AUSTRALIAN VETERINARY JOURNAL
AUSTRALIA'S PREMIER VETERINARY SCIENCE TEXT
EDUCATION, ETHICS & WELFARE

ORIGINAL ARTICLE

Antimicrobial-resistant *Salmonella* is detected more frequently in feed milling equipment than in raw feed components or processed animal feed

EM Parker,[a]* M Valcanis,[b] LJ Edwards,[c] P Andersson,[b] DF Mollenkopf[a] and TE Wittum[a]

Food for human and animal consumption can provide a vehicle for the transfer of pathogenic and antimicrobial-resistant bacteria into the food chain. We investigated the antimicrobial susceptibility of 453 *Salmonella* isolates collected from raw feed components, equipment and finished feed from 17 commercial feed mills in Australia between 2012 and 2021. Previous studies have found *Salmonella* prevalence and the diversity of *Salmonella* serotypes are greatest in the raw feed components. We, therefore, hypothesised that we would find a greater proportion of antimicrobial-resistant *Salmonella* isolates in the raw feed components compared to other sample types. We found that of 453 isolates tested, 356 (0.80) were susceptible to all antimicrobials tested, 49 (0.11) were nonsusceptible to streptomycin only and 48 (0.11) were resistant to two or more antimicrobials. Of the 48 antimicrobial-resistant isolates, 44 were found in feed milling equipment, two in raw feed components and two in finished feed. Statistical analysis, using a logistic regression with random effects model, found that the population-adjusted mean probability of detecting antimicrobial-resistant *Salmonella* isolates from feed milling equipment of 0.39, was larger than the probability of detecting resistant isolates in raw feed components 0.01, ($P < 0.001$) and in finished feed, 0.11, ($P = 0.006$). This propensity for antimicrobial-resistant bacteria to colonise feed milling equipment has not been previously reported. Further studies are required to understand the ecology of antimicrobial-resistant *Salmonella* in the feed milling environment.

Keywords animal feed; antibiotics; antimicrobial resistance; feed mill; food safety; public health

Aust Vet J 2022;100:213–219 doi: 10.1111/avj.13146

As modern livestock production systems intensify, the reliance on processed animal feed increases as does the role of the feed mill in maintaining microbial safety in the food supply chain.[1] The global trade in raw feed components and finished feed may contribute to the spread of food-borne pathogens, such as *Salmonella* that infect animals and then humans via the consumption of animal food products.[2] *Salmonella* is listed by the World Health Organisation (WHO) as a major cause of diarrheal disease worldwide. The most common cause of salmonellosis in humans is the consumption of contaminated foods of animal origin such as meat, milk and eggs.[3] The microbial safety of animal feed is critical for the health of both humans and animals.

Salmonellosis in humans and animals may be complicated by *Salmonella* organisms that are resistant to medically important antibiotics.[3] Antimicrobial resistance (AMR), can spread throughout the food chain as both pathogenic and nonpathogenic bacteria carry and share AMR genes.[4] AMR is considered by the WHO as one of the greatest threats to global health, food security and development.[5] Previous studies have found *Salmonella* isolates resistant to medically important antibiotics in raw feed components[6,7] and compound animal feed.[8] In a study of pig feed mills in the United States, whole-genome sequence analysis of *Salmonella* isolated from milling equipment and the feed mill environment found 40% of the isolates carried at least one AMR gene.[9] The importance of feed safety is recognised by the United Nations interagency coordination group on AMR. The interagency coordination group prioritised the importance of ensuring the safety of feed and food production in a sustained One Health response to AMR.[10]

To ensure the safety of feed for food-producing animals, commercial feed mills in Australia are required by law to participate in feed safety accreditation programs.[11] An important aspect of these programs is regular microbial monitoring of the raw feed components, feed milling equipment and finished feed. A previous analysis of microbial monitoring data from 22 Australian commercial feed mills, owned by a single feed mill company and collected between January 2003 and May 2018, found the prevalence of *Salmonella* contamination and the diversity of serotypes was greatest in the raw feed component samples (11.7% of 4932 samples).[12] *Salmonella* in raw feed components may occasionally survive heat or other processing treatments and contaminate feed milling equipment.[13] Contaminated equipment can then become a source of post processing contamination of animal feed.[14] In the Australian study, 2.6% of 15,209 samples from feed milling equipment and 2.3% of 3822 samples from finished feed were *Salmonella* positive.[12] The AMR patterns of *Salmonella* isolates from Australian feed mills are currently unknown. Worldwide, previous studies have characterised the phenotypic antimicrobial susceptibility of *Salmonella* isolates

