2012; Rafii et al., 2008; Willing et al., 2011) and also a small number of molecular studies (Cotter et al., 2012; Ianiro et al., 2016; Jernberg et al., 2010; Langdon et al., 2016). However, it is unclear as to which antimicrobial agents consistently cause significant changes in microbial diversity and composition, and which bacterial taxa are frequently affected by the antibiotics. In this review we explore and summarise the impact of antibiotics on the gut microbiome in humans, laboratory animals and food production animals.

Definitions and methods

We use microbial ecology terms described previously by Robinson et al. (2010). Microbial diversity refers to the extent of variety (bacterial taxa) in a community, consisting of two components: richness and evenness. Microbial richness refers to the number of types in a community, while microbial evenness refers to the distribution of individuals across types. Microbial diversity in a local pool or in a particular ecosystem (alpha diversity) can be expressed using some diversity indices such as Shannon, Simpson or Chao1 (Dhariwal et al., 2017). The change in community structure (consisting of composition and abundance) is usually visualised by using principal coordinate analysis (PCoA) or non-metric multidimensional scaling (NMDS), calculated using beta diversity metrics such as Bray Curtis, Jaccard index or unifrac distances (Dhariwal et al., 2017). For some studies, the abundance or copy number of 16Sr RNA genes has also been assessed using real-time PCR of 16S rRNA genes, which is usually termed the microbial load. In this review, changes in microbial diversity refer to changes in either richness, evenness or overall diversity, while changes of microbial structure refer to differences in microbial membership and abundance. Not all metagenomic studies on the gut microbiota are included in this review. The antibiotics were grouped based on their significance in affecting microbial richness and/or diversity and microbial structure, as measured by alpha and beta diversity. Those studies with unspecified antibiotics or from single case patients were excluded, focusing on studies that performed a

statistical analysis from multiple samples or multiple objects. A bacterial taxa indicator for each antibiotic was summarised, based on its significance on suppression or proliferation, not based on dominant taxa. The ability of the gut microbial community to recover (resilience) following antibiotic treatment was obtained from several studies that include at least three time points, including pre-treatment, during treatment and after treatment, with multiple sampling. We categorised the antibiotics based on therapeutic vs non-therapeutic use, bactericidal vs bacteriostatic (Leekha et al., 2011; Nemeth et al., 2015) and broad- vs narrow-spectrum (Sarpong and Miller, 2014).

Microbial diversity and structure

Normally, antibiotics affect the gut microbiota negatively either by decreasing the bacterial abundance or creating a shift in the relative abundance of certain bacterial taxa, even during short periods of antibiotic exposure (McDonald, 2017). Investigated by alpha and beta diversity and/or relative abundance of 16S rRNA genes, we found that only 77% (50/65) of antibiotic studies in human and animal models indicated a significant reduction in microbial richness and/or diversity. In food animals, only 24% (4/17) of studies were associated with reduced microbial diversity. Groups of antibiotics, based on the severity of their effect on the gut microbiota, are presented in Fig 1. We observed that the reduction of microbial diversity was associated with therapeutic (to treat infections) and prophylactic (to prevent and control common diseases) use of antibiotics, while most antibiotics used as growth promoter (non-therapeutic) were not associated with reduction of microbial diversity.

Cefotaxime, ciprofloxacin, clindamycin and vancomycin were among the antibiotics that were highly associated with a reduction in gut microbial diversity and changes in microbial structure, as demonstrated in both human (Dethlefsena and Relman, 2011; Gibson et al., 2016; Pop et al., 2016; Rashid et al., 2015; Reijnders et al., 2016; Zaura et al., 2015) and murine studies (Buffie et al., 2012; Choo et al., 2017; Hansen et al., 2012; Lankelma et al., 2017; Tulstrup et al., 2015; Yao et al., 2016; Yin et al., 2015). A marked decrease of microbial richness is usually followed by a decrease in microbial diversity, leading to community structure changes. However, this effect does not apply to all antibiotic treatments. In some cases, antibiotic use may decrease the richness without significantly decreasing the overall diversity, as demonstrated by rifampin-combination drugs for treating *Mycobacterium* infections in humans (Wipperman et al., 2017), although these eventually showed directional changes in microbial structure, reflecting different microbial memberships and actual

Table 1. Current metagenomic studies related to antibiotic effect on gut microbiome in humans, animal models and food animals.

Antibiotics	Subject	Sampling time	Sample type	Gene marker	NGS platform	Add. study	References
Clarithromycin, metronidazole, omeprazole	Human (dyspepsia, ulcer n=6)	Day 0, 8-13, year 1, 4	throat, faeces	V6 16S rRNA	454 GS-FLX	2	(Jakobsson et al., 2010)
Ciprofloxacin	Human (healthy n=3)	~40 time points: pre-, during and after	faeces	V1-V3 16S rRNA	454 GS-FLX	-	(Dethlefsen et al., 2008; Dethlefsena and Relman, 2011)
Ampicillin and gentamicin	Human (infant n=18))	Day 2, week 4, 8	faeces	16S rRNA	454 GS-FLX		(Fouhy et al., 2012)
Amoxicillin-clavulanate, levofloxacin, ceftriaxone, ciprofloxacin	Human (non-digestive diseases n=21)	Day 0, 7	faeces	V4 16S rRNA	454 GS-Junior	3	(Panda et al., 2014)
Ampicillin and gentamicin	Human (infants n=74)	Week 1, 2, 3	faeces	V3-V5 16S rRNA	454 GS-FLX	-	(Greenwood et al., 2014)
Ciprofloxacin, nitrofurantoin	Human (UTI n=40)	Day 0, 7, week 4	faeces	V3-V4 16S rRNA	454 GS-FLX	-	(Stewardson et al., 2015)
Ciprofloxacin and clindamycin	Human (healthy n=30)	Day 0, 11, month 1, 2, 4, 12	faeces	V5-V7 16S rRNA	454 GS-FLX	-	(Rashid et al., 2015)
Rifaximin	Human (IBS n=20)	Day 0, 14, week 6	faeces	V3-V6 16S rRNA	Illumina MiSeq	-	(Soldi et al., 2015)
Clindamycin, ciprofloxacin, amoxicillin, and minocycline	Human (healthy n=66)	Day 0, 7, month 1, 2, 4, 12	saliva, faeces	V2 16S rRNA	Illumina MiSeq	1, 2	(Zaura et al., 2015)
Rifaximin	Human (GI and liver disorders n=20)	Day 0, day 11, day 31	faeces	16S rRNA	454 GS-Junior	-	(Ponziani et al., 2016)
Meropenem, cefotaxime, ticarcillin/clav, vancomycin & gentamicin	Human (preterm infants n=84)	before and after (within 48 hrs)	faeces	V4 16S rRNA	Illumina MiSeq	1	(Gibson et al., 2016)
Cefprozil	Human (healthy n=18)	Day 0, 7, month 3	faeces	V3 16S rRNA	Illumina HiSeq	1	(Raymond et al., 2016a; Raymond et al., 2016b)
Ciprofloxacin	Human (ETEC challenge)	Day 0, 1-7, 9, 28, 84	faeces	V1-V2 16S rRNA	454 GS-FLX	-	(Pop et al., 2016)
Vancomycin and amoxicillin	Human (obese n=57)	Day 0, 7, week 8	faeces	16S rRNA	HITchip	2, 3, 4	(Reijnders et al., 2016; Vrieze et al., 2014)

Amoxicillin and azithromycin	Human (healthy n=56)	Day 0, 3, 7, week 8, month 6	saliva, faeces	V1-V2 16S rRNA	Ion Torrent PGM	1	(Abeles et al., 2016)
AB cocktail	Human (healthy n=16)	Day 0, 9	faeces	V1-V2 16S rRNA	Illumina MiSeq	4	(Lankelma et al., 2017)
Vancomycin	Human (RA)	Day 0, week 2, 6, 14, 22	faeces				(Isaac et al., 2017)
Ridinilazole and fidaxomicin	Human (CDI)	-	faeces	V4 16S rRNA	Illumina MiSeq		(Mitra et al., 2017)
Isoniazid, rifampin, pyrazinamide, ethambutol (HRZE)	Human (TB n=139)	-	faeces	V4-V5 16S rRNA	Illumina MiSeq		(Wipperman et al., 2017)
Cefoperazone, vancomycin, AB cocktail	Murine (healthy)	Before, during, after	intestine	V6 16S rRNA	454 GS-FLX	-	(Antonopoulos et al., 2009)
AB cocktail	Murine	Before, after	intestine, faeces	16S rrNA	454 GS-FLX	4	(Hill et al., 2010)
Vancomycin	Murine (DM)	1 time-point	feces	V3-V4 16S rRNA	454 GS-FLX	1, 4	(Hansen et al., 2012)
Clindamycin	Murine (CDI)	Day -2, 1-7, 10, week 2-4	intestine, faeces	V1-V3 16S rRNA	454 GS-FLX	-	(Buffie et al., 2012)
Penicillin and vancomycin	Murine (weaning)	Week 3, 6	intestine, faeces	V1-V3 16S rRNA	454 GS-FLX	4	(Cho et al., 2012; Cox et al., 2014)
Tigecycline	Murine (CDI n=28)	Day 0, 3, 5, 10, week 1-5	faeces	V3-V5 16S rRNA	454 GS-FLX	-	(Bassis et al., 2014)
Amoxicillin, cefotaxime, vancomycin, metronidazole	Murine (healthy n=60)	During and after	intestine, faeces	V3 16S rRNA	Ion Torrent PGM	-	(Tulstrup et al., 2015)
β-Lactams, Quinolones, Glycopeptides and Macrolides	Mice (n=56)	~17 time-points: pre-, during and post	faeces	16S rRNA	Illumina MiSeq	-	(Yin et al., 2015)
Streptomycin and antibiotic cocktail	Mice (CDI, *S. typhimurium*)	Day 0, 1, 3	faeces	V4 16S rRNA	Illumina MiSeq	3, 4	(Lichtman et al., 2016)
Linezolid, clindamycin, moxifloxacin, amoxicillin, afibacin	Mice (healthy)	Day 0, 2, 6, 10, 12, 17, 23, 30, 37	faeces	V1-V3 16S rRNA	Illumina MiSeq	-	(Yao et al., 2016)
Azithromycin, amoxicillin and cefaclor	Rats (pregnant n=48)	Day 7	intestine	V4 16S rRNA	Illumina MiSeq	-	(Khan et al., 2016)
Florfenicol & azithromycin	Mice (healthy)	Week 1-4	faeces	V3-V5 16S rRNA	Illumina MiSeq	4	(Li et al., 2017c)

Ciprofloxacin & vancomycin-imipenem	Mice (healthy)	Day 0, 14, 23	faeces	V4 16S rRNA	Illumina MiSeq	2	(Choo et al., 2017)
Penicillin	Mice (healthy n=20)	Day 28	intestine, faeces	V3-V4 16S rRNA	Illumina MiSeq	4	(Daniluk et al., 2017)
Isoniazid-rifampin-pyrazinamide (HRZ)	Mice (TB)	Week 1-4, 6, 8, 12, 16, 20, 24, 28, 32	faeces	V3-V5 16S rRNA	Illumina MiSeq	2	(Namasivayam et al., 2017)
Chlortetracycline, sulfamethazine, & penicillin	Swine	Day 0, 3, 14, 21	intestine, faeces	V3 16S rRNA	454 GS-FLX	1	(Looft et al., 2014; Looft et al., 2012)
Tylosin	Swine	Age week 10, 13, 16, 19, 22	faeces	V3 16S rRNA	454 GS-FLX	-	(Kim et al., 2012)
Tulathromycin	Swine	Day 1, 5, 14	intestine	16S rRNA	PITChip	3, 4	(Schokker et al., 2014)
Chlortetracycline	Swine	Day 10	intestine	V3-V4 16S rRNA	Illumina MiSeq	-	(Zhang et al., 2016)
Colistin and bacitracin	Swine	Day 28	intestine	V3-V4 16S rRNA	Illumina MiSeq	-	(Li et al., 2017b)
Virginiamycin and bacitracin	Chickens	Age day 44	caecum	V6-V8 16S rRNA	454 GS-FLX	-	(Neumann and Suen, 2015)
Chlortetracycline	Chickens	-	faeces	16S rRNA	454 GS-FLX	1	(Hegde et al., 2016)
Amoxicillin	Chickens	Day 0, 5, 14	jejunum	V3 16S rRNA	Illumina MiSeq	3, 4	(Schokker et al., 2017)
Zinc bacitracin, enramycin, halquinol, virginiamycin & avilamycin	Chickens	Age day 43	caecum	V4 16S rRNA	Illumina MiSeq	-	(Costa et al., 2017)
Chlortetracycline and salinomycin	Chickens	Day 7,28, 42	faeces	V3-V4 16S rRNA	454 GS-FLX	-	(Gao et al., 2017)
Enrofloxacin	Chickens	16 time-points	intestine	V4 16S rRNA	Illumina MiSeq	1	(Li et al., 2017a)
Monensin & tylosin	Cattle	-	rumen, intestine	16S rRNA	Illumina MiSeq	1, 2	(Thomas et al., 2017)

*Additional study: 1) resistome; 2) metabolome; 3) trascriptome; 4) immunohistochemistry.

abundance. Additionally, severe perturbation caused by ciprofloxacin and clindamycin was not in accordance with the copy number of 16S rRNA genes, which was relatively stable or slightly increased by the treatment (Buffie et al., 2012; Panda et al., 2014). The decrease in microbial diversity with an increase in microbial load indicates that bacteria sensitive to these groups of antibiotics provide an opportunity for a small number of resistant strains to overgrow and dominate the niche.

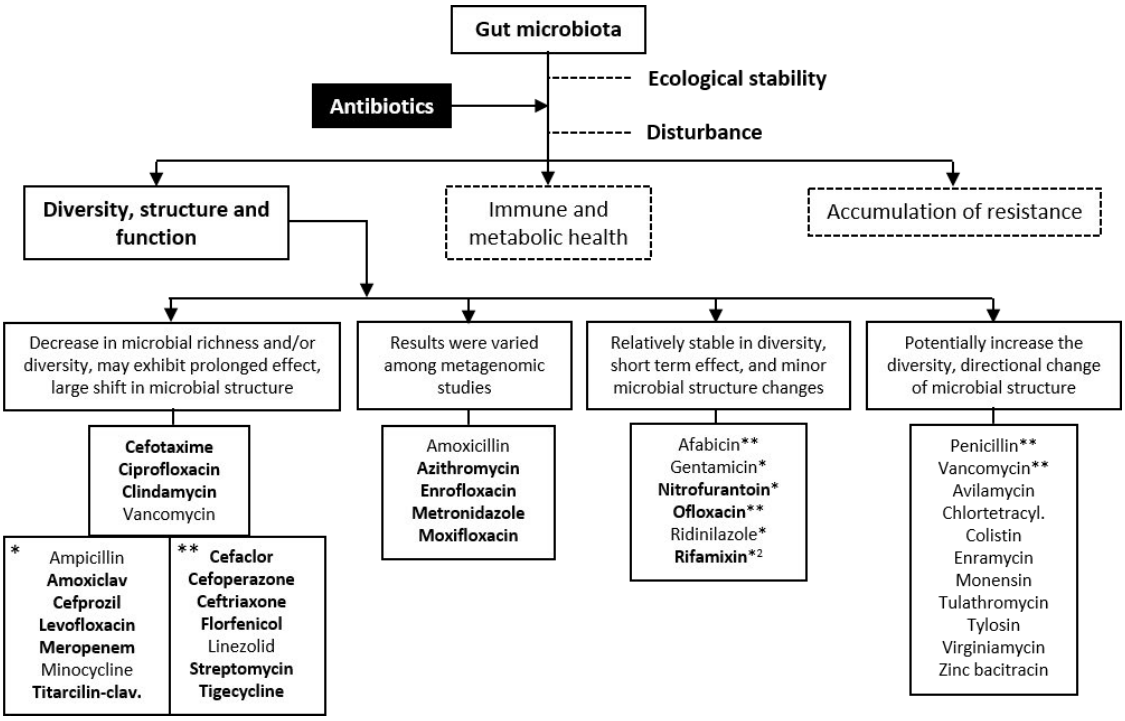

Figure 1. A chart describing the effect of single antibiotic use in regards to microbial richness, diversity and structure of the gut microbiota.* Human study only; ** Animal study only. Antibiotics in bold are categorised as broad-spectrum antibiotics. [1]Rifaximin is considered as "eubiotic": a positive modulator on gut microbiota.

It is well known that the human gut microbiota presents high inter-individual variability, while murine exhibit a more controlled, high similarity across individuals (Nguyen et al., 2015). Therefore, it is important to note that normal gut microbiota in murine may not fully reflect the real human gut microbiota (Nguyen et al., 2015). As a result, we observed several differences among the metagenomics studies in response to antibiotics. For example, amoxicillin, which did not significantly affect microbial diversity in healthy humans, as described in a UK and Netherlands study (Reijnders et al., 2016; Zaura et al., 2015) but significantly reduced gut microbial diversity in murine studies (Khan et al., 2016; Tulstrup et al., 2015; Yao et al., 2016). Three out of four azithromycin studies showed a significant effect,

including in both human (Abeles et al., 2016) and murine (Khan et al., 2016; Li et al., 2017c) studies, but showed no significant effect in another murine study (Yin et al., 2015) even though a human dosage was applied. Administered at different concentrations, enrofloxacin showed variable effects on gut microbiome changes in chickens (Li et al., 2017a). Metronidazole showed no significant association with reduced diversity in a murine study (Tulstrup et al., 2015) but significantly reduced microbial diversity in the human distal colon model (Rea et al., 2011). Moxifloxacin showed changes in composition and microbial load but the microbiota recovered quickly despite ongoing treatment in a murine study (Yao et al., 2016), whilst showing a fluctuating result (an increasing trend during treatment but decreasing after treatment) in a bronchitis patient (Perez-Cobas et al., 2013).

Narrow-spectrum antibiotics, such as afabicin and gentamicin demonstrated minor impacts on the gut microbiome. Afabicin (Debio1450) did not reduce the microbial load and showed a similarity in microbial structure with the control group in a murine study (Yao et al., 2016) with ofloxacin (Yin et al., 2015). A stable or slight increase in microbial richness was observed following gentamicin administration as demonstrated in an infant study (Gibson et al., 2016). A broad spectrum rifaximin produced high efficacy treatments in inflammatory bowel disease (IBD) patients, without disrupting the gut microbiota (Ponziani et al., 2016; Soldi et al., 2015). Nitrofurantoin also treats urinary tract infections effectively (Stewardson et al., 2015), while ridinilazole is effective against *C. difficile* infection (Mitra et al., 2017), with only a minor impact on the gut's microbial community.

In contrast to the therapeutic and prophylactic use of antibiotics in humans, we found that the sub therapeutic use of antibiotics in food production animals as a growth promoter most likely did not decrease microbial richness and diversity. Instead, it has the potential to increase microbial diversity. Across 14 studies of food-producing animals, only enramycin (Costa et al., 2017) and virginiamycin (Neumann and Suen, 2015) were associated with a significant reduction in microbial diversity. In contrast, another study in broiler chickens found that microbial diversity was significantly higher in chicken which fed with virginiamycin compared to the control group (Costa et al., 2017). As a prophylaxis, enrofloxacin and amoxicillin significantly reduced diversity but the microbial diversity level recovered quickly after antibiotic administration ceased (Li et al., 2017a; Schokker et al., 2017). It is important to note that although sub-therapeutic use of antibiotics resulted in minor effects on the diversity and structure, gut microbiota may accumulate greater numbers of resistance genes, as reported previously in a human study (Zaura et al., 2015) and food animal studies (Hegde et al., 2016; Li et al., 2017a; Looft et al., 2014; Looft et al., 2012). Additionally, increasing trends

of diversity during gut microbial colonisation in early life in food animals is assumed to be detrimental for immune development (Schokker et al., 2017).

Resilience and recovery time

Despite this variation in bacterial taxa shift, it appears that the human and murine gut microbiota exhibits resilience after antibiotic administration, returning to the initial state of the ecosystem. The recovery speed of gut microbiota varied across individuals and bacterial species but in most cases, usually stabilised at a distinctive microbial structure. The stages of treatment and recovery time summarised from several studies are presented in Table 2-3. Since the studies of recovery time are sampling-time-dependent, there is a risk of underestimation in the exact turning point for recovery after exposure, in particular with minimum sampling times. When the duration of the response is short-lived, it is possible that changes in gut microbiota may occur between sample collections. Thus, the time point indicating recovery time is only approximate. Frequent sampling is crucial for more accurate comparison of changes associated with antibiotic administration (Stokell et al., 2015).

Most of the gut microbiota started to return to the initial composition after antibiotic cessation. For some cases, the gut microbiota demonstrates an immediate recovery from the first exposure and continue to return to the baseline, despite the treatment being ongoing. A quick initial start of recovery was observed in afabicin and moxifloxacin, which also resulted in a low impact by the antibiotics on the microbial composition in mice (Yao et al., 2016). On the other hand, long lasting effects of antibiotics were also reported, causing a delayed and difficult return to the initial composition, up to 2 months after the treatment. This evidence can be seen for ciprofloxacin and clindamycin (bactericidal and bacteriostatic agents, respectively) (Rashid et al., 2015; Zaura et al., 2015), and narrow-spectrum vancomycin (Reijnders et al., 2016) as reported in human studies and a murine study (Yao et al., 2016). Additionally, the longer term impact of microbial richness reduction can also be observed in macrolides (azithromycin and clarithromycin), which did not reach the level of the control samples even 12–24 months after the course (Korpela et al., 2016). In one case, the increase in resistance genes persisted for up to 4 years (Jakobsson et al., 2010).

Table 2. Microbial community changes during and after treatment with various doses (BID, twice a day; TID, three times a day; and QID four times a day) antibiotics.

Antibiotics and dosage	*Time points*					*Ref.*
	≤Week 1	Week 2	Month 1	Month 6	≥Year 1	
Amoxicillin (250 mg, BID) for 7 days	showed dissimilarity but no significant decrease in microbial richness	no significant effect on overall microbiome diversity				(Zaura et al., 2015)
Ciprofloxacin (500mg, BID) for 10 days			differed from the placebo group up to 1 month in faeces		diversity significantly reduced up to 12 months	(Zaura et al., 2015)
Ciprofloxacin (500 mg, BID) for 5 days	loss of diversity and shift in composition after 3–4 days of treatment	communities began to return to their initial state, but incomplete		continued to show directional change up to month 2; some showed complete recovery but stabilized in a distinct composition	several taxa failed to recover within 6 months	(Dethlefsen et al., 2008)
Ciprofloxacin (500 mg, BID) for 10 days		no significant changes in composition but the species richness decreased significantly at day 11	the most pronounced change in composition observed at month 1	richness remained reduced up to month 2	a single OTU being affected even at month 12	(Rashid et al., 2015)
Clindamycin (150 mg, QID) for 10 days		significant changes in composition observed and decreased richness at day 11		richness remained reduced up to month 3	Bifidobacteria did not normalize until month 12	(Rashid et al., 2015)
Clindamycin (150 mg, QID) for 10 days				diversity was significantly reduced for up to 4 months		(Zaura et al., 2015)
Minocycline (100 mg, BID) for 5 days	minor shift in composition was observed at week 1		differed from the placebo group up to 1 month			(Zaura et al., 2015)
Rifaximin (550 mg, TID) for 14 days	did not significantly affect the overall composition of the core microbiota	decreased Firmicutes/ Bacteroidetes ratio after 14 days of treatment but bacterial profiles with higher biodiversity were observed		slight increasing trend of diversity at month 2, recovery of IBS patients after rifaximin intervention		(Soldi et al., 2015)
Vancomycin (250 mg, QID) for 14 days		Significantly reduced richness and diversity	Slowly recover, richness significantly lower than baseline	Microbial richness failed to recover but not significant based on Shanon index		(Isaac et al., 2017)
Ampicillin/sulbactam and cefazolin single dose and continued with intravenous cefazolin alone for the next 14 days	reduced diversity at day 3	lowest diversity index at day 11 but started to recover at day 14		exhibited the most divergent microbiota among the samples analysed at day 40		(Perez-Cobas et al., 2013)
Metronidazole 400 mg, clarithromycin 250 mg, and omeprazole 20 mg (in combination, BID) for 7 days	decreased bacterial diversity (Shannon diversity index)	bacterial diversity slowly recovered			microbial communities had partially recovered, resistance genes persisted up to 4 years	(Jakobsson et al., 2010)

Table 3. Microbial richness and diversity changes during and after antibiotic treatment in murine model studies

Antibiotics and dosage	*Time points*					*Ref.*
	≤Week 1	Week 2	Month 1	Month 6	≥Year 1	
Afabicin 29.4 mg/ml for 10 days	no effect on microbial load, minor shift on Bacteriodales	microbiome composition was evaluated on day 12	indistinguishable from that of untreated mice			(Yao et al., 2016)
Amoxicillin (48 mg/ml), Linezolid (40 mg/ml), Clindamycin (40 mg/ml) (as single treatment) for 10 days	significantly reduced microbial load		the bacterial abundance did not recover until day 17, but day 23 for clindamycin; minor taxa did not recover			(Yao et al., 2016)
Cefoperazone (0.5 mg/ml) for 10 days	significantly decreased microbial diversity	continued reduction of richness despite withdrawal, decrease in Bacteriodetes		microbial diversity returned to baseline at week 6 but distinct microbial composition		(Antonopoulos et al., 2009)
Clindamycin (200ug) by injection (single dose)	markedly reduce the diversity			caecal biodiversity rebounded modestly but remained persistently low up to 4 weeks 87% of all OTUs initially present in the caecum were depleted		(Buffie et al., 2012)
Moxifloxacin (13 mg/ml) for 10 days	decreased microbial load at day 2 but rapidly recovered despite ongoing treatment	nearly reaching the baseline after 2 days of withdrawal	minor taxa remaining unstable and continuing to fluctuate at day 37			(Yao et al., 2016)
Tigecycline (6.25 mg/kg) by injection twice a day for of 10 days	the faecal bacterial community had shifted dramatically	after tigecycline treatment stopped, the faecal bacterial community continued to change		community structure after 5 weeks of recovery was significantly different compared with prior treatment		(Bassis et al., 2014)
Ceftriaxone, cefoperazone/sulbactam, meropenem, vancomycin (as single treatment) for 4 days	clearly declined from the second day after drug delivery	different microbial structure since day 2, more divergent for vancomycin, Shannon index returned to normal at day 14		observed OUT returned to normal at day 90 but showed distinct community		(Yin et al., 2015)
Ofloxacin and azithromycin (as single treatment) for 4 days	did not significantly differ from the control group	returned to normal on the 4th day after drug withdrawal, no change on beta diversity				(Yin et al., 2015)
Isoniazid (25 mg/ml), rifampin (1 mg/ml), and pyrazinamide (150 mg/ml) first 2 months and isoniazid and rifampin only for an additional 2 months	significantly decreased microbial richness (Chao1) and diversity (Shannon)	microbial diversity slowly recovered, slowly changed microbial structure	incomplete recovery of diversity at week 8 while microbial richness recovered at week 20	Shannon index similar to baseline but slightly lower in richness, microbial structure clustered separately		(Namasivayam et al., 2017)

A mechanism for resilience in gut microbiota has been proposed previously, which includes the ability to return to initial richness and functional diversity, as well as competition and a feedback loop in the community (Lozupone et al., 2012). Where there is a prolonged reduction in the level of diversity, antibiotics may have a stronger effect than the ability of gut microbiota itself to recover, resulting in incomplete recovery and a distinctive microbial structure when compared with the initial state of the community. However, it is also possible that during antibiotic administration, when particular bacterial taxa are suppressed during the treatment, the ecosystem may able to support the growth of other bacterial taxa because there is less competition among the species. For example, one individual had a 20 % decrease in observed species following antibiotic treatment, while over the same period they had a 10% increase of other species, indicating the emergence of new populations that had not been detected prior to antibiotic exposure and so had a low impact on the observed microbial richness and diversity.

