Surveillance for antimicrobial resistance in enteric commensals and pathogens in Australian meat chickens

October 2018

Foreword

The Commonwealth Government has been actively progressing the development of a coordinated plan for the management of antimicrobial resistance and antimicrobial use (AU) in humans and animals. Broad support for the development of the “National Antimicrobial Resistance Strategy” was obtained from key stakeholders across the medical, health, veterinary, agricultural and pharmaceutical communities at the ‘Australian One Health Antimicrobial Resistance Colloquium’ in 2013.

A surveillance model for use in the Australian chicken meat industry was developed and implemented, which is closely in-line with the OIE Chapter 6.7 recommendations.

Acknowledgements

The Australian Chicken Meat Federation would like to thank The Department of Agriculture and Water Resources for the funding to complete this survey, AgriFutures Australia for funding the *Salmonella* serotyping, Birling Avian Laboratories for exceptional coordination of bacterial isolation Dr Sam Abraham (Murdoch University) and Dr Darren Trott (University of Adelaide) and their research teams for their dedication to precision, transparency and accuracy with the AMR analyses and reporting.

ABBREVIATIONS

ACMF	Australian Chicken Meat Federation
AMR	Antimicrobial resistance
APL	Australian Pork Limited
BPW	buffered peptone water
CI	Clinically-Intermediate
CLSI	Clinical and Laboratory Standards Institute
CS	Clinically-Susceptible
CR	Clinically-Resistant
DAFF	Department of Agriculture, Fisheries and Forestry
DANMAP	Danish Programme for surveillance of antimicrobial consumption and resistance
DAWR	Department of Agriculture and Water Resources
ECOFF	Epidemiological Cut-off Values
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MIC	minimum inhibitory concentration
MOA	mechanism of action
MLA	Meat and Livestock Australia
MS	Microbiologically-Susceptible
MR	Microbiologically-Resistant
MDR	Multi-drug resistance (clinical resistance to three or more classes)
MLST	Multilocus sequence type
NARMS	National Antimicrobial Resistance Monitoring System
NRS	National Residue Survey

CONTENTS

Foreword 2
Abbreviations 3
Table List 5
Figure List 6
Executive summary 7
Introduction 11
AMR survey in Australian Meat Chickens 14
Objective 14
Roles and responsibilities 14
Materials and methods 15
- Animal population under study 15
- Sampling of caecal contents from chickens at processing for AMR surveillance 15
- Randomisation – Reducing bias in sample selection 17
- Data obtained at specimen collection 18
- Act of specimen collection 18
- Isolation and confirmation of target organisms (to species level) at the primary laboratory 19
- Dispatch to AMR laboratories 21
- AMR Testing 21
- Interpretation 22
- Genetic analysis 26
- Statistical analysis 27

Results 28
- Bacterial isolation 28
- MIC distributions 29
- Multi-drug resistance profiles 44
- Genetic analysis of non-susceptible isolates 50

Discussion 55
Appendix 1 Sample collection form 60
Appendix 2 Tables 22 – 32 61
References 71

TABLE LIST

Table 1. Antibiotics that are permitted for use in the Australian meat chicken industry 13
Table 2. The number of samples to be collected from each plant 16
Table 3. Breakpoints used for susceptibility testing of *Enterococcus* species....24
Table 4. Breakpoints used for susceptibility testing of *Escherichia coli* and *Salmonella* species 25
Table 5. Breakpoints used for susceptibility testing of *Campylobacter* species 26
Table 6. Isolates recovered....29
Table 7. Distribution of minimum inhibitory concentrations for *Enterococcus faecalis* 31
Table 8. Distribution of minimum inhibitory concentrations for *Enterococcus faecium* 33
Table 9. Distribution of minimum inhibitory concentrations for other *Enterococcus* spp. 35
Table 10. Distribution of minimum inhibitory concentrations for commensal *Escherichia coli*....37
Table 11. Distribution of minimum inhibitory concentrations for *Salmonella* spp.. 39
Table 12. Distribution of minimum inhibitory concentrations for *Campylobacter jejuni* 41
Table 13. Distribution of minimum inhibitory concentrations for *Campylobacter coli*....43
Table 14. Clinical antimicrobial resistance profiles of *Enterococcus faecalis*....44
Table 15. Clinical antimicrobial resistance profiles of *Enterococcus faecium* 45
Table 16. Clinical antimicrobial resistance profiles of other *Enterococcus* spp 46
Table 17. Clinical antimicrobial resistance profiles of *Escherichia coli* 47
Table 18. Clinical antimicrobial resistance profiles of Salmonella spp.....48
Table 19. Clinical antimicrobial resistance profiles of *Campylobacter jejuni* 49
Table 20. Clinical antimicrobial resistance profiles of *Campylobacter coli* 49
Table 21. Isolates selected for genetic analysis....50
Table 33. Antimicrobial (microbiological) resistance in commensal *E. coli* isolates from meat chickens from Australian surveys.* 58
Table 22. MLST and resistance profile of *Enterococcus faecalis*....61
Table 23. MLST and resistance profile of *Enterococcus faecium* 62
Table 23 Cont. MLST and resistance profile of *Enterococcus faecium* 63
Table 24. Quinupristin-dalfopristin resistant genes detected and corresponding broth dilution result of *Enterococcus faecium* 64
Table 25. Resistance profile of *Enterococcus durans*....65
Table 26. Quinupristin-dalfopristin resistant genes detected and corresponding broth dilution result of *Enterococcus durans* 66
Table 27. Resistance profile of *Enterococcus hirae*....67
Table 28. Quinupristin-dalfopristin resistant genes detected and corresponding broth dilution result of *Enterococcus hirae* 67
Table 29. MLST and profile of resistance genes in commensal *E. coli* 68
Table 30. MLST and profile of resistance genes in *Salmonella* 68
Table 31. MLST and resistance profile of *Campylobacter jejuni* 69
Table 32. MLST and resistance profile of *Campylobacter coli* 70

FIGURE LIST

Figure 1. Components in the sample collection kits. 17
Figure 2. Antimicrobial resistance patterns for *Enterococcus faecalis* (n=41) based on microbiological (ECOFF) break points. 30
Figure 3. Antimicrobial resistance patterns for *Enterococcus faecium* (n=77) based on microbiological (ECOFF) break points. 32
Figure 4. Microbiological resistance patterns for other *Enterococcus* spp. (n=87) comprising: *Enterococcus hirae* (n= 25), *Enterococcus durans* (n= 61) and *Enterococcus gallinarum* (n=1) based on microbiological (ECOFF) break points. 34
Figure 5. Antimicrobial resistance patterns for commensal *Escherichia coli* (n=206) based on microbiological (ECOFF) break points. 36
Figure 6. Antimicrobial resistance patterns for *Salmonella* spp. (n=53) based on microbiological (ECOFF) break points. 38
Figure 7. Microbiological resistance patterns for *Campylobacter jejuni* (n=108) based on microbiological (ECOFF) break points. 40
Figure 8. Microbiological resistance patterns for *Campylobacter coli* (n=96,) based on microbiological (ECOFF) break points. 42

Background

Surveillance for antimicrobial resistance (AMR) can help identify new developments and provide valuable feedback on how antimicrobial stewardship programs should be conducted. In Australia, a pilot program in food-producing animals was commissioned by DAFF (Department of Agriculture, Fisheries and Forestry) in 2003/2004. Recently, the Commonwealth Government has been actively progressing the development of a coordinated plan for the management of AMR and antimicrobial use in humans and animals. Increasing global interest in AMR prompted the ACMF to approach DAFF to discuss potential inclusion of the chicken meat industry in AMR surveillance activities. This report defines a surveillance model for use in the Australian chicken meat industry based on the recommendations in OIE Chapter 6.7 "Harmonisation of national AMR surveillance and monitoring programmes" and is closely in line with the surveillance project undertaken in other industries such as pork. The outcomes of this project will assist the Department of Agriculture and Water Resources in international and national discussions regarding AMR and the Australian chicken meat industry in progressing antimicrobial stewardship efforts.

Approach

The project design was to account as much as possible for the variation in antimicrobial resistance present in the population of commercially-raised meat chickens in an efficient and practical way that could be replicated into the future. This approach aimed to achieve economies of scale, to maximize the number of isolates evaluated and hence the accuracy of findings, and to maximise comparability with data from the medical sector, other industries and internationally. The study was overseen by representatives from AMR experts, the Australian chicken meat industry and the Australian Government Department of Agriculture and Water Resources (DAWR).

The study focused on AMR in bacteria of meat chickens at slaughter from meat chicken slaughtering plants around Australia. To prioritise the resources to keep within budget, the companies that produce the bulk (> 95%) of Australian chicken meat were included in this study and the number of caecal samples collected from meat chickens was limited to no more than 220 in total (200 primary samples) to be affordable, provide reasonable confidence limits, and to be approximately the same as many international surveillance programs that evaluate AMR in commensal bacteria from food animals. This excluded samples that were negative for all target pathogens, which were recollected.

To align with the USA 'National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria' protocol, a single 'sample' constituted a composite of five chicken caeca. The number of samples collected at each plant was proportionally distributed based on the approximate number of chickens processed by each plant in each category each week and the most accurate estimate of the total number of chickens processed in Australia in 2015 and samples were collected between June and November 2016. To reduce bias, only one sample from any single batch on a specific farm was collected. The methods were established to remove bias in isolate selection but align with relevant Australian Standards.

For *E. coli* and *Salmonella* spp., the antimicrobials tested were: amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, colistin,

streptomycin, tetracycline and trimethoprim/sulfamethoxazole. For *Enterococcus*, the antimicrobials tested were: ampicillin, chloramphenicol, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, penicillin, quinupristin/dalfopristin, streptomycin, teicoplanin, tetracycline, vancomycin and virginiamycin. For *Campylobacter* spp., the antimicrobials tested were based on the standard *Campylobacter* minimum inhibitory concentration (MIC) plate available for the Sensititre system: azithromycin, ciprofloxacin, erythromycin, gentamicin, tetracycline, florfenicol, nalidixic acid, telithromycin, and clindamycin.

Antimicrobial susceptibility for the isolates was determined by the broth microdilution method either on veterinary reference card panels according to the manufacturers' guidelines or in-house panels prepared according to Clinical and Laboratory Standards Institute (CLSI) standards. Isolates were subjected to analysis using both Clinical Breakpoints and Epidemiological Cut-off Values (ECOFF).

Genetic analysis was used to clarify the resistance profiles of all *Campylobacter* and *Enterococcus* isolates and key isolates of *Salmonella* and *E.coli.*

Key results

Reporting of the results is in line with recommendations in OIE chapter 6.7 which states that "*For surveillance purposes, use of the microbiological breakpoint (also referred to as epidemiological cut-off point), which is based on the distribution of MICs or inhibition zone diameters of the specific bacterial species tested, is preferred*.". In this report, the clinical resistance results are also reported because of their relevance to public health, but the focus of the reporting is on defining rates of microbiological resistance with these results supported by genetic analysis where possible. No direct comparison between results for commensal isolates from chickens in this report and clinical isolates from humans has been made due to inherent differences in sample and bacterial characteristics of isolates from healthy chickens and septic human patients. Where isolates were both clinically and microbiologically resistant, the term 'resistance' alone is used.

A total of 668 bacterial isolates were collected – 205 *Enterococcus*, 206 *E.coli*, 53 *Salmonella* and 204 *Campylobacter*.

Enterococcus

No resistance was detected to aminoglycosides or chloramphenicol and low resistance was detected to linezolid and vancomycin, however these phenotypes were not supported by the presence of known resistance genes. Among the enterococci isolates, 17.5% isolates were classified as MDR (clinical resistance to three or more drug classes). Resistance and presence of resistance genes to tetracycline (40.3-46.3%) was common among *Enterococcus spp.* reflecting historical use in the chicken industry. Elevated frequency of quinupristin-dalfopristin (54.5%) resistance among *E. faecium* may be a consequence of past virginiamycin use, however quinupristin-dalfopristin resistance in general may require further evaluation as isolates with MIC ≥16mg/L for quinupristin-dalfopristin did not carry the *vatE* gene.

Although not entirely comparative, it can be highlighted that there has been a significant reduction in phenotypic resistance to erythromycin in *Enterococcus* isolates from Australian meat chickens since the earlier study in 2004. This could reflect the reduction in use of macrolides in the industry since the introduction of the *Mycoplasma* vaccines in the 1990s.

E.coli

The microbiological resistance of commensal *E. coli* isolates demonstrated that 47% were susceptible to all tested antimicrobials and only 5.8% of isolates were classified as MDR. No resistance was detected to amoxicillin, ceftiofur, chloramphenicol, florfenicol, colistin or gentamicin. Two isolates demonstrated microbiological resistance to ciprofloxacin at MICs (0.13 and 0.25 mg/L) near the breakpoint. Quinolones have never been registered for use in food-producing animals in Australia and whole genome sequencing revealed that these two isolates carried a single point mutation in the QRDR of GyrA (Ser-83-Leu or Asp-87-Gly), shown to be associated with low level fluoroquinolone resistance. The absence of ceftiofur resistance among *E. coli* isolated from Australian meat chickens is noteworthy in both 2017 and 2004. Compared to the 2004 survey, resistance to tetracycline, ampicillin, and trimethoprim/sulfamethoxazole were substantially reduced.

Salmonella

Susceptibility to all antimicrobials tested was observed in 92.5% of the 53 *Salmonella* isolates. No multi-drug resistant bacteria were detected. None of the *Salmonella* were microbiologically resistant to ceftiofur, ciprofloxacin, chloramphenicol, florfenicol, colistin, gentamicin or tetracycline. Resistance was detected at low frequency to ampicillin, streptomycin and trimethoprim. None of the six isolates that were microbiologically resistant to cefoxitin carried any beta lactam genes required for cefoxitin resistance which suggests that there is measurement variation in the assay, the breakpoints may be inappropriate, or there exists previously uncharacterised resistance mechanisms.

Campylobacter

No resistance was detected to any of the antibiotics tested in 63% of *C. jejuni* isolates and 86.5% *C. coli* isolates. MDR phenotype were identified in one *C. jejuni* and four *C. coli*. All *Campylobacter* isolates were microbiologically susceptibile to florfenicol and gentamicin. Resistance to tetracycline (22.2% *C. jejuni*; 3.1% *C. coli*), nalidixic acid (14.8% *C. jejuni*; 5.2% *C. coli*) or ciprofloxacin (14.8% *C. jejuni*; 5.2% *C. coli*) were the most commonly detected forms of resistance. For isolates with fluoroquinolone resistance no other resistance to any other drug class was identified. The finding of some isolates with fluoroquinolone resistance was unexpected, since fluoroquinolones are not approved for use, and are not used, in Australian livestock and the isolates therefore unlikely to have evolved as a result of local selection pressure. The level of ciprofloxacin resistance detected in *Campylobacter* are similar to the levels of resistance to fluoroquinolones detected in meat chickens in other countries that also don't use fluoroquinolones. For isolates with fluoroquinolone resistance no other resistance to any other drug class was identified, suggesting they are likely to have evolved from use in a situation where fluoroquinolones were used as a first-line therapy. The isolates potentially entered the chickens through anthropozoonosis i.e. human-chicken transmission, or some other transmission pathway such as wild birds and rodents. Subsequently, the National Biosecurity Manual for Chicken Growers is being updated to include the potential for transfer of AMR bacteria from humans to chickens.

Only one *C. jejuni* (0.9%) and five *C. coli* (5.2%) were resistant to macrolides; one of the key antimicrobials used for treating human campylobacteriosis. The overall frequency of erythromycin resistance among *Campylobacter* spp. in the 2004 survey was 19.9%. Despite the lack of speciation in the 2004 study, the current survey showed a decisive reduction in the carriage of macrolide resistance among *Campylobacter* isolates.

Conclusion

In general, the results of this survey demonstrate either nil or substantially low carriage of resistance to antimicrobials used in human medicine. The findings are extremely favourable compared to resistance profiles for chicken isolates described internationally. While the fluoroquinolone resistance in the *Campylobacter* isolates deserves further investigation, there was a general reduction in AMR observed in comparison with the 2004 study. These results highlight the efficacy of the chicken industry's past and current antimicrobial stewardship efforts and identify further areas for investigation and improvement.

INTRODUCTION

Antimicrobial resistance is a serious threat to public health globally. The cornerstone of national and international efforts to deal with AMR is antimicrobial stewardship – programs and activities broadly designed to halt the emergence of resistance and its spread in animal and human populations. Whilst the development of AMR impacting on public health is foremost a consequence of antimicrobial use in human medicine, the use of antimicrobials in food-producing animals and companion animals has been found in other countries to play a part. Therefore, the application of antimicrobial stewardship across both human and animal populations offers the community the greatest protection from the harmful consequences of AMR.