*Corresponding author.
[a]The Department of Veterinary Preventive Medicine, The Ohio State University, 1920, Coffey Road, Columbus, Ohio, 43210, USA; parker.1224@osu.edu
[b]Microbiological Diagnostic Unit–Public Health Laboratory, Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Victoria, Australia
[c]Ridley AgriProducts Pty Ltd, 70-80, Bald Hill Road, Pakenham, Victoria, 3810, Australia

Table 1. Probability of antimicrobial susceptible, nonsusceptible and resistant *Salmonella* isolates recovered from Australian feed mills

Antimicrobial susceptibility testing result	Number of *Salmonella* isolates	Probability 95% confidence interval
Susceptible	356	0.79 (0.75–0.82)
Nonsusceptible to streptomycin only	49	0.11 (0.08–0.14)
Resistant to two or more antimicrobials	48	0.11 (0.08–0.13)
Total	453	

Table 2. Probability of antimicrobial-resistant *Salmonella* isolates by sample type from Australian feed mills

Sample type	Number of isolates	Number of resistant *Salmonella* isolates	Probability (95% confidence interval)
Raw material	228	2	0.01 (0.00–2.1)
Equipment	124	44	0.36 (0.27–0.44)
Finished feed	101	2	0.02 (0.0–0.05)
Total	453	48	0.11 (7.8–13.4)

from raw feed components[6,7] and finished feed,[8] however, the results of antimicrobial susceptibility testing (AST) of isolates from feed milling equipment have not been previously published. The objective of this study was to characterise the phenotypic antimicrobial susceptibility of *Salmonella* isolates collected from feed mills owned by a single commercial feed milling company. The risk of detection and the diversity of *Salmonella* in feed mills is reported to be greatest in raw feed component samples,[12] therefore, we hypothesised that a greater proportion of the *Salmonella* isolates from raw feed components would be AMR compared to the proportion of AMR isolates from equipment and finished feed.

Materials and methods

Sample collection

Microbial monitoring for the purpose of feed safety accreditation requires collection of samples of feed and raw feed components. Swabs are also collected from specified feed mill equipment sites associated with each step of the production process, from intake to dispatch. Samples are collected once per month. If a positive sample is detected, corrective action is initiated with follow-up sampling. *Salmonella* isolates included in this study were collected between November 2012, when AST began, and February 2021, from commercial feed mills located in four Australian States and submitted to a single enteric reference laboratory. Twelve restricted animal material (RAM) mills and five non-RAM mills were included in the study. RAM mills manufacture feeds for monogastric animals, predominantly pigs and poultry and when required will include rendered animal products in the raw feed components. Non-RAM mills manufacture feed that is free of RAMs and do not include rendered animal products in the production of animal feed.

Mill personnel, trained in sample collection and sterile technique, collected surface, feed and raw feed component samples. Procedures for sample collection have been previously described.[12]

Salmonella isolation and serotyping

Samples were analysed at a National Association of Testing Authorities (NATA), Australian accredited food safety laboratory. Detection of *Salmonella* spp. followed the Australian Standard 5013.10-2009 protocol with pre-enrichment of the sample in buffered peptone water (37°C for 18 h) followed by enrichment in Rappaport-Vassiliadis medium with soya (41.5°C for 24 h), and Muller-Kauffmann tetrathionate and novobiocin broth (37°C for 24 h). The enrichment cultures were then plated on xylose lysine desoxycholate agar and Brilliance *Salmonella* Agar (or another appropriate selective medium). At least four colonies were taken to confirm that the isolates were *Salmonella*. One colony was sent to the Microbiological Diagnostic Unit Public Health Laboratory (MDU) at the Peter Doherty Institute for Infection and Immunity, the University of