Bacterial taxa indicators

It has been suggested that disruption of the gut microbiota can be significantly affected by pathological intestinal conditions. Although antibiotics possess the potential to kill pathogenic bacteria, their application is frequently followed by the occurrence of other bacteria groups. Enhanced susceptibility to infection has frequently been associated with the negative impacts of broad-spectrum antimicrobials on the gut microbiota. We surveyed the bacterial taxa that were suppressed/underrepresented (negative effect) and proliferated/overrepresented (positive effect), as indicated in each study. The analysis commonly performed determined significant changes mainly based on LefSe, ANOVA/AMOVA, or t-tests between time points. We found that the percent change in composition of bacterial taxa varied considerably across the metagenomics studies. The antibiotic-responsive bacterial taxa were affected in unique ways by different compounds. The common patterns of gut microbiota affected by antibiotic treatments are presented in Table 4. The proliferated bacteria may indicate their ability to resist antibiotic exposure or be a result of lower nutrient competition between bacterial species that allows for increased in abundance of other bacterial species. Supressed bacteria represent bacterial taxa susceptible to certain antibiotics.

We observed that most of the administration of antibiotics negatively impacted on *Bifidobacterium, Alistipes, Faecalibacterium*, Lachnospiraceae and Ruminococcaceae. These bacterial taxa are regarded as indicative of gastrointestinal good-health and are highly

abundant in human and animal guts (Ellis et al., 2013; Greenhalgh et al., 2016; Lloyd-Price et al., 2016; Stanley et al., 2016). Unfortunately, these beneficial bacterial taxa were depleted significantly by various antibiotics, including fluoroquinolones, beta-lactams, lincosamides and glycopeptides. Suppressed *Alistipes* and *Faecalibacterium* were reported by many studies as a result of fluoroquinolone use, while lincosamides and beta-lactams mainly affected Lachnospiraceae and Ruminococcaceae. Inflammatory and immunomodulatory properties of the gut may be affected by a reduction of *Faecalibacterium* and *Bifidobacterium* (Ponziani et al., 2017). Lachnospiraceae and Ruminococcaceae may disrupt carbohydrate metabolism in the gut, as those families of bacteria are specialised for the degradation of complex carbohydrate (Biddle et al., 2013).

On the other hand, antibiotics are positively associated with some bacterial taxa, including *Bacteriodes* and *Parabacteriodes*, Enterobacteriaceae and *Akkermansia*. The most reported bacterial taxa are Enterobacteriaceae and *Bacteroides* which are associated with beta-lactam, lincosamide and glycopeptide use, while an increase in *Akkermansia* was associated with fluoroquinolones and glycopeptide use (Dubourg et al., 2013). Abundant Enterobacteriaceae have been associated with the microbiota of patients with ulcerative colitis (UC) and Crohn's disease (CD). Increasing abundance of *Bacteriodes* and *Akkermansia* has been associated with type 2 diabetes (Guinane and Cotter, 2013) while *Parabacteriodes* may affect modulation B and T cells in peripheral blood (Abecia et al., 2017).

While some beneficial bacteria were supressed in human and animal studies involving administration of therapeutic doses, sub therapeutic use of antibiotics in feed animal has given markedly different results. *Lactobacillus* and *Faecalibacterium* were significantly overgrown in pigs and chickens after antibiotic administration (Neumann and Suen, 2015; Schokker et al., 2017; Schokker et al., 2014). However, other studies show that in-feed antibiotics also increase Enterobacteriaceae levels (Looft et al., 2012), which in turn leads to an increase in the risk of infections, usually associated with multidrug-resistant strains (Matar, 2017). It is believed that the antibiotic resistance genes that are enriched after specific antibiotic treatments are generally unique and are highly correlated with the single species that are overrepresented (Gibson et al., 2016).

Table 4. Bacterial taxa indicator in response to antibiotics (AB) administered to humans, animal models and food animals

Bacterial taxa (Gram-stain)	No of study	Antibiotic negatively affect the abundance	No of investigations	Antibiotic positively affect the abundance
Bifidobacterium (+)	8	ciprofloxacin (n=3), cefprozil, clindamycin, ampicillin-gentamicin, HRZE, MCO	3	cefotaxime, tulathromycin, HZR
Bacteroides (-)	6	amoxicillin, streptomycin, tigecycline, vancomycin-imipenem, AB cocktail	12	ciprofloxacin (n=3), levofloxacin, amoxicillin, amoxiclav, cefprozil, cefotaxime, penicillin, vancomycin, AB cocktail (n=2)
Parabacteroides (-)	3	cefoperazone, vancomycin, vancomycin-imipenem	4	amoxicillin, penicillin, cefrpozil, AB cocktail
Alistipes (-)	9	ciprofloxacin (n=4), vancomycin, vancomycin-imipenem, azithromycin, florfenicol, AB cocktail	2	amoxicillin, minocycline
Lactobacillus (+)	6	azythomycin (n=3), amoxicillin, ciprofloxacin, HZRE	10	vancomycin (n=2), vancomycin-imipenem, tylosin, rifaximin, bacitracin, amoxicillin, ciprofloxacin, enrofloxacin, cefoperazone
Faecalibacterium (-)	7	ciprofloxacin (n=4), ampicillin, vancomycin, AB cocktail	5	Nitrofurantoin, rifaximin, bacitracin, virginiamycin, thulathromycin
Lachnospiraceae (+)	9	clindamycin (n=3), amoxicillin, ciprofloxacin, vancomycin, vancomycin-imipenem, monensin-tylosin, AB cocktail	6	cefprozil, virginiamycin, penicillin, vancomycin, penicillin-vancomycin, ASP250
Blautia (+)	4	Enrofloxacin, levofloxacin, chlortetracycline-sulfa, HZRE	3	ciprofloxacin (n=3)
Coprococcus (+)	7	clindamycin (n=2), vancomycin, amoxicillin, HZRE, AB cocktail	2	ciprofloxacin, levofloxacin
Roseburia (+)	6	clindamycin (n=2), rifaximin, ciprofloxacin, vancomycin-imipenem, chlortetracycline-sulfa	1	ciprofloxacin
Ruminococcaceae (+)	13	ciprofloxacin (n=2), vancomycin, vancomycin-imipenem, monensin-tylosin, clindamycin, cefotaxime, cefoperazone, clindamycin, AB cocktail (n=2), HZRE, HZR	3	streptomycin, ciprofloxacin, chlortetracycline-sulfa
Enterobacteriaceae (-)	4	amoxicillin, meropenem, cefotaxime, enrofloxacin, ciprofloxacin, colistin,	17	cefprozil, amoxicillin, cefaclor, cefotaxime, ampicillin, titarcillin-clav, ampicillin-gentamicin (n=2), tigecycline, clindamycin, azithromycin, vancomycin, vancomycin-imipenem chlortetracycline-sulfa, chlortetracycline-salinomycin, AB cocktail (n=2)
Akkermansia (-)	0	-	6	vancomycin (n=2), tigecycline, moxifloxacin, ciprofloxacin, AB cocktail

The effect of antibiotic categories

The initial microbial structure that shapes the changes in microbiota during and after an antibiotic course is crucial. Apart from that, internal and external factors, such as the target specificity and spectrum, pharmacokinetic and pharmacodynamic properties, route of administration, and dosage and duration of treatment with antibiotics also greatly affect the gut microbiota (Jernberg et al., 2010; Rashid et al., 2015; Zhao et al., 2014). There are a number of studies with different classes of antibiotics which further emphasize this point (Yao et al., 2016; Yin et al., 2015; Zaura et al., 2015). We hypothesized that bactericidal and broad-spectrum antibiotics would affect gut microbiota severely, in contrast with bacteriostatic and narrow-spectrum antibiotics. However, that generalised opinion is not relevant in some cases. We observed microbial diversity was more affected by the dosage (the therapeutic or sub therapeutic dosage of antibiotics) rather than other factors, with some exceptions. Therapeutic antibiotic dosage is associated with decreasing microbial richness and diversity, while sub therapeutic levels of use are most likely to increase microbial richness. In therapeutic antibiotic usage, broad-spectrum antibiotics are most likely to have a prolonged effect after cessation, with variable recovery times. When narrow-spectrum antibiotics were administered, gut microbiota were able to recover easily, despite ongoing treatment.

Indeed, we also observed that specific antibiotic classes also affect specific bacterial taxa, as discussed previously. For instance, fluoroquinolones negatively affect Gram-negative *Alistipes* and *Faecalibacterium*, while beta-lactams were associated with proliferation of Bacteriodes and Enterobacteriaceae, as reported by many studies (Table 4). In another study, the class of antibiotics significantly shaped the microbiota on the basis of the antimicrobial effect (bactericidal or bacteriostatic) and the mode of action (Perez-Cobas et al., 2013). The categorisation is not absolute as some antibiotics that are bactericidal against certain organisms may only be bacteriostatic against others and vice versa.

In terms of directional changes of microbial structure (beta diversity), antibiotic class may have a greater role in any changes. Antibiotics in the same class were nearest neighbours: for instance, administration of tetracyclines (doxycycline and tetracycline) and fluoroquinolones (ciprofloxacin and norfloxacin) resulted in divergent responses (Maurice et al., 2013). Different patterns of microbial structure changes were also observed in another murine study. Ofloxacin and azithromycin were closely clustered as they have a minor impact on gut microbiota, while beta-lactams (ceftriaxone, cefoperazone-sulbactam and meropenem) and vancomycin (glycopeptides) showed clearly different trajectories as visualised through

principal coordinates analysis (Yin et al., 2015). Based on redundancy analysis (RDA), gut microbiota in individuals treated with beta-lactam antibiotics (ampicillin-sulbactam, cefazolin, ceftazidime and ceftriaxone) were significantly different from those treated with fluoroquinolones (ciprofloxacin and moxifloxacin) (Knecht et al., 2014).

Conclusion

Antibiotics are agents that naturally kill bacteria, providing not only beneficial clinical outcomes by treating infectious disease but may also adversely affect gut affecting gut microbial communities and structures. Even though exact comparisons of metagenomics studies seem difficult because of the differences in experimentation, methodologies and analysis, we observed reproducible results in terms of the effects of antibiotic use on microbial diversity and structure, and common bacterial taxa. In general, each antibiotic affects gut microbiota differently, mainly depending on the therapeutic use and the antibiotic's properties (i.e. pharmacodynamics and pharmacokinetics). The resilience and recovery time for gut microbiota after antibiotic administration were varied across individuals and bacterial taxa, which in most cases settled into a new steady abundance profile. The results from the studies stressed that the general health of gut microbiota should be taken into consideration when prescribing antibiotics, as they may induce proliferation of opportunistic bacteria, leading to changes in microbial function and immune development.

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Competing interest

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References

Abecia, L., Rodríguez-Romero, N., Martínez-Fernández, G., Martínez-Vallespín, B., Fondevila, M., 2017. Pyrosequencing study of caecal bacterial community of rabbit does and kits from a farm affected by epizootic rabbit enteropathy. World Rabbit Sci. 25, 261.

Abeles, S.R., Jones, M.B., Santiago-Rodriguez, T.M., Ly, M., Klitgord, N., Yooseph, S., Nelson, K.E., Pride, D.T., 2016. Microbial diversity in individuals and their household contacts following typical antibiotic courses. Microbiome 4, 39.

Allali, I., Arnold, J.W., Roach, J., Cadenas, M.B., Butz, N., Hassan, H.M., Koci, M., Ballou, A., Mendoza, M., Ali, R., Azcarate-Peril, M.A., 2017. A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. BMC Microbiol. 17, 194.

Antonopoulos, D.A., Huse, S.M., Morrison, H.G., Schmidt, T.M., Sogin, M.L., Young, V.B., 2009. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect. Immun. 77, 2367-2375.

Backhed, F., Fraser, C.M., Ringel, Y., Sanders, M.E., Sartor, R.B., Sherman, P.M., Versalovic, J., Young, V., Finlay, B.B., 2012. Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. Cell Host Microbe 12, 611-622.

Bassis, C.M., Theriot, C.M., Young, V.B., 2014. Alteration of the murine gastrointestinal microbiota by tigecycline leads to increased susceptibility to *Clostridium difficile* infection. Antimicrob. Agents Chemother. 58, 2767–2774.

Biddle, A., Stewart, L., Blanchard, J., Leschine, S., 2013. Untangling the genetic Basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity 5, 627-640.

Bragg, L., Tyson, G.W. 2014. Metagenomics using next-generation sequencing, In: Paulsen, I., Holmes, A. (Eds.) Environmental Microbiology: Methods and Protocols. Humana Press, Sydney, Australia, 183-201.

Buffie, C.G., Jarchum, I., Equinda, M., Lipuma, L., Gobourne, A., Viale, A., Ubeda, C., Xavier, J., Pamer, E.G., 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. Infect. Immun. 80, 62-73.

Cho, I., Yamanishi, S., Cox, L., Methe, B.A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I., Li, H., Alekseyenko, A.V., Blaser, M.J., 2012. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature 488, 621-626.

Choo, J.M., Kanno, T., Zain, N.M., Leong, L.E., Abell, G.C., Keeble, J.E., Bruce, K.D., Mason, A.J., Rogers, G.B., 2017. Divergent relationships between fecal microbiota and metabolome following distinct antibiotic-induced disruptions. mSphere 2, 1-16.

Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkila, J., Marchesi, J.R., Smidt, H., de Vos, W.M., Ross, R.P., O'Toole, P.W., 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4, e6669.

Costa, M.C., Bessegatto, J.A., Alfieri, A.A., Weese, J.S., Filho, J.A., Oba, A., 2017. Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. PLoS One 12, e0171642.

Cotter, P.D., Stanton, C., Ross, R.P., Hill, C., 2012. The impact of antibiotics on the gut microbiota as revealed by high throughput DNA sequencing. Discov. Med. 13, 193-199.

Cox, L.M., Yamanishi, S., Sohn, J., Alekseyenko, A.V., Leung, J.M., Cho, I., Kim, S.G., Li, H., Gao, Z., Mahana, D., Zarate Rodriguez, J.G., Rogers, A.B., Robine, N., Loke, P., Blaser, M.J., 2014. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell 158, 705-721.

Daniluk, J., Daniluk, U., Rusak, M., Dabrowska, M., Reszec, J., Garbowicz, M., Huminska, K., Dabrowski, A., 2017. The effect of penicillin administration in early life on murine gut microbiota and blood lymphocyte subsets. Anaerobe 47, 18-24.

Dethlefsen, L., Huse, S., Sogin, M.L., Relman, D.A., 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6, e280.

Dethlefsena, L., Relman, D.A., 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. PNAS 108, 4554–4561.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic. Acids. Res. 45(W1), W180-W188.

Dubourg, G., Lagier, J.C., Armougom, F., Robert, C., Audoly, G., Papazian, L., Raoult, D., 2013. High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment. Int. J. Antimicrob. Agents 41, 149-155.

Ellis, R.J., Bruce, K.D., Jenkins, C., Stothard, J.R., Ajarova, L., Mugisha, L., Viney, M.E., 2013. Comparison of the distal gut microbiota from people and animals in Africa. PLoS One 8, 1-11.

Fouhy, F., Guinane, C.M., Hussey, S., Wall, R., Ryan, C.A., Dempsey, E.M., Murphy, B., Ross, R.P., Fitzgerald, G.F., Stanton, C., Cottera, P.D., 2012. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob. Agents. Chemother. 56, p. 5811–5820.

Francino, M.P., 2015. Antibiotics and the human gut microbiome: dysbioses and acumulation of resistances. Front Microbiol. 6, e1543.

Fuerst, J.A., 2014. Microorganisms-a Journal and a anifying concept for the science of microbiology. Microorganisms 2, 140-146.

Gao, P., Ma, C., Sun, Z., Wang, L., Huang, S., Su, X., Xu, J., Zhang, H., 2017. Feed-additive probiotics accelerate yet antibiotics delay intestinal microbiota maturation in broiler chicken. Microbiome 5, 91.

Gasparrini, A.J., Crofts, T.S., Gibson, M.K., Tarr, P.I., Warner, B.B., Dantas, G., 2016. Antibiotic perturbation of the preterm infant gut microbiome and resistome. Gut Microbes. 7, 443-449.

Gibson, M.K., Crofts, T.S., Dantas, G., 2015. Antibiotics and the developing infant gut microbiota and resistome. Curr. Opin. Microbiol. 27, 51-56.

Gibson, M.K., Wang, B., Ahmadi, S., Burnham, C.A., Tarr, P.I., Warner, B.B., Dantas, G., 2016. Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. Nat. Microbiol. 1, 16024.

Greenhalgh, K., Meyer, K.M., Aagaard, K.M., Wilmes, P., 2016. The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. Environ. Microbiol. 18, 2103-2116.

Greenwood, C., Morrow, A.L., Lagomarcino, A.J., Altaye, M., Taft, D.H., Yu, Z., Newburg, D.S., Ward, D.V., Schibler, K.R., 2014. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of *Enterobacter*. J. Pediatr. 165, 23-29.

Guinane, C.M., Cotter, P.D., 2013. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Therap. Adv. Gastroenterol. 6, 295 –308.

Hansen, C.H., Krych, L., Nielsen, D.S., Vogensen, F.K., Hansen, L.H., Sorensen, S.J., Buschard, K., Hansen, A.K., 2012. Early life treatment with vancomycin propagates *Akkermansia muciniphila* and reduces diabetes incidence in the NOD mouse. Diabetologia 55, 2285-2294.

Hegde, N.V., Kariyawasam, S., DebRoy, C., 2016. Comparison of antimicrobial resistant genes in chicken gut microbiome grown on organic and conventional diet. Vet. Anim. Sci. 1-2, 9-14.

Hill, D.A., Hoffmann, C., Abt, M.C., Du, Y., Kobuley, D., Kirn, T.J., Bushman, F.D., Artis, D., 2010. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol. 3, 148-158.

Ianiro, G., Tilg, H., Gasbarrini, A., 2016. Antibiotics as deep modulators of gut microbiota: between good and evil. Gut 65, 1906-1915.

Isaac, S., Scher, J.U., Djukovic, A., Jimenez, N., Littman, D.R., Abramson, S.B., Pamer, E.G., Ubeda, C., 2017. Short- and long-term effects of oral vancomycin on the human intestinal microbiota. J. Antimicrob. Chemother. 72, 128-136.

Jakobsson, H.E., Jernberg, C., Andersson, A.F., Sjolund-Karlsson, M., Jansson, J.K., Engstrand, L., 2010. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS One 5, e9836.

Jernberg, C., Lofmark, S., Edlund, C., Jansson, J.K., 2010. Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiol. 156, 3216-3223.

Khan, I., Azhar, E.I., Abbas, A.T., Kumosani, T., Barbour, E.K., Raoult, D., Yasir, M., 2016. Metagenomic analysis of antibiotic-induced changes in gut microbiota in a pregnant rat model. Front Pharmacol. 7, 104.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J., Isaacson, R.E., 2012. Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. Proc. Natl. Acad. Sci. USA 109, 15485-15490.

Knecht, H., Neulinger, S.C., Heinsen, F.A., Knecht, C., Schilhabel, A., Schmitz, R.A., Zimmermann, A., dos Santos, V.M., Ferrer, M., Rosenstiel, P.C., Schreiber, S., Friedrichs, A.K., Ott, S.J., 2014. Effects of beta-lactam antibiotics and fluoroquinolones on human gut microbiota in relation to *Clostridium difficile* associated diarrhea. PLoS One 9, e89417.

Korpela, K., Salonen, A., Virta, L.J., Kekkonen, R.A., Forslund, K., Bork, P., de Vos, W.M., 2016. Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children. Nat. Commun. 7, 10410.

Lagier, J.C., Million, M., Hugon, P., Armougom, F., Raoult, D., 2012. Human gut microbiota: repertoire and variations. Front Cell Infect. Microbiol. 2, 136.

Langdon, A., Crook, N., Dantas, G., 2016. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. Genome Med. 8, 39.

Lankelma, J.M., Cranendonk, D.R., Belzer, C., Vos, A.F.d., Vos, W.M.d., Poll, T.v.d., Wiersinga, W.J., 2017. Antibiotic-induced gut microbiota disruption during human endotoxemia: a randomised controlled study. Gut 66, 1623–1630.

Leekha, S., Terrell, C.L., Edson, R.S., 2011. General principles of antimicrobial therapy. Mayo Clin. Proc. 86, 156-167.

Li, J., Hao, H., Cheng, G., Liu, C., Ahmed, S., Shabbir, M.A.B., Hussain, H.I., Dai, M., Yuan, Z., 2017a. Microbial shifts in the intestinal microbiota of *Salmonella* infected chickens in response to enrofloxacin. Front Microbiol. 8, 1711.

Li, K., Xiao, Y., Chen, J., Chen, J., He, X., Yang, H., 2017b. Microbial composition in different gut locations of weaning piglets receiving antibiotics. Asian-Australas. J. Anim. Sci. 30, 78-84.

Li, R., Wang, H., Shi, Q., Wang, N., Zhang, Z., Xiong, C., Liu, J., Chen, Y., Jiang, L., Jiang, Q., 2017c. Effects of oral florfenicol and azithromycin on gut microbiota and adipogenesis in mice. PLoS One 12, e0181690.

Lichtman, J.S., Ferreyra, J.A., Ng, K.M., Smits, S.A., Sonnenburg, J.L., Elias, J.E., 2016. Host-microbiota interactions in the pathogenesis of antibiotic-associated diseases. Cell Rep. 14, 1049-1061.

Lloyd-Price, J., Abu-Ali, G., Huttenhower, C., 2016. The healthy human microbiome. Genome Med 8, 51.

Looft, T., Allen, H.K., Cantarel, B.L., Levine, U.Y., Bayles, D.O., Alt, D.P., Henrissat, B., Stanton, T.B., 2014. Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. ISME J 8, 1566-1576.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M., Stanton, T.B., 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci USA 109, 1691-1696.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., Knight, R., 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489, 220-230.

Matar, G.M., 2017. Editorial: Emerging Enterobacteriaceae infections: antibiotic resistance and novel treatment options. Front Microbiol. 8, 509.

Maurice, C.F., Haiser, H.J., Turnbaugh, P.J., 2013. Xenobiotics shape the physiology and gene expression of the active human gut microbiome. Cell 152, 39-50.

McDonald, L.C., 2017. Effects of short- and long-course antibiotics on the lower intestinal microbiome as they relate to traveller's diarrhea. J. Travel. Med. 24, S35-S38.

Mitra, S., Chilton, C., Freeman, J., Henry Wood, Quirke, P., Taylor, M., Vickers, R., Wilcox, M., 2017. Preservation of gut microbiome following ridinilazole vs. fidaxomicin treatment of *Clostridium difficile* infection. Open Forum Infect. Dis. 4, S526–S527.

Montandon, S.A., Jornayvaz, F.R., 2017. Effects of antidiabetic drugs on gut microbiota composition. Genes (Basel) 8.

Morowitz, M.J., Carlisle, E.M., Alverdy, J.C., 2011. Contributions of intestinal bacteria to nutrition and metabolism in the critically ill. Surg. Clin. North Am. 91, 771-785, viii.

Namasivayam, S., Maiga, M., Yuan, W., Thovarai, V., Costa, D.L., Mittereder, L.R., Wipperman, M.F., Glickman, M.S., Dzutsev, A., Trinchieri, G., Sher, A., 2017. Longitudinal profiling reveals a persistent intestinal dysbiosis triggered by conventional anti-tuberculosis therapy. Microbiome 5, 71.

Nemeth, J., Oesch, G., Kuster, S.P., 2015. Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: systematic review and meta-analysis. J. Antimicrob. Chemother. 70, 382-395.

Neumann, A.P., Suen, G., 2015. Differences in major bacterial populations in the intestines of mature broilers after feeding virginiamycin or bacitracin methylene disalicylate. J. Appl. Microbiol. 119, 1515-1526.

Nguyen, T.L., Vieira-Silva, S., Liston, A., Raes, J., 2015. How informative is the mouse for human gut microbiota research? Dis. Model Mech. 8, 1-16.

Oulas, A., Pavloudi, C., Polymenakou, P., Pavlopoulos, G.A., Papanikolaou, N., Kotoulas, G., Arvanitidis, C., Iliopoulos, I., 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. Bioinform. Biol. Insights 9, 75-88.

Panda, S., El khader, I., Casellas, F., Lopez Vivancos, J., Garcia Cors, M., Santiago, A., Cuenca, S., Guarner, F., Manichanh, C., 2014. Short-term effect of antibiotics on human gut microbiota. PLoS One 9, e95476.

Perez-Cobas, A.E., Artacho, A., Knecht, H., Ferrus, M.L., Friedrichs, A., Ott, S.J., Moya, A., Latorre, A., Gosalbes, M.J., 2013. Differential effects of antibiotic therapy on the structure and function of human gut microbiota. PLoS One 8, e80201.

Ponziani, F.R., Scaldaferri, F., Petito, V., Paroni Sterbini, F., Pecere, S., Lopetuso, L.R., Palladini, A., Gerardi, V., Masucci, L., Pompili, M., Cammarota, G., Sanguinetti, M., Gasbarrini, A., 2016. The role of antibiotics in gut microbiota modulation: the eubiotic effects of rifaximin. Dig. Dis. 34, 269-278.

Ponziani, F.R., Zocco, M.A., D'Aversa, F., Pompili, M., Gasbarrini, A., 2017. Eubiotic properties of rifaximin: Disruption of the traditional concepts in gut microbiota modulation. World J. Gastroenterol. 23, 4491-4499.

Pop, M., Paulson, J.N., Chakraborty, S., Astrovskaya, I., Lindsay, B.R., Li, S., Bravo, H.C., Harro, C., Parkhill, J., Walker, A.W., Walker, R.I., Sack, D.A., Stine, O.C., 2016. Individual-specific changes in the human gut microbiota after challenge with enterotoxigenic *Escherichia coli* and subsequent ciprofloxacin treatment. BMC Genomics 17, 440.

Rafii, F., Sutherland, J.B., Cerniglia, C.E., 2008. Effects of treatment with antimicrobial agents on the human colonic microfl ora. Therapeutics and Clinical Risk Management 4, 1343–1357.

Rashid, M.-U., Zaura, E., Buijs, M.J., Keijser, B.J.F., Crielaard, W., Nord, C.E., Weintraub, A., 2015. Determining the long-term effect of antibiotic administration on the human normal intestinal microbiota using culture and pyrosequencing methods. CID 60, S77-S84.

Raymond, F., Deraspe, M., Boissinot, M., Bergeron, M.G., Corbeil, J., 2016a. Partial recovery of microbiomes after antibiotic treatment. Gut Microbes 7, 428-434.