Surveillance for AMR can help identify new developments and provide valuable feedback on how stewardship programs should be conducted. European and North American countries stand out as having well established surveillance systems that incorporate data from food animals on an ongoing basis. These include, for example, DANMAP (Denmark) (1), CIPARS (Canada) (2), and NARMS (USA) (3). In Australia, a pilot program in food-producing animals was commissioned by DAFF (Department of Agriculture, Fisheries and Forestry) in 2003/2004 (4).

The complexities of bacterial disease in humans and animals dictate that AMR stewardship programs are customized for each sector. In Australia, there has been careful management of the type and class of antimicrobials available for each food-animal industry and the conditions under which they may be used. Indeed, Australia was one of the first (and remains amongst the minority of) countries to have adopted AMR risk analysis as part of regulatory processes involved in registering veterinary medicines. The Australian chicken meat industry is an approximately $2.8 billion industry, producing >650 million chickens annually, that is dominated by seven companies that supply the bulk (>95%) of the domestically produced chicken meat. Less than 1% of total chicken meat consumed in Australia is imported.

The industry is highly vertically integrated, and the chicken farmers are predominately contractors to the processing companies, who ultimately own the chickens. This dynamic means that the processing companies are responsible for the inputs to the farm that relate directly to the chickens – the feed, management advice and health management. The health aspect is always managed by at least one registered veterinarian specialising in poultry, often directly employed by a company, who oversee and manage disease surveillance, diagnosis and treatment. This veterinarian supervises the administration of antibiotics, for all company flocks including breeder flocks. It's important to note that the Australian chicken industry's national representative body, the ACMF, has since 2007 had a policy of no antimicrobials to be used for growth promotion purposes and the ACMF has been actively working with registrants to remove growth promotion claims from product labels. The antibiotics available for use in meat chickens in Australia are listed in Table 1. Owing to the similarity between the mechanism of action of chemically similar antimicrobials within the same class, use of the drugs listed in Table 1 by the meat chicken industry can potentially give rise to resistance to some drugs that are exclusively used in humans and aren't used in chickens. For example, virginiamycin and quinupristin-dalfopristin are two streptogramin A and B combinations with similar MOA. The use of virginiamycin selects for virginiamycin-resistant *E. faecium* which are cross-resistant to quinupristin-dalfopristin which is used in human medicine but not animal medicine (5, 6).

The Commonwealth Government has been actively progressing the development of a coordinated plan for the management of AMR and antimicrobial use in humans and animals. Broad support for the development of the “National Antimicrobial Resistance Strategy” was obtained from key stakeholders across the medical, health, veterinary, agricultural and pharmaceutical communities at the ‘Australian One Health Antimicrobial Resistance Colloquium’ in 2013. The then Department of Agriculture sponsored a review of the national surveillance programs in place for monitoring AMR and antimicrobial use in animals around the world with a view to defining a program suitable for Australia and combined this with roundtable discussions with key stakeholders in the agriculture and veterinary sectors. The review ‘Surveillance and reporting of antimicrobial resistance and antibiotic usage in animals and agriculture in Australia’ (the AMRIA report) (7) identified one of the major components of surveillance being the assessment of AMR in commensal bacteria and pathogens present in the gut of food animals at slaughter.

Increasing global interest in AMR prompted the ACMF to approach the then Australian Government Department of Agriculture to discuss potential inclusion of the chicken meat industry in AMR surveillance activities. In March 2015, a one-day meeting convened by the then Department of Agriculture established the “Antimicrobial Resistance Surveillance Task Group”. Present at the meeting were representatives from the then Department of Agriculture, Animal Health Australia, scientists working in the area of AMR, most of the major Research and Development Corporations or industry bodies involved in animal production (MLA, APL, ACMF, Dairy Australia) and representatives from the Australian pharmaceutical industry. The Task Group reviewed the recommendations from the surveillance report and provided advice from technical and industry perspectives for developing an AMR surveillance component based on the collection of faecal samples from food animals at slaughter. As a result of this meeting, a plan was developed to build on experience in the beef industry to deliver a proof-of-concept project for surveillance for AMR in pigs that may also be applied to other major food industries in the future. A subsequent meeting of the Task Group discussed the extension of this concept to the chicken meat sector. This project is the result of that meeting. It defines a surveillance model for use in the Australian chicken meat industry based on the OIE Chapter 6.7 “Harmonisation of national antimicrobial resistance surveillance and monitoring programmes” and is closely in line with the surveillance project undertaken in other industries such as pork and beef cattle.

The outcomes of this project will assist the Department of Agriculture and Water Resources in discussions nationally and internationally concerning the AMR status of Australia’s animal populations. The outcomes are also vital to the Australian chicken industry for defining cost-effective approaches to antimicrobial stewardship.

Table 1. Antibiotics that are permitted for use in the Australian meat chicken industry

Antimicrobial class	Antimicrobial	Route of administration	Registered use
Aminocyclitol, Lincosamide	Spectinomycin + Lincomycin	Water, Injection	treatment or prevention
Aminoglycoside	Apramycin	Water	treatment or prevention
	Neomycin	Feed, water	treatment or prevention
Arsenical	Roxarsone	Feed	growth promotion [a]
Glycophospholipid	Flavophospholipol	Feed	growth promotion [b]
Ionophore	Lasalocid	Feed	treatment or prevention
	Maduramicin	Feed	treatment or prevention
	Monensin	Feed	treatment or prevention
	Narasin	Feed	treatment or prevention
	Salinomycin	Feed	treatment or prevention
	Semduramicin	Feed	treatment or prevention
Macrolide	Erythromycin	Water	treatment or prevention
	Tylosin	Feed, water	treatment or prevention
Orthosomycin	Avilamycin	Feed	treatment or prevention + growth promotion [c]
Pleuromutilin	Tiamulin	Feed, water	treatment or prevention
Polypeptide	Bacitracin	Feed	treatment or prevention
Streptogramin	Virginiamycin	Feed	treatment or prevention
Sulfonamide, Diaminopyrimidine	Sulfadiazine + Trimethoprim	Water	treatment or prevention
	Sulfadimidine + Trimethoprim	Water	treatment or prevention
Sulfonamide	Sulfadimidine	Water	treatment or prevention
	Sulfaquinoxaline	Water	treatment or prevention
Tetracycline	Chlortetracycline	Feed, water	treatment or prevention
	Oxytetracycline	Feed, water	treatment or prevention
β lactam penicillin	Amoxicillin	Water	treatment or prevention

[a] Registration discontinued in 2018; [b] Used off-label as a therapeutic treatment for necrotic enteritis or enteritis when other medications are inappropriate.; [c] Although the avilamycin formulation having a growth promotion claim is approved for use there are presently no such products available for sale in Australia. (Source: Industry report; ACMF and Dr. Stephen Page)

Objective

The primary aim of the work was to estimate the proportion of isolates resistant to specified antimicrobials amongst *E. coli, Salmonella* spp., *Enterococcus* spp. and *Campylobacter* spp. isolated from the gut of Australian meat chickens at slaughter).

Roles and responsibilities

Successful completion of this work required collaboration amongst several individuals and institutions. A number of people involved in the Technical Group and the Antimicrobial Resistance Surveillance Task Group have given freely of their time and expertise to assist this collaboration between the chicken meat industry and the DAWR, and their contributions are gratefully acknowledged.

- Australian Chicken Meat Federation (ACMF), Dr. Kylie Hewson; Project coordinator – Overall coordination of the project and first contact point for stakeholders. Establish and provide protocols to laboratories and for sample collection. Primary responsibility for the project and authorship of the report. kylie.hewson@chicken.org.au

- Company coordinator for each company involved in the study – coordinated collection of samples in each plant associated with that company and training, as needed, for those collecting the samples. Trained quality assurance staff or poultry veterinarians at the participating chicken processing plants. Responsibility for ensuring samples are collected and shipped as per the protocol.

- Birling Avian Laboratories, Dr. Sue Sharpe and Dr Tony Pavic; Primary laboratory – NATA accreditation, general expertise in veterinary microbiology with capacity and infrastructure for collation of caecal samples, isolation and identification of target organisms, storage of isolates and collation of data sent to the AMR laboratories in coordination with the project coordinator. Responsibility for ensuring only one sample from each farm collected at processing was submitted, isolation protocol was followed, and isolates are characterised, stored and shipped appropriately. Maintains a copy of all isolates for reference. Sue_Sharpe@baiada.com.au; Tony_Pavic@baiada.com.au

- Antimicrobial Resistance and Infectious Diseases Laboratory, School of Veterinary Life Sciences, Murdoch University[1] (Dr. Sam Abraham[1]) / ACARE Laboratory, University of Adelaide[2] (Dr. Darren Trott[2]); AMR testing laboratories – specialist ability at performing phenotypic AMR testing on bacterial isolates by broth microdilution. Responsible for providing scientific and technical advice to the project as requested and assist the project coordinator in analysis and interpretation of results and compilation of the report. Additional technical support was provided by Mark O'Dea[1], Terence Lee[1], Tanya Laird[1], Jan Bell[2] and David Jordan (NSW Department of Primary Industries). S.Abraham@murdoch.edu.au; darren.trott@adelaide.edu.au

- Dr. Leigh Nind (DAWR), Dr. Vivien Kite (ACMF), Dr. David Jordan (NSW DPI); Management group – General oversight of the entire project. Responsible for making final decisions on protocols and reporting. Leigh.nind@agriculture.gov.au; vivien.kite@chicken.org.au; david.jordan@dpi.nsw.gov.au .

Materials and methods

The methods followed for this study are in line with recommendations from the OIE Chapter 6.7 "Harmonisation of national antimicrobial resistance surveillance and monitoring programmes", which also align with the approaches taken for other DAWR-funded AMR surveillance projects in livestock.

Animal population under study

The work focused on AMR in bacteria of meat chickens at slaughter from meat chicken slaughtering plants around Australia. To prioritise the resources to keep within budget, the companies that produce the bulk (> 95%) of Australian chicken meat were included in this study, which is aligned with the AMRIA report recommendation that surveillance proceeds on a 'risk' basis and a major component of risk is the volume of product/extent of human exposure.

There was a company coordinator for each of the seven companies involved in the study, and in some cases, coordinators took the samples themselves, or arranged for other trained personnel to take the samples as per the below protocol. The ACMF project coordinator was the intermediary between the company coordinators and Birling Avian Laboratories to enable an additional level of anonymity and scrutiny. Smaller processors were regarded as out of scope of this study.

Sampling of caecal contents from chickens at processing for AMR surveillance

Number of samples

The number of caecal samples collected from meat chickens was limited to no more than 220 (200 primary samples collected with resources available for another 20 in case repeats were required) in total to be affordable, provide reasonable confidence limits, and to be comparable to many similar surveillance programs reported internationally. This excluded samples that were negative for all target pathogens, which were recollected. The numbers of samples positive for at least one pathogen (200 for a single major production system on the grounds of 'international comparability') was considered to give acceptable statistical accuracy within the scope of the allocated budget to achieve the required objectives.

To align with the USA National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria protocol, a single 'sample' constituted a composite of five chicken caeca. Each processing plant (total of 20) had a target number of samples to submit for surveillance which was based on estimated weekly throughput and subsequent proportion of the total national flock size. The processing plants were grouped into four categories: <300,000 chickens/week (four plants); 300,000 – 450,000 chickens/week (three plants); 450,000 – 600,000 chickens/week (six plants); >600,000 chickens/week (seven plants).

The number of samples to be collected at each plant was proportionally distributed based on the approximate number of chickens processed by each plant in each category each week and the most accurate estimate of the total number of chickens processed in Australia in 2015 (estimated at 11,295,000/week) (8). This is the method used for calculating sampling requirements for the National Residue Survey as actual number of chickens processed by each plant is commercially sensitive data and was therefore not available to ACMF. Calculations are provided in Table 2.

Table 2. The number of samples to be collected from each plant

	Chickens processed/week (no. of plants)				
	<300,000 (4)	300,000 – 450,000 (3)	450,000 – 600,000 (6)	>600,000 (7)	Total
Processed estimate* / total in category	280,000/ 1,120,000	425,000 / 1,275,000	550,000 / 3,300,000	800,000 / 5,600,000	11,295,000
% of overall total	9.9	11.3	29.2	49.6	100
Samples required per category	20	23	58	99	200
Samples per plant (total)[a]	5;5;5;5 (20)	8;8;8 (24)	9;9;9;10;10;10[b] (57)	13;13;14;14;15; 15;15[b] (99)	200

*Number of chickens estimated to have been processed in a week at each of the plants in that category

[a] Note that the total samples per category may be slightly different than that calculated in the row above to account for calculated part samples and have therefore been rounded accordingly.

[b] Samples have been distributed using a best estimate of which plants may have higher throughput than others within this category

Sample collection kits

The project coordinator and Birling Avian Laboratories coordinated the assembly of the collection kits to ensure consistency with sample collection and shipping. This also ensured traceability of the samples in case they were not received if sent, that the samples were received within 24hrs of collection and to reduce variability in shipping conditions between samples. Birling Avian Laboratories coordinated the distribution of the required number of sample kits to each processing plant (one kit per sample). No more than four kits at a time was sent to each processing plant to reduce the chance of “sampling-by-convenience”. Once samples were returned to Birling Avian Laboratories, additional kits were dispatched until the required number of (viable) samples had been collected from each processing plant.

Each collection kit contained (Figure 1): 2 sterile 120ml yellow screw-top sample containers; Permanent marker; 1 pair of scissors (1 pair should be sufficient for each processing plant as long as appropriate sterilisation can be undertaken in between samplings); 2 zip-loc bags; 2 pairs of examination gloves; 1 large plastic pad to prepare the samples on; disposable alcohol wipes; buffer to go between the samples and the gel pack (absorbent paper 5ply); 1 plastic sleeve (for the sample collection form); 2 sample collection forms; 2 gel coolant packs; 1 insulated shipping container (esky); 1 pre-printed shipping consignment note; 1 stamped envelope addressed to ACMF.

Figure 1. Components in the sample collection kits. Each sample had an individual kit.

Randomisation – Reducing bias in sample selection

Flock/farm selection

To reduce the chance for bias in results it was imperative to avoid sampling on the basis of convenience, for example, all at once, or multiple chickens from the same farm or flock. Each processing plant generally processes chickens from more than one farm on a single day, but no more than four. To reduce bias, only one sample from any single batch on a specific farm was collected, until such time as the requisite number of samples allocated for the plant had been collected. For example, if a processing plant typically processed chickens from three farms in one day, then that plant would collect three samples i.e. one sample from each farm processed that day. In order to meet sampling quotas, each participating plant collected samples on more than one day and the sample number (described below) was used to ensure that only one sample from a farm was submitted. In a small number of cases the number of samples required was more than the number of farms that supply the processing plant. In these cases, an additional sample was collected from the farm but from a different batch of chickens.

Chicken selection for sample collection

Multiple pick-ups from the same batch of chickens over the course of one to two weeks is common practice in the chicken meat industry. Bias from sampling chickens all the same age was reduced by not specifying which pick-up from a batch was to be sampled at the processing plant. Due to the speed of chicken processing it was not possible to specify a carcass number on the line to be sampled. Therefore, a chicken was selected from approximately mid-way through that farm's intake through the plant that day (i.e. not the first or last chicken to be processed in that batch).

The presentation of chickens for slaughter from a particular pick-up is completely random. Chickens are harvested by 'pick-up' crews who enter sheds and randomly pick-up the nearest chickens from the shed entry point (the proportion of chickens harvested from the flock that day will depend on the company's pick-up policy, and whether it is the only or last pick-up). Multiple chickens will be placed in transport containers at random. These crates are then loaded onto trucks which transport the chickens to the processing plant and the containers of chickens are then unloaded in the lairage area awaiting processing. The order in which containers from a single farm are unloaded from their containers and processed will broadly take into account the time that they were originally picked up (i.e. first ones in, first ones processed) but otherwise the procedures involved in pick-up, loading of containers at the farm, unloading of the containers in the lairage at the processing plant, and unloading of the chickens from their containers for slaughtering, ensures randomisation of the order in which chickens from a particular farm on any particular day are slaughtered. It is considered that this randomisation prior to processing, was sufficient to ensure that a chicken collected somewhere in the middle of a batch being processed was a randomly collected sample.