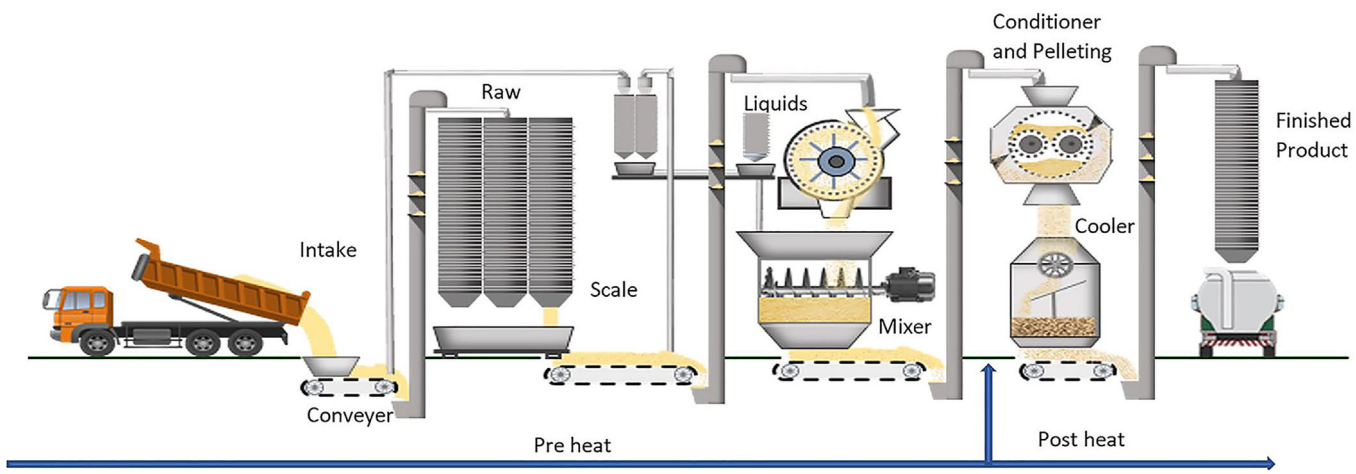

Figure 1. The feed processing chain. Forty-four antimicrobial-resistant *Salmonella* isolates were from environmental swabs of equipment in the post heat treatment area of the mill.

Melbourne, Australia, for serotyping according to the Kauffmann-White scheme[15] from November 2012 to September 2018 and *Salmonella* In Silico Typing Resource (SISTR)[16] from September 2018.

Antimicrobial susceptibility testing

The AST of the isolates included in this study was completed at the MDU using agar breakpoint dilution, generating qualitative categorical AST data.[17] Breakpoint concentrations are provided in the supplemental material (Table S1). Routine testing for the following agents was performed from November 2012 to February 2021: ampicillin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfathiazole, trimethoprim and tetracycline; azithromycin and meropenem from 2015 and trimethoprim-sulfathiazole (co-trimoxazole) from 2018. Isolates displaying intermediate resistance were classified as nonsusceptible.[17] When possible, contemporaneous Clinical and Laboratory Standards Institute (CLSI) breakpoints were used for interpretation, as described previously.[18] There are no CLSI breakpoints for azithromycin resistance in nontyphoidal *Salmonella*; therefore, in accordance with other *Salmonella* AMR surveillance systems azithromycin nonsusceptibility was defined as ≥32 μg/mL.[19]

Data analysis

AMR patterns were described in tables by year, mill and sample type. A logistic regression with random effects model, was developed to estimate the association between sample type, (finished feed, equipment or raw feed components) and detection of AMR *Salmonella*. For this model, the dependent variable was categorised as either

Table 3. Antimicrobial resistance patterns for antimicrobial-resistant *Salmonella* isolates representing six serotypes with phenotypic resistance to antimicrobials recovered from Australian feed mills

Serotype	Sample type	Mill no.[a]	Year	*N*	Resistance patterns
Anatum	Equipment	2	2019	1	ACCtSuTTmSp (nsS)
Number of isolates = 32	Equipment	2	2018	1	ACSuTmSp (nsST)
Number AMR = 16	Equipment	5	2015	8	ACSuTTmSp (nsC, 3 x nsS)
	Equipment	5	2015	1	CTSu
	Equipment	4	2018–19	3	CtSpSuTTm (nsS)
	Equipment	1	2018	1	CtSpSuTTm
	Equipment	1	2020	1	SuT (nsS)
Mbandaka	Equipment	7	2015	1	AGSSpSuTTM
Number of isolates = 35	Equipment	7	2015	1	AGSpSuTm
Number AMR = 16	Equipment	7	2015	1	ASpSuTTm
	Feed	7	2016	1	AAzCSSpSuTTm
	Equipment	7	2012	1	SpSuTTm
	Equipment	5	2018	2	ASuT (nsCp)
	Equipment	1	2015	1	SpSuTTm
	Equipment	6	2015	8	SpSuTTm
Singapore	Canola meal	3	2014	1	SSpSuT
Number of isolates = 13	Meat meal	3	2015	1	SSpSuT
Number AMR = 11	Equipment	3	2016–19	9	SSpSuT
Subsp. I ser. 4,[5],12:i:-	Equipment	3	2019	1	ASSuT
Number of isolates = 2	Equipment	5	2020	1	ASSuT
Number AMR = 2					
Orion	Feed	6	2020	1	SSuTSp
Number of isolates = 57					
Number AMR = 1					
Senftenberg	Equipment	2	2020	1	ACSuTTmSp (nsSCp)
Number of isolates = 24					
Number AMR = 1					
Worthington	Equipment	6	2018	1	SuTTmSp
Number of isolates = 2					
Number AMR = 1					