Raymond, F., Ouameur, A.A., Deraspe, M., Iqbal, N., Gingras, H., Dridi, B., Leprohon, P., Plante, P.L., Giroux, R., Berube, E., Frenette, J., Boudreau, D.K., Simard, J.L., Chabot, I., Domingo, M.C., Trottier, S., Boissinot, M., Huletsky, A., Roy, P.H., Ouellette, M., Bergeron, M.G., Corbeil, J., 2016b. The initial state of the human gut microbiome determines its reshaping by antibiotics. ISME J 10, 707-720.

Rea, M.C., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P.D., Shanahan, F., Kiely, B., Hill, C., Ross, R.P., 2011. Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. Proc. Natl. Acad. Sci. USA 108 Suppl 1, 4639-4644.

Reijnders, D., Goossens, G.H., Hermes, G.D., Neis, E.P., van der Beek, C.M., Most, J., Holst, J.J., Lenaerts, K., Kootte, R.S., Nieuwdorp, M., Groen, A.K., Olde Damink, S.W.,

Boekschoten, M.V., Smidt, H., Zoetendal, E.G., Dejong, C.H., Blaak, E.E., 2016. Effects of gut microbiota manipulation by antibiotics on host metabolism in obese humans: A randomized double-blind placebo-controlled trial. Cell Metab. 24, 63-74.

Robinson, C.J., Bohannan, B.J., Young, V.B., 2010. From structure to function: the ecology of host-associated microbial communities. Microbiol. Mol. Biol. Rev. 74, 453-476.

Rogers, M.A.M., Aronoff, D.M., 2016. The influence of non-steroidal anti-inflammatory drugs on the gut microbiome. Clin. Microbiol. Infect. 22, 178 e171-178 e179.

Sarpong, E.M., Miller, G.E., 2014. Narrow- and broad spectrum antibiotic use among U.S. children. Health Services Research 50, 830-846.

Schokker, D., Jansman, A.J., Veninga, G., de Bruin, N., Vastenhouw, S.A., de Bree, F.M., Bossers, A., Rebel, J.M., Smits, M.A., 2017. Perturbation of microbiota in one-day old broiler chickens with antibiotic for 24 hours negatively affects intestinal immune development. BMC Genomics 18, 241.

Schokker, D., Zhang, J., Zhang, L.L., Vastenhouw, S.A., Heilig, H.G., Smidt, H., Rebel, J.M., Smits, M.A., 2014. Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. PLoS One 9, e100040.

Sender, R., Fuchs, S., Milo, R., 2016. Revised estimates for the number of human and bacteria cells in the body. PLoS Biol 14, e1002533.

Soldi, S., Vasileiadis, S., Uggeri, F., Campanale, M., Morelli, L., Fogli, M.V., Calanni, F., Grimaldi, M., Gasbarrini, A., 2015. Modulation of the gut microbiota composition by rifaximin in non-constipated irritable bowel syndrome patients: a molecular approach. Clin. Exp. Gastroenterol. 8, 309-325.

Stanley, D., Hughes, R.J., Geier, M.S., Moore, R.J., 2016. Bacteria within the gastrointestinal tract microbiota correlated with improved growth and feed conversion: challenges presented for the identification of performance enhancing probiotic bacteria. Front Microbiol. 7, 187.

Stewardson, A.J., Gaia, N., Francois, P., Malhotra-Kumar, S., Delemont, C., Martinez de Tejada, B., Schrenzel, J., Harbarth, S., Lazarevic, V., Saturn, W.P., Groups, W.P.S., 2015. Collateral damage from oral ciprofloxacin versus nitrofurantoin in outpatients with urinary tract infections: a culture-free analysis of gut microbiota. Clin. Microbiol. Infect. 21, 344 e341-311.

Stokell, J.R., Gharaibeh, R.Z., Hamp, T.J., Zapata, M.J., Fodor, A.A., Steck, T.R., 2015. Analysis of changes in diversity and abundance of the microbial community in a cystic fibrosis patient over a multiyear period. J. Clin. Microbiol. 53, 237-247.

Thomas, M., Webb, M., Ghimire, S., Blair, A., Olson, K., Fenske, G.J., Fonder, A.T., Christopher-Hennings, J., Brake, D., Scaria, J., 2017. Metagenomic characterization of the effect of feed additives on the gut microbiome and antibiotic resistome of feedlot cattle. Sci. Rep. 7, 12257.

Tulstrup, M.V., Christensen, E.G., Carvalho, V., Linninge, C., Ahrne, S., Hojberg, O., Licht, T.R., Bahl, M.I., 2015. Antibiotic treatment affects intestinal permeability and gut microbial composition in wistar rats dependent on antibiotic class. PLoS One 10, e0144854.

van den Bogert, B., de Vos, W.M., Zoetendal, E.G., Kleerebezem, M., 2011. Microarray analysis and barcoded pyrosequencing provide consistent microbial profiles depending on the source of human intestinal samples. Appl. Environ. Microbiol. 77, 2071-2080.

Vrieze, A., Out, C., Fuentes, S., Jonker, L., Reuling, I., Kootte, R.S., van Nood, E., Holleman, F., Knaapen, M., Romijn, J.A., Soeters, M.R., Blaak, E.E., Dallinga-Thie, G.M., Reijnders, D., Ackermans, M.T., Serlie, M.J., Knop, F.K., Holst, J.J., van der Ley, C., Kema, I.P., Zoetendal, E.G., de Vos, W.M., Hoekstra, J.B., Stroes, E.S., Groen, A.K., Nieuwdorp, M., 2014. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. J. Hepatol. 60, 824-831.

Willing, B.P., Russell, S.L., Finlay, B.B., 2011. Shifting the balance: antibiotic effects on host-microbiota mutualism. Nat. Rev. Microbiol. 9, 233-243.

Wipperman, M.F., Fitzgerald, D.W., Juste, M.A.J., Taur, Y., Namasivayam, S., Sher, A., Bean, J.M., Bucci, V., S., M., Glickman, M.S., 2017. Antibiotic treatment for Tuberculosis induces a profound dysbiosis of the microbiome that persists long after therapy is completed. Sci. Rep. 7, 1-11.

Yao, J., Carter, R.A., Vuagniaux, G., Barbier, M., Rosch, J.W., Rock, C.O., 2016. A pathogen-selective antibiotic minimizes disturbance to the microbiome. Antimicrob. Agents Chemother. 60, 4264-4273.

Yin, J., M, P., Wang, S., Liao, S.X., Peng, X., He, Y., Chen, Y.R., Shen, H.F., Su, J., Chen, Y., Jiang, Y.X., Zhang, G.X., Zhou, H.W., 2015. Different dynamic patterns of beta-Lactams, quinolones, glycopeptides and macrolides on mouse gut microbial diversity. PLoS One 10, e0126712.

Zaura, E., Brandt, B.W., Teixeira de Mattos, M.J., Buijs, M.J., Caspers, M.P., Rashid, M.U., Weintraub, A., Nord, C.E., Savell, A., Hu, Y., Coates, A.R., Hubank, M., Spratt, D.A., Wilson, M., Keijser, B.J., Crielaard, W., 2015. Same exposure but two radically different responses to antibiotics: resilience of the salivary microbiome versus long-term microbial shifts in feces. MBio 6, e01693-01615.

Zhang, D., Ji, H., Liu, H., Wang, S., Wang, J., Wang, Y., 2016. Changes in the diversity and composition of gut microbiota of weaned piglets after oral administration of Lactobacillus or an antibiotic. Appl. Microbiol. Biotechnol. 100, 10081-10093.

Zhao, J., Murray, S., Lipuma, J.J., 2014. Modeling the impact of antibiotic exposure on human microbiota. Sci. Rep. 4, 4345.

Chapter 6

Faecal microbiota profiling in rabbits following single and multiple doses of oral pradofloxacin

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Faecal microbiota profiling in rabbits following single and multiple doses of oral pradofloxacin

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Abstract

Pradofloxacin is a third-generation veterinary fluoroquinolone with an enhanced spectrum of activity against a wide range of bacteria, including anaerobes. *In vitro* activity and clinical efficacy of pradofloxacin has been widely reported, however, the effect of pradofloxacin on gut microbiota has not been investigated. This study aimed to determine the effects of pradofloxacin on the rabbit faecal microbiota. A single dose of 7.5mg/kg pradofloxacin was administered per os to four female rabbits in one treatment group, while three daily doses of 7.5 mg/kg pradofloxacin were administered to four male rabbits in a second treatment group. Hard and soft pellets were collected during the trial period and microbial community changes were assessed by V3-V4 16S rRNA gene sequencing. Overall, pradofloxacin significantly affected the relative abundance of 34% of the initial composition of the gut microbial community. After treatment, microbial richness was reduced to below detectable limits for 28% and 30% of the autochthonous microbial species in single and multiple dosing groups, respectively. Significantly decreased abundance was observed in several genera belonging to the Gammaproteobacteria (*Pseudomonas* spp. and *Oceanisphaera* spp.) while significantly increased abundance was detected in genera belonging to Ruminococcaceae and Lachnospiraceae. Between faecal types, hard faeces contained higher microbial richness than soft faeces after pradofloxacin treatment, however, soft faeces were more enriched with Ruminococcaceae. Clinically adverse effects such as diarrhoea or impaction were observed in neither single nor multiple pradofloxacin treatment groups. Additionally, no overproliferation of bacteria (i.e. *Clostridium spiriforme* and *Escherichia coli*) that cause enteritis in lagomorphs was observed. This study confirmed that short-term administration of oral pradofloxacin 2.5% suspension appears to be safe in rabbits

at a dose of 7.5 mg/kg. However, oral pradofloxacin treatment does result in significant changes in approximately one third of the bacterial taxa that comprise the faecal microbiota.

Keywords: pradofloxacin, metagenomics, microbiota, microbial diversity, rabbit

Introduction

Pradofloxacin is a third-generation veterinary fluoroquinolone with an improved activity against a wide range of bacteria, including both Gram-positive and -negative organisms, anaerobes, *Mycoplasma* spp. and some intracellular organisms such as *Rickettsia* spp. and *Mycobacterium* spp. (Lees, 2013; Sykes and Blondeau, 2014). It is administered orally and has been used effectively for treating urinary tract, skin and soft tissue, oral and respiratory infections in dogs and cats (Papich, 2016). Pradofloxacin differs from the other fluoroquinolones registered for use in veterinary medicine by inclusion of a bicyclic amine, *S*,*S*-pyrrolidinopiperidine in place of an ethylpiperazine moiety at position C7; and differs from moxifloxacin, a similar third-generation fluoroquinolone registered for human use, by possession of a cyano group at position C8 (Wetzstein and Hallenbach, 2011). These differences in chemical structure contribute to a wider spectrum of activity, and increased potency of pradofloxacin, resulting in both lower minimum inhibitory concentrations (MICs) and mutant prevention concentrations (MPCs) for both *Staphylococcus* spp. and *Escherichia coli* compared to the other veterinary fluoroquinolones (Silley et al., 2012; Wetzstein and Hallenbach, 2011).

Pradofloxacin has been approved in the European Union, Canada and Australia for the treatment of bacterial infections in dogs and cats (Pallo-Zimmerman et al., 2010; Papich, 2016; Schink et al., 2013), but is only approved for use in cats in the USA (Papich, 2016). The *in vitro* activity, physico-chemical, pharmacological, toxicological and therapeutic properties of pradofloxacin have been well studied (Lees, 2013; Silley et al., 2012). However, compared to the other fluoroquinolones, the effect of pradofloxacin on the gastrointestinal tract microbiota has not previously been evaluated. Fluoroquinolones are generally regarded as safe for use in rabbits, due to their lack of activity against strict anaerobes, but it is possible that pradofloxacin with its enhanced spectrum of activity may have unintended consequences on the gut microbiota of rabbits, and potentially other caecotrophic and/or hindgut fermenting mammals such as cavies and other rodent species that are also prone to antibiotic induced gut dysbiosis (Campbell-Ward, 2012; DeCubellis and Graham, 2013).

As a caecothropic mammal, the rabbit excretes two types of faeces; hard faeces that contains indigestible fiber and soft faeces (caecotrophs) that are enriched with nutrients as a

result of caecal fermentation (Campbell-Ward, 2012; Halls, 2008). The microbial community that comprises the rabbit hard faeces and caecotroph microbiota has been reported in several recent studies and is mainly dominated by Firmicutes and Bacteriodetes (Crowley et al., 2017; Eshar and Weese, 2014; Zeng et al., 2015). Compared to rabbit hard faeces, several bacterial taxa including members of the Ruminococcaceae and Barnesiellaceae families, *Akkermansia* spp., *Blautia* spp., and *Lactococcus* spp. were more abundant in caecotrophs (soft faeces) (Zeng et al., 2015). Here, we aimed to characterise the microbial community changes in both hard and soft rabbit faeces following single and multiple doses of pradofloxacin oral suspension using molecular-based methods (metagenomics). In particular, we focused on populations of bacteria known to cause enteritis in rabbits resulting from oral antibiotic-induced gut microbiota dysbiosis such as *Clostridium spiriforme* and *E. coli* (Agnoletti et al., 2009; Borriello and Carman, 1983; Oglesbee and Jenkins, 2012; Raw, 2017). Furthermore, we predicted the functional properties of the microbial community in both types of faecal samples for a deeper exploration of the effects of pradofloxacin exposure on rabbit gastrointestinal physiology and nutrition.

Materials and Methods

Animal experiments. All experimental procedures were conducted with the approval of the University of Adelaide Animal Ethics Committee (S-2015-165). Eight healthy New Zealand White crossbred rabbits were sourced from the control group of a previous feeding trial and had been fed a commercial pelleted diet (Barastoc; crude protein 16.5%, crude fat 3%, and crude fibre 21% with added vitamins and minerals) for at least one month prior to trial commencement and had a starting weight of between 2.8-3.5kg. The trial was conducted at the Animal Desexing Clinic, 604 Port Rd, Allenby Gardens, Adelaide, Australia. A single dose of 7.5 mg/kg of pradofloxacin oral suspension 25 mg/ml (Veroflox™) was administered to four female rabbits in one treatment group while a single dose of 7.5 mg/kg pradofloxacin was given to four male rabbits daily for three days in the other treatment group. T0 samples were collected before the administration of the first dose of pradofloxacin (Day 1). Hard pellets were aseptically collected from the floor below the cages of individually housed animals each morning of the trial immediately after passage. Soft faeces samples were collected opportunistically each morning during the trial for four days. To obtain soft faeces samples, collars were made from malleable plastic hose and placed around the neck of the rabbit to prevent it from being able to reach the anus to ingest the soft faeces. During the trial, the

rabbits were monitored and examined for any clinical signs related to gastrointestinal tract problems such as such diarrhoea, inappetance or gut pain/impaction.

Library preparation and sequencing. In order to define changes in the faecal microbiota, we characterized the microbial communities of caecal and faecal samples of rabbits by targeting the V3-V4 region of the 16S rRNA gene using the Illumina MiSeq platform. Total bacterial DNA was extracted by using the PowerFecal™ DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). DNA concentration was standardised to 5ng/µL before amplification using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA). V3-V4 forward primer 5'ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG3' and reverse primer 5'TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC3' were used for Illumina 16S metagenomics library preparation according to the manufacturer's protocol. The underlined regions are the CS1 and CS2 Fluidigm adapter nucleotide sequences, followed by the locus specific sequences targeting conserved regions within the V3 and V4 (Klindworth et al., 2013). Amplified libraries were sequenced on an Illumina MiSeq platform at South Australian Health and Medical Research Institute (SAHMRI) Adelaide, Australia.

Metagenomic and statistical analysis. Bioinformatic analysis of bacterial 16S rRNA gene amplicon data was performed using the Quantitative Insights Into Microbial Ecology (QIIME) version 1 pipeline (Caporaso et al., 2010) and CLC Microbial Genomics Module in CLC Genomics Workbench (QIAGEN). Sequences were clustered into Operational Taxonomic Units (OTUs) based on 97% sequence similarity and then aligned against the SILVA reference database, as previously described (Alfano et al., 2015; Barker et al., 2013). Alpha diversity analysis was performed based on Shannon index and Chao1. Relative abundance of the observed species were compared between treatment and control groups with Mann-Whitney/Kruskal-Wallis tests to determine statistical significance. Comparative analysis was performed at different phylogenetic levels: OTU, species, genus, family, order, class, and phylum. Beta diversity of each sample group was assessed using Analysis of Group Similarities (ANOSIM), including both unweighted and weighted Unifrac distances and visualized by Principal Coordinate Analysis (PCoA). Differential abundance between time points was assessed using metagenomeSeq and normalised with cumulative sum scaling (CSS), the zero-inflated Gaussian method and fitFeatureModel and reach significant when $P<0.5$ and false discovery rate (FDR) <0.5 . LDA (Linear Discriminant Analysis) effect size (LEfSe) was performed to discover differentially abundant features as a biomarker in both hard and soft faeces. The metabolic potentials of microbial communities were predicted using Tax4Fun by transforming 16S rRNA gene sequencing data that have been annotated to a taxonomic profile of the

prokaryotic KEGG (Kyoto Encyclopedia of Genes and Genomes) organisms. Relative abundance of KEGG orthology IDs (KO) was produced and then subjected to shotgun data profiling (SDP). All statistical analysis was performed in Microbiomeanalyst (Dhariwal et al., 2017).

Results

16S rDNA profiling. A total of 2,416,867 V3-V4 16S rRNA gene sequences were obtained from 41 faecal samples (30 from hard faeces and 11 from soft faeces) as presented Table S1. After filtration (quality control, chimera checking, and trimming of reads with low quality), 1,884,224 high quality sequences were aligned and resulted in a total of 532,643 reads in OTUs with an average 12,991 reads per sample. The proportion of unclassified sequences was 2.7% of total reads (14,288 reads) and excluded in from the following analyses. Overall, 11 phyla were identified consisting of 20 classes, 63 families and 205 genera. Firmicutes was the dominant phyla in each sample group, with relative abundance ranged from 47.02%-90.87%, followed by Bacteriodetes (7.9%-24.38%). There was no impact of time point and type of treatment on the total number of OTUs that were identified in each sample group.

Population dynamics of bacterial community changes in the single pradofloxacin dose treatment group. Following a single dose of pradofloxacin on Day 1 (after T0 sampling), a trend of decreasing microbial diversity (indicated by both the observed number of species and reduced Shannon diversity index) in hard faeces was observed in the successive sampling time points, eventually reaching significance on Day 3 ($p<0.05$) (Fig 1 A-B). Microbial community structure also differed significantly on Day 3 as showed by Unifrac distance metric. At the phylum level, a significant change was observed in Proteobacteria ($p=0.002$, FDR=0.02), with the number of observed species decreasing by up to 28%. After data filtering and normalisation for metagenomeSeq analysis, we observed significant changes for approximately 27% of the total initial bacterial abundance, with a total of 21 genera supressed following pradofloxacin administration while only four genera were significantly overrepresented (Fig 2 A-B). The suppressed bacterial taxa with the most significant drop after treatment belonged to the Gammaproteobacteria class (*Pseudomonas* spp. and *Oceanisphaera* spp). Interestingly, all overrepresented bacterial genera occurred in Firmicutes belonging to the Clostridia class (Ruminococcaceae and Lachnospiraceae) and a small number of Actinobacteria (Coriobacteriaceae).

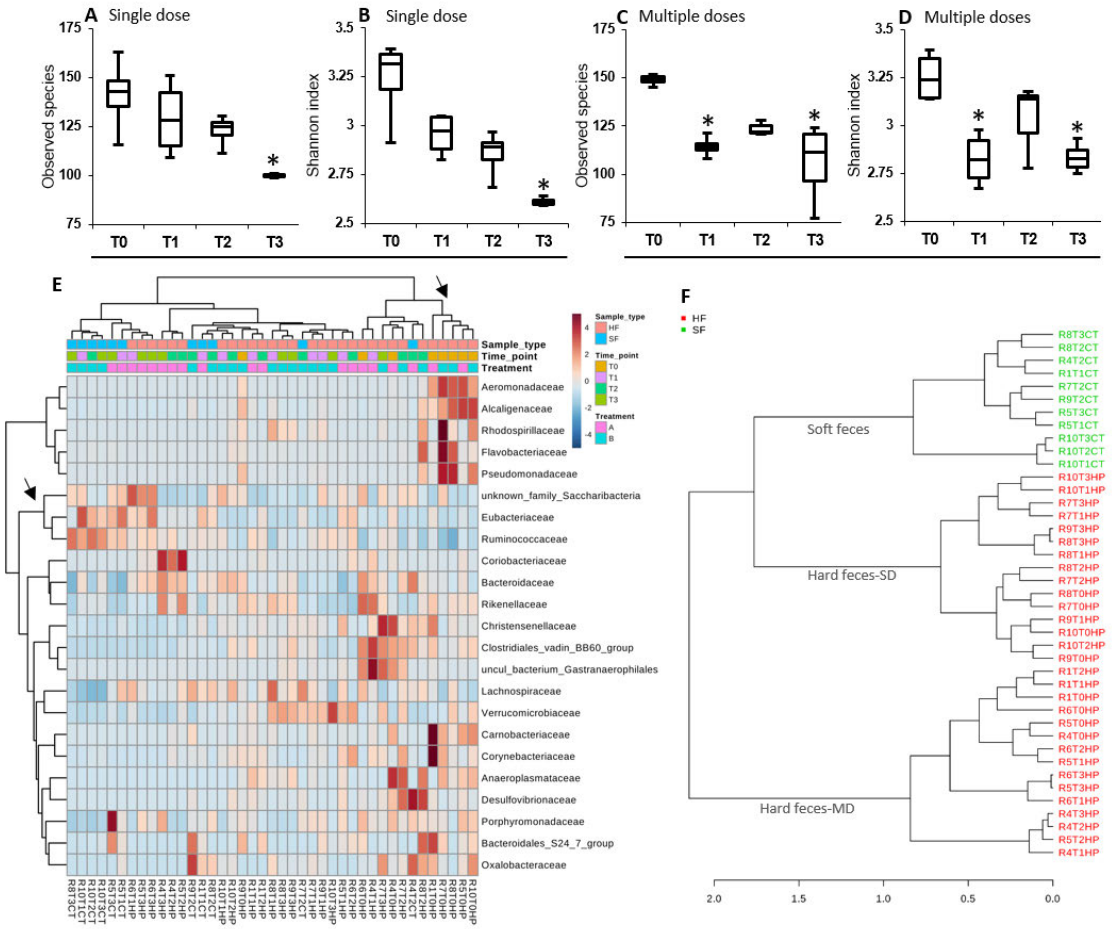

Figure 1. Effect of single dose and multiple doses pradofloxacin on microbial diversity and the clustering analysis of each sample. Alpha diversity measurement in faecal samples after single dose treatment (A-B) and multiple doses treatment (C-D). Asterisks (*) showed significantly different features when compared to before treatment samples (T0) using Mann-Whitney/Kruskal-Wallis methods ($p<0.05$). (E) Heatmap clustering analysis generated from selected bacterial taxa with proportion >1%. The arrows showed clustered bacterial that significantly different between before and after treatment and faecal types. The distances between data points and clusters were measured by Euclidean and Ward method. (F) Ward-clustered dendogram generated at OUT level using Bray-Curtis distance measure.

Population dynamics of bacterial communities in the multiple pradofloxacin dose treatment group. A fluctuation of diversity was observed in the multiple dose group where alpha diversity (observed species and Shannon index) was significantly decreased ($p<0.05$) on Day 1 and then slightly increased on Day 2 but eventually was also significantly decreased on Day 3 (Fig 1 C-D), leading to the emergence of a distinct microbial community structure compared to T0 samples. In general, multiple doses of pradofloxacin affected up to 34% of total abundance with the number of observed species decreased by up to 30% compared to pre-treatment samples. A significant reduction at the phylum level was observed in the

Proteobacteria (p=0.001 FDR=0.01). Analysis with metagenomeSeq showed a significant drop in a large variety of Gram-positive and -negative bacteria, but similar to the single dose treatment, the main bacterial taxa that were suppressed were from the Gammaproteobacteria while overrepresented bacterial taxa were Gram-positive bacteria from the Clostridia class only (Fig 2 C-D). In both single and multiple dose treatments, overproliferation of Enterobacteriaceae and Clostridiaceae, bacterial families containing species associated with enteritis in rabbits (i.e. *Escherichia coli* and *Clostridium spiriforme*), were not detected following pradofloxacin administration.

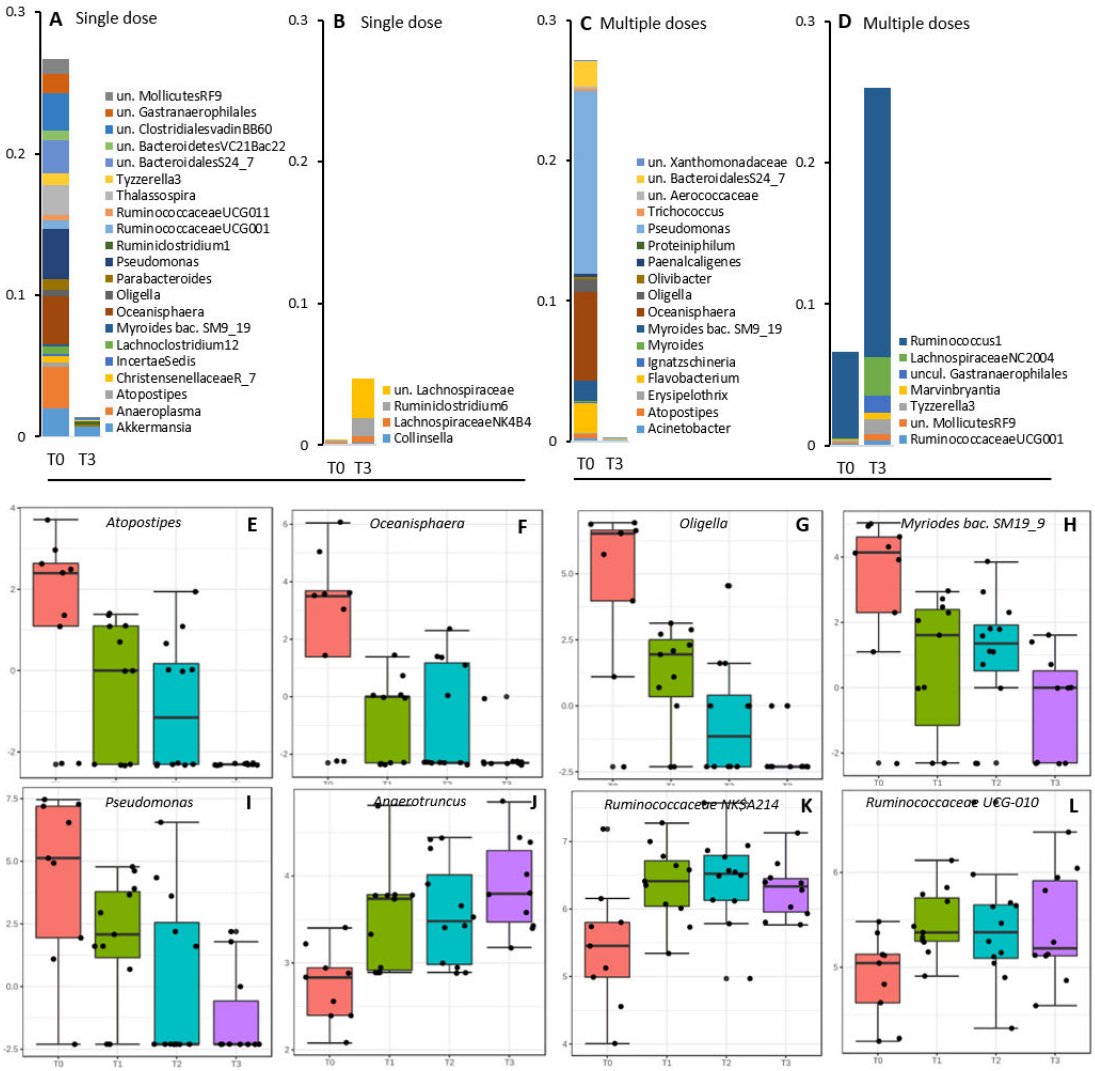

Figure 2. Bacterial taxa that were significantly (FDR p<0.05) affected by pradofloxacin treatment. Abundance of genus detected in hard faeces which significantly decreased (A) and increased (B) after single dose treatment; and abundance of genus which significantly decreased (C) and increased (D) after multiple doses treatment. (E-I) Log-transformed counts of bacterial taxa that consistently depleted in both single and multiple doses groups. (J-L) Bacterial taxa that increased after pooling both treatment samples. Analysis was performed using metagenomSeq-based method and the zero inflated-Gaussian fit statistical model.

