frontiers | Research Topics

Antimicrobial resistance in food-producing environment: A One Health approach

Edited by
Kebede Amenu and Getahun E. Agga

Published in
Frontiers in Antibiotics

ISSN 1664-8714
ISBN 978-2-8325-5255-1
DOI 10.3389/978-2-8325-5255-1

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Antimicrobial resistance in food-producing environment: A One Health approach

Topic editors

Kebede Amenu — Addis Ababa University, Ethiopia

Getahun E. Agga — Food Animal Environmental Systems Research, Agricultural Research Service (USDA), United States

Citation

Amenu, K., Agga, G. E., eds. (2024). *Antimicrobial resistance in food-producing environment: A One Health approach*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-5255-1

Table of contents

frontiers | Frontiers in Antibiotics

TYPE Editorial
PUBLISHED 19 June 2024
DOI 10.3389/frabi.2024.1436987

OPEN ACCESS

EDITED AND REVIEWED BY
Jianping Xie,
Southwest University, China

*CORRESPONDENCE
Getahun E. Agga
Getahun.agga@USDA.Gov
Kebede Amenu
kebede.amenu@aau.edu.et;
k.amenu@cgiar.org

RECEIVED 23 May 2024
ACCEPTED 27 May 2024
PUBLISHED 19 June 2024

CITATION
Agga GE and Amenu K (2024) Editorial: Antimicrobial resistance in food-producing environments: a One Health approach. *Front. Antibiot.* 3:1436987. doi: 10.3389/frabi.2024.1436987

Editorial: Antimicrobial resistance in food-producing environments: a One Health approach

Getahun E. Agga[1]* and Kebede Amenu[2,3]*

[1]Food Animal Environmental Systems Research Unit, Agricultural Research Service, United States Department of Agriculture, Bowling Green, KY, United States, [2]College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, Ethiopia, [3]Animal and Human Health Program, International Livestock Research Institute (ILRI), Addis Ababa, Ethiopia

KEYWORDS

antimicrobial resistance, One Health, antimicrobial use, antimicrobial stewardship, animal manure, probiotics, extended-spectrum β-lactamases, flies

Editorial on the Research Topic
Antimicrobial resistance in food-producing environments: a One Health approach

Introduction

The One Health High-Level Expert panel comprised of the United Nations Food and Agriculture Organization (FAO), the United Nations Environment Program (UNEP), the World Health Organization (WHO), and the World Organization for Animal Health (WOAH; founded as OIE) defines One Health as "an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems. It recognizes that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent. The approach mobilizes multiple sectors, disciplines and communities at varying levels of society to work together to foster well-being and tackle threats to health and ecosystems, while addressing the collective need for clean water, energy and air, safe and nutritious food, taking action on climate change, and contributing to sustainable development." (Mettenleiter et al., 2023). There is nothing more fitting than antimicrobial resistance (AMR) to the principles of One Health, which provides a framework for an interdisciplinary approach to dealing with this global challenge (FAO, 2016; Robinson et al., 2016; Lancet, 2023).

Globally, bacterial AMR has been associated with an estimated 4.95 million human deaths in 2019, including 1.27 million deaths directly attributable to bacterial AMR (Murray et al., 2022). In the United States, bacterial AMR causes more than 2.9 million infections each year, with an estimated 35,000 deaths (CDC, 2019). Such estimates on the burden of antimicrobial-resistant pathogens to animal health do not exist (Robinson et al., 2016). Nevertheless, a recent ecological study (Allel et al., 2023) reported a 24.8% AMR mean prevalence in bacteria associated with food-producing animals. The authors also reported significant associations between animal antimicrobial consumption and AMR in bacteria associated with food-producing animals, and between human antimicrobial consumption and AMR in human pathogens. They also found bidirectional associations between veterinary antimicrobial sales and AMR in human pathogens, and between human

antimicrobial sales and AMR in animals. The study highlighted that AMR in food-producing animals is associated with the quantity of antimicrobials sold for use in animals and in humans. Seven articles published under this theme discuss (1) baseline occurrence of AMR under cow-calf production; (2) estimation of national antimicrobial use in food-producing animals; (3) standardization of variables and parameters used to quantify on-farm antimicrobial use (AMU); (4) animal manure storages such as lagoons that serve as a reservoir for antimicrobial resistance genes (ARGs); (5) dissemination of AMR through flies; (6) probiotics as a source of AMR determinants; and (7) potential interventions to reduce the burden of AMR.