A, ampicillin; Az, azithromycin, C, chloramphenicol; Cp, ciprofloxacin; Ct, trimethoprim-sulphathiazole (co-trimoxazole); G, gentamicin; ns, nonsusceptible; S, streptomycin; Sp, spectinomycin; Su, sulphathiozole; T, tetracycline; Tm, trimethoprim.
[a] Mills 1 to 5 are RAM mills, Mills 6 and 7 are non-RAM mills.

Table 4. Results of the multivariable logistic regression with random effects model to assess the odds of detection of an antimicrobial-resistant *salmonella* isolate from raw material, equipment and finished feed in Australian feed mills

Variable	Coefficient[a] (95% CI)	P-value[b]	Population adjusted predicted probability (95% CI)
β_0	−6.47 (−8.70 to 4.26)	<0.001	
Sample type			
Raw materials	0[A]		0.01 (0.00–0.04)
Equipment	5.74[B] (3.91–7.56)	<0.001	0.39 (0.19–0.60)
Finished feed	3.03[C] (0.72–5.34)	0.004	0.11 (0.00–0.27)
Mill site		Residual intraclass correlation (95% CI)	
Variance	5.08 (1.45–17.56)	0.61 (0.31–0.84)	

[a] Coefficients within variables with different superscripts, (A, B or C), differ (P < 0.05).
[b] P-value based on the likelihood ratio chi square test statistic.
β^0 the logistic regression model intercept.
CI, Confidence interval.

resistant or not resistant. The not-resistant category included both susceptible and nonsusceptible results. The initial model included the fixed effects of sample type, year, (2012–2015, 2016–2018 or 2019–2021) and type of mill, (RAM or Non-RAM) and random effect of feed mill site. The final model was developed using a backward selection process[20] to test each independent variable so that only variables with a P-value < 0.05 would remain. The model with the smallest Bayesian Information Criterion (BIC)[20] was selected. The intraclass correlation coefficient was calculated to assess the correlation between samples collected from the same mill. Population-adjusted mean probabilities were calculated for each independent variable in the final model. All data analyses were completed using StataCorp software (StataCorp Software, version 16; StataCorp LP, College Station, TX).

Results

Between November 2012 and February 2021, 453 *Salmonella* isolates detected in samples from 17 feed mills were submitted to the MDU enteric reference laboratory for *Salmonella* serotyping and AST. All *Salmonella* isolates tested were susceptible to nalidixic acid, kanamycin, cefotaxime and meropenem and 356 were susceptible to all antimicrobials tested. Forty-nine isolates were nonsusceptible to streptomycin only, and 48 were resistant to two or more medically important antimicrobials (Table 1). Of the 48 AMR isolates, 44 were from equipment, two from raw feed components and two from finished feed (Table 2). All AMR *Salmonella* isolates were collected from seven of the 17 mills, two non-RAM mills and five RAM mills.

All environmental samples positive for AMR *Salmonella* were collected from the post heat treatment milling equipment, 17 from the cooler, 15 from the pelleting press, seven from the finished product bins, three from the coater (between the conditioner and cooler) and two from the conditioner (Figure 1).

Isolates from only seven of the 39 different *Salmonella* serotypes detected were resistant to antimicrobials. A full list of the serotypes found in non-RAM and RAM mills for different time periods is included as supplementary material (Table S2). All 48 AMR *Salmonella* isolates were resistant to sulphathiazole and 46 were resistant to tetracycline. Both sulphathiazole and tetracycline are listed by the WHO as 'highly important' antimicrobials. Twenty isolates were resistant to at least one 'critically important' antimicrobial, including ampicillin, azithromycin, ciprofloxacin, gentamycin and streptomycin.[21] Forty-three of the 48 AMR isolates were either *Salmonella* Anatum, *S.* Mbandaka or *S.* Singapore (Table 3).