Differences between single and multiple doses treatment. In both single and multiple dose treatment groups, we observed decreasing abundance of the genera *Atopostipes, Myriodes*, *Oceanisphaera, Oligella,* and *Pseudomonas*. Alpha diversity measurement showed that the reduction of microbial richness did not differ significantly between single and multiple dose treatment groups in both hard and soft faeces. However, based on ANOSIM unweighted Unifrac distance as visualised by PCoA (Fig 3 C-D), we observed dissimilarity in microbial community structure between the two treatment groups, in particular for microbial species composition.

Microbial community differences between hard and soft faecal samples. Due to the low number of caecotrophs obtained, it was neither possible to show the differences between before and after pradofloxacin treatments in soft faeces nor to directly compare soft and hard faeces over the majority of time points. However, comparative analysis could be performed on soft (n=11) and hard faecal samples (n=36) that were collected immediately following cessation of pradofloxacin treatment in the single and multiple dose groups. As demonstrated by observed species, Shannon index and Chao1, hard faeces contained significantly ($P<0.05$) more microbial richness and diversity compared to soft faeces in the multiple dose treatment group based on Mann-Whitney/Kruskal-Wallis tests (Figure 3 A-B and Table S3). In the single dose treatment group, a significantly higher microbial richness in hard faeces was confirmed by observed species only. In total, we identified 37 bacteria families in soft faeces that were also present in hard faeces, except for Clostridiaceae 1 that were uniquely identified in soft faeces only with total relative abundance of <0.2%. Additionally, microbial structure between hard and soft faeces was also distinct, as verified by the separated cluster identified by weighted Unifrac distance (Fig 3 D-E). Furthermore, LefSe performed after pooling all soft faeces and had faeces from both treatment groups confirmed that soft faeces were enriched with six out of seven genera from the Ruminococcaceae family (Fig 3 G).

Prediction of functional features in rabbit faecal microbial communities. Prediction of functional feature analysis in general metabolic pathways using Tax4Fun identified several significant features between sample groups. We observed enrichment of the citrate cycle (TCA cycle) functional capacity, but reduction of biotin metabolism and several xenobiotic degradations in post-treatment compared to pre-treatment samples (Table S5).

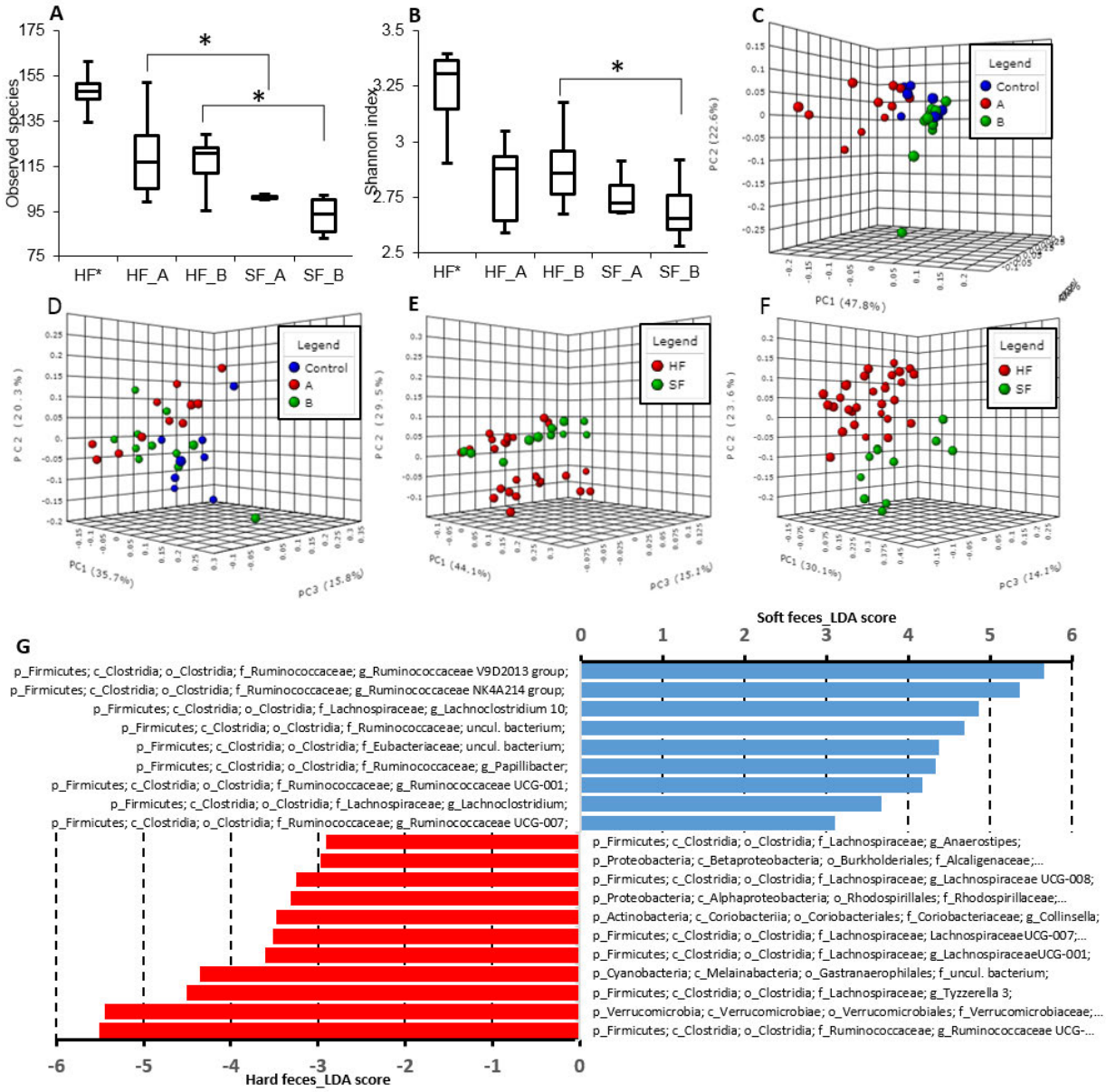

Figure 3. Different features observed between treatment groups and faecal types. Observed species in hard faeces in both single treatment (HF_A) and multiple treatment (HF_B) significantly higher compared to soft faeces (SF_A and SF_B) (Fig 3. A). Using Shannon index, alpha diversity of hard faeces also higher compared to soft faeces as showed in treatment B (multiple) group (* p<0.05, Mann-Whitney/Kruskal-Wallis test) (Fig 3.B.). Unweighted ([ANOSIM] R: 0.70214; p-value < 0.001, Fig 3. C.) and weighted ([ANOSIM] R: 0.53804; p-value < 0.001, Fig 3. D.) unifrac distance showing different microbial structure between single (A) and multiple doses (B) treatment groups. Unweighted ([ANOSIM] R: 0.63671; p-value < 0.001, Fig 3. E.) and weighted ([ANOSIM] R: 0.43978; p-value < 0.001, Fig 3. F.) uniftrac distance showing distinctive microbial tructure between fecal types after treatment. (G) Bacterial taxa significantly enriched in soft feces and hard feces based on LefSe, showed as log LDA score.

Discussion

This study reports the molecular assessment of faecal microbiota in rabbits in response to pradofloxacin treatment using an Illumina MiSeq platform. The main findings of this study were as follows: 1) pradofloxacin significantly altered up to 34% of the faecal microbial community and resulted in reduced microbial richness of up to 30%, leading to alterations in the predicted abundance of genes in several bacterial metabolic pathways; 2) pradofloxacin effectively supressed the abundance of both Gram-negative and Gram-positive bacteria, but did not induce proliferation of opportunistic bacteria associated with gut dysbiosis and enteritis in rabbits (e.g. *Clostridium* spp. and coliforms); 3) both single and multiple doses of pradofloxacin equally reduced faecal microbial diversity; and 4) following pradofloxacin treatment, soft faeces were lower in microbial richness when compared to hard faeces, however they still shared many similar microbial taxa.

To the best of our knowledge, this is the first study to investigate the effect of pradofloxacin treatment on the faecal microbiota in any animal species. Significant alterations in the faecal microbiota after pradofloxacin treatment confirms the ecological consequences associated with administration of antimicrobial agents in the rabbit gastrointestinal tract. As reported previously, administration of long term oral meloxicam, a non-steroidal anti-inflammatory drug (NSAID) for 21 days, did not adversely affect the faecal microbiota of New Zealand White rabbits (Eshar and Weese, 2014), in contrast with previous studies involving oral administration of several classes of antimicrobial agent (Katz et al., 1978) and our study of pradofloxacin. Oral treatment with other members of the fluoroquinolone class has been reported to cause a significant reduction in gut microbial richness and evenness in several previous studies. Ciprofloxacin, an earlier generation fluoroquinolone with a narrower spectrum of activity compared to pradofloxacin, had a significant inhibitory effect on faecal microbial diversity in several studies undertaken in humans (Pop et al., 2016; Rashid et al., 2015; Stewardson et al., 2015) and laboratory animals (Choo et al., 2017). Another study in healthy humans showed that ciprofloxacin affected the abundance of 30% of the bacteria in the faecal microbial community (Dethlefsen et al., 2008), while another study on levofloxacin showed that microbial richness was reduced by 25%, largely resulting from a decrease of Firmicutes and an increase of Bacteroidetes (Panda et al., 2014). In a murine model, moxifloxacin, an analogue of pradofloxacin used in human medicine that also has a wide spectrum of activity, caused a significant reduction of faecal bacterial 16S rRNA gene copy numbers after the first dose was administered, and throughout the 10-day treatment period.

However, bacterial populations recovered 2 days after cessation of treatment (Yao et al., 2016).

The shifts in the gut microbiota caused by inappropriate diet or selective antibiotic administration in rabbits are known to result in proliferation of opportunistic pathogens, leading to secondary bacterial enteritis and/or enterotoxemia, especially in young and immunocompromised animals (DeCubellis, 2016; DeCubellis and Graham, 2013; Huynh and Pignon, 2013). In the present study, we observed no clinical symptoms such as diarrhoea, inappetance, gut pain/impaction, or mucoid faeces during the trial. 16S rDNA profiling revealed overrepresentation of several bacterial taxa after the pradofloxacin treatment, however, proliferation of *Clostridium spp.* (including *C. spiroforme* the main agent associated with enterotoxaemia in rabbits) and other coliform bacteria was not observed. This may indicate that while pradofloxacin caused significant changes in the rabbit faecal microbiota, dysbiosis within the specialised caecum, resulting in overproliferation of pathogenic organisms likely did not occur. We are currently analysing the rabbit caecal microbiota to confirm this hypothesis. A recent study monitored changes in the murine gut microbiota following oral treatment with the earlier generation veterinary fluoroquinolone, enrofloxacin, which has a predominantly Gram-negative spectrum of activity and does not affect strictly anaerobic bacteria. Notable changes in the murine gut microbiota included increased proportions of *Clostridium* spp., Bacteriodetes and *Bifidobacterium* spp. (Strzepa et al., 2017). This contrasts with our study in rabbits where bacterial overgrowth was mainly associated with taxa from the families Ruminococcaceae and Lachnospiraceae. This may indicate that pradofloxacin has a reduced risk of causing enteritis in rabbits, even compared to the earlier generation fluoroquinolones, but it could also indicate host microbiota differences between mice and rabbits.

We showed that a single dose treatment of pradofloxacin caused a very similar reduction in microbial diversity compared to multiple dose treatments. The significant reduction in faecal microbial diversity in the single dose group may also indicate that pradofloxacin may exhibit a prolonged suppressive effect on the faecal microbial community after cessation of treatment, a finding supported by studies in healthy humans treated with ciprofloxacin (Rashid et al., 2015). A further study incorporating a longer period of sampling following cessation of antimicrobial treatment would be required to confirm this. The suppressive effect of fluoroquinolones on the faecal microbiota was suggested in another study conducted in humans, which might be caused by higher elimination of moxifloxacin in faeces (approximately 37-38% of the administered dose) compared to urine with only 2.5% of

administered dose (Stass and Kubitza, 1999). Pradofloxacin is eliminated from the body viaurine and faeces but the proportion were varied among animals; in dogs at equal parts, in cats to a greater extent via the faeces (Bayer, 2004). This may suggest that pradofloxacin is also eliminated in high concentrations via rabbit faeces, resulting greater impact on faecal microbiota.

Despite single and multiple dose treatments having a similar effect on microbial diversity, we did show that the microbial structure in both treatment groups differed, as visualised by PCoA. In line with our study, a study in New Zealand-Californian rabbits indicated that in general, hard faeces possessed higher microbial diversity than soft faeces, although no significant differences were identified (Michelland et al., 2010). Several bacterial taxa such as Ruminococcaceae, and *Akkermansia* spp. were enriched in soft faeces compared to hard faeces in previous comparative studies (Zeng et al., 2015). Although we compared between faecal types from the pradofloxacin-treated group only, soft faeces remained enriched with Ruminococcaceae which play an important role in the breakdown of complex carbohydrates. Ruminococcaceae and Lachnospiraceae were among the main families previously identified in rabbit faecal samples (Crowley et al., 2017; Eshar and Weese, 2014), however, the abundance of other bacterial taxa has varied considerably between studies. In the present study, Bacteriodetes was the second most abundance phyla after Firmicutes, which is in agreement with previous work (Crowley et al., 2017) but in contrast with another study describing that Verrucomicrobiota was the second most abundant taxa in faecal microbiota after Firmicutes (Eshar and Weese, 2014). The differences may reflect differences in diets and/or lack of variation in diets that included timothy hay or timothy-based pelleted diet in one study (Eshar and Weese, 2014) compared to a wider variety of diets including rabbit muesli, vegetables, fruits, willow bark, hay, straw and wild leaves in another study (Crowley et al., 2017).

The present study has some limitations. Obtaining enough soft faeces samples in both groups of rabbits was quite challenging. Not all collars were successful in preventing rabbits from ingesting soft faeces each day so the collection of these samples during the trial was quite sporadic. As a result, only 11 soft faeces samples were obtained, which did not generate enough data to analyse and compare soft faeces over all time points and/or between faecal types. Additionally, low quality of sequencing was observed in some samples, resulting in reduced library size (OTU number).

In conclusion, this is the first study describing the impact of pradofloxacin on gut microbiota in animals. In rabbits, pradofloxacin treatment affected up to a third of microbial community in hard faecal samples with significant adaptive changes, including decreased

abundance in a large variety of Gram-positive and -negative bacteria but increased abundance in some genera of Gram-positive bacteria. Pradofloxacin did not induce proliferation of bacterial taxa associated with enteritis in rabbits, indicating that it can be safely used in rabbits for short duration therapy. Furthermore, microbiota profiling of stomach and caecum contents together with gross and histological assessment of mucosal tissues could provide additional detailed assessment of the safety of pradofloxacin in lagomorphs. Establishing that pradofloxacin is safe in rabbits will enable clinical pharmacokinetic trials to be undertaken to confirm dose rates to achieve concentrations resulting in bacteriological cure in diseased rabbits.

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Competing interest

SA and DJT have received research grants and contracts from Zoetis, Novartis, Bayer, Luoda Pharma and Neoculi. All other authors declare no competing interests.

Supplementary information

Table S1. Summary of metagenomics analysis from 30 hard feces and 11 soft feces from eight rabbits.

Table S2. Relative abundance of microbial taxa per fecal sample group from eight rabbits.

Table S3. Alpha diversity measurement including observed species, Chao1 and Shannon index.

Table S4. Bacterial taxa significantly different between T0 vs T3 in single dose treatment (A) and multiple doses treatment (B) group.

Table S5. Metabolic pathways significantly enriched in faecal sample groups, compared to references' faecal samples obtained from Microbiomeanalyst.

References

Agnoletti, F., Ferro, T., Guolo, A., Marcon, B., Cocchi, M., Drigo, I., Mazzolini, E., Bano, L., 2009. A survey of *Clostridium spiroforme* antimicrobial susceptibility in rabbit breeding. Vet. Microbiol. 136, 188-191.

Alfano, N., Courtiol, A., Vielgrader, H., Timms, P., Roca, A.L., Greenwood, A.D., 2015. Variation in koala microbiomes within and between individuals: effect of body region and captivity status. Sci. Rep. 5, 10189.

Barker, C.J., Gillett, A., Polkinghorne, A., Timms, P., 2013. Investigation of the koala (*Phascolarctos cinereus*) hindgut microbiome via 16S pyrosequencing. Vet. Microbiol. 167, 554-564.

Bayer 2004. Veraflox® Summary of Product Characteristics (Bayer Animal Health).

Borriello, S.P., Carman, R.J., 1983. Association of iota-like toxin and *Clostridium spiroforme* with both spontaneous and antibiotic-associated diarrhea and colitis in rabbit. J. Clin. Microbiol. 17, 414-418.

Campbell-Ward, M.L. 2012. Gastrointestinal Physiology and Nutrition. In Ferrets, Rabbits, and Rodents: Clinical Medicine and Surgery, Quesenberry, K.E., Carpenter, J.W., eds. (St. Louis, Missouri, Elsevier Saunders), 183-192.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high throughput community sequencing data. Nat. Methods 7, 335-336.

Choo, J.M., Kanno, T., Zain, N.M., Leong, L.E., Abell, G.C., Keeble, J.E., Bruce, K.D., Mason, A.J., Rogers, G.B., 2017. Divergent relationships between fecal microbiota and metabolome following distinct antibiotic-induced disruptions. mSphere 2, 1-16.

Crowley, E.J., King, J.M., Wilkinson, T., Worgan, H.J., Huson, K.M., Rose, M.T., McEwan, N.R., 2017. Comparison of the microbial population in rabbits and guinea pigs by next generation sequencing. PLoS One 12, e0165779.

DeCubellis, J., 2016. Common emergencies in rabbits, guinea pigs, and chinchillas. Vet. Clin. North. Am. Exot. Anim. Pract. 19, 411-429.

DeCubellis, J., Graham, J., 2013. Gastrointestinal disease in guinea pigs and rabbits. Vet. Clin. North. Am. Exot. Anim. Pract. 16, 421-435.

Dethlefsen, L., Huse, S., Sogin, M.L., Relman, D.A., 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6, e280.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic. Acids. Res. 45(W1), W180-W188.

Eshar, D., Weese, J.S., 2014. Molecular analysis of the microbiota in hard feces from healthy rabbits (*Oryctolagus cuniculus*) medicated with long term oral meloxicam. BMC Vet. Res. 10, 1-9.

Halls, E.H. 2008. Caecotrophy in rabbits. In Nutrifax (Canada, Nutreco Canada Inc.).

Huynh, M., Pignon, C., 2013. Gastrointestinal disease in exotic small mammals. J. Exo. Pet Med. 22, 118-131.

Katz, L., LaMont, T., Trier, J.S., Sonnenblick, E.B., Rothman, S.W., Briotman, S.A., Rieth, S., 1978. Experimental clindamycin-associated colitis in rabbits. Gastroenterology 74, 246-252.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glockner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1.

Lees, P., 2013. Pharmacokinetics, pharmacodynamics and therapeutics of pradofloxacin in the dog and cat. J. Vet. Pharmacol. Ther 36, 209-221.

Michelland, R.J., Combes, S., Monteils, V., Cauquil, L., Gidenne, T., Fortun-Lamothe, L., 2010. Molecular analysis of the bacterial community in digestive tract of rabbit. Anaerobe 16, 61-65.

Oglesbee, B.L., Jenkins, J.R. 2012. Gastrointestinal Diseases. In Ferrets, Rabbits, and Rodents: Clinical Medicine and Surgery, Quesenberry, K.E., Carpenter, J.W., eds. (St. Louis, Missouri, Elsevier Saunders), 193-204.

Pallo-Zimmerman, L.M., Byron, J.K., Graves, T.K., 2010. Fluoroquinolones: Then and Now. Compendium 32, E1-E9.

Panda, S., El khader, I., Casellas, F., Lopez Vivancos, J., Garcia Cors, M., Santiago, A., Cuenca, S., Guarner, F., Manichanh, C., 2014. Short-term effect of antibiotics on human gut microbiota. PLoS One 9, e95476.

Papich, M.G. 2016. Pradofloxacin. In Saunders Handbook of Veterinary Drugs (Fourth Edition): Small and Large Animal (St. Louis, Missouri, Elsevier), 660-662.

Pop, M., Paulson, J.N., Chakraborty, S., Astrovskaya, I., Lindsay, B.R., Li, S., Bravo, H.C., Harro, C., Parkhill, J., Walker, A.W., Walker, R.I., Sack, D.A., Stine, O.C., 2016. Individual-specific changes in the human gut microbiota after challenge with enterotoxigenic *Escherichia coli* and subsequent ciprofloxacin treatment. BMC Genomics 17, 440.

Rashid, M.-U., Zaura, E., Buijs, M.J., Keijser, B.J.F., Crielaard, W., Nord, C.E., Weintraub, A., 2015. Determining the long-term effect of antibiotic administration on the human normal intestinal microbiota using culture and pyrosequencing methods. CID 60, S77-S84.

Raw, C., 2017. Infectious diseases in rabbits. Vet. Nurs. J. 32, 298-300.

Schink, A.K., Kadlec, K., Hauschild, T., Brenner Michael, G., Dorner, J.C., Ludwig, C., Werckenthin, C., Hehnen, H.R., Stephan, B., Schwarz, S., 2013. Susceptibility of canine and feline bacterial pathogens to pradofloxacin and comparison with other fluoroquinolones approved for companion animals. Vet. Microbiol. 162, 119-126.

Silley, P., Stephan, B., Greife, H.A., Pridmore, A., 2012. Bactericidal properties of pradofloxacin against veterinary pathogens. Vet. Microbiol. 157, 106-111.

Stass, H., Kubitza, D., 1999. Pharmacokinetics and elimination of moxifloxacin after oral and intravenous administration in man. J. Antimicrob. Chemother. 43, 83-90.

Stewardson, A.J., Gaia, N., Francois, P., Malhotra-Kumar, S., Delemont, C., Martinez de Tejada, B., Schrenzel, J., Harbarth, S., Lazarevic, V., Saturn, W.P., Groups, W.P.S., 2015. Collateral damage from oral ciprofloxacin versus nitrofurantoin in outpatients with urinary tract infections: a culture-free analysis of gut microbiota. Clin. Microbiol. Infect. 21, 344 e341-311.

Strzepa, A., Majewska-Szczepanik, M., Lobo, F.M., Wen, L., Szczepanik, M., 2017. Broad spectrum antibiotic enrofloxacin modulates contact sensitivity through gut microbiota in a murine model. J. Allergy. Clin. Immunol. 140, 121-133 e123.

Sykes, J.E., Blondeau, J.M., 2014. Pradofloxacin: a novel veterinary fluoroquinolone for treatment of bacterial infections in cats. Vet. J. 201, 207-214.

Wetzstein, H.G., Hallenbach, W., 2011. Tuning of antibacterial activity of a cyclopropyl fluoroquinolone by variation of the substituent at position C-8. J. Antimicrob. Chemother. 66, 2801-2808.

Yao, J., Carter, R.A., Vuagniaux, G., Barbier, M., Rosch, J.W., Rock, C.O., 2016. A pathogen-selective antibiotic minimizes disturbance to the microbiome. Antimicrob. Agents Chemother. 60, 4264-4273.

Zeng, B., Han, S., Wang, P., Wen, B., Jian, W., Guo, W., Yu, Z., Du, D., Fu, X., Kong, F., Yang, M., Si, X., Zhao, J., Li, Y., 2015. The bacterial communities associated with fecal types and body weight of rex rabbits. Sci. Rep. 5, 9342.

Suppelementary information

Table S1. Summary of metagenomics analysis from 30 hard feces and 11 soft feces from eight rabbits.

Group	Rabbit ID	Time point	Fecal type	Treatment dose	Sample ID	Number of reads	Chimeric reads	Reads in OTUs
1 (HFT0A)	1	T0- Day 0	Hard	Single	R1T0HP	62116	48464	13652
	4	T0- Day 0	Hard	Single	R4T0HP	55199	44031	11168
	5	T0- Day 0	Hard	Single	R5T0HP	55311	43174	12137
	6	T0- Day 0	Hard	Single	R6T0HP	68816	53135	15681
2 (HFT1A)	1	T1- Day 1	Hard	Single	R1T1HP	54914	43199	11715
	4	T1- Day 1	Hard	Single	R4T1HP	69888	54475	15413
	5	T1- Day 1	Hard	Single	R5T1HP	69225	55121	14104
	6	T1- Day 1	Hard	Single	R6T1HP	62619	49305	13314
3 (HFT2A)	1	T2- Day 2	Hard	Single	R1T2HP	61882	48185	13697
	4	T2- Day 2	Hard	Single	R4T2HP	58755	44810	13945
	5	T2- Day 2	Hard	Single	R5T2HP	65514	50582	14932
	6	T2- Day 2	Hard	Single	R6T2HP	59119	46862	12257
4 (HFT3A)	4	T3- Day 3	Hard	Single	R4T3HP	62817	47670	15147
	5	T3- Day 3	Hard	Single	R5T3HP	77707	60019	17688
	6	T3- Day 3	Hard	Single	R6T3HP	43944	34018	9926
5 (HFT0B)	7	T0- Day 0	Hard	Multiple	R7T0HP	48748	37975	10773
	8	T0- Day 0	Hard	Multiple	R8T0HP	39668	30441	9227
	9	T0- Day 0	Hard	Multiple	R9T0HP	54007	42190	11817
	10	T0- Day 0	Hard	Multiple	R10T0HP	62526	48733	13793
6 (HFT1B)	7	T1- Day 1	Hard	Multiple	R7T1HP	49096	38388	10708
	8	T1- Day 1	Hard	Multiple	R8T1HP	47703	36015	11688
	9	T1- Day 1	Hard	Multiple	R9T1HP	45223	35173	10050
	10	T1- Day 1	Hard	Multiple	R10T1HP	48127	38197	9930
7 (HFT2B)	7	T2- Day 2	Hard	Multiple	R7T2HP	59013	46381	12632
	8	T2- Day 2	Hard	Multiple	R8T2HP	57537	44773	12764
	10	T2- Day 2	Hard	Multiple	R10T2HP	64770	50413	14357
8 (HFT3B)	7	T3- Day 3	Hard	Multiple	R7T3HP	58808	45289	13519
	8	T3- Day 3	Hard	Multiple	R8T3HP	56405	43222	13183
	9	T3- Day 3	Hard	Multiple	R9T3HP	63324	47906	15418
	10	T3- Day 3	Hard	Multiple	R10T3HP	51549	40921	10628
9 (SFT1A)	1	T1- Day 1	Soft	Single	R1T1CT	66771	52157	14614
	5	T1- Day 1	Soft	Single	R5T1CT	65339	52154	13185
10 (SFT2A)	4	T2- Day 2	Soft	Single	R4T2CT	66369	51045	15324
11 (SFT3A)	5	T3- Day 3	Soft	Single	R5T3CT	68739	54701	14038
12 (SFT1B)	10	T1- Day 1	Soft	Multiple	R10T1CT	54537	42242	12295
13 (SFT2B)	7	T2- Day 2	Soft	Multiple	R7T2CT	54855	43065	11790
	8	T2- Day 2	Soft	Multiple	R8T2CT	67509	53554	13955
	9	T2- Day 2	Soft	Multiple	R9T2CT	58006	44819	13187
	10	T2- Day 2	Soft	Multiple	R10T2CT	70219	54902	15317
14 (SFT3B)	8	T3- Day 3	Soft	Multiple	R8T3CT	61093	48198	12895
	10	T3- Day 3	Soft	Multiple	R10T3CT	49100	38320	10780

Table S2. Relative abundance of OUT reads (%) per fecal sample group from eight rabbits.