Data obtained at specimen collection

The project coordinator assigned sample codes to each sample to allow for anonymity and traceability. Each company and plant were assigned an identifier, and the farm number was provided by the company. The codes were assigned as "company-plant-farm-sampling number.container" e.g. BAF12.1 – company B, plant A, farm F, sample 12, container 1 (is either '1' or '2' which refers to the two separate sample containers used for each collection; see below). The farm identifier and the sample number were used as internal controls for traceability purposes. Data obtained and recorded at the time of sample collection included (the sample collection form is included as Appendix 1): date and time of collection (to allow for subsequent confirmation that each sample is from a different farm, if necessary), establishment ID number (for confidentiality purposes only the project coordinator knew which processing plant had which ID number), age of flock, the name of the specimen collector, and the within-establishment sample number (a unique number within each establishment written on the label identifying each). This data accompanied the sample to the primary laboratory, with a duplicate copy of the data sent to the project coordinator. This process allowed for the project coordinator to also keep track of sample collection and ensure only one sample from each farm had been submitted.

Act of specimen collection

Sample collection was undertaken between June and November 2016. Sampling was carried out by persons suitably trained in the collection procedure described and had previous experience with specimen collection at slaughter (e.g. those trained to collect samples for the NRS program). Additional training was provided specific to the below procedure by the company veterinarians as required.

Five random viscera (which constituted a single sample) were removed post mechanical evisceration, with intact caeca, as per the sample collection requirements for NARMS (9). Viscera that were not visibly contaminated with feed, digesta etc. were selected. The caecal pair was removed using sterile scissors at the sphincter between the caeca and the small intestines. New consumables (tubes, gloves etc.) were used

for each collection. If the scissors were to be reused on a day when more than one sample was being collected then they were sterilized in ethanol to reduce the opportunity for cross contamination (one pair of scissors was sent with each kit, with one kit per sample, to minimize this). Each caecal pair was separated and placed into individual containers (70 mL sterile screw top containers), so that each sample constituted two containers with five caeca each. This allowed for efficient sample processing in the laboratory due to the different requirements for isolating *Campylobacter*. The containers were placed in the shipping container (Esky/foam-box) with a buffer of absorbent paper to prevent direct contact of the samples with the ice-packs used to keep the samples cool (< 8°C), but not frozen, during transport.

Instructions were provided to each person collecting samples that allowance must be made for time to dispatch samples at the end of the day. The time of collection was recorded so management of the time lag to bacterial isolation could be managed. Samples were shipped on the same day as collection and were required to arrive at the primary laboratory within 24hrs of collection. To ensure this, samples were collected on Mondays, Tuesdays and Wednesdays only, with some on Thursdays if the processing plant was in close proximity to Birling Avian Laboratories.

Isolation and confirmation of target organisms (to species level) at the primary laboratory

The processing of samples inevitably involves strenuous mixing of the caecal material with diluent (e.g. vortexing) so it is reasonable to assume the target organisms were completely randomly distributed throughout the test matrix (diluted caecal material). Duplicate copies of all isolates were retained in on-site storage at Birling Avian Laboratories with single copies dispatched to the AMR testing laboratories.

Sample receival and preparation

Upon receival of the samples, the time and temperature inside the shipping container was recorded. Any samples that arrived more than 24hrs after collection or at a temperature above 8°C were deemed unacceptable and discarded. In these instances, the collection staff at the processing plant were notified and sent additional sampling kits to collect replacement samples.

The caeca in each of the two containers for each sample were placed into individual stomacher bags and stomached to homogenise for 60 seconds as per the Australian Standard AS 5013.20-2004 (12.2) and left at room temperature for 5 min for gravity settling of large particles. For the caeca from one container, 25g of homogenised sample was combined with 225 ml of sterile buffered peptone water (BPW) and mixed well. These caeca were used for isolation of *E. coli*, *Enterococcus* spp. and *Salmonella* spp.. For the caeca from the second container, 10g of homogenised sample was combined with 90ml of Bolton broth and mixed well. These caeca were used for isolation of *Campylobacter*.

Bacterial isolation and typing

Enterococcus isolation and typing

The prepared sample was shaken to resuspend the particles, and then streaked direct from BPW onto BEA agar. The agar plates were incubated at 42°C for 48 h and speciated using Vitek 2 (BioMerieux) mass spectrometry. From a pure subculture from the original colony, bacteria were harvested for storage at -20°C on cryo-beads in two separate, identical containers labelled with the sample code and the laboratory reference number.

E. coli isolation and typing

The prepared sample was shaken to resuspend the particles, and then streaked direct from BPW onto *E. coli* chromogenic agar which achieved both bacterial isolation and type confirmation. The agar plates were incubated at 37°C for 18h and then one clone was selected and subcultured onto Coli ID for purity. *E. coli* isolation was confirmed using an indole test. From a pure subculture from the original colony, bacteria were harvested for storage at -20°C on cryo-beads (Cryobank, Mast Diagnostics) in two separate, identical containers labelled with the sample code and the laboratory reference number.

Salmonella isolation and typing

Salmonella was isolated using the AS 5013.10-2009 method (ISO 6579:2002) for *Salmonella* spp. using RV and MK media with two different selective and differential plates (XLD as the primary and Hektone as a secondary selective).

The remaining homogenate from the first container was mixed well and incubated at 37°C for 24h. A post incubation screen using Atlas PCR (validated to AS 5013. 10-2009 and NATA approved) was conducted to screen for *Salmonella* in addition to the AS method. Samples positive for both methods will be confirmed using the AS reference method stated above with the following validated and NATA approved modification. A *Salmonella* specific chromogenic media (SMID2, BioMereriux) was used in place of biochemical testing by subculturing any suspect colonies onto nutrient agar for serological confirmation. From a pure subculture from the original colony, bacteria were harvested for storage at -20°C on cryo-beads in two separate, identical containers labelled with the sample code and the laboratory reference number.

Campylobacter isolation and typing

Campylobacter was isolated as per the AS 5013.6-2015 method using *Campylobacter* selective Bolton broth. The caecal homogenate from the second container was shaken to suspend the particles and for samples that were <12hrs post-sampling, 100uL was streaked direct from Bolton broth/homogenate onto CSK (Skirrow, BioMerieux) and CFA (Campy food Agar, BioMerieux) agar and incubated at 42°C for 48hrs. For samples that were >12hrs post-sampling, the direct streaking method was performed along with a preliminary incubation of the Bolton broth/homogenate sample at 42°C for 48hrs under microaerophilic conditions, prior to streaking onto CSK and CFA agar. The *Campylobacter* was speciated using Vitek 2 (BioMerieux) mass spectrometry. From a pure subculture from the original colony, bacteria were harvested for storage at -20°C on cryo-beads, using a proprietal suspension media* which prevents damage to the

bacteria from freezing, in two separate, identical containers labelled with the sample code and the laboratory reference number.

*The Campylobacter cryo-beads were the same as used for the other bacteria however the suspension fluid was removed and replaced with a proprietal suspension fluid which preserves Campylobacter when frozen, and will be made available for use in future studies.

Dispatch to AMR laboratories

One vial of cryo-beads for each isolate was shipped to the reference laboratories for species identification/confirmation using MALDI-TOF MS (Microflex, Bruker, MA, USA) and antimicrobial susceptibility testing, at the School of Veterinary and Life Science, Murdoch University, Perth (*Enterococcus* spp. and *Campylobacter* spp.) which coordinated shipping of *E. coli* and *Salmonella* spp. to ACARE at the University of Adelaide.

AMR Testing

Recovery of isolates for AMR testing

For *E. coli*, *Salmonella* and *Enterococcus*, one cryo-bead from each vial was placed onto Columbia sheep blood agar (Micromedia, Australia) and rolled with a loop in a circle, to create the initial streak zone. Further streaking from the initial zone was done prior to aerobic incubation at 37°C for 24hrs. A single colony was again sub-cultured on Columbia sheep blood agar at 37°C for 24hrs before performing antimicrobial susceptibility testing. For *Campylobacter*, one cryo-bead from each vial was placed onto a Columbia sheep blood agar and incubated microaerophilically at 37°C for 48hrs. A single colony was streaked on to another Columbia sheep blood agar and incubated at 42°C for24 hrs before performing antimicrobial susceptibility testing.

Susceptibility testing of isolates in specialist AMR laboratories

For *E. coli* and *Salmonella* spp., the antimicrobials tested were: amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, colistin (replaces kanamyocin in previous studies), streptomycin, tetracycline and trimethoprim/sulfamethoxazole. For *Enterococcus*, the antimicrobials tested were: ampicillin, chloramphenicol, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, penicillin, quinupristin/dalfopristin, streptomycin, teicoplanin, tetracycline, vancomycin and virginiamycin. For *Campylobacter* spp., the antimicrobials tested were based on the standard *Campylobacter* minimum inhibitory concentration (MIC) Plate available for the Sensititre system: azithromycin, ciprofloxacin, erythromycin, gentamicin, tetracycline, florfenicol, nalidixic acid, telithromycin, and clindamycin.

Antimicrobial susceptibility for the isolates was determined by the broth microdilution method either on veterinary reference card panels (NARMS, Sensititre®, Trek Diagnostics, East Grinstead, UK) according to the manufacturers' guidelines or in-house panels prepared according to Clinical and Laboratory Standards Institute (CLSI) standards (10). For reference card panels, the CMV3AGNF plate format was used to test *E. coli* and *Salmonella spp*.; CMV3AGPF for *Enterococcus* spp., and CAMPY for *Campylobacter* spp.. Antimicrobials that were not available on reference card panels, colistin and florfenicol for *E. coli* and

Salmonella spp. and ampicillin, teicoplanin and virginiamycin for *Enterococcus* spp., were tested on in-house broth microdilution panels. The complete list of antimicrobials along with the concentration ranges that were tested are listed according to their antimicrobial classes in Table 3, 4 and 5 for *Enterococcus* spp., *E. coli* / *Salmonella* spp. and *Campylobacter* spp. respectively.

Quality control was performed on control strains Staphylococcus aureus ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Campylobacter jejuni* ATCC 33560 throughout the study period.

Interpretation

Antimicrobial susceptibility testing is commonly undertaken for diagnostic or surveillance purposes and therefore it is important to appreciate the different ways in which the data can be interpreted. The overarching principle of interpreting susceptibility data is to classify data into distinct and meaningful categories by using breakpoint values. When laboratories measure the expression of resistance to a drug by a bacterial isolate the results are given along a continuous scale. The breakpoint is an agreed position along that scale such that all isolates can be classified as being either above or below the breakpoint. The breakpoint classifies the isolate as sensitive or resistant to the tested antimicrobial. There are two types of breakpoints used for classifying antimicrobial susceptibility of a bacterial isolate. This includes **Clinical Breakpoints** and **Epidemiological Cut-off Values** (ECOFF). To allow for comparability between other studies that may only use one or the other of these, both have been used in this study. Briefly, Clinical resistance to an antimicrobial refers to isolates that, in a clinical setting, would not be successfully removed by use of that antimicrobial, and microbiologically resistant refers to isolates that have potentially been exposed to an antimicrobial and while potentially not clinically resistant, may show signs of emerging resistance.

Clinical Breakpoints

These are values provided by CLSI in document VET01S (11) that are used to guide clinicians with regards to antimicrobial treatment options for their patients. As such, they include considerations such as clinical outcome data and in vitro pharmacological properties of the antimicrobial drug in addition to susceptibility data. Therefore, clinical breakpoints have a limited role in surveillance studies looking for emerging resistances. In tables 3, 4 and 5, two clinical breakpoint values are provided which creates a maximum possibility of three categories; Clinically Susceptible (CS), Clinically Intermediate (CI) [between CS and CR, not shown] and Clinically Resistant (CR). These terms are defined as follows:

Clinically-Susceptible (CS): Bacterial isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

Clinically-Intermediate (CI): Susceptibility of isolates approach attainable blood and tissue levels and response rates may be lower than for susceptible isolates. Implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher than normal dosage can be prescribed.

Clinically-Resistant (CR): Bacterial isolates are not inhibited by the usually achievable concentrations or when susceptibility results indicate the likelihood of specific AMR mechanisms and the success of treatment by the agent has not been reliably shown.

Multi-drug resistance (MDR): Isolates that are resistant to three or more classes of antimicrobials based on clinical breakpoint (where one is available) is classified as multi-drug resistant (MDR) phenotype.

Epidemiological Cut-off Values (ECOFF)

Besides the clinical breakpoint, the other applicable system of classification is ECOFF provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (12). The ECOFF is referred to as the "Microbiological Breakpoint" in this report for clarity. In recent years, "Microbiological Breakpoint" or ECOFF values are encouraged to be used in AMR surveillance since it allows for the detection of emerging resistance in a bacterial population. As a result, large surveillance systems such as DANMAP uses ECOFFs as a standard breakpoint for classifying AMR phenotype (1). As such, the microbiological breakpoints are more often used for identifying emerging resistances in surveillance studies than clinical breakpoints. Both the clinical and microbiological breakpoints for each bacteria-antimicrobial pair are listed in Tables 3, 4 and 5. The microbiological breakpoint consists of a single breakpoint value which classifies isolates into two categories; Microbiologically-Susceptible (MS, Wild Type) and Microbiologically-Resistant (MR, Non-Wild Type). These terms are defined as follows:

Microbiologically-Susceptible (MS): Wildtype isolates which are the typical form of bacteria as it occurs in nature. These bacteria have not been exposed to antimicrobial selection pressures and therefore have no need for AMR.

Microbiologically-Resistant (MR): Non-Wildtype isolates which are the mutated form of bacteria that are expressing some elevated levels of AMR. These isolates do not necessarily indicate that they are expressing clinical levels of resistance.

Table 3. Breakpoints used for susceptibility testing of *Enterococcus* species

Class	Agent	Species	Range (mg/L)	Microbiological Breakpoint [c]	Clinical Breakpoint [a] [b]	
					CS	CR
Aminoglycosides	Gentamicin	All	128 - 1024	- [d]	≤500	>500
	Kanamycin [d]	All	128 - 1024	-	≤512	>512
	Streptomycin	All	512 - 2048	-	≤1000	>1000
Glycopeptides	Vancomycin	All	0.25 - 32	4	≤4	>16
	Teicoplanin	All	0.25 - 128	2	≤8	>16
Lincosamide	Lincomycin [d]	All	1 - 8	-	≤2	>4
Lipopeptides	Daptomycin	All	0.25 - 16	4	≤4	-
Macrolides	Erythromycin	*E. faecium*, *E. faecalis*	0.25 - 8	4	≤0.5	>4
		E. hirae	0.25 - 8	2	≤0.5	>4
Oxazolidinones	Linezolid	All	0.5 - 8	4	≤2	>4
Penicillins	Ampicillin	All	0.25 - 64	4	≤8	>8
	Benzylpenicillin	*E. faecium*, *E. faecalis*	0.25 - 16	16	≤8	>8
Phenicols	Chloramphenicol	*E. faecium*, *E. faecalis*	2 - 32	32	≤8	>16
		E. hirae	2 - 32	8	≤8	>16
Streptogramins	Quinupristin-Dalfopristin	*E. faecium*	0.5 - 32	-	≤1	>2
	Virginiamycin	*E. faecium*	0.25 - 128	4	-	-
		E. faecalis	0.25 - 128	32	-	-
		E. hirae	0.25 - 128	-	-	-
Tetracyclines	Tetracycline	All	1 – 32	4	≤4	>8

[a] CLSI VETO1S(8) or M100S(10) breakpoints (mg/L), CS = Clinically-Sensitive; ; CI = Clinically-Intermediate (between CS and CR, not shown); CR = Clinically-Resistant

[b] NARMS(3) breakpoints (mg/L) (green text)

[c] EUCAST epidemiological cut-off values (mg/L)

[d] Not defined

Table 4. Breakpoints used for susceptibility testing of *Escherichia coli* and *Salmonella* species

Class	Agent	Range (mg/L)	Microbiological Breakpoint [a]		Clinical Breakpoint [b c]	
			E. coli	*Salmonella*	CS	CR
Aminoglycosides	Gentamicin	0.25 - 16	2	2	≤4	>8
	Streptomycin	2 - 64	16	16	≤32	>32
β-lactam / β-lactam inhibitor combination	Amoxicillin-Clavulanate (2:1 ratio)	1 - 32	- [d]	-	≤8	>16
Cephems	Cefoxitin	0.5 - 32	8	8	≤8	>16
	Ceftiofur	0.12 - 8	1	2	≤2 [e]	>4
	Ceftriaxone	0.25 - 64	0.12	-	≤1	>2
Fluoroquinolones	Ciprofloxacin (*E. coli*)	0.015 - 4	0.06	-	≤1	>2
	Ciprofloxacin (*Salmonella*)	0.015 - 4	-	0.06	≤0.06	>0.5
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole (1:19)	0.12 - 4	1	1	≤2	>2
Macrolides	Azithromycin (*Salmonella*)	0.12 - 16	-	-	≤16	>16
Penicillins	Ampicillin	1 - 32	8	8	≤8	>16
Phenicols	Chloramphenicol	2 - 32	16	16	≤8	>16
	Florfenicol	1 - 128	16	16	≤4 [f]	>8
Polymyxins	Colistin	0.12 - 8	2	-	-	-
Tetracyclines	Tetracycline	4 - 32	8	8	≤4	>8

[a] EUCAST epidemiological cut-off values (mg/L)

[b] CLSI VETO1S,(8) or M100S(10) breakpoints (mg/L), CS = Clinically-sensitive ;CI = Clinically-Intermediate (between CS and CR, not shown); CR =Clinically-resistant

[c] NARMS(3) breakpoints (mg/L) (green text)

[d] Not defined

[e] *E. coli* only

[f] *Salmonella* Choleraesuis only

Table 5. Breakpoints used for susceptibility testing of *Campylobacter* species

Class	Agent	Species	Range (mg/L)	Microbiological Breakpoint [a]	NARMS Breakpoint [b]	
					S	R
Aminoglycosides	Gentamicin	All	0.12 - 32	2	≤2	>2
Ketolides	Telithromycin	*C. jejuni*	0.015 - 8	4	≤4	>4
Lincosamide	Clindamycin	*C. coli* [c]	0.03 - 16	1	≤1	>1
		C. jejuni	0.03 - 16	0.5	≤0.5	>0.5
Macrolides	Azithromycin	*C. coli*	0.015 - 64	0.5	≤0.5	>0.5
		C. jejuni	0.015 - 64	0.25	≤0.25	>0.25
	Erythromycin	*C. coli*	0.03 - 64	8	≤8	>8
		C. jejuni	0.03 - 64	4	≤4	>4
Phenicols	Florfenicol	All	0.03 - 64	4	≤4	>4
Quinolones	Ciprofloxacin	All	0.015 - 64	0.5	≤0.5	>0.5
	Nalidixic acid	All	4 - 64	16	≤16	>16
Tetracyclines	Tetracycline	*C. coli*	0.06 - 64	2	≤2	>2
		C. jejuni	0.06 - 64	1	≤1	>1

[a] EUCAST epidemiological cut-off values (mg/L)

[b] NARMS(3) breakpoints, adapted from microbiological breakpoints, (mg/L , S = Sensitive; I = Intermediate (between S and R, not shown) R = Resistant

[c] *C. coli* and species other than *C. jejuni*

Genetic analysis

Genetic analysis was undertaken to investigate the molecular mechanisms responsible for unexpected resistance profiles in a subset of isolates.