The logistic regression with random effects model with the smallest BIC included sample type as a fixed effect and mill site as a random effect. The population-adjusted mean probability of detecting AMR *Salmonella* in equipment of 0.39 was greater than detection in raw feed components, 0.11 (P < 0.001) and in finished feed, 0.11 (P = 0.006). The intraclass correlation coefficient was 0.61 indicating moderate correlation[22] between samples taken from the same mill (Table 4). The likelihood ratio test indicated that the mixed model was different from the fixed effects only model (P < 0.001).

Discussion

We found that all 453 *Salmonella* isolates included in this study were susceptible to meropenem, cefotaxime, nalidixic acid and kanamycin, 356 (79.6%) were susceptible to all antimicrobials tested, 49 (10.8%) were nonsusceptible to streptomycin only and 48 (10.6%) were resistant to two or more antimicrobials. In contrast to our original hypothesis, we found the population-adjusted mean probability of detecting AMR *Salmonella* isolates from equipment of 0.39, was greater (P < 0.01) than from raw materials (0.01) and finished feed (0.11). The results of phenotypic AST of *Salmonella* isolates from raw feed components[7,8,23,24] and finished feed[8,25–28] have been previously reported, whereas AST results of *Salmonella* isolates from feed milling equipment have not. In one study in the United States, 3% of 365 isolates collected from raw materials and finished feed, were resistant to one or more antimicrobials[28] and in another, 33% of nine isolates collected from raw materials were resistant to at least one antimicrobial.[24] A study in India found that 100% of 34 isolates from cattle feed were resistant to one or more antimicrobials with

53% of the isolates resistant to cefotaxime, 21% to ceftazidime and 6% to imipenem.[25] In this study, four (1.2%) of the 329 *Salmonella* isolates from finished feed and raw feed components were AMR. The detection of AMR *Salmonella* in animal feed produced in an Australian commercial feed mill is rare, despite vigilant microbial monitoring, with only one isolate detected in 2016 and one in 2020.

There is clustering of AMR *Salmonella* positive samples within mills. Of the 48 AMR *Salmonella* isolates detected in this study, 44 were from equipment samples and all were collected from only seven of the 17 mills included in this study. This clustering of AMR *Salmonella* isolates within mills may be related to both isolate and mill characteristics.

Characteristics of the *Salmonella* isolate may determine its ability to persist in the mill equipment. Forty-three of the AMR *Salmonella* isolates were serotypes *S*. Anatum (16), *S*. Mbandaka (16) and *S*. Singapore (11). Previous studies have found that *Salmonella* serotypes such as those reported here are '*mill adapted*' and are frequently detected in the feed mill environment.[12,29–31] *Salmonella* use multiple mechanisms to survive in the hostile food processing environment, including upregulation of the production of osmoprotectants, filamentation and biofilm formation that protect them from desiccation, heat treatment and biocides.[32] A previous study found that the biofilm-forming ability of mill-adapted *Salmonella* serotypes was associated with persistence in the feed mill environment.[33] Once biofilms are formed they are difficult to remove, persist in the feed mill environment, sometimes for years and become a source of post processing contamination.[14] Phylogenetic analysis is required to determine if the AMR *Salmonella* strains of the same serotype detected in this study are genetically related with a gradual accumulation of AMR markers, or if the strains are unrelated, suggesting ongoing contamination from raw feed components.

Mill characteristics such as the microbial quality of raw feed components utilised by the mill, the age of the mill and the production of medicated feed may also be associated with the risk of AMR *Salmonella* detection. First, the microbial quality of the raw feed components determines the risk of introducing *Salmonella* to the feed mill. *Salmonella* from raw feed components that survive heat treatment may persist in the mill equipment.[13] Keeping the feed mill environment and equipment dry is an important aspect of *Salmonella* control and limits the use of liquid disinfectants.[34] Thorough cleaning relies on other processes such as physical scraping to remove organic matter and dust extraction. Modern mill design enhances the ability to thoroughly clean equipment using these methods. Therefore, the second characteristic that may increase the susceptibility of a mill to AMR *Salmonella* detection is the age of the mill. Wear and tear of older equipment surfaces may promote *Salmonella* attachment and biofilm formation,[14] exacerbated by the design of older mills that may hinder thorough cleaning. Finally, selection for AMR bacteria relies on exposure to antimicrobial residues. In Australia, in 2010, 130.5 tonnes of antimicrobials were sold for administration in feed for poultry, 67.7 tonnes for pigs and 25.8 tonnes for cattle and sheep.[35] Antimicrobials are added to the raw feed components in the mixer (Figure 1), therefore, mill adapted *Salmonella* serotypes residing in the downstream feed mill equipment are often exposed to feed medicated with antimicrobials. For example, 11 *Salmonella* Singapore isolates with identical AMR patterns were detected in one mill. This strain was first detected in 2014 and 2015 in raw feed components and then in equipment from 2015 to 2019. It is possible that resistance to common in feed antimicrobials such as streptomycin, spectinomycin, tetracycline and sulpha-based antimicrobials allowed this strain to outcompete susceptible strains and persist in the mill equipment. Both *Salmonella* strain type and mill characteristics such as age and design, sources and quality of raw feed components and the addition of antimicrobials to feed may be associated with AMR *Salmonella* detection in feed mill equipment.