Phylum	HFT0A	HFT0B	HFT1A	HFT1B	HFT2A	HFT2B	HFT3A	HFT3B	SFT1A	SFT1B	SFT2A	SFT2B	SFT3A	SFT3B
Actinobacteria	0.125	0.055	0.099	0.019	0.308	0.047	0.195	0.011	0.009	0	0.01	0.009	0	0.006
Bacteroidetes	24.15	20.24	14.75	14.58	18.03	23.96	24.38	15.42	10.76	12.5	30.84	13.2	14.79	7.937
Cyanobacteria	1.597	0.016	1.675	0.117	0.067	0.578	0.008	1.229	0	0.005	0.041	0.003	0	0
Euryarchaeota	0.01	0	0.015	0	0.059	0	0.039	0	0.005	0	0	0	0.024	0
Firmicutes	65.95	47.02	76.26	73.28	75.79	62.2	74.45	67.2	87.04	82.56	67.92	85.32	81.66	90.87
Proteobacteria	5.599	22.48	0.281	1.105	0.056	3.23	0.003	0.159	0.149	0.025	0.377	0.137	0.072	0.017
Saccharibacteria	0.092	0.097	0.134	0.115	0.024	0.117	0.195	0.082	0.099	0.1	0.153	0.074	0.161	0.102
Tenericutes	0	3.594	0	0.891	0	5.624	0	1.6	0	0	0	0	0	0
Verrucomicrobia	2.483	6.494	6.784	9.884	5.661	4.242	0.741	14.3	1.944	4.803	0.653	1.252	3.295	1.066
Family														
Aeromonadaceae	4.249	7.351	0.072	0.128	0.004	0.305	0	0.004	0.023	0.005	0	0.003	0	0
Alcaligenaceae	0.501	1.083	0.072	0.125	0.028	0.252	0	0.042	0.005	0	0.01	0.071	0	0.017
Anaeroplasmataceae	0	3.594	0	0.891	0	5.624	0	1.6	0	0	0	0	0	0
Bacteroidaceae	16.61	12.05	11.55	11.88	15.14	17.89	20.78	13.08	7.872	10.85	28.05	9.496	3.327	6.962
Bacteroidales S247group	2.835	2.145	0.349	0.44	0.993	1.96	0.005	0.148	1.239	0.025	0.031	1.645	4.68	0.221
Carnobacteriaceae	0.164	0.1	0.02	0.024	0.015	0.003	0	0	0	0	0	0.02	0	0
Christensenellaceae	0.404	0.116	0.237	0.155	0.085	0.249	0	0.345	0.136	0.12	0.398	0.046	0.016	0.028
Clostridialesvadin BB60 group	2.155	1.002	1.578	0.464	0.277	1.552	0.083	1.611	0.213	0.345	1.938	0.259	0	0.023
Coriobacteriaceae	0.045	0	0.057	0	0.262	0	0.195	0	0	0	0	0	0	0
Corynebacteriaceae	0.08	0.055	0.042	0.019	0.046	0.047	0	0.011	0.009	0	0.01	0.009	0	0.006
Desulfovibrionaceae	0.047	0.011	0.026	0	0.004	0.15	0	0.013	0	0.015	0.286	0.009	0	0
Eubacteriaceae	0.279	0.026	0.336	0.04	0.362	0.026	0.543	0.042	1.026	0.955	0.082	0.582	0.822	0.437
Flavobacteriaceae	0.212	2.997	0.044	0.091	0.022	1.725	0	0.057	0.018	0.005	0.01	0.071	0.169	0.011
Lachnospiraceae	23.98	18.51	25.96	27.95	22.67	28.94	20.49	19.47	28.82	23.78	23.73	22.71	20.2	10.5
Methanobacteriaceae	0.01	0	0.015	0	0.059	0	0.039	0	0.005	0	0	0	0.024	0
Oxalobacteraceae	0.022	0.021	0.002	0.019	0	0.029	0	0.02	0.027	0.005	0.082	0.04	0	0
Porphyromonadaceae	2.526	1.659	1.385	1.185	0.57	1.52	2.237	0.789	1.347	0.905	2.336	1.044	6.332	0.164

Pseudomonadaceae	0.78	13.76	0.108	0.742	0.02	2.406	0.003	0.013	0.095	0	0	0.014	0.072	0
Rhodospirillaceae	0	0.258	0	0.091	0	0.088	0	0.066	0	0	0	0	0	0
Rikenellaceae	1.96	1.391	1.418	0.987	1.305	0.868	1.349	1.343	0.28	0.72	0.418	0.947	0.282	0.578
Ruminococcaceae	38.97	27.27	48.13	44.65	52.38	31.43	53.33	45.73	56.84	57.36	41.78	61.7	60.62	79.88
Verrucomicrobiaceae	2.483	6.494	6.784	9.884	5.661	4.242	0.741	14.3	1.944	4.803	0.653	1.252	3.295	1.066
Others	1.689	0.113	1.809	0.232	0.091	0.695	0.203	1.31	0.099	0.105	0.194	0.077	0.161	0.102

Table S3. Alpha diversity measurement including observed species, Chao1 and Shannon index.

Group#	Group ID	Sample ID	Observed	Chao1	Shannon
1	HFT0A	R1T0HP	143	186.59	3.28
		R4T0HP	143	178.06	3.36
		R5T0HP	163	190.39	3.39
		R6T0HP	111	130.46	2.90
2	HFT1A	R1T1HP	117	140.00	2.83
		R4T1HP	151	194.00	3.05
		R5T1HP	139	163.17	3.04
		R6T1HP	109	130.00	2.90
3	HFT2A	R1T2HP	126	159.00	2.90
		R4T2HP	111	128.55	2.66
		R5T2HP	124	166.50	2.88
		R6T2HP	130	175.00	2.97
4	HFT3A	R4T3HP	99	116.50	2.59
		R5T3HP	100	130.00	2.60
		R6T3HP	101	121.65	2.64
5	HFT0B	R7T0HP	152	185.21	3.33
		R8T0HP	145	163.60	3.14
		R9T0HP	149	205.00	3.15
		R10T0HP	150	167.55	3.39
6	HFT1B	R7T1HP	122	143.75	2.74
		R8T1HP	114	172.13	2.67
		R9T1HP	114	143.00	2.97
		R10T1HP	108	144.25	2.90
7	HFT2B	R7T2HP	128	153.20	3.17
		R8T2HP	122	170.46	3.14
		R10T2HP	121	142.94	2.78
8	HFT3B	R7T3HP	124	153.06	2.93
		R8T3HP	120	155.43	2.85
		R9T3HP	103	139.14	2.80
		R10T3HP	77	85.25	2.75
9	SFT1A	R1T1CT	100	117.77	2.77
		R5T1CT	102	121.25	2.68
10	SFT2A	R4T2CT	100	135.10	2.68
11	SFT3A	R5T3CT	99	113.25	2.91
12	SFT1B	R10T1CT	84	101.00	2.71
13	SFT2B	R9T2CT	101	114.15	2.81
		R8T2CT	86	97.38	2.92
		R7T2CT	99	128.25	2.66
		R10T2CT	100	118.07	2.53
14	SFT3B	R8T3CT	93	105.00	2.58
		R10T3CT	83	98.00	2.63

Table S4. Bacterial taxa significantly different between T0 vs T3 in single dose treatment (A) and multiple doses treatment (B) group.

Treatment A			Treatment B		
Genus	Pvalues	FDR	Genus	Pvalues	FDR
Pseudomonas	2.07E-10	1.68E-08	Oceanisphaera	1.413E-19	1.229E-17
Oceanisphaera	2.97E-07	8.65E-06	Pseudomonas	2.709E-16	1.178E-14
Thalassospira	4.74E-07	8.65E-06	uncul. bacterium Gastranaerophilales	1.807E-10	5.241E-09
uncul. bacterium BacteroidalesS24_7group	5.15E-07	8.65E-06	Oligella	4.108E-09	8.935E-08
uncul. bacterium Lachnospiraceae	5.34E-07	8.65E-06	MyroidesbacteriumSM9_19	8.604E-09	1.497E-07
Anaeroplasma	7.97E-07	1.08E-05	Flavobacterium	1.978E-07	2.869E-06
RuminococcaceaeUCG_001	5.21E-05	0.0006029	Atopostipes	1.13E-06	1.404E-05
Atopostipes	6.10E-05	0.0006181	LachnospiraceaeNC2004group	3.297E-05	0.0003586
uncul. bacterium BacteroidetesVC21Bac22	9.30E-05	0.0007843	Marvinbryantia	5.603E-05	0.0005416
uncul. bacterium MollicutesRF9	9.68E-05	0.0007843	Paenalcaligenes	6.322E-05	0.00055
ChristensenellaceaeR_7group	0.0001681	0.001238	uncul. bacterium BacteroidalesS24_7group	0.0001114	0.0008809
uncul. bacterium Gastranaerophilales	0.0002661	0.0017962	Tyzzerella3	0.0002268	0.0016445
Ruminiclostridium6	0.0003417	0.0020605	uncul. Aerococcaceae	0.0003072	0.0020556
RuminococcaceaeUCG_011	0.0003561	0.0020605	Trichococcus	0.0005156	0.0032042
Lachnoclostridium12	0.0005425	0.0029296	Olivibacter	0.0013659	0.0079222
Oligella	0.0008594	0.0041241	Erysipelothrix	0.0016875	0.0089047
Ruminiclostridium1	0.0008656	0.0041241	uncul. bacterium MollicutesRF9	0.00174	0.0089047
LachnospiraceaeNK4B4group	0.0028451	0.012803	Acinetobacter	0.0018905	0.0091373
IncertaeSedis	0.0068331	0.029131	uncul. Xanthomonadaceae	0.0035835	0.016132
Akkermansia	0.0098976	0.037475	Ruminococcus1	0.0037084	0.016132
Parabacteroides	0.010059	0.037475	Myroides	0.0064999	0.026928
Collinsella	0.010178	0.037475	RuminococcaceaeUCG_001	0.0080597	0.031872
MyroidesbacteriumSM9_19	0.011658	0.039397	Proteiniphilum	0.010259	0.038806
uncul. bacterium ClostridialesvadinBB60group	0.011951	0.039397	Ignatzschineria	0.012261	0.044447
Tyzzerella3	0.012159	0.039397			

*FDR, false discovery rate.

Table S5. Pathways significantly enriched in fecal sample groups, compared to references' fecal samples (rabbit and lion) obtained from Microbiomeanalyst.

PATHWAY	METABOLISM	HFT0A	HFT3A	HFT0B	HFT3B	SFT1A	SFT2B	SFT3B	RABBIT	LION
GLOBAL	Biosynthesis of amino acids	●	●	●	●	●	●	●	●	●
	Carbon metabolism	●	●	●	●	●	●	●	●	●
	Degradation of aromatic compounds	●	-	●	-	●	●	-	NS	NS
CARBOHYDRATE METABOLISM	Amino sugar and nucleotide sugar metabolism	NS	●	NS	NS	NS	NS	-	NS	NS
	Butanoate metabolism	NS	●	NS	NS	NS	NS	●	NS	NS
	C5-Branched dibasic acid metabolism	NS	-	NS	NS	NS	NS	NS	-	NS
	Citrate cycle (TCA cycle)	-	●	-	NS	NS	NS	NS	NS	NS
	Fructose and mannose metabolism	●	●	●	●	●	●	●	●	●
	Glycolysis / Gluconeogenesis	●	●	●	●	●	●	●	●	●
	Glyoxylate and dicarboxylate metabolism	●	●	●	●	●	●	●	●	●
	Pentose phosphate pathway	●	●	●	●	●	●	NS	●	●
	Propanoate metabolism	●	●	●	●	●	●	●	●	●
	Pyruvate metabolism	●	●	●	●	●	●	●	●	●
	Starch and sucrose metabolism	●	●	●	●	●	●	●	●	●
ENERGY METABOLISM	Carbon fixation in photosynthetic organisms	-	-	-	-	-	-	NS	-	-
	Carbon fixation pathways in prokaryotes	●	●	●	●	●	●	●	●	●
	Methane metabolism	●	●	●	●	●	●	●	●	●
	Nitrogen metabolism	NS	-	NS	●	-	●	-	-	-
LIPID METABOLISM	Steroid degradation	NS	●	NS	●	●	●	●	●	●
	Synthesis and degradation of ketone bodies	-	NS	-	-	-	-	NS	-	-
AMINO ACID METABOLISM	Arginine and proline metabolism	●	●	●	●	●	●	NS	●	●
	Cysteine and methionine metabolism	-	NS	-	-	NS	-	-	-	-
	D-Glutamine and D-glutamate metabolism	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Glycine, serine and threonine metabolism	●	●	●	●	●	●	●	●	●
	Histidine metabolism	NS	NS	NS	NS	NS	NS	NS	NS	-
	Lysine biosynthesis	NS	NS	NS	NS	NS	NS	-	NS	NS
	Phenylalanine, tyrosine and tryptophan biosynthesis	●	NS	●	●	●	●	●	●	●
	Valine, leucine and isoleucine biosynthesis	●	●	●	●	●	●	●	NS	●
METABOLISM OF OTHER AMINO ACIDS	Selenocompound metabolism	-	NS	-	-	-	-	NS	-	-
GLYCAN BIOSYNTHESIS AND METABOLISM	Lipopolysaccharide biosynthesis	●	●	●	●	●	●	●	●	●

	Peptidoglycan biosynthesis	●	●	●	●	●	●	●	NS	●
METABOLISM OF COFACTORS AND VITAMINS	Biotin metabolism	-	NS	-	NS	NS	NS	NS	NS	NS
	Folate biosynthesis	NS	NS	NS	NS	NS	NS	NS	-	-
	Pantothenate and CoA biosynthesis	●	NS	●	●	●	●	NS	NS	NS
	Porphyrin and chlorophyll metabolism	●	●	●	●	●	●	●	●	●
	Thiamine metabolism	NS	●	NS	NS	NS	NS	NS	NS	NS
METABOLISM OF TERPENOIDS AND POLYKETIDES	Terpenoid backbone biosynthesis	NS	●	NS	NS	NS	NS	●	NS	NS
BIOSYNTHESIS OF OTHER SECONDARY METABOLITES	Streptomycin biosynthesis	NS	●	NS	NS	NS	NS	●	NS	NS
XENOBIOTICS BIODEGRADATION AND METABOLISM	Atrazine degradation	NS	-	NS	NS	NS	NS	●	NS	NS
	Benzoate degradation	●	-	●	●	●	●	NS	●	●
	Chloroalkane and chloroalkene degradation	-	NS	-	-	-	-	NS	-	-
	Fluorobenzoate degradation	NS	●	NS	NS	NS	NS	NS	NS	NS
	Naphthalene degradation	NS	-	NS	-	●	●	-	●	●
	Nitrotoluene degradation	NS	-	NS	NS	-	-	-	NS	NS
	Toluene degradation	●	NS	●	NS	●	●	-	NS	NS

*NS, not significant.

Chapter 7

Oral pradofloxacin has limited impact on the gastric and caecal microbiota of rabbits

Statement of Authorship

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Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Oral pradofloxacin has limited impact on the gastric and caecal microbiota of rabbits

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Abstract

Rabbits are known to have a delicately balanced digestive system and microbiota, which are highly sensitive to the detrimental effects of certain classes of antibiotic. This study aimed to determine the microbial community changes in the rabbit stomach and caecum in response to pradofloxacin. Single and multiple doses of pradofloxacin were administered to New Zealand crossbred rabbits at a dose rate of 7.5 mg/kg body weight and bacterial community changes were assessed by 16S rRNA amplicon sequencing using Illumina MiSeq. Here, we demonstrate that a single dose of pradofloxacin only slightly affected the overall microbial diversity in the stomach and caecum. A greater effect was observed in the multiple doses group with decreased microbial richness of up to 8% and 12% in the stomach and caecum, respectively, however, this did not reach significance when compared to the control group. *Anaeroplasma, Tyzerella* and Bacteriodetes were among the most suppressed bacterial taxa while the abundance of *Akkermansia* and several genera from Ruminococcaceae significantly increased after pradofloxacin treatment. Significantly, no overproliferation of opportunistic bacterial pathogens such as *Clostridium* spp. and coliform bacteria which may cause antibiotic-induced enteritis were detected and no histological changes were detected in gut sections. The stomach and caecum shared similar core microbiota which was similar in composition to soft faeces (caecotroph) but not hard faeces. Apart from minor impacts on gastric and caecal microbiota, our findings suggest that pradofloxacin is a relatively safe antimicrobial agent for pet rabbits as we observed no abnormalities in gastrointestinal tract and histological appearance.

Keywords: pradofloxacin; microbiota; rabbit; stomach; caecum.

Introduction

Rabbits are monogastric, hindgut-fermenting herbivores with a unique digestive structure and physiology. The rabbit gastrointestinal (GI) tract harbours a complex microbial community that effectively processes and digests a plant-based diet and is characterised by distinct bacterial populations in each gut compartment (Campbell-Ward, 2012). The GI tract occupies a large proportion of the rabbit body cavity with the majority of digestible material contained within the stomach and caecum (Halls, 2008). The stomach is very acidic (pH 1-2), comprises up to 15% of GI volume and is composed of food, ingested hair and caecal pellets with a 3-6 hour gastric transit time (Halls, 2008). The caecum is the largest organ (containing approximately 40% of gut contents) where sorting and fermentation of digesta occurs (Campbell-Ward, 2012; Davies and Davies, 2003; Halls, 2008). Because the rabbit has a delicately balanced GI system, any environmental changes, for example, those induced by the diet being too high in energy and/or protein, or following antimicrobial therapy, may result in gut microbiota dysbiosis (Meredith, 2010). Although dynamic changes in rabbit gut microbial communities varies between individuals and over time, they appear to be largely dependent on food intake, coprophagy and overall health (Halls, 2008).

Gut microbiota dysbiosis in the rabbit is usually characterised by overgrowth of opportunistic autochthonous organisms such as *Clostridium spiriforme* and *Escherichia coli* (Carman and Borriello, 1984; Halls, 2005; Oglesbee and Jenkins, 2012; Raw, 2017). Diarrheal disease frequently results from dysbiosis, with symptoms ranging from softening of the stools, significant enteritis and life-threatening enterotoxemia (Campbell-Ward, 2012; DeCubellis and Graham, 2013). Antibiotics most likely to cause enteritis in rabbits include the β-lactams (cephalosporins and oral penicillin) and the macrolides (lincomycin, clindamycin, and erythromycin) (Carman and Borriello, 1984; Carman and Borriello, 1989; Ritzman, 2014).

Older generation fluoroquinolones such as enrofloxacin are among the few classes of antimicrobial agent that are relatively safe for use in pet rabbits (as opposed to rabbits classified as food-producing animals) (DeCubellis and Graham, 2013). However, the latest generation of the veterinary fluoroquinolones, pradofloxacin, has a much wider spectrum of activity and could be an important therapeutic option for pet rabbits. Pradofloxacin is active against Gram-negative, Gram-positive and strictly anaerobic bacteria, as well as *Mycoplasma* and rapid growing *Mycobacteria*, but it has limited effect against Actinobacteria (Govendir et al., 2011). The mechanism of action is dual targeting through inhibition of both DNA gyrase and topoisomerase IV in the majority of susceptible bacteria with much lower mutant

prevention concentration (MPC) and less chance of developing resistance during therapy compared to other fluoroquinolones (Silley et al., 2012; Wetzstein, 2005; Wetzstein and Hallenbach, 2011). However, information regarding the effect of pradofloxacin on gut microbial communities in animals, particularly in rabbits, is unknown. In previous work we analysed the effect of oral pradofloxacin on the rabbit faecal microbiota which suggested that pradofloxacin appears to be relatively safe to use in rabbit although overall microbial diversity was significantly reduced after treatment (Saputra et al., 2018, in submission). In this study we aimed to characterise the changes in the gastric and caecal microbial communities caused by pradofloxacin for both single dose and multiple dose treatments through a 16S phylogenetic approach, as well as histopathological analysis of the gastrointestinal tract.

Materials and methods

Animal experiments. Ethical approval was obtained from of the University of Adelaide Animal Ethics Committee (S-2015-165). Twelve healthy New Zealand White crossbred rabbits (body weight 2.8-3.5kg) were sourced from the control group of a previous feeding trial, and divided into three groups. A single dose of pradofloxacin was given to four female rabbits in Day 1 (single dose group) while a single dose of pradofloxacin was given to four male rabbits daily for four days in the other treatment group (multiple doses group). Two female and two male rabbits remained as untreated controls (control group). All rabbits had been fed a commercial pelleted diet (Barastoc) for at least a month prior to the trial. The pelleted diets contains crude protein (16.3%), fat (3%) and fibre (21%) with additional minerals (salt, copper, selenium, calcium and phosphorus) and vitamin A, D and E. The trial was conducted at the Animal Desexing Clinic, 604 Port Rd, Allenby Gardens, Adelaide Australia. Any clinical signs related to gastrointestinal tract problems were monitored and recorded. On Day 4, all twelve rabbits were anaesthetized using isoflurane gas via a face mask and euthanized by intravenous barbiturate overdose (60mg/kg pentobarbitone). Post mortems were conducted approximately two hours later. Body condition and GI tract organs of each rabbit were recorded. For microbiota profiling, samples of gut contents were aseptically collected from the lumen of the caecum and stomach and immediately placed at -80^{0}C. Full thickness sections of stomach and caecal wall tissue, and liver and kidney were immediately placed in 10% buffered formalin. Following fixation, the tissue was trimmed, fixed in wax and 4 µm sections cut using a microtome. Sections were mounted on glass slides, stained with Haematoxylin and Eosin and examined under a light microscope at 400x magnification.

Library preparation and sequencing. DNA extraction was performed using PowerFecal™ DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). DNA concentration

was standardised to 5ng/µL before amplification of the V3-V4 region of the 16S rRNA was performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA). The primers (F 5'ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG3' and R 5'TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC3') consist of adapter nucleotide sequences (CS1 and CS2 Fluidigm, underlined) and the locus specific sequences targeting conserved regions within the V3 and V4 (Klindworth et al., 2013). Metagenomics library preparation was performed according to manufacturer's protocol. Sequencing was performed using the Illumina MiSeq platform in the South Australian Health and Medical Research Institute (SAHMRI) Adelaide, Australia.

Bioinformatics and statistical analysis. Overall sequences were retained after primer and quality trimming. Quality trimmed reads were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline soſtware (Caporaso et al., 2010). Sequences were clustered into OTUs at a 3% dissimilarity level, and taxonomy was aligned and classified against the SILVA reference database (Alfano et al., 2015). All statistical analysis was performed in Microbiomeanalyst (Dhariwal et al., 2017). Significance test was performed for alpha diversity (Shannon index, Chao1 and observed species) using Mann-Whitney/Kruskal-Wallis test ($P<0.05$). Beta diversity was assessed at genus level or higher by unweighted and unweighted Analaysis of Group Similarities (ANOSIM) and visualized by Principal Coordinate Analysis (PCoA). Differential abundance between sample groups were assessed using metagenomeSeq. LDA (Linear Discriminant Analysis) effect size (LEfSe) was performed to discover distinct abundance of bacterial taxa in gastric and caecal microbiota. Further, annotated 16S rRNA sequencing data were transformed to a taxonomic profile of the prokaryotic KEGG (Kyoto Encyclopedia of Genes and Genomes) organisms. Relative abundance of each KEGG orthology ID (KO) was produced and then subjected to shotgun data profiling (SDP) to predict metabolic potentials of microbial communities. A P-value of <0.05 with a FDR (false discovery rate) of <0.05 was considered significant (Korpela et al., 2016).

Results

Gross anatomy and histopathology. During the trial, the rabbits showed no clinical symptoms such as diarrhoea, inappetance, gut pain/impaction, or mucoid faeces. All rabbits appeared normal anatomically on post mortem. All rabbits had a body condition score of four out of five and adequate fat covering. The stomach and caeca of all rabbits were full and there were no pathological lesions in any organs. Some rabbits in all treatment groups had post mortem changes not attributable to the trial, including atelectasis of the lung (most likely due to intra-cardiac euthanasia) and necrosis of the stomach adjacent to the oesophagus due to a

build-up of acid post mortem. There were no pathological changes or detectable differences between the gross appearance of the gastro-intestinal systems of pradofloxacin treated groups and the control group. Histological appearance of the liver, kidney, stomach and caecal mucosa were identical between the prdofloxacin treated groups and the control group, with no abnormal findings. An example of a histological section of the caecal mucosa from a control rabbit (rabbit 3) and a rabbit from the multiple doses group (rabbit 10) is provided in Fig 1 A-C.