DNA extraction and library preparation

DNA extraction was performed on all isolates using the MagMAX Multi-sample extraction kit (Thermofisher Scientific, USA) as per the manufacturer's instructions. DNA library preparation was conducted using an Illumina Nextera XT Library Preparation kit, with variation from the manufacturer's instructions for an increased time for tagmentation to 7 mins. Library preparations were sequenced via Illumina Nextseq platform with a high output 2x150 kit.

DNA sequencing and analysis

The genomic data was de novo assembled using SPAdes. All isolates were analysed using the Centre for Genomic Epidemiology for the screening of multi-locus sequence type, AMR genes, virulence genes and plasmids. *Campylobacter* with an unknown sequence type were additionally searched against the pubMLST database. The presence of various known mutations was detected using the SNIPPY tool in the Nullarbor bioinformatics pipeline.

Statistical analysis

Data from automated reading of broth microdilution plates were electronically captured and checked for conformance with design parameters and correctness of isolate identification. Scripting programs were used to generate standardised MIC tables and plots using the accepted breakpoint values and dilution ranges for each combination of drug and commensal bacteria. Confidence intervals of proportions were calculated using exact binomial confidence intervals derived by the Clopper-Pearson method. All analysis occurred in Stata version15.1 (StataCorp LLC, College Station, Texas USA, www.stata.com).

Results

Reporting of the results is in-line with recommendations in OIE chapter 6.7 which states that "*For surveillance purposes, use of the microbiological breakpoint (also referred to as epidemiological cut-off point), which is based on the distribution of MICs or inhibition zone diameters of the specific bacterial species tested, is preferred*.". The clinical resistance results are also reported (since these have relevance to public health) but the focus of the reporting is on microbiological results, with these results supported by genetic analysis where possible.

No direct comparison between resistance results from chicken commensal bacteria and reported human clinical cases has been provided as these results are not considered to be comparative due to inherent differences in sample and bacterial characteristics of isolates from healthy chickens and septic human patients. Where isolates were both clinically and microbiologically resistant, the term 'resistance' alone is used.

Bacterial isolation

As the primary aim of the work was to estimate the prevalence of resistance amongst commensal bacteria at a population level, not a company or flock level, and as such, the methods and description of the results is a reflection of this. As each single sample constituted five caeca, estimation of prevalence of these bacteria in Australian meat chickens is not possible. A total of 668 bacterial isolates were recovered for susceptibility testing as indicated in Table 6. Five *Enterococcus* species contributed to 30.7% of the total isolates recovered, three of which represented 87.3% of isolates from the genus (*E. durans* 29.7%, *E. faecalis* 20%, *E. faecium* 37.6%, *E. gallinarum* 0.5% and *E. hirae* 12.2%). *E. coli* and *Salmonella* spp. contributed to 30.8% and 7.9% respectively. Two *Campylobacter* species contributed to 30.5% of the total isolates recovered (*C. coli* 47% and *C. jejuni* 53%). Characterisation of the isolates at the primary laboratory using Vitek 2 mass spectrometry and the AMR laboratories using MALDI-TOF matched 100%.

Table 6. Isolates recovered

Genus	Species	Number (% of genus)
Escherichia	*coli*	206
Salmonella	various	53
Enterococcus	*E. faecium*	77 (37.6)
	E. hirae	25 (12.2)
	E. faecalis	41 (20.0)
	E. durans	61 (29.7)
	E. gallinarum	1 (0.5)
Campylobacter	*C. coli*	96 (47)
	C. jejuni	108 (53)

MIC distributions

Enterococcus species

Enterococcus spp. are intrinsically resistant to lincosomides and aminoglycosides. In addition, *E. faecalis* is intrinsically resistant to the streptogramin class (virginiamycin and quinupristin-dalfopristin). Microbiological susceptibility results varied widely among the enterococcus genus. All *Enterococcus* isolates were clincially susceptible to vancomycin and only one *Enterococcus* isolate (*E. faecalis*) demonstrated microbiological resistance (8mg/L). One isolate each of *E. faecium* and *E. faecalis* demonstrated clinical and microbiological resistance to linezolid. Microbiological and clinical resistance to ampicillin in *E. faecium* was 55.8% and 20.8% respectively. Among the *Enterococcus sp.* tetracycline resistance was common (40-46%). A large proportion of *E. faecium* isolates were resistant to quinuprisrin-dalfopristin (54.5%). All *E. faecalis* and *E. faecium* expressed clinical and microbiological susceptibility to chloramphenicol. Only two *Enterococcus* isolates (*E. faecium*) were clinically resistant to kanamycin and no other *Enterococcus* isolates demonstrated clinical resistance to the aminoglycosides class (currently no established microbiological breakpoints). Refer to Figures 2-4 and Tables 7-9 for the complete description of results. Note that after speciation of *Enterococcus*, three smaller groupings are made with this required due to differences amongst the individual species with respect to breakpoints and inhernet resistance to drug classes. .

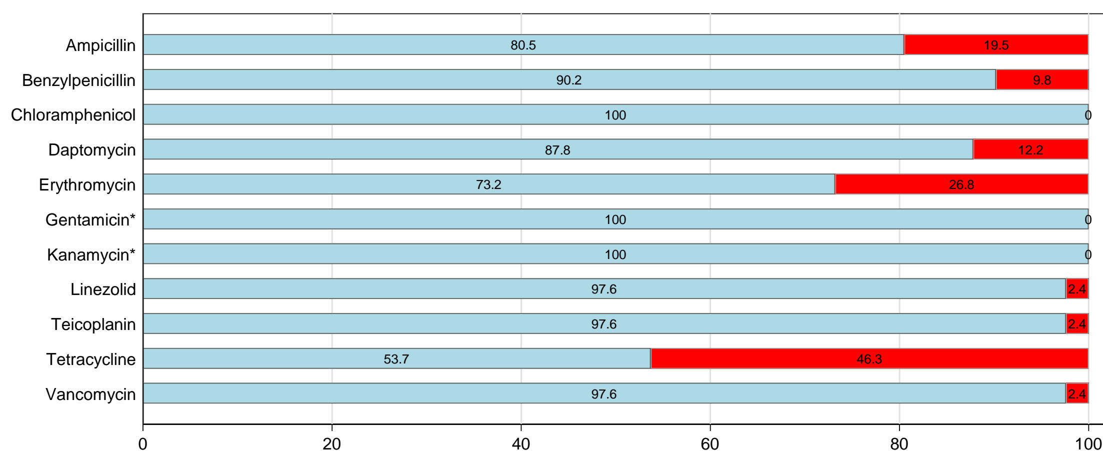

Figure 2. Antimicrobial resistance patterns for *Enterococcus faecalis* (n=41) based on microbiological (ECOFF) break points. Clinical break points are used when microbiological break points were unavailable. The proportion of susceptible is shown in blue and the proportion resistant in red. * Denotes use of clinical breakpoints where no microbiological breakpoints are available.

Table 7. Distribution of minimum inhibitory concentrations for *Enterococcus faecalis* (n=41) isolated from Australian meat chickens

Percentage of isolates classified as microbiologically resistant with corresponding 95% confidence intervals (CI) limits and percentage classified as clinically resistant. For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Microbiological breakpoints are not presently available for antimicrobials noted with* and blank boxes in the table also indicate lack of relevant breakpoints. Note that *E. faecalis* are intrinsically resistant to lincomycin and quinupristin-dalfopristin.

	Minimum inhibitory concentration (mg/L)													Microbiological resistant (%) (95% CI)	Clinical resistant (%)
Antimicrobial	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		
Ampicillin	0.0	2.4	14.6	2.4	61.0	9.8	9.8	0.0	0.0	0.0	0.0	0.0	0.0	19.5 (8.8 – 34.9)	9.8
Chloramphenicol	0.0	0.0	0.0	2.4	2.4	78.0	17.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 8.6)	0.0
Daptomycin	12.2	4.9	12.2	34.1	24.4	12.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.2 (4.1 – 26.2)	
Erythromycin	48.8	2.4	17.1	4.9	0.0	0.0	26.8	0.0	0.0	0.0	0.0	0.0	0.0	26.8 (14.2 – 42.9)	26.8
Gentamicin*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0		0.0
Kanamycin*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	87.8	12.2	0.0	0.0		0.0
Lincomycin*	0.0	0.0	4.9	0.0	0.0	4.9	90.2	0.0	0.0	0.0	0.0	0.0	0.0		
Linezolid	0.0	2.4	0.0	65.9	29.3	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0	2.4 (0.1 – 12.9)	2.4
Penicillin(benzyl)	22.0	2.4	12.2	9.8	34.1	7.3	2.4	9.8	0.0	0.0	0.0	0.0	0.0	9.8 (2.7 – 23.1)	12.2
Quinupristin-Dalfopristin*	0.0	7.3	0.0	34.1	9.8	22.0	19.5	7.3	0.0	0.0	0.0	0.0	0.0		
Teicoplanin	87.8	9.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	0.0	0.0	0.0	2.4 (0.1 – 12.9)	2.4
Tetracycline	0.0	0.0	51.2	0.0	2.4	0.0	0.0	7.3	39.0	0.0	0.0	0.0	0.0	46.3 (30.7 – 62.6)	46.3
Vancomycin	7.3	43.9	31.7	12.2	2.4	2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4 (0.1 – 12.9)	0.0
Virginiamycin	0.0	0.0	2.4	17.1	48.8	17.1	7.3	2.4	0.0	4.9	0.0	0.0	0.0	4.9 (0.6 – 16.5)	

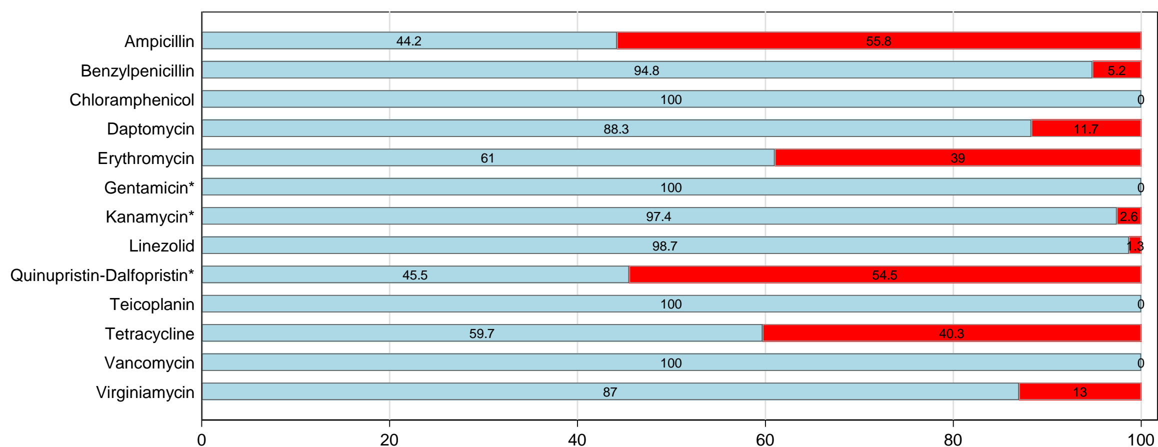

Figure 3. Antimicrobial resistance patterns for *Enterococcus faecium* (n=77) based on microbiological (ECOFF) break points. Clinical break points are used when microbiological break points were unavailable. The proportion of susceptible is shown in blue and the proportion resistant in red. *Denotes use of clinical breakpoints where no microbiological breakpoints are available.

Table 8. Distribution of minimum inhibitory concentrations for *Enterococcus faecium* (n=77) isolated from Australian meat chickens.

Percentage of isolates classified as microbiologically resistant with corresponding 95% confidence intervals (CI) and percentage classified as clinically resistant. For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Microbiological breakpoints are not presently available for antimicrobials noted with * and blank boxes in the table also indicate lack of relevant breakpoints. Note that E. faecium are intrinsically resistant to lincomycin.

	Minimum inhibitory concentration (mg/L)															Microbiological resistant (%) (95% CI)	Clinical resistant (%)
Antimicrobial	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048		
Ampicillin	0.0	9.1	7.8	5.2	9.1	13.0	35.1	14.3	5.2	1.3	0.0	0.0	0.0	0.0	0.0	55.8 (44.1 – 67.2)	20.8
Chloramphenicol*	0.0	0.0	0.0	0.0	0.0	3.9	75.3	20.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 4.7)	0.0
Daptomycin	0.0	14.3	11.7	5.2	33.8	23.4	11.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.7 (5.5 – 21.0)	
Erythromycin	0.0	35.1	3.9	13.0	9.1	0.0	3.9	35.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	39.0 (28.0 – 50.8)	39.0
Gentamicin*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0		0.0
Kanamycin*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	79.2	14.3	3.9	1.3	1.3		2.6
Lincomycin*	0.0	0.0	0.0	11.7	0.0	0.0	2.6	85.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
Linezolid	0.0	0.0	0.0	0.0	55.8	42.9	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3 (0.0 – 7.0)	1.3
Penicillin (benzyl)	0.0	27.3	11.7	7.8	7.8	31.2	3.9	5.2	5.2	0.0	0.0	0.0	0.0	0.0	0.0	5.2 (1.4 – 12.8)	10.4
Quinupristin-Dalfopristin*	0.0	0.0	10.4	6.5	28.6	7.8	15.6	19.5	10.4	1.3	0.0	0.0	0.0	0.0	0.0		54.5
Teicoplanin	0.0	98.7	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 4.7)	0.0
Tetracycline	0.0	0.0	0.0	58.4	1.3	0.0	0.0	0.0	3.9	36.4	0.0	0.0	0.0	0.0	0.0	40.3 (29.2 – 52.1)	40.3
Vancomycin	0.0	2.6	53.2	33.8	3.9	6.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 4.7)	0.0
Virginiamycin	0.0	53.2	9.1	5.2	9.1	10.4	10.4	1.3	1.3	0.0	0.0	0.0	0.0	0.0	0.0	13.0 (6.4 – 22.6)	

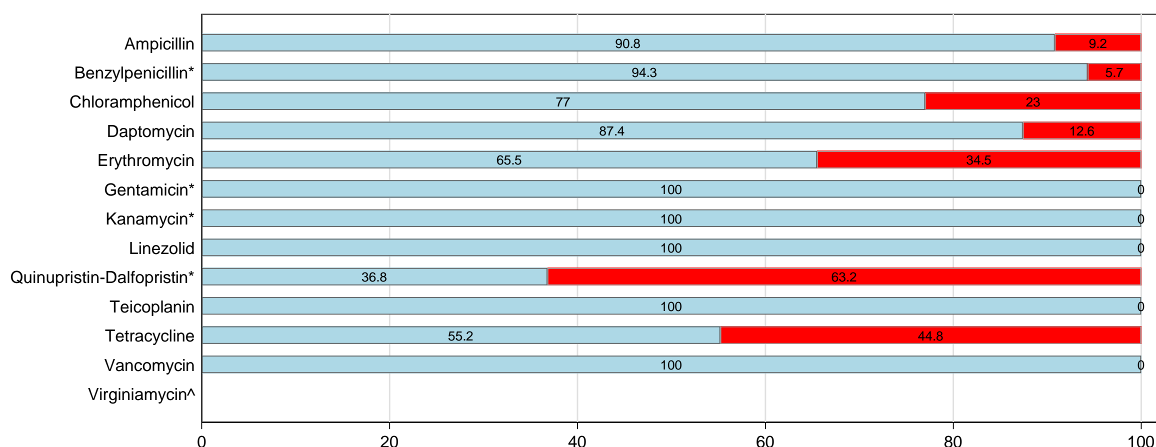

Figure 4. Microbiological resistance patterns for other *Enterococcus* spp. (n=87) comprising: *Enterococcus hirae* (n= 25), *Enterococcus durans* (n= 61) and *Enterococcus gallinarum* (n=1) based on microbiological (ECOFF) break points. Clinical break points are used when microbiological break points were unavailable. The proportion of susceptible is shown in blue and the proportion resistant in red. * Denotes use of clinical breakpoints where no microbiological breakpoints are available. ^ Denotes neither microbiological or clinical breakpoints available for interpretation.