Mitigating the risk of contamination of the feed mill equipment and environment with AMR *Salmonella* relies on good manufacturing practice to 1. Limit contamination of equipment and 2. Limit exposure of resident microbes to antimicrobials. The most important source of *Salmonella* contamination of the feed mill is the raw feed components and good manufacturing processes assumes that all raw feed components are potentially contaminated. Prevention of contamination of equipment and finished feed, therefore, relies on segregation of the dirty areas (pre-kill step equipment and raw feed components), and clean areas (post kill-step equipment and finished feed). This is achieved by controlling the flow of air, dust, equipment and personnel.[36] Feed safety accreditation also requires microbial monitoring of raw feed components so that follow up corrective action can be implemented by the supplier when a *Salmonella* positive sample is detected. In addition to regular sampling, equipment surfaces, especially of high risk milling equipment, such as the cooler, should be regularly inspected and cleaned to remove aggregates of feed and dust from the crevices and equipment seams. While the livestock industries continue to require the production of medicated feed, there is a risk that feed will be contaminated with AMR bacteria. Exposure of any resident microbes, including *Salmonella*, to antimicrobials, will select for AMR, therefore, cleaning methods to effectively remove antimicrobial residues from equipment surfaces need to be developed and implemented. Ongoing microbial monitoring and vigilant follow up corrective action in the event of a positive sample is the key to reducing the risk of detecting AMR *Salmonella* in feed.

Conclusion

The risk of detection of AMR *Salmonella* in Australian commercially prepared livestock feed mills is small compared to previously reported studies from other countries. In this study, most of the AMR *Salmonella* isolates were detected in samples from the post heat treatment feed mill equipment. Mitigating the risk of AMR *Salmonella* entering the food chain via animal feed relies on the microbial quality of raw feed components, reduction of in feed antimicrobial use, strategies to remove contaminants, including antimicrobial residues from feed mill equipment, and on-going surveillance with follow up cleaning and disinfection of *Salmonella* positive mill equipment. Further investigations are required to determine if the hostile environment of the feed mill, combined with trace amounts of antimicrobials from medicated feed, contribute to selection for AMR *Salmonella* in the milling equipment. It would also be interesting to evaluate to

what extent *Salmonella* strains with similar AMR patterns are observed in downstream animals and human cases.

Acknowledgments

The authors would like to thank Ridley AgriProducts for their support that included open access to data, the Microbiological Diagnostic Unit Public Health Laboratory, the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia for serotyping the isolates and ACE Laboratory Services, East Bendigo, Australia for *Salmonella* culture.

Conflicts of interest and funding

Ridley AgriProducts supported this work. Dr LJ Edwards was an employee of Ridley AgriProducts during this study but did not receive any funding or financial support for this project.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.13146/suppinfo.

Table S1 Microbiological Diagnostic Unit Public Health Laboratory antimicrobial concentrations and Clinical and Laboratory Standards Institute (CLSI) breakpoints interpretive standard[1] for antimicrobial susceptibility testing of *Salmonella* using agar breakpoint dilution.

Table S2 Frequency of *Salmonella* serotypes recovered from Australian feed mills listed by type of mill, year and antimicrobial resistance (AMR). Restricted animal material (RAM) mills manufacture feeds for monogastric animals, predominantly pigs and poultry and when required will include rendered animal products in the raw feed components used to produce feed. Non-RAM mills do not use RAMs/rendered animal products in the production of animal feeds.

(Accepted for publication 6 January 2022)