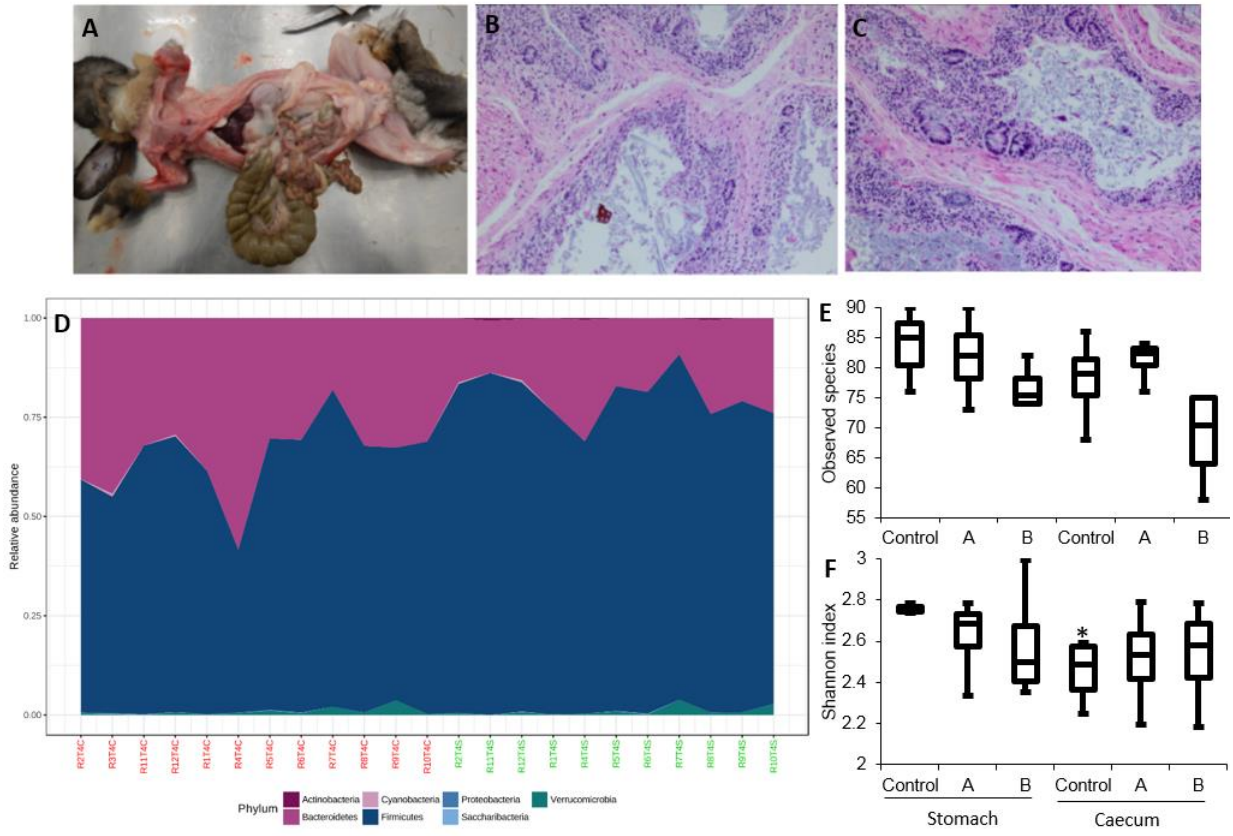

Figure 1. Anatomy and histology of gastrointestinal tract of rabbit and microbial diversity profiles in stomach and caecum. (A) Gross anatomy in post mortem showing no pathological lesion in the organs in both control group and treatment group. (B-C) Hematoxylin and eosin stain of mid caecum demonstrates no significant difference in histology appearance between control rabbit (B) and pradofloxacin treated rabbit (C). (D) Main phylum observed in stomach and caecum among all samples. (E-F) Species richness and Shannon diversity index in control rabbit and pradofloxacin-treated rabbit, (*) showed significant difference by Man-Whitney/Kruskal-Wallis ($p<0.05$) compared to stomach control group. No significant difference in observed species between stomach and caecum or treatment groups. Microbial diversity in stomach was significantly higher compared to caecum as showed by Shannon index.

16S rDNA profiling. A total of sequence 1,275,075 were obtained from 23 samples, resulting in 294515 reads after data filtering (Table S1). Approximately 2.3% of total reads were excluded (not classified) and only 287807 included in the next analysis with 12513 average counts per sample (range 19258-6263) and 768 OTUs in total. Firmicutes and Bacteriodetes were the most dominant phyla across all sample groups, ranged from 59%-83% and 15%-40%, respectively (Fig 1 D, table S2). Organisms belonging to the Actinobacteria were only detected in stomach samples. Fifteen classes were identified, with Ruminococcaceae and Lachnospiraceae being the dominant families, ranging from 25-50% and 20%-32% of the total population in the three respective sample groups.

Microbial community changes in the stomach. The rabbit gastric microbial community was dominated by Ruminococcaceae, Lachnospiraceae and Anaeroplasmataceae (Fig 2 A, Table S2). After pradofloxacin treatment, a slight decrease of observed species was identified in both single dose and multiple dose treatment groups with reductions of 2% and 8%, respectively, and a 4%-5% decrease in the Shannon index (Table 1 E-F). When examining the proportion of the total bacterial abundance at commencement of the trial affected by the treatment, suppressed bacterial taxa were observed in 25%-34% of the total abundance in both single and multiple dose treatment groups. Concomitantly, population increases in other bacterial taxa representing 14%-22% of total abundance were also observed in both single and multiple dose treatment groups. For the single dose treatment group, pradofloxacin had a negative effect on two main phyla, Tenericutes and Cyanobacteria which were mainly comprised of *Anaeroplasma* spp., *Thalassophiria* spp., Clostridiales and Bacteriodetes (Fig 2 C-D). For the multiple dose treatment group, a significant decrease of abundance at the phylum level was observed in Tenericutes and Proteobacteria, while Verrucomicrobia (*Akkermansia* spp.) were significantly overrepresented.

Microbial community changes in the caecum. Surprisingly, based on alpha diversity measurement (Shannon index), microbial diversity in the stomach was significantly higher (P=0.042) compared to the caecum in the control group (Fig 1 F). The caecal microbiota of all groups was dominated by Lachnospiraceae, Bacteroidaceae and Ruminocaoccaceae (Fig 2 A, Table S2). In general, the microbial community in the caecum was not affected by a single dose of pradofloxacin but a greater effect was observed in the multiple dose treatment group, with 12% loss of observed species, however this loss did not reach significance compared to the control group. The single dose treatment affected up to 6% of total abundance, mainly due to decreased abundance of *Anaeroplasma* spp., Bacteriodetes, and *Parasuturella* spp. A similar but greater effect was observed in the multiple dose treatment with a wider range of

bacterial taxa affected as presented in Fig 2 E-F. Similar to the stomach, several bacterial taxa such as *Akkermansia* spp. and Ruminococcaceae were more abundant after treatment. In line with no observation of antibiotic-induced diarrhoea, overproliferation of bacterial taxa from the Clostridiaceae and Enterobacteriaceae in both stomach and caecum samples was not detected.

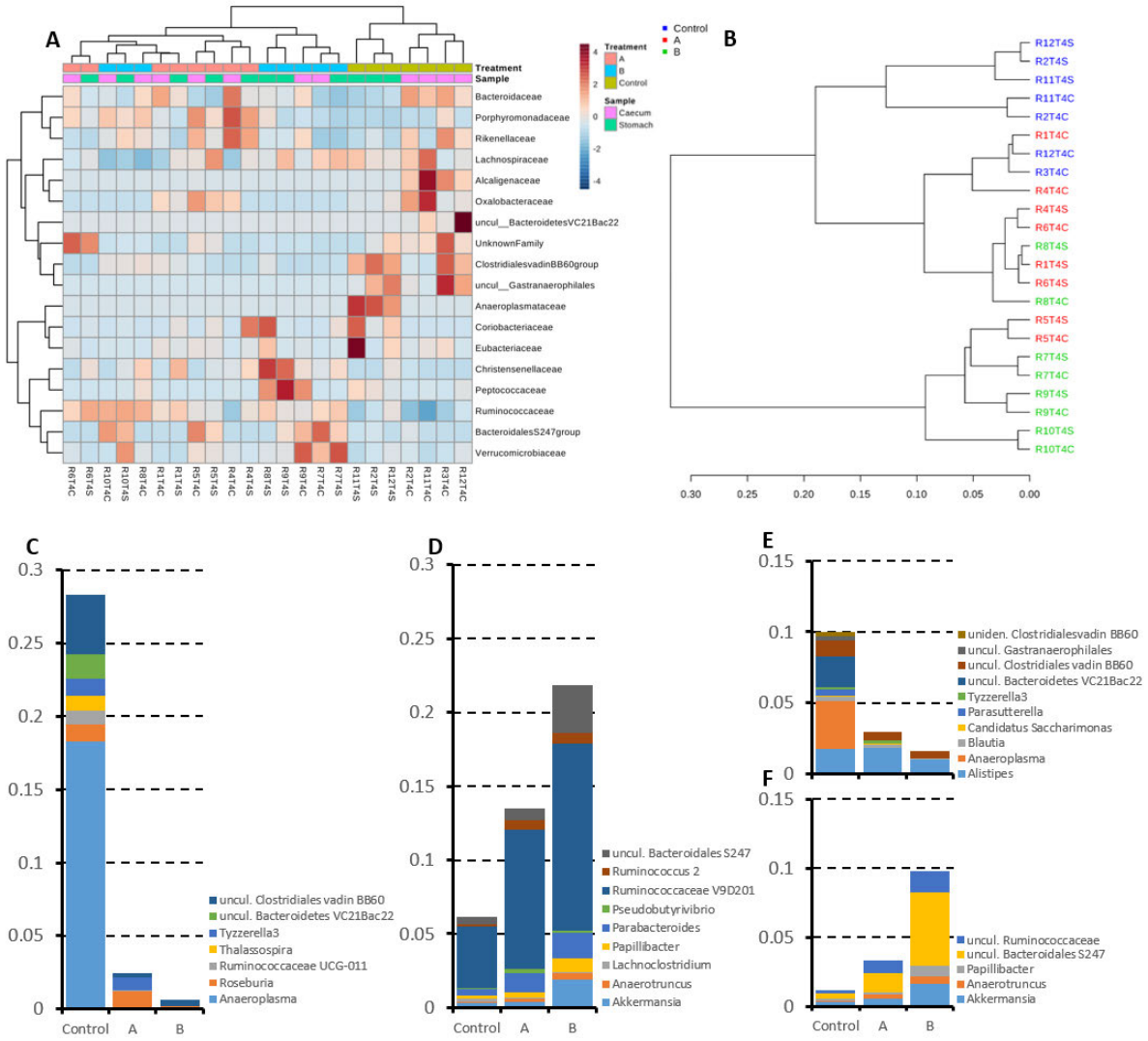

Figure 2. Heatmap, dendogram and histogram describe significant shift of bacterial taxa in each sample group. (A) Euclidean heatmap and clustering analysis of dominant families among stomach and caecum samples showing the abundance of bacterial families based on treatment types and sample types. (B) Dendogram developed at genus level using Jensen-Shannon divergence as distance measure, showing that stomach and caecum samples were clustered into the same individual rabbit. Bacterial taxa that significantly decreased (C) and increased (D) in stomach samples after single dose (A) and multiple doses (B) treatment. Bacterial taxa that significantly decreased (E) and increased (F) in caecum samples after single dose (A) and multiple doses (B) treatment. Statistical significant obtained from metagenomeSeq analysis where P<0.05, FDR p<0.005.

Microbial community structure. Based on heatmap clustering and dendogram analysis using the Jensen-Shannon distance metric (Fig 2 A-B), we found that stomach and caecum samples from the same individual were clustered together, in particular for the multiple doses group which indicate the uniqueness of the autochthonous microbial community present in each rabbit. In the control group, the stomach was enriched in Tenericutes and Firmicutes while the caecum was dominated by Bacteriodia but it was not significantly differentiated when analysed using LefSe. Similarly, comparative analysis within the same group showed no significant features between stomach and caecal samples. After pooling of all samples from both single and multiple dose treatment groups from the same rabbits, we found that the stomach was significantly enriched with Actinobacteria and Firmicutes while the caecum was enriched with Bacteriodetes ($p=<0.001$, FDR<0.01). Tenericutes were no longer enriched in the stomach due to a significant drop of *Anaeroplasma* spp. after treatment. At the class level, Coriobacteria and Clostridia were enriched in the stomach while Bacteroidia was enriched in the caecum ($P<0.005$, FDR <0.05). When LefSe analysis was performed at the genus level, nine enriched genera were significantly differentiated ($p<0.003$, FDR<0.03), including *Fusicatenibacter, Collinsella, Syntrophococcus, Lachnoclostridium*, Lachnospiraceae (n=3) in the stomach while Ruminoclostridum 9 and Ruminococcaceae UCG007 were enriched in caecum.

In contrast with Jensen-Shannon distance metric, analysis using unweighted Unifrac distance metric showed that gastric and caecal microbiota from all samples were clustered together (ANOSIM R= 0.82507; p-value < 0.001 (Fig 3 A)). Since the unweighted Unifrac distance only considers the presence and absence of bacterial taxa, the dissimilarity may be influenced by the presence of 14 genera and five genera that were uniquely present in stomach and caecum only, respectively. Assessed using weighted Unifrac distance which considers the actual abundance, microbial community structure for each sample group did not cluster clearly.

Before the rabbits were euthanized, we collected several faecal samples, including hard faeces and soft faeces (Saputra et al., 2018, in submission). Unfortunately, we are unable to perform statistical analysis due to low sample number. However, the reduction of microbial diversity caused by pradofloxacin in hard faeces was relatively higher (33%) than in the stomach (4%) and caecum (12%), when compared to the baseline. Further, using the Jensen-Shannon distance metric, we observed that the microbiota present in hard faeces was clustered differently from gastric, caecal and soft faeces samples (Fig 3C).

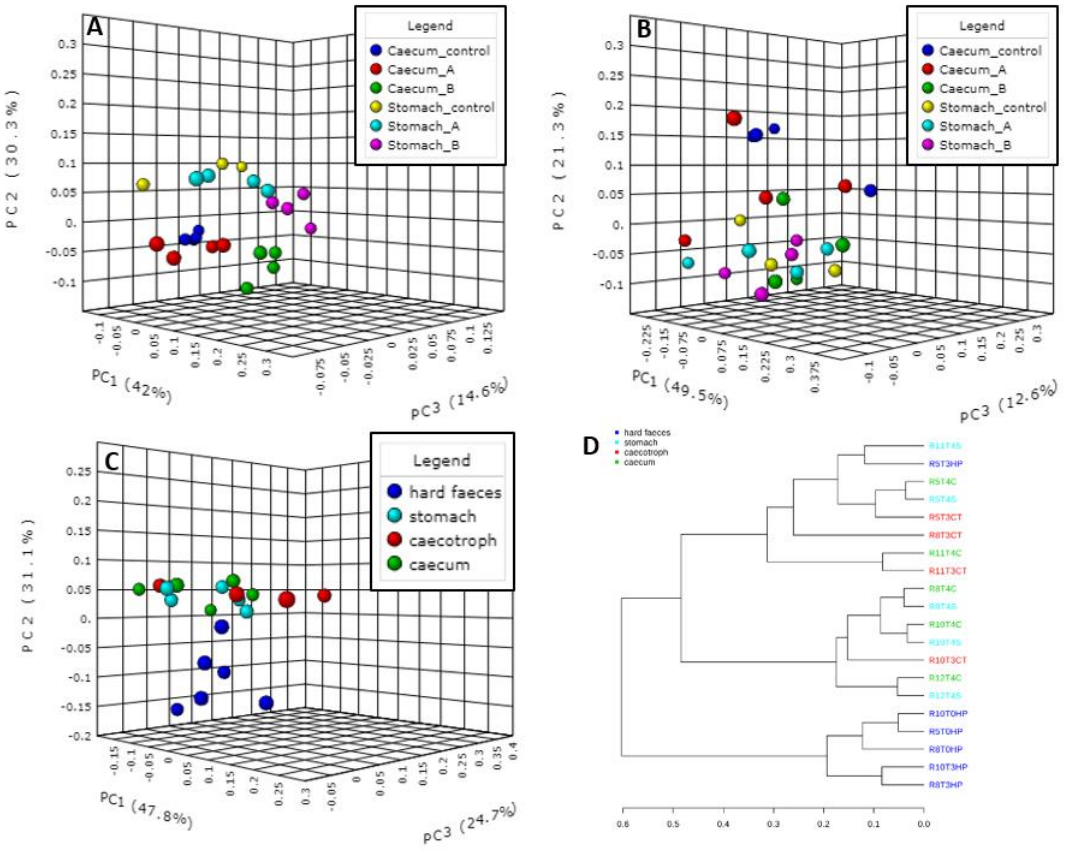

Figure 3. Principles coordinate analysis (PCoA) showing the dissimilarity between sample groups. (A-B) Unweighted ([ANOSIM] R: 0.82507; p-value < 0.001) and weighted ([ANOSIM] R: 0.5449; p-value < 0.001) Unifrac distance, respectively, showed that stomach and caecum were clustered separately among control and pradofloxacin treated samples. (C) Stomach and caecum samples were plotted with caecotroph (soft feces) and hard feces from the same rabbit, showing separate cluster for hard feces ([ANOSIM] R: 0.6163; p-value < 0.001). (D) Each sample from PCoA (C) were represented in dendogram (Jensen-Shannon divergence). *Control, control group; A, single dose treatment; B, multiple doses treatment.

Prediction of metabolic potentials. Although generally the caecum harboured lower microbial diversity compared to the stomach, prediction of functional diversity showed that several metabolic pathways such as thiamine, propanoate and biotin metabolism and folate biosynthesis were actually enriched in the caecum. No significant difference in metabolic pathways between control and treatment groups were identified in stomach and caecum. The most significantly abundant of the metabolic pathways in both stomach and caecum included biosynthesis of amino acids, pyruvate metabolism, carbon fixation pathways in prokaryotes, and amino sugar, nucleotide sugar, alanine, aspartate and glutamate metabolism (Table S3).

Discussion

This is the first study describing gastric and caecal microbial community changes resulting from pradofloxacin administration in animals generally and rabbits specifically. The notable findings of this study are 1) pradofloxacin caused minimal changes in the gastric and caecal microbiota with no detection of proliferation of bacterial taxa typically associated with enteritis in this species; 2) multiple daily doses resulted in greater changes to microbiota compared to the single dose treatment, but these differences were not significant when compared to the control group; 3) the rabbit stomach harboured surprisingly greater microbial diversity compared the caecum, but both compartments shared a similar core microbiota.

The minor effect of pradofloxacin on gastric and caecal microbiota was in contrast with several studies that have shown fluoroquinolones, as broad-spectrum, bactericidal antimicrobial agents, have a significant effect on some gastrointestinal tract bacterial communities. In studies involving ciprofloxacin in humans (Dethlefsen et al., 2008; Pop et al., 2016; Zaura et al., 2015) and mice (Choo et al., 2017); moxifloxacin in mice (Yao et al., 2016); and levofloxacin in humans (Panda et al., 2014); the effect on microbial diversity was much more dramatic. However, it is important to note that these studies characterised microbial communities in faecal samples only, not in other compartments of the gastrointestinal tract. There are only a few studies that have profiled gastric and caecal microbiota in response to fluoroquinolone therapy, but several studies have described the effect of other antibiotic classes. These have also shown that faecal microbiota is most likely to be markedly affected by antibiotics compared to other gastrointestinal tract compartments. For example, a study in healthy humans exposed to clindamycin and ciprofloxacin treatment showed significant perturbation of the faecal compared the salivary microbial community (Zaura et al., 2015). Amoxicillin, ceftriaxone and vancomycin also had significant effects on mouse faecal microbiota but bacterial populations in the ileum remained relatively stable (Tulstrup et al., 2015). Likewise, in the present study, we observed that pradofloxacin had a relatively greater effect on faecal compared to gastric and caecal microbiota. The reasons for these contrasting results may be due to the differences in resilience of the microbial community against stress in different gut compartments such as the caecum which also may include the ability to recover from antibiotic exposure as well as differences in gut transit time (Zaura et al., 2015). Another possibility is that pradofloxacin may be eliminated in faeces at much higher concentrations compared to those present in the stomach and caecum during transit. This has been previously observed in rabbits treated with moxifloxacin, an analogue to pradofloxacin. This study demonstrated that microbially active moxifloxacin was excreted unchanged through a

trans-epithelial mechanism, contributing 20% to the faecal concentration while the remaining 80% is excreted via the bile but only a small fraction of this proportion reaches the caecum (Musafija et al., 2000).

This study confirmed that the effect of both single and multiple doses treatment on gastric and caecal microbiota did not differ significantly, as demonstrated by alpha and beta diversity indices, and it must be remembered that a high concentration of pradofloxacin was administered in this study (7.5 mg/kg, the labelled dose recommendation in cats in some countries). Generally, pradofloxacin is given orally at a dose of 3-6 mg/kg orally in dogs, with label claims (Bayer, 2017) for the treatment of canine staphylococcal pyoderma, urinary tract and *Mycoplasma* infection (Dowers et al., 2009; Hartmann et al., 2008a; Hartmann et al., 2008b; Papich, 2016). Regardless, of the minor effect on the overall microbial communities inhabiting the stomach and caecum, this study did confirm that pradofloxacin effectively kills and/or inhibits the growth of several Gram-positive and-negative genera including strict anaerobes. The genus of bacteria that were the most significantly killed and/or suppressed by pradofloxacin were the *Anaeroplasma* spp., which are denoted as "anaerobic *Mycoplasma*" within the Mollicutes class (Brown et al., 2015). Furthermore, overrepresentation of Clostridiaceae and Enterobacteriaceae which potentially causing enteritis in rabbits was not observed. Instead, we observed proliferation of Akkermansia which are regarded as mucin degraders (Derrie et al., 2015) and Ruminococcaceae which play an active role in the gut as plant cellulose degraders (Biddle et al., 2013). These two taxa were overrepresented after pradofloxacin treatment. Our observations suggest that pradofloxacin rapidly kills specific bacterial taxa in these gut compartments, even following a single dose only, but in general, pradofloxacin (following single and multiple doses) has minimal effects on the autochthonous microbial community. It did not cause severe dysbiosis in the caecum and may have in fact benefited some nutritionally important communities.

Several previous studies undertaken in rabbits have assessed the microbial community in faeces and caecum (Abecia et al., 2017; Combes et al., 2013; Crowley et al., 2017; Eshar and Weese, 2014; Huybens et al., 2013; Michelland et al., 2010; Yang et al., 2016; Zeng et al., 2015), but the microbial community in the rabbit stomach remains unexplored. A metagenomics study in wild and domesticated rabbits has only described the microbial composition in stomach at the phyla level (Crowley et al., 2017) while other studies only describe the differences in microbial structure in gastric, caecal and faecal microbiota based on DGGE and TRFLP analysis (Skrivanova et al., 2010). In agreement with other studies, we showed that the caecal microbiota was dominated by Firmicutes (Ruminococcaceae and

Lachnospiraceae) and Bacteriodetes of varied proportions (Abecia et al., 2017; Crowley et al., 2017).

It is widely known that the gastric pH of the rabbit is very acidic compared to that of other species, and the stomach has previously been regarded as a sterile environment (Campbell-Ward, 2012; Davies and Davies, 2003). This statement may not be entirely true as our data suggest that the rabbit stomach in fact contains a similar core microbiota as that found in the caecum. In addition, using Shannon index for alpha diversity measurement, microbial diversity in the rabbit stomach was significantly higher compared to the caecum. Further, since the core microbiota in the stomach is also similar to that described in caecotrophs as visualised by PCoA in this study and previous studies (Michelland et al., 2010; Skrivanova et al., 2010), we hypothesize that the microbial community structure in the rabbit stomach is most likely influenced by caecotroph intake which may assist the fermentation process. In fact, it is difficult to ensure that the caecotrophs are swallowed without breaking the mucin coat within the stomach for at least 6–8 hours after ingestion (Davies and Davies, 2003).

In alignment with our functional predictions of microbial community structure, metabolic pathways were generally similar among the three rabbit treatment groups. However, the metabolic function described in this present study was generated based on metagenome predictions which only provide information on general function within the microbial community. It is believed that, even without significant effects on microbiota composition and general metabolic function, the KEGG orthologous groups might have been significantly affected (Zaura et al., 2015). A holistic approach which includes integration of metagenomic (V3-V4 16S rRNA gene sequencing) and metabolomic (nuclear magnetic resonance, NMR) as well as phenotypic data (culture-based) should be employed to more fully understand the consequences of the shift in microbial communities due to antibiotic intervention (Choo et al., 2017). The limitations of this study include low numbers of animals per treatment group, our inability to obtain matching caeocotroph and faecal samples for all time points and the requirement for the trial to be terminated to obtain caecum and stomach samples before the autochthonous microbiota could recover post-cessation of antimicrobial therapy. To address these, further analysis with a complete set of gastrointestinal compartment samples including soft and hard faeces samples is required.

In conclusion, this is the first report describing the effect of pradofloxacin on intestinal microbiota within the stomach and caecum in animals. The short term exposure of pradofloxacin only slightly affected the microbial community in the rabbit stomach and caecum. Considering other factors such as no observation of antibiotic-induced diarrhoea, toxicoses and/or other abnormalities at the gross post-mortem and histopathological level, which was confirmed by microbiota profiling, we suggest that pradofloxacin is a very safe short term antimicrobial treatment for use in pet rabbits not destined for human consumption.

Acknowledgement

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Competing interest

SA and DJT have received research grants and contracts from Zoetis, Merial, Luoda Pharma and Neoculi. All other authors declare no competing interests.

Supplementary information

Table S1. Clinical records, samples ID and overall read obtained from Illumina sequencing.

Table S2. Relative abundance of dominant bacterial taxa in control group, single dose (A) and multiple doses (B) treatment groups.

Table S3. Prediction of metabolic functional based on Tax4Fun in control group, single dose (A) and multiple doses (B) treatment groups.

Figure S1. Bar graph of dominant genera showing similar composition of caecotroph and stomach microbiota in control group (R11T3CT-caecotroph and R11T4S-stomach) and multiple doses group (R10T3CT-caecotroph and R10T4S-stomach).

References

Abecia, L., Rodríguez-Romero, N., Martínez-Fernández, G., Martínez-Vallespín, B., Fondevila, M., 2017. Pyrosequencing study of caecal bacterial community of rabbit does and kits from a farm affected by epizootic rabbit enteropathy. World Rabbit Sci. 25, 261.

Alfano, N., Courtiol, A., Vielgrader, H., Timms, P., Roca, A.L., Greenwood, A.D., 2015. Variation in koala microbiomes within and between individuals: effect of body region and captivity status. Sci. Rep. 5, 10189.

Bayer 2017. Veraflox® Summary of Product Characteristics (Bayer Animal Health).

Biddle, A., Stewart, L., Blanchard, J., Leschine, S., 2013. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity 5, 627-640.

Brown, D.R., Bradbury, J.M., Johansson, K.-E. 2015. *Anaeroplasma*, In: Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Inc.

Campbell-Ward, M.L. 2012. Gastrointestinal physiology and nutrition. In Ferrets, Rabbits, and Rodents: Clinical Medicine and Surgery, Quesenberry, K.E., Carpenter, J.W., eds. (St. Louis, Missouri, Elsevier Saunders), 183-192.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high throughput community sequencing data. Nat. Methods 7, 335-336.

Carman, R.J., Borriello, S.P., 1984. Infectious nature of *Clostridium spiroforme*-mediated rabbit enterotoxaemia. Vet. Microbiol. 9, 497-502.

Carman, R.J., Borriello, S.P., 1989. Alternative animal models of *Clostridium spiroforme* mediated diarrhoea of rabbits. Microbial Ecology in Health and Disease 2, 69-73.

Choo, J.M., Kanno, T., Zain, N.M., Leong, L.E., Abell, G.C., Keeble, J.E., Bruce, K.D., Mason, A.J., Rogers, G.B., 2017. Divergent relationships between fecal microbiota and metabolome following distinct antibiotic-induced disruptions. mSphere 2, 1-16.

Combes, S., Fortun-Lamothe, L., Cauquil, L., Gidenne, T., 2013. Engineering the rabbit digestive ecosystem to improve digestive health and efficacy. Anim. 7, 1429-1439.