Table 9. Distribution of minimum inhibitory concentrations for other *Enterococcus* spp. (n=87) comprising: *Enterococcus hirae* (n= 25), *Enterococcus durans* (n= 61) and *Enterococcus gallinarum* (n=1) isolated from Australian meat chickens.

Percentage of isolates classified as microbiologically resistant with corresponding 95% confidence intervals (CI) and percentage classified as clinically resistant. For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Microbiological breakpoints are not presently available for antimicrobials noted with * and blank boxes in the table also indicate lack of relevant breakpoints. *E. hirae* breakpoints were used for this table. Note that *Enterococcus* spp. are intrinsically resistant to lincomycin.

	Minimum inhibitory concentration (mg/L)													Microbiological resistant (%) (95% CI)	Clinical resistant (%)
Antimicrobial	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		
Ampicillin	31.0	6.9	17.2	16.1	19.5	8.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	9.2 (4.1 – 17.3)	1.1
Chloramphenicol*	0.0	0.0	0.0	1.1	6.9	69.0	23.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 4.2)	0.0
Daptomycin	10.3	9.2	11.5	24.1	32.2	12.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.6 (6.5 – 21.5)	
Erythromycin	35.6	6.9	13.8	9.2	0.0	0.0	34.5	0.0	0.0	0.0	0.0	0.0	0.0	34.5 (24.6 – 45.4)	34.5
Gentamicin*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0		0.0
Kanamycin*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	83.9	13.8	2.3	0.0		0.0
Lincomycin*	0.0	0.0	9.2	1.1	0.0	0.0	89.7	0.0	0.0	0.0	0.0	0.0	0.0		
Linezolid	0.0	1.1	0.0	59.8	39.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 4.2)	0.0
Penicillin (benzyl)	17.2	11.5	5.7	20.7	32.2	6.9	1.1	4.6	0.0	0.0	0.0	0.0	0.0	4.6 (1.3 – 11.4)	5.7
Quinupristin-Dalfopristin*	0.0	8.0	4.6	24.1	11.5	20.7	26.4	4.6	0.0	0.0	0.0	0.0	0.0		63.2
Teicoplanin	95.4	3.4	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 4.2)	0.0
Tetracycline	0.0	0.0	54.0	0.0	1.1	0.0	3.4	3.4	37.9	0.0	0.0	0.0	0.0	44.8 (34.1 – 55.9)	44.8
Vancomycin	5.7	46.0	36.8	8.0	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 4.2)	0.0
Virginiamycin*	36.8	8.0	14.9	11.5	8.0	6.9	6.9	6.9	0.0	0.0	0.0	0.0	0.0		

E. coli

All commensal *E. coli* isolates tested were microbiologically susceptibile to amoxicillin-clavulanate, ceftiofur, chloramphenicol, colistin, florfenicol and gentamicin. Microbiological resistance was observed for ampicillin (14.1%), streptomycin (9.7%), tetracycline (19.4%) and trimethoprim/sulfamethoxazole (8.7%). For ceftriaxone, which currently does not have an established microbiological breakpoint (for commensal *E. coli* from animals), no isolates were found to be clinically resistant. Only two isolates demonstrated microbiological resistance to the fluroquinolone class (ciprofloxacin MIC 0.13 and 0.25 mg/L). However, these two isolates were classified as susceptable based on clinical breakpoints. Of the 206 *E.coli* isolates, 63.1% were susceptible to all of the antibiotics tested. The AMR patterns for *E. coli* based on microbiological (ECOFF) break points is shown in Figure 5. Comprehensive distribution of MIC concentrations for *E. coli* including frequency of clinical resistance is shown in Table 10.

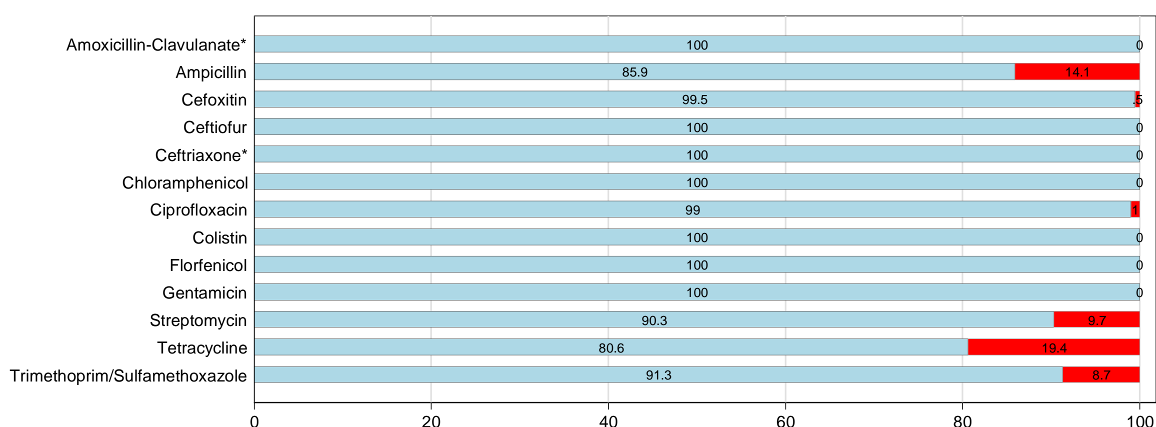

Figure 5. Antimicrobial resistance patterns for commensal *Escherichia coli* (n=206) based on microbiological (ECOFF) break points. Clinical break points are used when microbiological break point is unavailable. The proportion of susceptible is shown in blue and the proportion resistant in red. * denotes no microbiological breakpoints available, therefore clinical breakpoints were used.

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Table 10. Distribution of minimum inhibitory concentrations for commensal *Escherichia coli* (n=206) isolated from Australian meat chickens.

Percentage of isolates classified as microbiologically resistant with corresponding 95% confidence intervals (CI) and percentage classified as clinically resistant. For each antimicrobial, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Microbiological breakpoints are not currently available for antimicrobials noted with *.

	Minimum inhibitory concentration (mg/L)														Microbiological resistant (%) (95% CI)	Clinical resistant (%)
Antimicrobial	**0.016**	**0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**		
Amoxicillin-Clavulanate*	0.0	0.0	0.0	0.0	0.0	0.0	5.3	39.3	42.7	12.6	0.0	0.0	0.0	0.0		**0.0**
Ampicillin	0.0	0.0	0.0	0.0	0.0	0.0	19.9	44.2	21.4	0.5	0.0	0.0	14.1	0.0	14.1 (9.6 – 19.6)	**14.1**
Cefoxitin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.9	70.4	24.3	0.5	0.0	0.0	0.0	0.5 (0.0 – 2.7)	**0.0**
Ceftiofur	0.0	0.0	0.0	1.5	45.1	52.4	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 1.8)	**0.0**
Chloramphenicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.4	43.7	51.9	0.0	0.0	0.0	0.0	0.0 (0.0 – 1.8)	**0.0**
Ciprofloxacin	95.1	3.9	0.0	0.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 (0.1 – 3.5)	**0.0**
Colistin	0.0	0.0	0.0	21.8	73.3	3.4	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 1.8)	
Ceftriaxone*	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		**0.0**
Florfenicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.7	76.2	14.1	0.0	0.0	0.0	0.0 (0.0 – 1.8)	**14.1**
Gentamicin	0.0	0.0	0.0	0.0	5.8	79.1	15.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 1.8)	**0.0**
Streptomycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	50.0	36.9	2.4	4.9	2.4	2.4	9.7 (6.0 – 14.6)	**4.9**
Tetracycline	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	80.6	0.0	0.0	0.0	19.4	0.0	19.4 (14.2 – 25.5)	**19.4**
Trimethoprim/ Sulfamethoxazole	0.0	0.0	0.0	87.9	1.5	1.5	0.5	0.0	0.0	8.7	0.0	0.0	0.0	0.0	8.7 (5.3-13.5)	**8.7**

Salmonella species

All *Salmonella* isolates tested were microbiologically susceptible to ceftiofur, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin and tetracycline. Six *Salmonella* isolates were clinically resistant to cefoxitin. Microbiological and clinical resistance was identified for ampicillin (two isolates), streptomycin (one isolate) and trimethoprim/sulfamethoxazole (1 isolate). Two isolates demonstrated clinical resistance to ampicillin. For ceftriaxone, which currently does not have an established microbiological breakpoint, no isolates were found clinically resistant. No dichotomised results were shown for colistin as no interpretive standards are currently available. Of the 53 isolates, 92.5% were susceptible to all antibiotics tested. The phenotypic patterns of resistance for *Salmonella* spp*.* based on microbiological (ECOFF) break points is shown in Figure 6. None of the resistant *Salmonella* were typed as *Salmonella Typhimurium*. Comprehensive distribution of MIC concentrations for S*almonella* sp including frequency of clinical resistance is shown in Table 11.

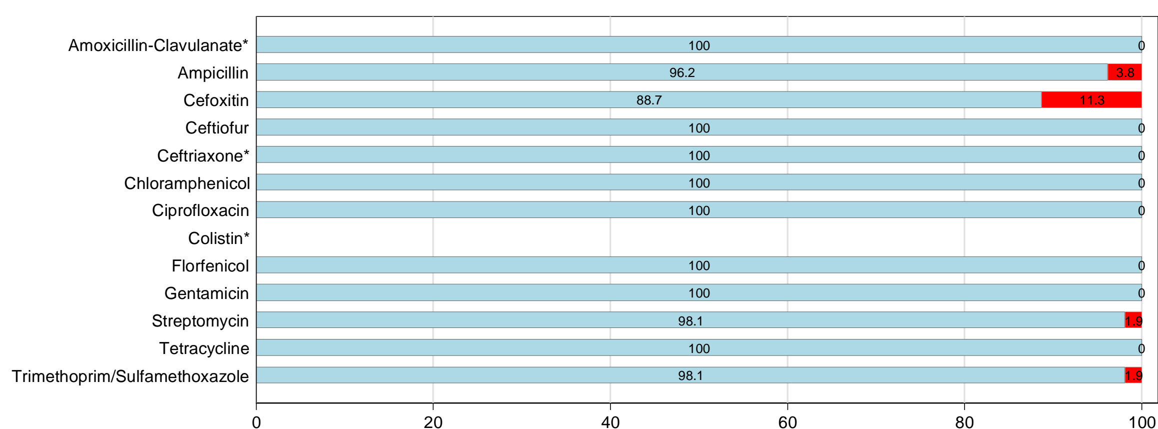

Figure 6. Antimicrobial resistance patterns for *Salmonella* spp. (n=53) based on microbiological (ECOFF) break points. Clinical break points are used when microbiological break point is unavailable. The proportion of susceptible is shown in blue and the proportion resistant in red. * Denotes use of clinical breakpoints where no microbiological breakpoints are available. Neither microbiological or clinical breakpoints available for colistin interpretation.

Table 11. Distribution of minimum inhibitory concentrations for *Salmonella* spp. (n=53) isolated from Australian meat chickens.

Percentage of isolates classified as microbiologically resistant with corresponding 95% confidence interval (CI) and percentage classified as clinically resistant. For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Microbiological breakpoints are not presently available for antimicrobials noted with *.

	Minimum inhibitory concentration (mg/L)														Microbiological resistant (%) (95% CI)	Clinical resistant (%)
Antimicrobial	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128		
Amoxicillin-Clavulanate*	0.0	0.0	0.0	0.0	0.0	0.0	77.4	17.0	1.9	1.9	1.9	0.0	0.0	0.0		0.0
Ampicillin	0.0	0.0	0.0	0.0	0.0	0.0	67.9	28.3	0.0	0.0	0.0	0.0	3.8	0.0	3.8 (0.5 – 13.0)	3.8
Cefoxitin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	34.0	41.5	13.2	11.3	0.0	0.0	0.0	11.3 (4.3 – 23.0)	0.0
Ceftiofur	0.0	0.0	0.0	0.0	1.9	18.9	64.2	15.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 6.7)	0.0
Chloramphenicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	45.3	54.7	0.0	0.0	0.0	0.0	0.0 (0.0 – 6.7)	0.0
Ciprofloxacin	49.1	50.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 6.7)	0.0
Colistin	0.0	0.0	0.0	0.0	9.4	60.4	30.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
Ceftriaxone*	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0
Florfenicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.5	71.7	3.8	0.0	0.0	0.0	0.0 (0.0 – 6.7)	3.8
Gentamicin	0.0	0.0	0.0	0.0	66.0	34.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 6.7)	0.0
Streptomycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.8	60.4	17.0	0.0	0.0	1.9	1.9 (0.0 – 10.1)	1.9
Tetracycline	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 6.7)	0.0
Trimethoprim/ Sulfamethoxazole	0.0	0.0	0.0	96.2	1.9	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	1.9 (0.0 – 1.9)	1.9

Campylobacter species

All *Campylobacter* isolates tested were microbiologically susceptibile to florfenicol and gentamicin. Microbiological and clincial resistance to ciprofloxacin was detected in 14.8% of *C. jejuni* isolates and 5.2% of *C. coli*. One isolate of *C. jejuni* and five isolates of *C. coli* were microbiologically and clinically resistant to the macrolides azithomycin and erythromycin. No resistance was detected to any of the antibiotics tested in 63% of *C. jejuni* isolates and 86.5% *C. coli* isolates. The AMR patterns for *Campylobacter* spp. based on microbiological (ECOFF) break points is shown in Figure 7 and 8. Comprehensive distribution of MIC concentrations for *Campylobacter* spp. including frequency of clinical resistance is shown in Tables 12 and 13.

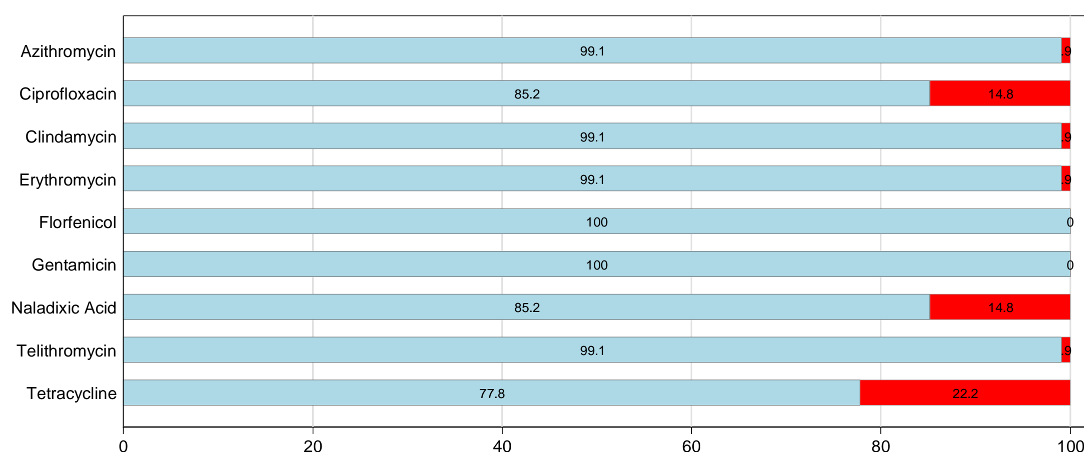

Figure 7. Microbiological resistance patterns for *Campylobacter jejuni* (n=108) based on microbiological (ECOFF) break points. Clinical break points are used when microbiological break points were unavailable. The proportion of susceptible is shown in blue and the proportion resistant in red.