Crowley, E.J., King, J.M., Wilkinson, T., Worgan, H.J., Huson, K.M., Rose, M.T., McEwan, N.R., 2017. Comparison of the microbial population in rabbits and guinea pigs by next generation sequencing. PLoS One 12, e0165779.

Davies, R.R., Davies, J.A.E.R., 2003. Rabbit gastrointestinal physiology. Vet. Clin. Exot. Anim. 6, 139–153.

DeCubellis, J., Graham, J., 2013. Gastrointestinal disease in guinea pigs and rabbits. Vet. Clin. North. Am. Exot. Anim. Pract. 16, 421-435.

Derrie, M., Plugge, C.M., Vos, W.M.d., Zoetandal, E.G. 2015. Akkermansia, In: Bergey's Manual of Systematics of Archaea and Bacteria Wiley Online Library.

Dethlefsen, L., Huse, S., Sogin, M.L., Relman, D.A., 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6, e280.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic. Acids. Res. 45(W1), W180-W188.

Dowers, K.L., Tasker, S., Radecki, S.V., Lappin, M.R., 2009. Use of pradofloxacin to treat experimentally induced *Mycoplasma hemofelis* infection in cats. Am. J. Vet. Res. 70, 105-111.

Eshar, D., Weese, J.S., 2014. Molecular analysis of the microbiota in hard feces from healthy rabbits (*Oryctolagus cuniculus*) medicated with long term oral meloxicam. BMC Vet. Res. 10, 1-9.

Govendir, M., Norris, J.M., Hansen, T., Wigney, D.I., Muscatello, G., Trott, D.J., Malik, R., 2011. Susceptibility of rapidly growing *Mycobacteria* and *Nocardia* isolates from cats and dogs to pradofloxacin. Vet. Microbiol. 153, 240-245.

Halls, A.E. 2005. Enteric Diseases in Rabbits. In Nutrifax (Canada, Nutreco Canada Inc.).

Halls, E.H. 2008. Caecotrophy in rabbits. In Nutrifax (Canada, Nutreco Canada Inc.).

Hartmann, A., Krebber, R., Daube, G., Hartmann, K., 2008a. Pharmacokinetics of pradofloxacin and doxycycline in serum, saliva, and tear fluid of cats after oral administration. J. Vet. Pharmacol. Ther. 31, 87-94.

Hartmann, A.D., Helps, C.R., Lappin, M.R., Werckenthin, C., Hartmann, K., 2008b. Efficacy of pradofloxacin in cats with feline upper respiratory tract disease due to *Chlamydophila felis* or *Mycoplasma* infections. J. Vet. Intern. Med. 22, 44-52.

Huybens, N., Houeix, J., Licois, D., Mainil, J., Marlier, D., 2013. Pyrosequencing of epizootic rabbit enteropathy inocula and rabbit caecal samples. Vet. J. 196, 109-110.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glockner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1.

Korpela, K., Salonen, A., Virta, L.J., Kekkonen, R.A., Forslund, K., Bork, P., de Vos, W.M., 2016. Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children. Nat. Commun. 7, 10410.

Meredith, A. 2010. The rabbit digestive system: a delicate balance. In Rabbiiting On (Rabbit Welfare Association & Fund, UK).

Michelland, R.J., Combes, S., Monteils, V., Cauquil, L., Gidenne, T., Fortun-Lamothe, L., 2010. Molecular analysis of the bacterial community in digestive tract of rabbit. Anaerobe 16, 61-65.

Musafija, A., Ramon, J., Shtelman, Y., Yoseph, G., Rubinovitz, B., Segev, S., Rubinstein, E., 2000. Trans-epithelial intestinal elimination of moxifloxacin in rabbits. J. Antimicrob. Chemother. 45, 803-805.

Oglesbee, B.L., Jenkins, J.R. 2012. Gastrointestinal diseases. In Ferrets, Rabbits, and Rodents: Clinical Medicine and Surgery, Quesenberry, K.E., Carpenter, J.W., eds. (St. Louis, Missouri, Elsevier Saunders), 193-204.

Panda, S., El khader, I., Casellas, F., Lopez Vivancos, J., Garcia Cors, M., Santiago, A., Cuenca, S., Guarner, F., Manichanh, C., 2014. Short-term effect of antibiotics on human gut microbiota. PLoS One 9, e95476.

Papich, M.G. 2016. Pradofloxacin. In Saunders Handbook of Veterinary Drugs (Fourth Edition): Small and Large Animal (St. Louis, Missouri, Elsevier), 660-662.

Pop, M., Paulson, J.N., Chakraborty, S., Astrovskaya, I., Lindsay, B.R., Li, S., Bravo, H.C., Harro, C., Parkhill, J., Walker, A.W., Walker, R.I., Sack, D.A., Stine, O.C., 2016. Individual-specific changes in the human gut microbiota after challenge with enterotoxigenic *Escherichia coli* and subsequent ciprofloxacin treatment. BMC Genomics 17, 440.

Raw, C., 2017. Infectious diseases in rabbits. Vet. Nurs. J. 32, 298-300.

Ritzman, T.K., 2014. Diagnosis and clinical management of gastrointestinal conditions in exotic companion mammals (rabbits, guinea pigs, and chinchillas). Vet. Clin. North. Am. Exot. Anim. Pract. 17, 179-194.

Silley, P., Stephan, B., Greife, H.A., Pridmore, A., 2012. Bactericidal properties of pradofloxacin against veterinary pathogens. Vet. Microbiol. 157, 106-111.

Skrivanova, E., Worgan, H.J., Pinloche, E., Marounek, M., Newbold, C.J., McEwan, N.R., 2010. Changes in the bacterial population of the caecum and stomach of the rabbit in response to addition of dietary caprylic acid. Vet. Microbiol. 144, 334-339.

Tulstrup, M.V., Christensen, E.G., Carvalho, V., Linninge, C., Ahrne, S., Hojberg, O., Licht, T.R., Bahl, M.I., 2015. Antibiotic treatment affects intestinal permeability and gut microbial composition in wistar rats dependent on antibiotic class. PLoS One 10, e0144854.

Wetzstein, H.G., 2005. Comparative mutant prevention concentrations of pradofloxacin and other veterinary fluoroquinolones indicate differing potentials in preventing selection of resistance. Antimicrob. Agents Chemother. 49, 4166-4173.

Wetzstein, H.G., Hallenbach, W., 2011. Tuning of antibacterial activity of a cyclopropyl fluoroquinolone by variation of the substituent at position C-8. J. Antimicrob. Chemother. 66, 2801-2808.

Yang, C., Mi, L., Hu, X., Liu, J., Wang, J., 2016. Investigation into host selection of the cecal acetogen population in rabbits after weaning. PLoS One 11, e0158768.

Yao, J., Carter, R.A., Vuagniaux, G., Barbier, M., Rosch, J.W., Rock, C.O., 2016. A pathogen-selective antibiotic minimizes disturbance to the microbiome. Antimicrob. Agents Chemother. 60, 4264-4273.

Zaura, E., Brandt, B.W., Teixeira de Mattos, M.J., Buijs, M.J., Caspers, M.P., Rashid, M.U., Weintraub, A., Nord, C.E., Savell, A., Hu, Y., Coates, A.R., Hubank, M., Spratt, D.A., Wilson, M., Keijser, B.J., Crielaard, W., 2015. Same exposure but two radically different responses to antibiotics: resilience of the salivary microbiome versus long-term microbial shifts in feces. MBio 6, e01693-01615.

Zeng, B., Han, S., Wang, P., Wen, B., Jian, W., Guo, W., Yu, Z., Du, D., Fu, X., Kong, F., Yang, M., Si, X., Zhao, J., Li, Y., 2015. The bacterial communities associated with fecal types and body weight of rex rabbits. Sci. Rep. 5, 9342.

Supplementary information

Table S1. Clinical records, samples ID and overall read obtained from Illumina sequencing.

Treatment group	Sample type	Rabbit ID	Sex	Start weight	Euthanasia weight	Dose	Number of dose	Sample ID	Total number of reads	Filtered or chimeric reads	Reads in OTUs
Control	Caecum	2	F	2.82	2.26	0	0	R2T4C	53435	39975	13460
	Stomach							R2T4S	62354	48953	13401
	Caecum	3	F	2.96	2.36	0	0	R3T4C	57846	44078	13768
	Caecum	11	M	3.12	2.5	0	0	R11T4C	55291	41731	13560
	Stomach							R11T4S	31287	24773	6514
	Caecum	12	M	3.2	2.56	0	0	R12T4C	45611	34994	10617
	Stomach							R12T4S	30357	23690	6667
A	Caecum	1	F	3.25	2.56	0.96	1	R1T4C	77243	57639	19604
	Stomach							R1T4S	60917	46857	14060
	Caecum	4	F	3.47	-	1.04	1	R4T4C	63404	47983	15421
	Stomach							R4T4S	53468	41850	11618
	Caecum	5	F	2.95	2.36	0.89	1	R5T4C	51816	40615	11201
	Stomach							R5T4S	53650	42798	10852
	Caecum	6	F	3	2.4	0.9	1	R6T4C	63451	47980	15471
	Stomach							R6T4S	51937	41075	10862
B	Caecum	7	M	3.1	2.48	0.93	4	R7T4C	58012	43867	14145
	Stomach							R7T4S	49594	38775	10819
	Caecum	8	M	3.4	2.71	1	4	R8T4C	59667	45544	14123
	Stomach							R8T4S	46679	36845	9834
	Caecum	9	M	3.2	2.56	0.96	4	R9T4C	55067	42017	13050
	Stomach							R9T4S	69020	52113	16907
	Caecum	10	M	3.13	2.48	0.94	4	R10T4C	63665	49275	14390
	Stomach							R10T4S	61304	47133	14171

Table S2. Relative abundance of dominant bacterial taxa in control group, single dose (A) and multiple doses (B) treatment groups.

Kingdom	Phylum	Class	Ordo	Family	Stomach			Caecum		
					Control	A	B	Control	A	B
Arcaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	0.008	0.004	0.002	0	0.003	0.002
Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	0.255	0.266	0.152	0.034	0.049	0.018
	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	8.844	14.556	12.179	29.278	27.757	16.938
				Bacteroidales S24-7 group	0.439	0.835	3.212	0.358	1.354	5.187
				Porphyromonadaceae	1.018	4.890	3.009	2.561	7.728	4.542
				Rikenellaceae	0.623	1.085	0.925	1.718	1.780	1.019
		Bacteroidetes VC2.1 Bac22	uncul. bacterium	uncul. bacterium	1.676	0.002	0.008	2.127	0.015	0.002
	Cyanobacteria	Melainabacteria	Gastranaerophilales	uncul. bacterium	0.889	0.004	0.777	0.334	0.002	0
				uncul. rumen bacterium 4C0d-2	0.008	0.013	0	0.004	0.003	0
	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	0.349	0.054	0.089	0.038	0.012	0.029
				Clostridiales vadinBB60 group	4.132	0.320	0.425	2.225	0.633	0.532
				Eubacteriaceae	1.046	0.333	0.348	0.411	0.338	0.139
				Family XIII	0.059	0.019	0.012	0.004	0.015	0.013
				Lachnospiraceae	27.774	31.519	28.312	31.887	20.004	20.088
				Peptococcaceae	0.047	0.002	0.130	0.030	0.005	0.053
				Ruminococcaceae	33.062	45.597	48.495	24.646	39.569	49.812
				Ruminococcaceae	0	0	0.006	0.002	0.007	0.005
		Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	0.020	0.026	0	0.008	0.045	0
		unknown			0	0.006	0.002	0	0	0
	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	0.078	0.004	0	0.429	0.005	0
				Oxalobacteraceae	0.020	0.026	0	0.094	0.056	0
		Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	0	0	0	0.024	0.018	0
		Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	1.007	0	0.002	0.056	0	0
			Rickettsiales		0.012	0.002	0.016	0	0	0
	Saccharibacteria	Unknown Class	Unknown Order	Unknown Family	0.035	0.048	0.012	0.098	0.059	0.007
	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	18.229	0.006	0.008	3.292	0.010	0
			Mollicutes RF9	uncul. bacterium	0.039	0.009	0	0.038	0.013	0
				uncul. rumen bacterium	0.008	0	0	0.004	0	0
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	0.325	0.368	1.870	0.296	0.520	1.608
	unknown				0	0.004	0.010	0.002	0	0.005

Table S3. Prediction of metabolic functional based on Tax4Fun in control group, single dose (A) and multiple doses (B) treatment groups.

Pathway	Stomach									Caecum								
	Control			A			B			Control			A			B		
	Hits	Pval	FDR	Hits	Pval	FDR	Hits	Pval	FDR	Hits	Pval	FDR	Hits	Pval	FDR	Hits	Pval	FDR
Biosynthesis of amino acids	115	6.07E-22	8.98E-20	118	2.40E-24	3.56E-22	117	5.70E-25	8.43E-23	116	5.68E-23	8.41E-21	115	3 51E-22	5.19E-20	118	3 54E-24	5.24E-22
Pyruvate metabolism	39	3.83E-08	2.83E-06	39	2.71E-08	1.34E-06	39	1.12E-08	5.52E-07	36	1.34E-06	6.61E-05	38	1 22E-07	6 02E-06	39	3 05E-08	1.50E-06
Carbon fixation pathways in prokaryotes	33	1.18E-07	5.80E-06	34	1.98E-08	1.34E-06	34	8.83E-09	5.52E-07	35	4.43E-09	3.28E-07	37	1 83E-10	1 36E-08	36	9 31E-10	6.89E-08
Amino sugar and nucleotide sugar metabolism	33	8.80E-07	3.26E-05	33	6.62E-07	2.45E-05	32	1.19E-06	4.40E-05	31	8.44E-06	0.000312	30	2 90E-05	0 00086	32	2.61E-06	9.67E-05
Alanine, aspartate and glutamate metabolism	30	1.49E-05	0 000442	29	3.71E-05	0 000915	29	2.04E-05	0.000605	29	3.86E-05	0.000951	28	0.000123	0.0026	29	4 01E-05	0.000847
Valine, leucine and isoleucine biosynthesis	11	6.41E-05	0.00158	11	5.72E-05	0.00121	11	4.26E-05	0.000902	11	5.83E-05	0.00123	10	0.000457	0 00752	11	5 94E-05	0.0011
Carbon metabolism	83	0.00016	0.00338	85	3.06E-05	0 000906	83	3.24E-05	0.000799	86	1.76E-05	0.00052	88	5 55E-06	0.000205	86	1 91E-05	0.000471
Citrate cycle (TCA cycle)	24	0.000394	0.00646	25	0 000109	0.00201	25	6.49E-05	0.0012	25	0.000113	0.00208	26	3.75E-05	0.000925	27	1 04E-05	0.000309
Glycine, serine and threonine metabolism	32	0.000414	0.00646	30	0.00179	0.0162	30	0.00108	0.0114	30	0.00185	0 015	28	0 00852	0.0466	29	0 00409	0.0252
Porphyrin and chlorophyll metabolism	29	0.000478	0.00646	28	0 000961	0.0118	28	0.000582	0.00718	29	0.000402	0.00496	28	0 00106	0.0121	29	0.000416	0.00605
Selenocompound metabolism	10	0.00048	0.00646	10	0 000435	0.00643	10	0.000336	0.00498	10	0.000442	0.00503	9	0 00263	0.0229	10	0.000449	0.00605
Glycolysis / Gluconeogenesis	32	0.000707	0.00872	33	0 000237	0.00389	32	0.000324	0.00498	32	0.000591	0.00625	32	0.000635	0.0094	34	0.000101	0.00167
Cysteine and methionine metabolism	29	0.000841	0.00958	29	0 000688	0.00926	29	0.000408	0.00549	29	0.000712	0.00702	28	0 00179	0.0171	29	0.000736	0.00908
Starch and sucrose metabolism	27	0.000931	0.00984	26	0.00186	0.0162	25	0.00279	0.0197	26	0.00192	0 015	25	0 00461	0.031	26	0 00198	0.0172
Histidine metabolism	17	0.00107	0 0105	16	0 0029	0.0211	16	0.0021	0.0179	17	0.00095	0.00879	17	0.000996	0.0121	16	0 00303	0.022
One carbon pool by folate	13	0.00117	0 0108	13	0.00104	0.0118	13	0.00077	0.00877	14	0.00024	0.00355	14	0 00025	0 00463	13	0 00108	0.0123
Streptomycin biosynthesis	8	0.00187	0 0163	8	0.00173	0.0162	6	0.0352	0.141	9	0.000225	0.00355	8	0.0018	0.0171	8	0 00177	0.0169
Pantothenate and CoA biosynthesis	14	0.00317	0 0247	14	0.00283	0.0211	14	0.0021	0.0179	14	0.00288	0.0204	14	0 003	0.0238	14	0 00294	0.022
Polyketide sugar unit biosynthesis	4	0.00313	0 0247	4	0.00299	0.0211	4	0.00266	0.0197	4	0.00301	0.0204	4	0 00306	0.0238	4	0 00304	0.022
2-Oxocarboxylic acid metabolism	23	0.00346	0 0256	23	0.00296	0.0211	23	0.00196	0.0179	23	0.00304	0.0204	22	0 00734	0.0434	23	0 00312	0.022
Peptidoglycan biosynthesis	8	0.00387	0 0264	8	0.00358	0.023	8	0.00293	0.0197	8	0.00362	0.0224	8	0 00372	0.0262	8	0 00367	0.0236
Phenylalanine, tyrosine and tryptophan bios.	25	0.00393	0 0264	25	0.00333	0.0224	25	0.00217	0.0179	25	0.00343	0.0221	25	0 00362	0.0262	25	0 00352	0.0236
Fructose and mannose metabolism	18	0.00791	0 0496	18	0.00697	0.0397	16	0.0274	0.113	17	0.0167	0.0798				16	0.0366	0.139
Lipopolysaccharide biosynthesis	9	0.00839	0 0496	10	0.00177	0.0162	8	0.0232	0.104	11	0.00033	0.00443	10	0 00185	0.0171	10	0 00182	0.0169
Pentose and glucuronate interconversions	17	0.00834	0 0496	16	0 0177	0.0821	16	0.0134	0.0756	15	0.0395	0.158	15	0.0407	0.157	16	0.0184	0.0851
Thiamine metabolism	11	0.00954	0 0543	11	0.00872	0.0461	11	0.0069	0.0444	11	0.00885	0.0468	11	0 00912	0.0466	11	0 00898	0.0492
Propanoate metabolism	21	0 0107	0 0589	22	0.00412	0.0254	22	0.00279	0.0197	21	0.00959	0 049	24	0.000704	0 00947	23	0 00178	0.0169
Cyanoamino acid metabolism	6	0 0149	0.079	6	0 0141	0.072	6	0.0122	0.075	6	0.0142	0.0703	6	0.0145	0.0716	6	0.0144	0.076
Fatty acid biosynthesis	12	0.019	0 0971	12	0 0174	0.0821	12	0.0138	0.0756	13	0.00617	0.0351	12	0.0182	0.0866	12	0.0179	0.0851
Biotin metabolism	9	0 0201	0 0992	10	0.00529	0.0313	9	0.0154	0.0816	11	0.00126	0 011	10	0 00552	0.0341	10	0 00544	0.031
C5-Branched dibasic acid metabolism	6	0 0264	0.122				6	0.0217	0.104	7	0.0051	0.0302	7	0 00522	0.0336	7	0 00516	0.0306
Folate biosynthesis	12	0 0257	0.122	12	0 0235	0.102	12	0.0188	0.0959	13	0.00884	0.0468	13	0 00914	0.0466	12	0.0242	0.105
D-Glutamine and D-glutamate metabolism	4	0.031	0.127	4	0 0298	0.116	4	0.0268	0.113	4	0.03	0.123	4	0.0304	0.121	4	0.0302	0.117
Drug metabolism - other enzymes	9	0 0291	0.127	9	0 0271	0.113	9	0.0225	0.104	9	0.0274	0.121	9	0.0281	0.12	9	0.0277	0.115
Nitrogen metabolism	8	0 0293	0.127	8	0 0275	0.113	8	0.0232	0.104	8	0.0278	0.121	8	0.0284	0.12	8	0.0281	0.115
Tetracycline biosynthesis	4	0.031	0.127	4	0 0298	0.116	4	0.0268	0.113	4	0.03	0.123	4	0.0304	0.121	4	0.0302	0.117
Butanoate metabolism	21	0 0363	0.141	23	0.00796	0.0436	20	0.045	0.171				23	0 00857	0.0466	22	0.0173	0.0851
Pyrimidine metabolism	45	0.036	0.141															
Carbon fixation in photosynthetic organisms				14	0 0206	0.0924	13	0.0377	0.147	14	0.0209	0.0968	14	0.0216	0.097	14	0.0213	0.0954
Lipoic acid metabolism										3	0.0425	0.161	3	0 043	0.159	3	0.0427	0.158
Lysine biosynthesis				16	0 0177	0.0821	16	0.0134	0.0756				16	0.0187	0.0866	16	0.0184	0.0851
Vitamin B6 metabolism										6	0.0406	0.158	6	0.0414	0.157			

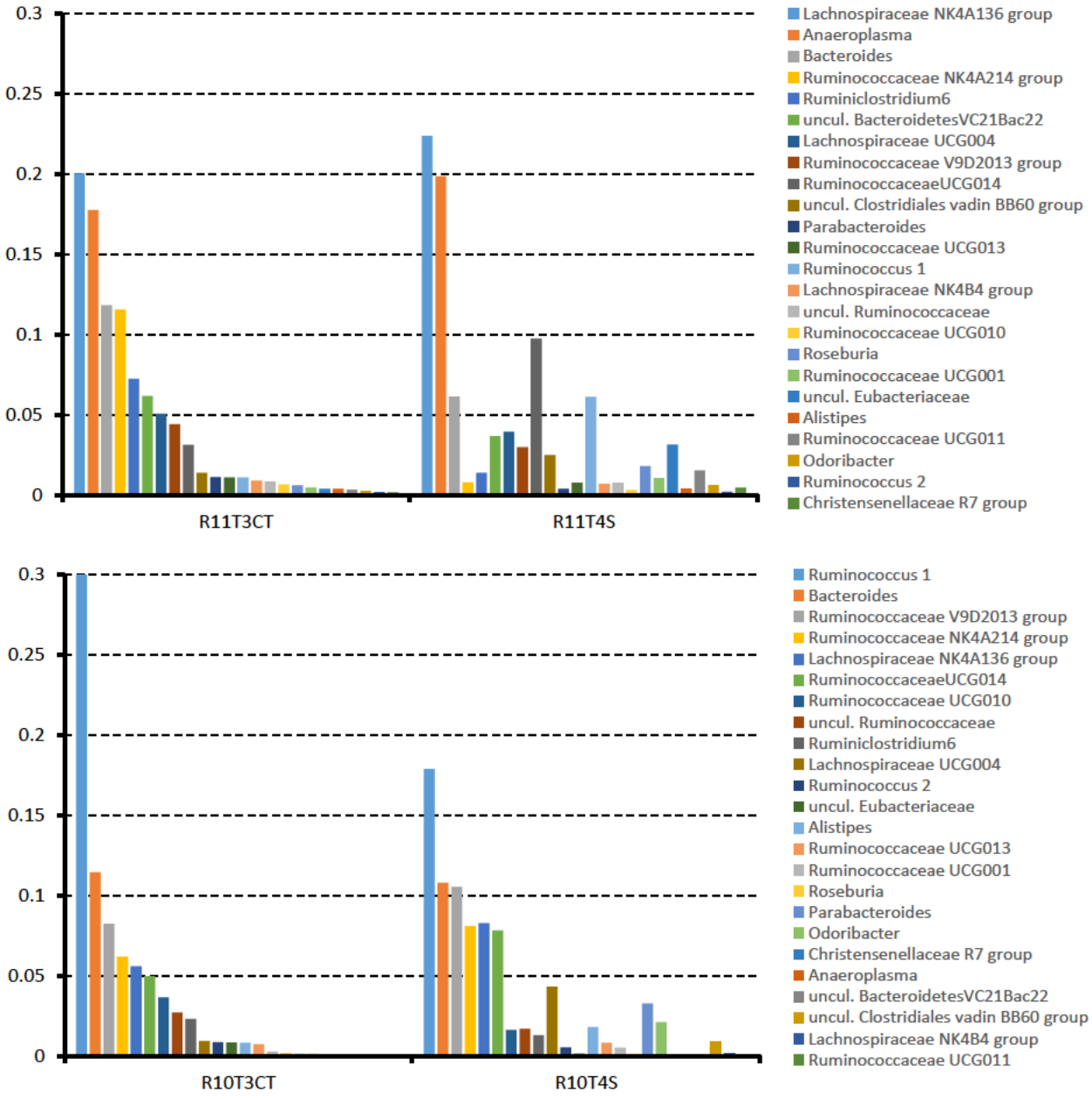

Figure S1. Bar graph of dominant genera showing similar composition of caecotroph and stomach microbiota in control group (R11T3CT-caecotroph and R11T4S-stomach) and multiple doses group (R10T3CT-caecotroph and R10T4S-stomach).

Chapter 8

General discussion

8.1. General summary

Antimicrobials are pharmaceutical drugs widely used to treat infections in humans and animals, contributing enormously to the modern medicine. Following the broad use of these antimicrobials for therapeutic (i.e. treatment, metaphylaxis and prophylaxis) and non-therapeutic use (i.e. growth promotion), there has been some unintended consequences that affect the individual as well as the community. This thesis focuses on two critical consequences of antimicrobial use, namely the development and measurement of antimicrobial resistance (AMR) and the disruptive effect of antimicrobial agents on gut microbiota (dysbiosis).

Forming a critical aspect of the first nation-wide survey of AMR in Australian animals, this thesis provides an important baseline on the current level of AMR among the two major groups of companion animal pathogens. In Chapters 2 and 3, frequency of resistance to 16 antimicrobials was determined among Gram-negative pathogens (*E. coli,* n=883) and Gram-positive pathogens (*Staphylococcus pseudintermedius* n=629 and *S. aureus* n=117) obtained from veterinary diagnostic laboratories throughout Australia. Statistical analysis was performed on epidemiological data accompanying the isolates, which revealed that prior antimicrobial use and recurrent diseases increase the risk of infection by multidrug-resistant (MDR) bacteria, in particular MDR *E. coli* causing urinary tract infections, while any surgical interventions increase the risk of infection by methicillin-resistant *S. pseudintermedius* (MRSP). Comparison with similar studies in other countries (Chapter 4) revealed that generally Australia was among the group of countries with comparative low levels of AMR among companion animal pathogens.

Chapter 6 and 7 focused on antimicrobial effect on gut microbial communities in rabbits after treatment with pradofloxacin, a new veterinary fluoroquinolone that has a much wider spectrum of activity, including anaerobes. Concern was expressed among veterinarians as to the safety of pradofloxacin given this much broader spectrum of activity and the fact that rabbits are predisposed to antibiotic-induced clostridial enteritis. Microbiota profiling revealed that disruption caused by pradofloxacin was far greater in faecal samples than in gastric and caecal samples, as indicated by alpha diversity measurement. Considered with other parameters, such as the absence of gross post mortem and histological evidence of pathology and clinical assessment during the trial, pradofloxacin oral suspension was considered safe for use in the rabbit for up to three days of treatment.