Table 12. Distribution of minimum inhibitory concentrations for *Campylobacter jejuni* (n=108) isolated from Australian meat chickens.

Percentage of isolates classified as non-wild type by EUCAST, and corresponding 95% confidence limits. For each drug, vertical bars show position of the interpretive breakpoint and shaded areas indicate the range of dilutions evaluated. EUCAST breakpoints are not presently available for antimicrobials noted with *.

	Minimum inhibitory concentration (mg/L)														Microbiological resistant (%) (95% CI)	Clinical resistant (%)
Antimicrobial	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128		
Azithromycin	56.5	38.0	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9 (0.0 – 5.1)	0.9
Ciprofloxacin	0.0	4.6	44.4	30.6	5.6	0.0	0.0	0.0	0.9	7.4	6.5	0.0	0.0	0.0	14.8 (8.7 – 22.9)	14.8
Clindamycin	0.0	17.6	43.5	34.3	3.7	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.9 (0.0 – 5.1)	0.9
Erythromycin	0.0	0.9	9.3	24.1	53.7	11.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9 (0.0 – 5.1)	0.9
Florfenicol	0.0	0.0	0.0	0.9	8.3	50.0	40.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 3.4)	0.0
Gentamicin	0.0	0.0	0.0	7.4	50.9	41.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 3.4)	0.0
Nalidixic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	65.7	19.4	0.0	0.0	2.8	12.0	14.8 (8.7 – 22.9)	14.8
Telithromycin	0.0	0.0	4.6	13.9	23.1	49.1	8.3	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.9 (0.0 – 5.1)	0.9
Tetracycline	0.0	0.0	20.4	33.3	18.5	4.6	0.9	2.8	0.0	0.9	10.2	6.5	0.9	0.9	22.2 (14.7 – 31.2)	22.2

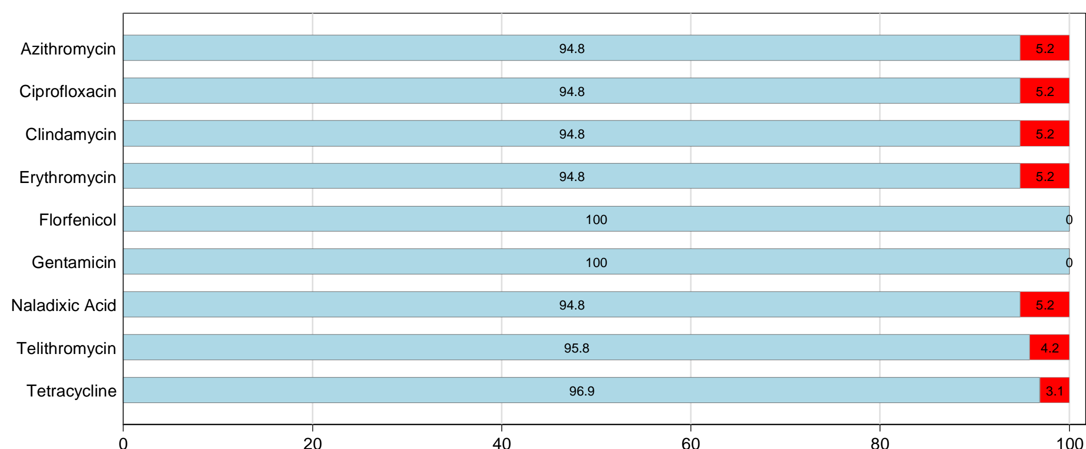

Figure 8. Microbiological resistance patterns for *Campylobacter coli* (n=96,) based on microbiological (ECOFF) break points. Clinical break points are used when microbiological break points were unavailable. The proportion of susceptible is shown in blue and the proportion resistant in red.

Table 13. Distribution of minimum inhibitory concentrations for *Campylobacter coli* (n=96) isolated from Australian meat chickens.

Percentage of isolates classified as microbiologically resistant with corresponding 95% confidence intervals (CI) and percentage classified as clinically resistant. For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Microbiological breakpoints are not presently available for antimicrobials noted with *.

Antimicrobial	Minimum inhibitory concentration (mg/L)														Microbiological resistant (%) (95% CI)	Clinical resistant (%)
	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128		
Azithromycin	7.3	42.7	36.5	8.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.2	5.2 (1.7 – 11.7)	5.2
Ciprofloxacin	0.0	4.2	39.6	42.7	8.3	0.0	0.0	0.0	2.1	3.1	0.0	0.0	0.0	0.0	5.2 (1.7 – 11.7)	5.2
Clindamycin	0.0	0.0	11.5	49.0	32.3	2.1	0.0	2.1	2.1	1.0	0.0	0.0	0.0	0.0	5.2 (1.7 – 11.7)	5.2
Erythromycin	0.0	0.0	0.0	9.4	42.7	28.1	10.4	4.2	0.0	0.0	0.0	0.0	0.0	5.2	5.2 (1.7 – 11.7)	5.2
Florfenicol	0.0	0.0	0.0	0.0	6.3	43.8	45.8	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 3.8)	0.0
Gentamicin	0.0	0.0	0.0	4.2	26.0	68.8	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0 – 3.8)	0.0
Nalidixic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	55.2	39.6	0.0	0.0	2.1	3.1	5.2 (1.7 – 11.7)	5.2
Telithromycin	0.0	0.0	0.0	11.5	24.0	33.3	15.6	7.3	4.2	0.0	4.2	0.0	0.0	0.0	4.2 (1.1 – 10.3)	4.2
Tetracycline	0.0	0.0	15.6	43.8	29.2	6.3	2.1	0.0	0.0	0.0	2.1	0.0	0.0	1.0	3.1 (0.6 – 8.9)	3.1

Multi-drug resistance profiles

Enterococcus

A total of 20 unique resistance profiles were identified among the 205 *Enterococci* isolates of which 17.5% were MDR (defined as being clinically resistant to three or more classes of antimicrobial). The frequency of MDR isolates was low among *E. faecalis* (2.4%) compared to *E. faecium* (23.4%) and other *Enterococcus* spp. (19.5%). The dominant MDR profiles were bla/mac/tet for *E.faecalis* and mac/str/tet for *E.faecium* and other *Enterococcus.* The MDR profiles for *Enterococcus* spp. is shown in Tables 14 – 16, and any isolates classified as MDR have been highlighted.

Table 14. Clinical antimicrobial resistance profiles of *Enterococcus faecalis* isolates (n=41)

No. of Resistances	Resistance	No. of isolates	% of total
0	nil	13	31.7
1	bla	1	2.4
1	mac	6	14.6
1	oxa	1	2.4
1	tet	9	22.0
2	bla_gly	1	2.4
2	bla_tet	5	12.2
2	mac_tet	4	9.8
3	bla_mac_tet	1	2.4

* bla= beta lactams, phe= phenicols, lip= lipopeptides, mac= macrolides, ami= aminoglycosides, oxa= oxazolidinones, str= streptogramins, gly= glycopeptides, tet= tetracyclines

Table 15. Clinical antimicrobial resistance profiles of *Enterococcus faecium* isolates (n=77)

No. of Resistances	Resistance	No. of isolates	% of total
0	nil	17	22.1
1	bla	5	6.5
1	mac	1	1.3
1	str	5	6.5
1	tet	2	2.6
2	ami_tet	1	1.3
2	bla_mac	1	1.3
2	bla_str	3	3.9
2	bla_tet	6	7.8
2	mac_str	11	14.3
2	mac_tet	1	1.3
2	str_tet	6	7.8
3	bla_mac_str	2	2.6
3	bla_mac_tet	1	1.3
3	bla_str_tet	2	2.6
3	mac_str_tet	9	11.7
4	ami_mac_oxa_str	1	1.3
4	bla_mac_str_tet	3	3.9

* bla= beta lactams, phe= phenicols, lip= lipopeptides, mac= macrolides, ami= aminoglycosides, oxa= oxazolidinones, str= streptogramins, gly= glycopeptides, tet= tetracyclines

Table 16. Clinical antimicrobial resistance profiles of other *Enterococcus* spp isolates (n=87; including *E.hirae, E.durans, E.gallinarum*)

No. of Resistances	Resistance	No. of isolates	% of total
0	nil	21	24.1
1	mac	4	4.6
1	str	13	14.9
1	tet	4	4.6
2	bla_str	1	1.1
2	mac_str	7	8.0
2	mac_tet	2	2.3
2	str_tet	18	20.7
3	bla_mac_str	2	2.3
3	bla_mac_tet	1	1.1
3	mac_str_tet	12	13.8
4	bla_mac_str_tet	2	2.3

* bla= beta lactams, phe= phenicols, lip= lipopeptides, mac= macrolides, ami= aminoglycosides, lin= lincosamide, oxa= oxazolidinones, str= streptogramins, gly= glycopeptides, tet= tetracyclines

E. coli

For commensal *E. coli*, a total of 19 resistance profiles were identified among the 206 isolates. Profiles list the antimicrobial classes for which resistance was detected at the clinical level. There were nine antimicrobial classes represented by the 13 antimicrobials evaluated. Among the *E. coli* isolates only 5.8% of isolates were classified as MDR. The most common multi-drug resistance profile was bla/fpi/tet. The MDR profiles for commensal *E.coli* spp. is shown in Table 17, and any isolates classified as MDR have been highlighted.

Table 17. Clinical antimicrobial resistance profiles of *Escherichia coli* isolates (n=206)

No. of Resistances	Resistance	No. of isolates	% total
0	nil	130	63.1
1	ami	1	0.5
1	bla	9	4.4
1	fpi	3	1.5
1	phe	17	8.3
1	tet	12	5.8
2	ami_tet	3	1.5
2	bla_phe	3	1.5
2	bla_tet	6	2.9
2	fpi_phe	1	0.5
2	fpi_tet	4	1.9
2	phe_tet	5	2.4
3	ami_bla_fpi	1	0.5
3	ami_bla_tet	1	0.5
3	ami_phe_tet	1	0.5
3	bla_fpi_tet	5	2.4
4	ami_bla_fpi_phe	1	0.5
4	ami_bla_fpi_tet	2	1.0
4	bla_fpi_phe_tet	1	0.5

* ami= aminoglycosides, bla= beta lactams, phe= phenicols, fpi= folate pathway inhibitors, tet=tetracycline

Salmonella

Among the 53 *Salmonella* spp isolates a total of five resistance profiles were identified (Table 18). There were nine drug classes represented by the 13 antimicrobials evaluated. No MDR phenotype was detected among the *Salmonella* isolates.

Table 18. Clinical antimicrobial resistance profiles of Salmonella spp. isolates (n=53)

No. of Resistances	Resistance	No. of isolates	% of total
0	nil	49	92.5
1	fpi	1	1.9
1	phe	1	1.9
2	ami_bla	1	1.9
2	bla_phe	1	1.9

* ami= aminoglycosides, bla= beta lactams, phe= phenicols, fpi= folate pathway inhibitors

Campylobacter

A total of six unique resistance profiles were identified among the 204 *Campylobacter* isolates. There were seven drug classes represented by the nine antimicrobials evaluated. Single class resistance and wild-type profiles made up 97.1% of all isolates. Only four isolates of *C. coli* and one isolate of *C. jejuni* were classified as MDR phenotype. The only multidrug resistant profile for *C. jejuni* was ket/lin/mac/tet and the only MDR profile for *C. coli* was the same except without the tetracycline resistance. The MDR profiles for *Campylobacters* spp. is shown in Tables 19 and 20, and any isolates classified as MDR have been highlighted.

Table 19. Clinical antimicrobial resistance profiles of *Campylobacter jejuni* isolates (n=108)

No. of Resistances	Resistance	No. of isolates	% of total
0	nil	68	63.0
1	qui	16	14.8
1	tet	23	21.3
4	ket_lin_mac_tet	1	0.9

* mac= macrolides, qui= quinolones, lin= lincosamide, phe= phenicols, ami= aminoglycosides, ket= ketolide, tet= Tetracycline

Table 20. Clinical antimicrobial resistance profiles of *Campylobacter coli* isolates (n=96)

No. of Resistances	Resistance	No. of isolates	% of total
0	nil	83	86.5
1	qui	5	5.2
1	tet	3	3.1
2	lin_mac	1	1.0
3	ket_lin_mac	4	4.2

* mac= macrolides, qui= quinolones, lin= lincosamide, phe= phenicols, ami= aminoglycosides, ket= ketolide, tet= tetracycline

Genetic analysis of non-susceptible isolates

The results from the study revealed the presence of elevated resistance to critically important antimicrobials in a small subset of isolates when compared to wildtype cut-off values. These results may not necessarily reflect the presence of resistance genes but instead a natural variation in tolerance towards these antimicrobials. One such example was an elevated MIC for ciprofloxacin for two commensal *E. coli* isolates. Another example was elevated non-susceptibility (83.1%) of *E. faecium* to quinupristin-dalfopristin despite only 22.6% non-wild type phenotype for virginiamycin which belongs to the same class. Similarly, a high proportion of lincomycin resistance to *Enterococcus* was also unexpected.

Break-point genomic characterization of the *Enterococcus* isolates was performed to identify if the elevated prevalence of resistance to quinupristin-dalfopristin and lincomycin was an artefact arising from application of an inappropriate break-point.

Another unexpected finding was the detection of ciprofloxacin resistance among *Campylobacter coli* (14.8%) and *C. jejuni* (5.2%). The fluoroquinolone resistance was the only resistance identified on those isolates, suggesting they are likely to be evolved from a situation where fluoroquinolone were used as a first-line therapy. Given the Australian chicken industry does not use the fluoroquinolone class of antimicrobials, this finding required further investigation. The most direct way to investigate this further was via determination of the MLST (multilocus sequence type), AMR genes/ mutations and core genome analysis.

The isolates selected for sequencing (Table 21) included critically important antimicrobial resistant *E. coli* and *Salmonella* and all *Enterococci and Campylobacter.*

Table 21. Isolates selected for genetic analysis

Species	Isolates (n)
Campylobacter	204
Enterococcus	205
E. coli	3
Salmonella	6
Total	418

Enterococcus species

Enterococcus faecalis

There were four prominent sequence types among the 41 sequenced *E. faecalis* isolates, ST314 (n=7), 16 (n=5), 502 (n=4) and 530 (n=4), with a total of 18 known sequence types. Sequence types 314, 16 and 502 have all been isolated from human clinical samples previously, in addition to various animal species (companion and livestock) (18).

E. faecalis is intrinsically resistant to lincosamides and quinupristin-dalfopristin through the *lsa* gene found in 97.6% of the isolates (absent in one isolate potentially due to low sequencing depth over the gene). There were no vancomycin resistant genes found, supporting the phenotypic data (Table 22; Appendix 2).

There were no resistance genes or mutations detected to convey the phenotypic resistance observed against the glycopeptides. For the single isolate that was resistant to linezolid there were no resistance genes or mutations detected. Similarly, there were no resistance genes detected that convey resistance to daptomycin and the penicillins in any *E. faecalis* isolates.

Phenotypic resistance was detected in 26.8% of *E. faecalis* isolates to erythromycin however resistant genes (ermB) were detected in 58.6% of isolates. Likewise, 46.3% of *E. faecium* isolates were resistant to tetracycline however 78% of *E. faecalis* isolates carried tetracycline resistance genes.

Disagreement between phenotypic and genetic classifications of resistance can be accounted for three reasons. Firstly, there is the possibility of measurement error in assessing either the MIC or occurrence of known resistance genes. Secondly there is the possibility that breakpoints for the interpretation of phenotypic data are inappropriate for the organism under assessment. Thirdly, it is possible that isolates possess resistance mechanisms for which the DNA sequence is yet to be discovered.

Enterococcus faecium

All 77 *E. faecium* isolates were successfully sequenced. Of these, 45 belonged to 18 different, known sequence types with the most common being ST492, ST195 and ST241 (Table 23; Appendix 2). ST492 has been reported in pigs with ST195 and 241 reported in poultry (18).

There were no genes detected that confer vancomycin resistance which reflects the lack of phenotypic expression of resistance. Clinical resistance to quinupristin-dalfopristin was identified in 54.5% of *E. faecium* isolates however, genotypically only 37.7% isolates carried resistance to the combination, and of these, 85.7% of isolates carrying genes for resistance to quinupristin (*ermA, ermB or msrC)* and 37.7% for dalfopristin (*vatE)* (Table 24; Appendix 2).

Resistance genes to lincosamides (*ermA, ermB, lnuB, lnuA, lsaA*) were detected in 59.7% of *E. faecium* isolates (Table 23; Appendix 2), for which there were no phenotypic or clinical breakpoints, but this could also represent inherent resistance.

For the single isolate that was resistant to linezolid there were no resistance genes or mutations detected. Similarly, there were no resistance genes detected that convey resistance to daptomycin and the penicillins

in any *E. faecium* isolates, however, the observed phenotypic and clinical resistance is suspected to be due to the presence of single nucleotide polymorphisms that adjust the efficacy of penicillin-binding proteins (19).

Phenotypic resistance was detected in 39% of *E. faecium* isolates to erythromycin, however, resistant genes (ermA, ermB, msrC) were detected in 85.7% of isolates. Likewise, 40.3% of *E. faecium* isolates were resistant to tetracycline however 61% of *E. faecium* isolates carried tetracycline resistance genes.

Explanations for differences between phenotypic and genetic classifications are discussed earlier.

Enterococcus durans

All *E. durans* (n=61) isolates were sequenced successfully. Lincosamide resistant genes *lnuA, lnuB* or *ermB* were identified in 82.0% of isolates (Table 25; Appendix 2), for which there were no phenotypic or clinical breakpoints, but this could also represent inherent resistance.

There were a high percentage of isolates with resistance genes to both streptogramin A and B, streptogramin A only, or streptogramin B only: 57.4%, 57.4% and 80.3% respectively (Table 26; Appendix 2). Despite this high percentage of streptogramin resistance, no vancomycin resistance genes were identified (Table 25; Appendix 2).

Enterococcus hirae

Twenty-five *E. hirae* isolates were sequenced. Lincosamide resistance genes were detected in 68.0% of isolates for which there were no phenotypic or clinical breakpoints, but this could also represent inherent resistance. Quinupristin-dalfopristin resistance genes were detected in 12.0% of isolates and no isolates carried vancomycin resistant genes (Table 27 and Table 28; Appendix 2).

Enterococcus gallinarum

The single *E. gallinarum* isolate was identified to contain vancomycin resistance associated genes (*vanC, vanS, vanR, vanT, van XY*) with an additional tetracycline resistance gene (*tet M*). Note *E. gallinarum* are intrinsically resistant to vancomycin at concentrations typically lower than or equal to 32 mg/mL and carry *vanC.*

Escherichia coli

Two of the commensal *E. coli* isolates were selected for whole genome sequencing based on elevated MICs and subsequent classification as microbiologically resistant to ciprofloxacin. These isolates, GBC3.1 (ST752) and GHD4.1 (ST4980), showed a slightly elevated MIC value towards ciprofloxacin (0.25 and 0.13 mg/L respectively). These two strains were identified as having a single point mutation in the QRDR of GyrA (Ser-83-Leu or Asp-87-Gly), shown to be associated with low level resistance as outlined above (Table 29; Appendix 2).

The remaining *E. coli* isolate BAH8.1 (MLST 38) was selected for genomic analysis based on slightly elevated MIC (16mg/L) to cefoxitin however no cefoxitin resistance genes were identified. Certain point mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase A subunit (GyrA) result in amino acid changes reducing the susceptibility of commensal *E.coli* to fluoroquinolones. The main two mutations in *E. coli* identified being point mutations resulting in amino acid changes of Ser-83-Leu and Asp-87-Gly (20, 21).

Other mutations in DNA gyrase subunit B (GyrB), Topoisomerase IV A subunit (ParC) and Topoisomerase IV B subunit (ParE) have been associated with reduced susceptibility with a single mutation in GyrA resulting in low-level resistant isolates compared to high level resistant isolates being associated with a combination of mutations (22). No QRDR mutations were detected in the BAH8.1 isolate so the potential mechanism for low-level fluoroquinolone resistance is unclear. Of the three *E. coli* sequence types present, ST38 is a global pathogenic strain commonly infecting humans and poultry and has been reported to produce extended spectrum beta lactamases (23). ST75 has been isolated globally from humans, animals and the environment with ST4980 only being isolated from poultry in Denmark and the Netherlands (23).

Both BAH8.1 and GHD4.1 also contained beta lactamase resistance genes.

Salmonella species

All six salmonella isolates that had elevated MICs for cefoxitin (16 mg/L) were subjected to whole genome sequencing. All sequenced belonged to the same sequence type, ST2116 (*S. Sofia*). There were no AMR genes detected among these isolates (Table 30; Appendix 2). Resistance without the presence of genes and vice versa suggests either that the breakpoints were inappropriate, there exists previously uncharacterised resistance mechanisms or both.

Campylobacter species

Campylobacter jejuni

Successful sequencing was achieved for 203 of the *Campylobacter* isolates, of which the 107 of the *C. jejuni* sequenced belonged to 32 known sequence types with the most prominent being ST7323 (n=9), 2083 (n=8), 535 (n=7) and 4896 (n=7) (Table 31; Appendix 2). All these sequence types have been found in humans and ST2083 and ST535 have also been found in poultry with ST7323 and ST535 previously reported in Australia (24).

Phenotypically 14.8% of *C. jejuni* isolates demonstrated resistance to fluoroquinolones and genetic analysis indicated that 16.6% possessed the mutation in the DNA gyrase A subunit (Thr (86) –Ile). This mutation has been reported to be associated with fluoroquinolone resistant *C. jejuni*. Two fluoroquinolone susceptible isolates also carried this mutation but when the MIC were repeated they were reclassified resistant, which is a common scenario for a number of reasons including multiple clones in a single freeze-down. The fluoroquinolone resistant *C. jejuni* belonged to sequence types ST732 (n=9), ST 2083 (n=8) and ST2343 (n=1). These sequence types have all previously been isolated from chickens in the United Kingdom (ST732), USA (ST2083) and New Zealand (ST2343). ST2083 and ST2343 have also been isolated from humans in Europe, America and Asia and in the United Kingdom and New Zealand respectively (24).

The single *C. jejuni* isolate that was resistant to macrolides and lincosamides did not contain the mutations that confer these resistances.

Campylobacter coli

From all 96 *C. coli* isolates, one was a mixed *C. jejuni* / *C. coli* culture and was subsequently not included in the analysis. Of the 95 remaining *C. coli* isolates, the predominant sequence types present were ST1181 (n=17), ST3985 (n=8), ST832 (n=8) and ST825 (n=7) with a further 11 known sequence types (Table 32; Appendix 2). The four main sequence types have all been isolated from humans and livestock previously. ST825, 1181 and 3985 have been isolated from Australian livestock and ST3985 isolated in an Australian human case. ST832 has not been reported in Australia previously. ST825, 832 and 1181 have been reported to cause gastroenteritis in humans (24).

Fluoroquinolone resistance was less common in *C. coli* compared to *C. jejuni* with only five (5.2%) isolates resistant. ST860 was the only sequence type identified among the fluoroquinolone resistant *C. coli* and this sequence type has been previously reported in chickens and humans from the United Kingdom and Germany (24). Only two of these five isolates had the point mutation in the DNA gyrase subunit associated with fluoroquinolone resistance. One of the resistant isolates without the mutation had no coverage across the GyrA gene. The basis for the resistance for the remaining two isolates is unknown.

All five *C. coli* isolates that demonstrated resistance to macrolides were also resistant to lincosamides and all five carried the point mutation A2075G in 23S rRNA known to confer a high-level of resistance towards macrolides with cross-resistance to lincosmides.

This study was undertaken to estimate the prevalence of resistance against specified antimicrobials amongst *Enterococcus* spp. (204 isolates), *E. coli* (206 isolates), *Salmonella* spp. (53 isolates), and *Campylobacter* spp. (204 isolates) isolated from the gut of Australian meat chickens at slaughter, from processing plants that produce >90% of Australian chicken meat.

The project design was to account as much as possible for the variation in antimicrobial resistance present in the population of commercially-raised meat chickens in an efficient and practical way that could be replicated into the future. This approach aimed to achieve economies of scale, to maximize the number of isolates evaluated and hence the accuracy of findings, and to maximise comparability with data from the medical sector, other industries and internationally.

Materials and methods

A trial was undertaken of the collection and isolation protocols prior to undertaking the study itself to allow for issues to be resolved prior to rolling the protocols out to all the participating processing plants. There were several issues with the collection protocol that had to be resolved, including determining the appropriate buffer material and modifying the protocol to ensure timing requirements were met so that samples were received within 24hrs of collection. These changes are reflected in the final protocol outlined in the methodology section. Additionally, and important to note for future studies, issues with shipping delays were experienced from the first week in November due to the high volume of packages being sent in the lead up to Christmas. All isolates were sent using overnight courier services, but even when the packages were sent priority guaranteed overnight delivery they were still not able to be received in time. Ultimately some samples had to be delivered by the companies affected directly to Birling labs to meet the required timeline.

The isolation protocols selected for the isolation of *Salmonella* and *Campylobacter* were the respective Australian Standards methods with minor validated modifications. These modifications were predominately in the speciations and/or confirmation of the isolate. An automated mass spectrometer Vitak 2 was used in the place of biochemical tests. As *Enterococcus* spp. and *E. coli* populations within the caeca are very high, direct plating onto specific selective and differential plates were used. The plates (ColiID and BEA) were selected in this instance due to their ease of interpretation.

It must be noted that the die-off period for *Campylobacter* is rapid and, in this study, it was found that *Campylobacter* was able to be isolated from direct culture from fresh (<24hrs) caecal samples. Because, enrichment is required for samples older than >24hrs these samples were discarded. This highlights the importance of maintaining the critical shipping period of <24hrs for future studies to ensure the enrichment step (and bias that this creates) is not required for *Campylobacter* isolation. Long term preservation of *Campylobacter* at -80°C is a challenge since most commercially available suspension fluids do not yield high resuscitation rates. The primary laboratory has developed a proprietal suspension fluid that protects Campylobacter from the freeze/thaw cycle during storage and the efficacy of this in preserving the *Campylobacter* was evidenced by the ability to recover the isolates at the AMR laboratories, and the 100% match in typing results between the primary and AMR laboratories. The design of Sensititre plates including drug choices for inclusion and concentrations have already been discussed amongst leading Australian experts in AMR testing. These are the same as used in the recent work performed in the cattle

and pork industries. Interpretation of resistance was performed with reference to break points published by CLSI and EUCAST after cross-checking with Australian experts on this topic.

Results

The methodology used in this study to determine the phenotypic and genotypic characteristics of the isolates is not that we have is not infallible however they are as close to the 'gold standard' as is currently available. Reporting of the results is in-line with recommendations in OIE chapter 6.7 which states that "*For surveillance purposes, use of the microbiological breakpoint (also referred to as epidemiological cut-off point), which is based on the distribution of MICs or inhibition zone diameters of the specific bacterial species tested, is preferred*.". The clinical resistance results are reported but the focus of the reporting is on microbiological results, with these results supported by genetic analysis where possible.

Given the known potential for discordancy between phenotypic and genotypic results, discrepancies should be expected, particularly in a study of this size involving 668 isolates. The outcome for genetic tests relies on several factors including the robustness of the sequencing and knowledge of the resistance gene profiles, which are still being determined for a number of combinations of antibiotics and bacteria. There is inherent variability in the MIC assay system, like all other laboratory assay systems. With large numbers of isolates being evaluated it is expected that some that are truly susceptible will exceed the breakpoint but will not be accompanied by a positive genotype result. Further, some of the genes detected may not have been functional reflecting the reduced percentage that were phenotypically resistant, however the presence of these genes does raise the possibility of those bacterial clones contributing to the total pool of resistance genes. Accurate interpretation of the MIC data also relies on the appropriateness of the breakpoints. When breakpoints are not appropriate it is expected that some misclassifications will occur.

In a small number of instances there was repeat testing of the MIC for bacteria after gene analysis to confirm the original results. These repeat results were not included in this report to avoid bias induced by selective inclusion of findings.

No direct comparison between rates of resistance in chicken commensal bacteria and related pathogens obtained from human clinical cases has been made. Such comparisons are not appropriate due to inherent differences in the context of sampling and bacterial characteristics of isolates from healthy chickens and septic human patients.

Enterococcus spp.

Two of the 205 *Enterococcus* isolates were resistant to vancomycin - a single *E.gallinarum* which is intrinsically resistant and one *E. faecalis* isolate which was microbiologically resistant to vancomycin with an MIC of 8mg/L, however this isolate did not carry any vancomycin resistance genes (*van* genes) strongly suggesting this was a false positive MIC result. Resistance and presence of resistance genes to the first line antimicrobial tetracycline was common among *Enterococcus spp.* reflecting historical use in the chicken industry. Elevated frequency of quinupristin–dalfopristin (54.5%) resistance among *E. faecium* is likely a consequence of past virginiamycin use, while the resistance in *E. faecalis* is acknowledged as intrinsic. Quinupristin- dalfopristin resistance may require further evaluation as isolates with MIC ≥16mg/L for

quinupristin–dalfopristin did not carry the *vatE* gene. This may be due to carriage of unidentified resistance mechanisms/resistance genes, an inappropriate breakpoint used for this antimicrobial complex, or both.

The ampicillin resistance in *E. Faecium* and *E. Faecalis* detected weren't supported by the presence of known resistance genes, however, the observed phenotypic and clinical resistance is suspected to be due to the presence of single nucleotide polymorphisms that adjust the efficacy of penicillin-binding proteins (19) and requires further investigation.

Despite differences in the methodology of this study and the pilot surveillance study of 2004, it appears there has been a substantial reduction in phenotypic resistance to erythromycin in *Enterococcus* isolates from Australian meat chickens (4). This could reflect the reduction in use of macrolides in the industry since the introduction of the *Mycoplasma* vaccines in the 1990s.

One *E. faecium* and one *E. faecalis* isolate demonstrated clinical resistance to linezolid at MIC >16 mg/L. Further investigation revealed that the *cfr gene* was likely not present in these isolates. However, linezolid resistance can be co-selected by the use of chloramphenicol or florfenicol by the acquisition of the *cfr* gene (12), although neither of these drugs are used in the chicken industry.

Among the enterococci isolates, 17.5% were classified as MDR, however the majority of resistance was to antimicrobial classes rated as of "low importance" by ASTAG and registered for use in meat chickens. These include beta-lactams, macrolides, and tetracyclines with an exception being streptogramins.

E. coli

The microbiological resistance among commensal *E. coli* isolates demonstrated that 47% were susceptible to all tested antimicrobials and only 5.8% of isolates were classified as MDR. Where breakpoints were available, none of the isolates demonstrated microbiological resistance to ceftiofur, chloramphenicol, florfenicol, colistin or gentamicin. Two isolates demonstrated microbiological resistance to ciprofloxacin at low MICs (0.13 and 0.25 mg/L) near the breakpoint. Quinolones have never been registered for use in food-producing animals in Australia and whole genome sequencing revealed that these two isolates carried a single point mutation in the QRDR of GyrA (Ser-83-Leu or Asp-87-Gly), shown to be associated with low level fluoroquinolone resistance. ST75 has been isolated globally from humans, animals and the environment with ST4980 only being isolated from poultry in Denmark and the Netherlands (23).

Interpretation of the data in this report is aided by comparison to the Australian DAFF study performed in 2004 (4) (Table 33). It should be noted that there are inevitably differences in the collection and testing methodologies used in different studies and that only general comparisons are possible. Data from each report were re-analysed using the microbiological breakpoint used in this study. The absence of ceftiofur resistance among *E. coli* isolated from Australian meat chickens is noteworthy in both 2017 and 2004. Resistance to tetracycline in 2017 (19.4%) was relatively lower compared to the 2004 Australian survey (44.3%). Similarly, ampicillin resistance in this survey among *E. coli* was comparatively lower (14.1%) compared to the previous Australian survey in 2004 (33.1%). Resistance to trimethoprim/sulfamethoxazole was also comparatively lower compared to the 2004 study.

Table 33. Antimicrobial (microbiological) resistance in commensal *E. coli* isolates from meat chickens from Australian surveys.*

	Frequency (%)	
Antimicrobial	Australia 2016 [a] n=206	Australia 2004 [b] n=269
Ampicillin	14.1	33.1
Cefoxitin	0.5	-
Ceftiofur	0	0
Chloramphenicol	0	1.8
Ciprofloxacin	1	2.9
Florfenicol	0	3.4
Gentamicin	0	0
Streptomycin	9.7	-
Tetracycline	19.4	44.3
Trimethoprim/ Sulfamethoxazole	8.7	27.9

*Note: due to the differences in collection method and testing methodologies the figures listed are for the purpose of general comparison only.

[a] Australia, 2016 – Current report

[b] Australia, 2004 – Department of Agriculture Fisheries and Forestry (4)

Salmonella spp.