8.2. Major findings

8.2.1. Comparative rates of AMR in Australia is low for Gram-negative pathogens but moderately high for Gram-positive pathogens

Since this is the first nationwide survey of AMR in companion animal pathogens conducted in Australia, no comparable data are available to conclude whether the frequency of AMR is increasing over time or not. Comparisons can be made between Australia and other countries for a selected number of resistances, with greatest interest in resistance to third generation cephalosporins (3GC), fluoroquinolones (FQN), aminoglycosides (AMG) and carbapenems in *E. coli* and the frequency of methicillin resistance among staphylococci. In most cases, the frequency of AMR reported in each country represents nationwide data, in particular for developed countries, while others may represent regional areas or derive their data from several veterinary diagnostic laboratory surveys.

Resistance to carbapenems was not detected while resistance to 3GC, FQN, and AMG was comparatively low in Australia, in particular for *E. coli* isolated from cats and dogs. Data on antimicrobial use in companion animals in Australia is limited (Hardefeldt et al., 2017), however, the low level of resistance may be attributed to the comparatively restricted use of these antimicrobials in Australia compared to other countries. Restricted use of carbapenems for exceptional circumstances in Australia resulted in zero frequency of resistance in clinical *E. coli* (AVA, 2017), while in other countries, such as the USA, carbepenem resistance in *E. coli* has been detected at low levels in both dogs (1.3%) and cats (1.8%) (Thungrat et al., 2015). Fluoroquinolones have not been registered for use in livestock in Australia, but several fluoroquinolones are registered for use in dogs and cats such as enrofloxacin, marbofloxacin, orbifloxacin, ibafloxacin and pradofloxacin as last resort antimicrobials (AVA, 2017). As a result, fluoroquinolone resistance has not been present or is detected at very low levels in food production animals in Australia (both in pathogens and commensals) (Abraham et al., 2015; APVMA, 2017; Barlow et al., 2015). Amongst animal isolates, resistance to fluoroquinolones is only likely to occur in clinical isolates from companion animals with a frequency <10% for both coagulase-positive staphylococci (Chapter 2) and *E. coli* isolates (Chapter 3) from dogs, cats and horses.

Among Gram-positive bacteria, the frequency of MRSP isolated from dogs was relatively high in Australia compared with many European countries (Chapter 4). Interestingly, a low rate of resistance in Gram-negative isolates but a high rate in Gram-positive isolates mirrors the situation in human medicine in Australia (AURA, 2016). In human medicine, the

frequency of MRSA isolates was higher, with 15%-17% frequency in non-invasive and invasive infections, respectively, compared with many developed countries in Europe (AURA, 2016). The reasons for the major difference are not clear, but it is likely that the drivers for these types of resistance are different between animals and humans.

8.2.2. High levels of AMR among bacterial isolates from veterinary healthcare-associated infections

It is confirmed that the infection site and prior antimicrobial treatment were strongly correlated with MRSP compared to MSSP infection in dogs (Chapter 2). Logistic regression analysis showed that surgical site infections were the most significant factor in the likelihood of isolation of MRSP with a frequency of 35.7% among clinical isolates from surgical sites. In contrast, the proportion of MRSP from other infection sites was significantly lower ($p<0.001$), for instance from urinary tract infections the proportion of MRSP was only 5.7%. Interestingly, a similar pattern was observed for other pathogens, i.e. *E. coli* and *S. aureus* (Figure 8.1). The proportion of isolates resistance to 3GCs (ceftriaxone) in *E. coli* isolated from skin and soft tissue infections in dogs was doubled when compared to urinary tract infection isolates (20.5% vs 10.2%) (Chapter 3), but these isolates represented the majority obtained from surgical site infections (52.3%). In *S. aureus*, methicillin-resistant strains were only isolated from skin and soft tissue infections, with a higher proportion in surgical sites (33.3%). With a total proportion of surgical site isolates representing only 5% of the total (81/1629), the proportion of isolates resistant to both 3GCs and FQNs among *E. coli*, and the proportion of isolates that were MRSP and MRSA was high and extremely high, respectively, ranging from 33.3%-52.3%. By contrast these isolates were underrepresented in other infection sites, being only recorded at low to moderate levels (<20%) (Figure 8.1). This phenomenon indicates that health care intervention (surgery, particularly orthopaedic surgery) is strongly correlated with high incidence of isolation of MDR bacteria, mirroring the situation observed in human medicine (Cohen et al., 2017; Dohmen, 2008). Greater attention to biosecurity and infection control, for example, swabbing hospital admissions with particular infections, could be a viable intervention strategy to keep the frequency of MDR infections low in companion animal veterinary practice. Only a small number of isolates were obtained from other health-care interventions, for instance isolates from catheter-associated urine in *E. coli,* with the frequency too low to perform any meaningful statistical analysis, although it is noted that three out of the five *E. coli* isolates obtained from catheterised companion animals also exhibited resistance to both 3GC and FQN.

Healthcare-associated infections (HAI) are defined as any infection which develops as a direct result of healthcare interventions. In human medicine, HAI are well recognised as contributors to high morbidity and mortality. These consist of several types of infections, including urinary tract infections (UTIs), pneumonia, surgical site infections (SSIs), and bloodstream infections (BSIs) (Horan et al., 2008). Veterinary data in this field are limited but have gained more attention after a US study reported the common occurrence of health-care associated infections and their risk factors in weaned dogs and cats (Ruple-Czerniak et al., 2013). Any surgical procedure, urinary catheterisation and duration of hospitalisation were among the main risk factors associated infections (Gibson et al., 2011; Nelson, 2011). Recent findings suggest that surgical site and other infections are increasingly complicated by multidrug resistance with potentially zoonotic pathogens, with particular concern about methicillin-resistant staphylococci (MRSP and MRSA) and ESBL-producing Enterobacteriaceae (Nelson, 2011; Wieler et al., 2011). Without protective isolation and nursing barrier protocols, the infections may spread in the health-care environment, increasing the risk of transmission between animal patients and veterinarians.

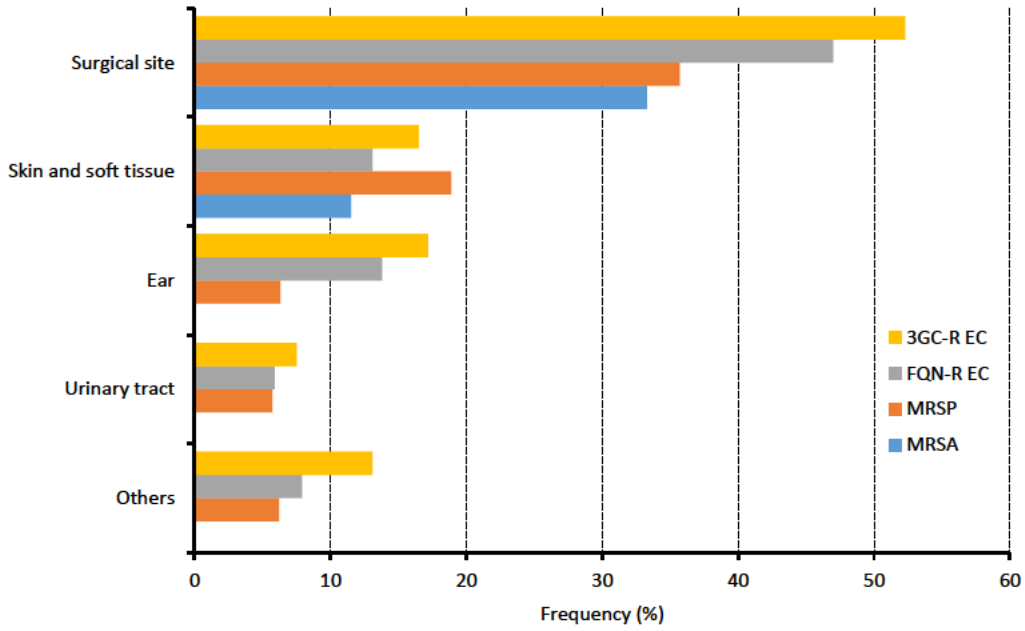

Figure 8.1. Frequency of AMR based on site of infection for isolates from companion animals obtained during the first nation-wide survey of AMR in animal pathogens. 3GC-R EC, resistance to 3GC in *E. coli*; FQN-R EC, resistance to fluoroquinolones in *E. coli*; MRSP, methicillin-resistant *S. pseudintermedius*; MRSA, methicillin-resistant *S. aureus*.

Although veterinary HAI cannot be completely eliminated, preventive strategies are preferable given that MDR infections can only be treated with a limited range of drugs that are often much more expensive and have greater significance in human medicine. Implementation of effective infection control practices is crucial, including adherence to aseptic principles during surgery, the prudent use of antimicrobial prophylaxis, management of the surgical wound in the postoperative period (Nelson, 2011) and continuous education on practical infection control training, such as hand disinfection procedures and proper patient care (Walther et al., 2017). There is a need for the development of strong policies on antibiotic stewardship, antimicrobial surveillance and infection control to help guide empirical antibiotic therapy and prevent the spread of MDR bacteria in companion animal medicine (Stull and Weese, 2015).

8.2.3. Resistance to antimicrobials that are critical to human care remains either undetected or low among companion animal isolates from Australia

One of the important results of the AMR survey of companion animal pathogens is that pandrug-resistant bacteria were not detected. Pandrug-resistant bacteria are defined as having non-susceptibility to all antimicrobial categories/classes. The term is mainly used to describe acquired resistance patterns in human isolates (Magiorakos et al., 2012). It should be noted that in the present study, only nine and twelve antimicrobial classes were assayed for *E. coli* and CoPS isolates respectively, while the number of antimicrobial categories suggested in the standard definition is 17 categories. Nevertheless, infection caused by MDR bacteria is most likely treatable, although the options are limited to highly important antimicrobials that must be used off-label for individual animals under exceptional circumstances, and confirmed as suitable for use by appropriate antimicrobial susceptibility testing. MRSA and MRSP are often MDR to other antimicrobial classes, with MRSP most likely to be resistant to more drugs than MRSA (Chapter 4).

It is confirmed that carbapenems (imipenem), aminoglycosides (amikacin and gentamicin), rifampin (rifampicin), phenicol (chloramphenicol) and lincosamides (clindamycin) are among the antimicrobial classes with the greatest potential for treating MDR bacterial infections (Chapter 2 and 3). In the current study, ESBL phenotype and MRSP isolates exhibited more co-resistance when compared with MRSA isolates, indicating that these two MDR bacteria may have more limitations in regards to viable antimicrobials. A zero rate of imipenem resistance in all clinical *E. coli* isolates indicates that this antimicrobial can be used as a last resort for treatment of MDR Gram-negative bacterial infections in dogs and cats

(Gibson et al., 2008). Nevertheless, use must be judicious and governed by antimicrobial stewardship principles (Abraham et al., 2014). Amikacin and rifampicin are among the antimicrobial agents that are also used off-label, with negligible levels of resistance among dog and cat isolates, and are therefore effective against ESBL-producing *E. coli* and MRSP infections, respectively. It should be noted however, that gentamicin, another aminoglycoside, also showed a low frequency of resistance (<5%) in *E. coli* isolates reflecting the fact that aminoglycosides have limited use in companion animals due to their injectable formulation and association with nephrotoxicity and ototoxicity if used for longer than five days. Several antimicrobials can be used as alternatives to critically important antimicrobials for the treatment of infections in dogs and cats. These include nitrofurantoin and fosfomycin which are highly efficacious against ESBL-producing *E. coli* causing urinary tract infections in dogs (Boothe, 2014; Fournier et al., 2013). Since MRSA exhibited less co-resistance, several options are available for treatment, including many of the broad-spectrum antimicrobials that ESBL *E. coli* and MRSP are resistant to, with the addition of chloramphenicol and clindamycin. The high co-resistance among ESBL *E. coli*, MRSP and MRSA to fluoroquinolones (Chapter 3 and 4) may indicate that these antimicrobials should not be recommended except as a last resort (AVA, 2017) as they have the potential to select for high-level resistant mutations if used inappropriately.

8.2.4. Oral pradofloxacin has different effects on gastric, caecal and faecal microbial diversity

Oral pradofloxacin administration (single and multiple doses) in rabbits did cause a significant reduction in microbial richness and diversity in faecal samples (Chapter 6). In contrast, after the treatment, gastric and caecal microbiota remained relatively stable in terms of microbial diversity (Chapter 7). The different response to pradofloxacin exposure may be due to different level of resilience of microbial communities in each gut compartment, which is defined as the amount of stress or perturbation that can be tolerated before reaching a different equilibrium state (Lozupone et al., 2012). The pharmacokinetic properties of pradoflxacin may also play a crucial role in particular its biliary excretion. It is suggested that pradofloxacin is eliminated in higher concentrations in faeces rather than in other gut compartments, as demonstrated by the similar results obtained for a moxifloxacin study, an analogue of pradofloxacin used in human medicine (Stass and Kubitza, 1999). Gastric and intestinal transit time could also play a role which can both be influenced by the presence of antibiotics (Davies and Davies, 2003).

Pradofloxacin has MIC values which are significantly lower than those of the other fluoroquinolones for the majority of bacterial pathogens (enrofloxacin, ciprofloxacin, marbofloxacin, orbifloxacin, difloxacin and ibafloxacin) (Saputra et al., 2017; Schink et al., 2013; Silley et al., 2012). Pradofloxacin has an enhanced spectrum of activity against a wide range of Gram-positive, Gram-negative, anaerobic and aerobic bacteria. Several opportunistic bacteria that were present in normal microbiota of rabbits were supressed after pradofloxacin treatment, such as *Pseudomonas* spp., *Anaeroplasma* (well known as anaerobic *Mycoplasma*) and *Oligella* spp. It was interesting to note that several bacterial pathogens which are commonly found in the gut microbiota in other animals such as coliforms were not detected in either treated group or control group by 16S rDNA analysis.

8.2.5. Pradofloxacin is safe for use in rabbits

It is confirmed that pradofloxacin 7.5% oral suspension at a dose of 7.5 mg/kg once daily for 3 days appeared to be well tolerated and is safe for rabbits. Several parameters were monitored for the evaluation of safety in the present study, including clinical observation, anatomical and histological analysis of the gut and microbiota profiling in the gastrointestinal tract. The rabbit gastrointestinal tracts is very sensitive to dietary and external changes. However, during the trial, no rabbit showed clinical signs of diarrhoea or enteritis, even though a higher dose was administered than is recommended for dogs (Chapter 4). A slight reduction in body weight was observed amongst all rabbits at post-mortem, which was most likely due to a reduction in feed intake during the trial due to the stress of daily handling. The stomachs and caecae of all rabbits were full and there were no pathological lesions in any organs. Histological analysis of the caecum revealed that there was no difference between the treatment and the control group (Chapter 5).

Clinical safety of pradofloxacin has been widely studied in dogs and cats. Mild gastrointestinal signs (diarrhoea and vomiting) were reported but are very rare, which suggests they are usually transient and resolved without corrective treatment (Sykes and Blondeau, 2014). Microbiota profiling of the stomach, caecum, and hard and soft faeces of rabbits following three days of pradofloxacin treatment revealed that a proliferation of bacteria causing diarrhoea and enteritis such as *Clostridium* spp. and *E. coli* was not detected. Additionally, compared with other veterinary fluoroquinolones, pradofloxacin does not cause retinal toxicity in cats, even when given at 6 and 10 times the recommended dose. In contrast, cats treated with high doses of enrofloxacin (30mg/kg) developed abnormalities suggestive of

severe rod and cone dysfunction (Messias et al., 2008) while blindness, has also been associated with marbofloxacin administration at high doses (Sykes and Blondeau, 2014).

8.3. Implication of the findings and future work

AMR can spread between different populations of humans and animals, developing rapidly and thereby increasing the threat to public health globally. It has long been recognised that better data on AMR and antimicrobial usage constitute a basis from which to develop strategies to combat this growing problem. This thesis reports on the first Australia-wide survey of AMR in companion animal pathogens. No comparable Australian data are available, thus, it is difficult to determine trends. However, the results allow for comparison between Australia and other countries at a baseline level. Ongoing surveys are now required to determine if resistance is increasing or decreasing among companion animal pathogens in Australia. It is important to bear in mind that results from different countries or laboratories may not be directly comparable, unless conducted using a similar methodology and method of interpretation. Furthermore, to detect the trend and emergence of AMR, continuous surveys on a regular basis with similar schema are very important for future benchmarking. A harmonised, standardised procedure for the monitoring program must be implemented to in order to compare data over the same time-points and between countries. As mentioned in the AURA report (AURA, 2016), effective surveillance systems require not only collections of AMR data but also need to provide links between data sources, and appropriate analyses for obtaining meaningful and accessible information as a foundation to derive action plans to prevent and combat AMR.

The resistance types of greatest concern focus on Enterobacteriacease that exhibit the extended-spectrum ß-lactamases (ESBLs) and plasmid-borne AmpC enzymes +/- fluoroquinolone resistance; and methicillin-resistant coagulase-positive staphylococci which confer resistance to all beta-lactams and are oftern MDR to other classes of antimicrobials. Ultimately, monitoring of the frequency and type of carbapenemases, which give resistance to carbapenems and almost all other ß-lactams, is also crucial because a recently published Australian study (on which I am a co-author) has identified carbapenem-resistant Enterobacteriaceae in a shelter cat (Abraham et al., 2016).

A body of evidence, based on clinical observation, culture-dependant methods and microbiota profiling demonstrates that pradofloxacin is safe for use in rabbits. Several lines of evidence based on clinical data suggest that pradofloxacin not only showed a lower risk of developing resistance compared with other veterinary fluoroquinolones (Liu et al., 2014) but

also successfully treated infections caused by strains resistant to some other fluoroquinolones, as predicted by pharmacodynamics and pharmacokinetics data, but depending on the specific MIC of the target strain (Lees, 2013). Therefore pradofloxacin should commonly be the first in class choice when fluoroquinolones are indicated as the best available treatment for an infection.

This is the first metagenomics study to assess the effect of pradofloxacin on the gut microbiota. Interestingly, the microbial community was relatively stable in the stomach and caecum but significantly disturbed in faecal samples. The reasons for these different responses are unknown and should be addressed in future studies. A potential explanation could be related to the pharmacodynamics and pharmacokinetics of pradofloxacin and the resilience level of microbial community (Zaura et al., 2015), biliary excretion during treatment (Koppel et al., 2017), combined with gut transit time in the rabbit (Davies and Davies, 2003).

8.4. Conclusions

Antimicrobial use is essential to treat life threatening illness in humans and animals such as bacterial sepsis. However antimicrobial use has become widespread in human and veterinary medicine, for inconsequential infections such as upper respiratory tract infection in humans, and in-feed prophylaxis in animals for diseases that can be controlled by effective vaccination and good management. Antimicrobial use has forced rapid evolutionary change amongst a number of pathogens, the most significant of which (*E. coli* and *Staphylococcus* spp.) are common inhabitants of the gut and tegument, respectively. This thesis aimed to determine the general situation regarding AMR in these major pathogenic bacteria causing infections in companion animals in Australia. The body of work constitutes the most comprehensive Australian data on resistance to human and veterinary antimicrobials as well as risk factors for infection by MDR strains, providing an important benchmark for policy recommendations in terms of monitoring the emerge of AMR in companion animals. The data produced in this present study have a number implications for future research, not only as a baseline that will allow AMR trends to be monitored over time, but also as the basis for an action plan and national strategy to combat this growing problem.

Antimicrobial choices for treating infections in animals are crucial, and ideally chosen after considering the risks of developing resistance and the dysbiosis effect on the gut. As part of maintaining public health, this thesis stresses the importance of monitoring for AMR and advocates the prudent use antibiotics with the final goal of preserving the effectiveness of

available antimicrobials for the benefit of future generations of both people and animals. As the latest generation of veterinary fluoroquinolones, pradofloxacin is the more rational choice than other fluoroquinolones for treating bacterial infections due to the broader spectrum activity with lesser risk of developing drug resistance as well as minor disturbance in the intestinal microbiota.

8.5. References

Abraham, S., Jordan, D., Wong, H.S., Johnson, J.R., Toleman, M.A., Wakeham, D.L., Gordon, D.M., Turnidge, J.D., Mollinger, J.L., Gibson, J.S., Trott, D.J., 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals. J. Glob. Antimicrob. Resist. 3, 273-277.

Abraham, S., O'Dea, M., Trott, D.J., Abraham, R.J., Hughes, D., Pang, S., McKew, G., Cheong, E.Y., Merlino, J., Saputra, S., Malik, R., Gottlieb, T., 2016. Isolation and plasmid characterization of carbapenemase (IMP-4) producing *Salmonella enterica* Typhimurium from cats. Sci. Rep. 6, 35527.

Abraham, S., Wong, H.S., Turnidge, J., Johnson, J.R., Trott, D.J., 2014. Carbapenemase-producing bacteria in companion animals: a public health concern on the horizon. J. Antimicrob. Chemother. 69, 1155-1157.

APVMA 2017. Antibiotic resistance in animals (Kingston, Australia, Australian Pesticides and Veterinary Medicines Authority).

AURA 2016. AURA 2016: First Australian report on antimicrobial use and resistance in human health (Sydney, Australian Commission on Safety and Quality in Health Care (ACSQHC)).

AVA 2017. Veterinary use of antibiotics critical to human health (NSW, Australia, Australian Veterinary Association).

Barlow, R.S., McMillan, K.E., Duffy, L.L., Fegan, N., Jordan, D., Mellor, G.E., 2015. Prevalence and antimicrobial resistance of *Salmonella* and *Escherichia coli* from Australian cattle populations at slaughter. J. Food. Prot. 78, 912-920.

Boothe, D. 2014. Multidrug antimicrobial resistance and its avoidance in dogs and cats. In: World Small Animal Veterinary Association World Congress Proceedings.

Cohen, M.E., Salmasian, H., Li, J., Liu, J., Zachariah, P., Wright, J.D., Freedberg, D.E., 2017. Surgical antibiotic prophylaxis and risk for postoperative antibiotic-resistant infections. J. Am. Coll. Surg. 225, 631-638 e633.

Davies, R.R., Davies, J.A.E.R., 2003. Rabbit gastrointestinal physiology. Vet. Clin. Exot. Anim. 6, 139-153.

Dohmen, P., 2008. Antibiotic resistance in common pathogens reinforces the need to minimise surgical site infections. J. Hosp. Infect. 70, 15-20.

Fournier, D., Chirouze, C., Leroy, J., Cholley, P., Talon, D., Plesiat, P., Bertrand, X., 2013. Alternatives to carbapenems in ESBL-producing *Escherichia coli* infections. Med. Mal. Infect. 43, 62-66.

Gibson, J.S., Morton, J.M., Cobbold, R.N., Filippich, L.J., Trott, D.J., 2011. Risk factors for multidrug-resistant *Escherichia coli* rectal colonization of dogs on admission to a veterinary hospital. Epidemiol. Infect. 139, 197-205.

Gibson, J.S., Morton, J.M., Cobbold, R.N., Sidjabat, H.E., Filippich, L.J., Trott, D.J., 2008. Multidrug-resistant *E. coli* and *Enterobacter* extraintestinal infection in 37 dogs. J. Vet. Intern. Med. 22, 844-850.

Hardefeldt, L.Y., Holloway, S., Trott, D.J., Shipstone, M., Barrs, V.R., Malik, R., Burrows, M., Armstrong, S., Browning, G.F., Stevenson, M., 2017. Antimicrobial prescribing in dogs and cats in Australia: results of the Australasian infectious disease advisory panel survey. J. Vet. Intern. Med. 31, 1100-1107.

Horan, T.C., Andrus, M., Dudeck, M.A., 2008. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am. J. Infect. Control 36, 309-332.

Koppel, N., Maini Rekdal, V., Balskus, E.P., 2017. Chemical transformation of xenobiotics by the human gut microbiota. Sci. 356.

Lees, P., 2013. Pharmacokinetics, pharmacodynamics and therapeutics of pradofloxacin in the dog and cat. J. Vet. Pharmacol. Ther. 36, 209-221.

Liu, X., Lazzaroni, C., Aly, S.A., Thungrat, K., Boothe, D.M., 2014. In vitro selection of resistance to pradofloxacin and ciprofloxacin in canine uropathogenic *Escherichia coli* isolates. Vet. Microbiol. 174, 514-522.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., Knight, R., 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489, 220-230.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18, 268-281.

Messias, A., Gekeler, F., Wegener, A., Dietz, K., Kohler, K., Zrenner, E., 2008. Retinal safety of a new fluoroquinolone, pradofloxacin, in cats: assessment with electroretinography. Doc. Ophthalmol. 116, 177-191.

Nelson, L.L., 2011. Surgical site infections in small animal surgery. Vet. Clin. North. Am. Small. Anim. Pract. 41, 1041-1056, viii.

Ruple-Czerniak, A., Aceto, H.W., Bender, J.B., Paradis, M.R., Shaw, S.P., Metre, D.C.V., J.S. Weese, Wilson, D.A., Wilson, J.H., Morley, P.S., 2013. Using syndromic surveillance to estimate baseline rates for healthcare-associated infections in critical care units of small animal referral hospitals. J. Vet. Intern. Med. 27, 1392-1399.

Saputra, S., Jordan, D., Worthing, K.A., Norris, J.M., Wong, H.S., Abraham, R., Trott, D.J., Abraham, S., 2017. Antimicrobial resistance in coagulase-positive staphylococci isolated from companion animals in Australia: A one year study. Plos One 12, e0176379.

Schink, A.K., Kadlec, K., Hauschild, T., Brenner Michael, G., Dorner, J.C., Ludwig, C., Werckenthin, C., Hehnen, H.R., Stephan, B., Schwarz, S., 2013. Susceptibility of canine and feline bacterial pathogens to pradofloxacin and comparison with other fluoroquinolones approved for companion animals. Vet. Microbiol. 162, 119-126.

Silley, P., Stephan, B., Greife, H.A., Pridmore, A., 2012. Bactericidal properties of pradofloxacin against veterinary pathogens. Vet. Microbiol. 157, 106-111.

Stass, H., Kubitza, D., 1999. Pharmacokinetics and elimination of moxifloxacin after oral and intravenous administration in man. J. Antimicrob. Chemother. 43, 83-90.

Sykes, J.E., Blondeau, J.M., 2014. Pradofloxacin: a novel veterinary fluoroquinolone for treatment of bacterial infections in cats. Vet. J. 201, 207-214.

Thungrat, K., Price, S.B., Carpenter, D.M., Boothe, D.M., 2015. Antimicrobial susceptibility patterns of clinical *Escherichia coli* isolates from dogs and cats in the United States: January 2008 through January 2013. Vet. Microbiol. 179, 287-295.

Walther, B., Tedin, K., Lubke-Becker, A., 2017. Multidrug-resistant opportunistic pathogens challenging veterinary infection control. Vet. Microbiol. 200, 71-78.

Wieler, L.H., Ewers, C., Guenther, S., Walther, B., Lubke-Becker, A., 2011. Methicillin-resistant staphylococci (MRS) and extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae in companion animals: nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. Int. J. Med. Microbiol. 301, 635-641.

Zaura, E., Brandt, B.W., Teixeira de Mattos, M.J., Buijs, M.J., Caspers, M.P., Rashid, M.U., Weintraub, A., Nord, C.E., Savell, A., Hu, Y., Coates, A.R., Hubank, M., Spratt, D.A., Wilson, M., Keijser, B.J., Crielaard, W., 2015. Same exposure but two radically different responses to antibiotics: resilience of the salivary microbiome versus long-term microbial shifts in feces. MBio 6, e01693-01615.