The recovery of *Salmonella* spp from pooled caeca (five caeca = one sample) obtained from meat chicken samples was 26.5% (53/200) with 92.5% demonstrating susceptibility to all antimicrobials tested. Overall, meat chicken *Salmonella* isolates demonstrated susceptibility to the majority of the antimicrobials tested and no MDR isolates were identified. None of the *Salmonella* were microbiologically resistant to ceftiofur, ciprofloxacin, chloramphenicol, florfenicol, colistin, gentamicin or tetracycline. Resistance was only detected at low frequency to ampicillin, streptomycin and trimethoprim. None of the six isolates that were microbiologically resistant to cefoxitin carried any beta lactam genes required for cefoxitin resistance which suggests an issue to do with inappropriate breakpoints, false positive measurement, or existence of previously uncharacterised resistance mechanisms. Recent NARMS data have demonstrated 8.3% of ceftiofur resistance among *Salmonella* spp. isolated from meat chickens from the USA (3).

Campylobacter spp.

No resistance was detected to any of the antibiotics tested in 63% of *C. jejuni* isolates and 86.5% *C. coli* isolates. Among the *Campylobacter* isolates, a low level of MDR phenotype was identified among *C. coli* (four isolates) and *C. jejuni* (one isolate). All *Campylobacter* isolates tested were microbiologically susceptibile to florfenicol and gentamicin. Only 0.9% (1/108) of *C. jejuni* and 5.2% (5/96) of *C. coli* were resistant to macrolides (erythromycin and azithromycin), one of the key antimicrobials used for treating human campylobacteriosis. The overall frequency of erythromycin resistance among *Campylobacter* spp. in the 2004 survey was 19.9% (4). However, in the 2004 survey speciation of *Campylobacte*r was not performed. Despite the lack of speciation, the current survey showed reduction in the carriage of macrolide resistance among *C. jejuni* and *C. coli*.

Resistance to tetracycline (22.2% *C. jejuni*; 3.1% *C. coli*), nalidixic acid (14.8% *C. jejuni*; 5.2% *C. coli*) or ciprofloxacin (14.8% *C. jejuni*; 5.2% *C. coli*) were the most commonly detected forms of resistance. The observed resistance to ciprofloxacin is unexpected since fluoroquinolones are not used in Australian livestock. In addition, ciprofloxacin resistant isolates were susceptible to all other tested antimicrobials with the exception of nalidixic acid suggesting they are likely to be evolved from a situation where fluoroquinolone were used as a first-line therapy. Recent reports from New Zealand (which also doesn't use fluoroquinolones in livestock) demonstrated that fluoroquinolone resistance in poultry was attributed to the emergence of a new clone of *C. jejuni* (ST 6964) that was resistant to both ciprofloxacin and tetracycline (16). The levels of resistance to fluoroquinolones is similar to that detected in meat chickens in other countries that also don't use fluoroquinolones (17).

A single point mutation (Thr-86-Ile) in the GyrA gene results in amino acid mutation that confers fluoroquinolone resistance in *Campylobacter* spp. (25) and whole genome sequence analysis demonstrated that all phenotypically resistant isolates possessed this mutation. The fluoroquinolone resistant *C. jejuni* belonged to the ST732 (n=9), ST 2083 (n=8) and ST2343 (n=1) sequence types which have all been previously isolated from chickens (ST732 in the United Kingdom, ST2083 in USA and ST2343 in New Zealand).

In the absence of fluoroquinolone use in the Australian chicken industry, the fluoroquinolone resistant isolates are unlikely to have evolved as a result of local selection pressure. It is likely that these isolates may have been introduced by anthropozoonosis i.e. human-chicken transmission. However, further longitudinal and genomic studies are required to fully validate this hypothesis as there may be 'bridge' species (such as wild birds or rodents) that transfer resistant bacteria directly to the chickens or to chickens via humans. Regardless, the National Biosecurity Manual for Chicken Growers is being updated to include transfer of AMR bacteria to chickens as a risk to be managed.

General conclusion

Overall, resistance to antimicrobials that are of critical importance to human health is considerably low in commensal bacteria from Australian meat chickens. These results of this study highlight the efficacy of the chicken industry's past and current antimicrobial stewardship efforts and identify further areas for investigation and improvement.

APPENDIX 1 SAMPLE COLLECTION FORM

SPECIMEN SUBMISSION FORM – ACMF AMR Survey

LAB NUMBER: [ ]

DATE RECEIVED: ______/______/____________

Sample code (*company-plant-farm-sampling number.container*): ______________________

SUBMITTER'S DETAILS

SUBMITTER: ____________________________ **SUBMITTER'S SIGNATURE:** ____________________

PHONE: ________________ **FAX:** ________________ **MOBILE:** ______________________

EMAIL ADDRESS: ________________________________

PLEASE CHARGE: **Australian Chicken Meat Federation**

COMPANY FLOCK CODE: ____________________

DATE COLLECTED: ____ / ____ / ____ **TIME COLLECTED** :________ AM / PM

DATE SUBMITTED: ____ / ____ / ____

FLOCK AGE (days): ________ **FREE-RANGE (y / n):** ________

REASON SUBMITTED: **AMR study**

TESTS REQUIRED: Campylobactor, Enterococcus, E.Coli and Salmonella isolation; speciation of Enterococcus and Campylobacter (mass spec).

SAMPLE TYPE:

________________Caeca____

RESULTS TO GO TO:

NAME	EMAIL / PHONE
Kylie Hewson	Kylie.hewson@chicken.org.au / (02 9929 4077)

LAB USE ONLY	Time Received:		Submission entered by:		Results Entered:	

AMR study form 12/04/2016

BIRLING AVIAN LABORATORIES
ABN 96 002 925 948
975 THE NORTHERN RD,
BRINGELLY, N.S.W, 2556
P.O. BOX 111 BRINGELLY, N.S.W 2556
PH: +61 2 4774 6100

APPENDIX 2 TABLES 22 – 32

Table 22. MLST and resistance profile of *Enterococcus faecalis* isolates (n=41)

MLST	Number of isolates	Resistance profile
16	1	*lsaA*
	3	*lsaA, tetO*
	1	*lsaA, tetO, tetM*
22	1	*ermB, lsaA, tetM*
59	1	*ermB, lsaA, tetL, tetM*
82	1	*lsaA, tetM*
100	1	*lsaA*
136	1	*lsaA*
202	1	*ermB, lsaA*
	1	*ermB, lsaA, tetL, tetM*
249	1	*lsaA, tetL, tetM*
287	1	*aadE, ermB, lsaA, tetO*
314	3	*lsaA, tetO*
	2	*ermB, lsaA, tetO*
	1	*ermB, lnuB, lsaA, tetL, tetM*
	1	*aadE, ermB, InuA, lsaA, tetM, tetO*
403	1	*aadE, ermB, lsaA, tetO*
444	1	*ant6-Ia, ermB, lsaA*
	1	*dfrG, ermB, lsaA*
477	1	*lsaA, tetO*
502	4	*lsaA, tetO*
530	4	*ermB, lsaA, tetL, tetM*
616	1	*ermB, lsaA*
634	1	*ermB, lsaA, tetL, tetM*
835	2	*ermB, lsaA*
-	2	*ermB, lsaA, tetL, tetM*
-	1	*dfrG, ermB, tetM, vatE*
-	1	*ant6-la, ermB, lnuB, lsaA, tetO*

- Sequence type not found

Table 23. MLST and resistance profile of *Enterococcus faecium* isolates (n=77)

MLST	Number of isolates	Resistance profile
8	1	*ermB, lnuB, msrC, vatE*
	1	*lnuB, msrC, tetL, tetM*
10	1	*None present*
	1	*tetL, tetM,*
	1	*aadE, ermB, lnuB, msrC, tetL, tetM, tetU, vatE*
124	1	*msrC, tetM*
	1	*lnuB, msrC, tetM*
	1	*aadE, erB, msrC, tetL, tetM*
	1	*ermB, lnuB, msrC, tetM, vatE*
158	1	*ermB, vatE*
190	1	*ermB, msrC, tetU, vatE*
194	1	*msrC, tetM*
195	2	*None present*
	1	*tetU, vatE*
	1	*lnuB, tetL, tetM,*
	1	*ermB, lnuB, tetL, tetM*
236	1	*ermA, spc*
	1	*aadE, ermA, ermB, lnuB, msrC, spc, vatE*
240	1	*msrC, tetM, tetS*
241	1	*None present*
	1	*lnuB*
	1	*msrC*
	1	*msrC, tetU*
	1	*ermB, lnuB, vatE*
245	1	*msrC*
492	2	*msrC*
	3	*msrC, tetU*
	1	*ermB, msrC, vatE*
	1	*lsaA, msrC, tetU*
507	2	*msrC,*
	1	*ermB, tetM*
511	1	*msrC, tetU*
517	1	*tetU*
	1	*lnuB, msrC, tetM*
	1	*ermB, lnuB, tetM, vatE*
640	2	*msrC, tetM*
	1	*ermB, msrC, tetL, tetM, vatE*
944	1	*dfrG, msrC*
1243	1	*msrC, tetM,*
-	2	*None present*
-	1	*msrC*
-	1	*ermB, vatE*
-	4	*lnuB, msrC*
-	1	*msrC, tetU*
-	1	*msrC, vatE*

Table 23 Cont. MLST and resistance profile of *Enterococcus faecium* isolates (n=77)

MLST	Number of isolates	Resistance profile
-	4	*ermB, msrC, vatE*
-	1	*ermB, lnuB, tetM*
-	2	*ermB, tetU, vatE*
-	1	*lnuB, msrC, tetM*
-	1	*msrC, tetL, tetM*
-	1	*ermB, msrC, tetL, tetM*
-	1	*ermB, msrC, tetM, vatE*
-	6	*ermB, msrC, tetU, vatE*
-	1	*aadE, ermA, lnuB, spc, tetL*
-	1	*aadE, ermB, lnuB, msrC, tetM, vatE*
-	1	*aadE, ermB, lnuB, tetL, tetM, vatE*
-	1	*ermA, ermB, lnuB, spc, tetL, tetM*
-	1	*ermA, ermB, msrC, spc, tetM, vatE*
-	1	*dfrG, ermB, lnuA, msrC, tetL, tetM, vatE*

- Sequence type not found

Table 24. Quinupristin-dalfopristin resistant genes detected and corresponding broth dilution result of *Enterococcus faecium* isolates (n=77).

Number of isolates	QD resistance genes	QD MIC result (mg/L)
1	-	<= 0.5
9	*msrC*	<= 0.5
1	*msrC, vatE*	<= 0.5
2	*msrC*	1
5	-	2
1	*ermA*	2
1	*ermB*	2
10	*msrC*	2
1	*ermB, msrC*	2
1	*ermB, vatE*	2
2	*ermB, msrC, vatE*	2
1	-	4
1	*ermA*	4
1	*ermB*	4
2	*msrC*	4
1	*ermA, ermB*	4
1	*ermB, msrC, vatE*	4
3	*msrC*	8
1	*vatE*	8
1	*ermB, vatE*	8
9	*ermB, msrC, vatE*	8
2	-	16
1	*ermB*	16
4	*msrC*	16
1	*ermB, msrC*	16
4	*ermB, vatE*	16
6	*ermB, msrC, vatE*	16
2	*ermA, ermB, msrC, vatE*	16
1	-	>32
1	*ermB, msrC, vatE*	>32

- Not present

Table 25. Resistance profile of *Enterococcus durans* isolates (n=61)

Number of isolates	Resistance profile
3	*None present*
1	*aadE*
2	*ermB*
1	*ermT*
2	*tetM*
1	*dfrG, tetM*
3	*ermB, tetM*
1	*ermB, tetU*
11	*ermB, vatE*
3	*tetL, tetM*
1	*dfrG, ermB, ermT*
1	*dfrG, ermB, lnuB*
1	*dfrG, ermB, tetM*
2	*dfrG, ermB, vatE*
1	*dfrG, tetM, tetS*
1	*ermB, ermT, vatE*
2	*ermB, tetM, vatE*
1	*lnuB, tetM, tetS*
1	*aadE, ermB, lnuB, tetS*
1	*dfrG, ermB, ermT, vatE*
4	*dfrG, ermB, tetM, vatE*
7	*dfrG, ermB, tetS, vatE*
1	*ermB, ermT, tetS, vatE*
1	*ermB, lnuA, tetM, vatE*
1	*ermB, lnuB, tetL, tetM*
1	*ermB, lsaA, tetM, tetS*
2	*ermB, tetM, tetS, vatE*
1	*aadE, ermB, lnuB, tetL, tetM*
2	*dfrG, ermB, tetM, tetS, vatE*
1	*ermB, ermT, tetM, tetS, vatE*

Table 26. Quinupristin-dalfopristin resistant genes detected and corresponding broth dilution result of *Enterococcus durans* isolates (n=61).

Number of isolates	QD resistance genes	QD MIC result (mg/L)
1	*ermT*	<= 0.5
9	-	2
9	*ermB*	2
3	*ermB, vatE*	2
1	*ermB, ermT*	4
7	*ermB, vatE*	4
1	-	8
2	*ermB*	8
9	*ermB, vatE*	8
1	-	16
1	*ermB*	16
6	*ermB, vatE*	16
3	*ermB, ermT, vatE*	16
1	*ermB*	>32
6	*ermB, vatE*	>32
1	*ermB, ermT, vatE*	>32

- Not present

Table 27. Resistance profile of *Enterococcus hirae* isolates (n=25)

Number of isolates	Resistance profile
4	*None present*
11	*lnuB*
3	*tetM*
1	*dfrG, lnuB*
1	*ermB, vatE*
3	*lnuB, tetU*
2	*ermB, lnuB, vatE*

Table 28. Quinupristin-dalfopristin resistant genes detected and corresponding broth dilution result of *Enterococcus hirae* isolates (n=25).

Number of isolates	QD resistance genes	QD MIC result (mg/L)
1	-	<= 0.5
6	-	1
11	-	2
1	-	4
2	*ermB, vatE*	4
3	-	16
1	*ermB, vatE*	16

- Not present

Table 29. MLST and profile of resistance genes in commensal *E. coli* isolates (n=3)

Isolate ID	MLST	Resistance profile	Ciprofloxacin MIC result (mg/L)	Cefoxitin MIC result (mg/L)	QRDR Mutations	
					QRDR	Amino Acid Substitution
BAH8.1	38	*bla_{TEM-1C}, sul2*	≤0.015	16	ND	ND
GBC3.1	752	*strA, strB*	0.25	≤8mg/L	GyrA ParC	Ser (83) - Leu Glu (475) - Asp
GHD4.1	4980	*Bla_{TEM-1B}, dfrA14, strA, strB, sul2, tetA*	0.12	≤8mg/L	GyrA ParC	Asp (87) - Asn Glu (475) - Asp

- ND Not detected

Table 30. MLST and profile of resistance genes in *Salmonella* isolates (n=6)

Isolate ID	MLST	Resistance profile	Cefoxitin MIC result (mg/L)
DEF6.1	2116	ND	16
DEG7.1	2116	ND	16
DEI9.1	2116	ND	16
GGJ10.1	2116	ND	16
GGM13.1	2116	ND	16
GGN14.1	2116	ND	16

- ND Not detected

Table 31. MLST and resistance profile of *Campylobacter jejuni* isolates (n=107)

Isolates grouped by MLST with the presence of either genotypic or phenotypic resistance are above the line. Gaps represent no presence. S-sensitive; R-resistant. *Phenotype for Fluoroquinolone resistance did not correspond to that of the genotype. QRDR; quinolone resistance-determining region

MLST	Number of isolates	Resistance profile	QRDR mutation	Ciprofloxacin (S/R)
48	3			S
	1	*tetO*		S
50	2			S
	2	*tetO*		S
449	2			S
	1*		yes	S
	1	*tetO*		S
791	1*	*tetO*	yes	S
2083	8		yes	R
2343	1		yes	R
6788	1	*tetO*		S
7323	9		yes	R
-	17	*tetO*		S
42	1			S
45	5			S
46	1			S
161	3			S
190	1			S
233	1			S
257	2			S
354	3			S
525	1			S
528	5			S
535	7			S
567	1			S
583	2			S
996	1			S
3804	1			S
4378	1			S
4896	7			S
6722	3			S
7013	1			S
7208	1			S
7572	3			S
7888	3			S
8470	2			S
8559	1			S
-	1			S

- Sequence type not found

Table 32. MLST and resistance profile of *Campylobacter coli* (n=95)

Isolates grouped by MLST with the presence of either genotypic or phenotypic resistance are above the line. Gaps represent no presence. S-sensitive; R-resistant. *Phenotype for Fluoroquinolone resistance did not correspond to that of the genotype.

MLST	Number of isolates	Resistance profile	QRDR mutation	Ciprofloxacin (S/R)
827	6			S
	1*			R
860	2			S
	2*			R
	2		yes	R
-	33			S
	2	*tetO*		S
832	8			S
825	7			S
1181	17			S
1243	2			S
1764	2			S
2534	1			S
3985	8			S
6755	2			S

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