AMR in beef cow-calf production system

Beef cow-calf production is an important and initial segment in commercial beef production system. First, it provides calves that are finished in feedlots for beef production; second, when culled from operation, beef cows are processed mainly for ground beef production. Compared to feedlot and dairy cattle production settings, antimicrobials are used infrequently. Understanding the epidemiology of AMR under the low antibiotic selection pressure that exists in cow-calf production can serve as a baseline for AMR risk that can arise when cows and calves enter the beef supply chain. Previous studies, including by the lead author of the paper by Agga et al., revealed that Gram-negative clinically important antimicrobial-resistant bacteria and associated genes can be found in cow-calf population (Agga et al., 2016a, 2019; Agga et al., 2022b). The study by Agga et al., conducted in a cow-calf operation, targeted enterococci as the indicator organism for Gram-positive bacteria and reported that while tetracycline-resistant enterococci were abundant, macrolide resistance occurred at low abundance. Furthermore, the two species, *E. faecalis* and *E. faecium*, most implicated in nosocomial infections, were widely detected in the cow-calf operation. The authors also pointed out that the use of wheat as a cover crop may have additional value in mitigating AMR in livestock raised under a grazing system.

Standardized methods for the quantification of AMU in food animals

Previous studies using randomized field trials indicated that AMU in feedlot cattle for approved indications increases AMR in bacteria and specific ARGs (Agga et al., 2016b, 2023). Collecting on-farm AMU data is challenging and only a few countries have on-farm AMU monitoring systems. Most other developed countries including the United States use national sales data. Magiri et al. used import data to quantify AMU in Fiji. Since many developing countries rely on imported antimicrobials from developed countries, this method may be a practical approach in quantifying national antimicrobial purchases intended for use in animals. The use of different measurements and analytical approaches without standardization hinders direct evaluation of intervention efforts to reduce AMR. To overcome this challenge, Lu et al. compared different metrics and developed standardized methods of quantifying AMU, primarily obtained from sales data for use in food animals. The authors identified various AMU indicators, generally grouped as count-based, mass-based, and dose-based, that have been used to quantify AMU in animals; developed them into standardized approaches; and evaluated them for accuracy without compromising privacy for on-farm use and for their applicability to antimicrobial stewardship programs. The authors also identified limitations of the AMU quantification approaches such as lack of strong causal evidence between AMU and AMR among animal pathogens and commensals, and failure to include important information such as disease conditions, routes of administration, and antimicrobial resistance information into the AMU metrics.

Environmental dissemination of AMR from animal farms

Animal manure is applied to fields following storage, and incentives are necessary for farmers to use existing manure-management technologies, which also have other added value such as biofuel production from anaerobic digestion and lagoon systems, and compost from animal manure composting, which can be used as organic fertilizer (Agga et al., 2022a). Animal manure removed from production facilities is stored in ponds, storage pits, or stockpiled between land applications. These storage units may act as reservoirs for AMR. Neher et al. compared the distribution of ARGs in manure storage pits across swine farms in Iowa. The study revealed that tetracycline and macrolide resistance genes were detected in all swine farms (n = 48) studied; their concentrations significantly varied among the farms, and by integrator type, with no significant effect of production type.

Caderhoussin et al. examined the role of flies in disseminating extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* on cattle farms. By applying advanced molecular techniques, the origin and maintenance of ESBL-producing *Enterobacteriaceae* were investigated in a farm that raised food-producing animals with no history of third-generation cephalosporin uses. The study found a similarity between plasmids and genes of ESBL-producing *E. coli* strains isolated from flies and cattle. The findings from the study suggest that flies can act as effective mechanical vectors in transferring ARGs across environments and to multiple hosts. The study uncovered the complexity of factors responsible for the transfer and maintenance of ARGs. To address the complexity of AMR spread, a comprehensive One Health approach that integrates human, animal, and environmental health aspects is needed.

Probiotics as a source of antibiotic-resistant bacteria and genes

Alternatives to antibiotics, including probiotics, are an important area of research to address the loss of effective treatments of infections caused by antimicrobial-resistant bacteria. Probiotics are direct-fed microbials that are added to animal feed to improve production efficiency and animal health

(Cameron and McAllister, 2019). However, probiotics such as lactobacilli may be resistant to antibiotics, and spread AMR elements to commensal or pathogenic bacteria. Nøhr-Meldgaard et al. characterized diverse strains of bacteria in the *Lactobacillaceae* family for AMR, genotypically using whole genome sequence data for the presence of ARGs, and phenotypically by minimum inhibitory concentration (MIC) method using epidemiological cutoffs (ECOFF). The authors used phylogenetic relatedness, rather than the traditional fermentation-based method, to propose a new ECOFF for the family of bacteria. Almost in all strains, acquired ARGs were not detected, thus fulfilling one of the theoretical requirements for a probiotic.

AMR interventions

Jacobsen et al. conducted a scoping review to summarize the literature reporting AMR interventions in animals. The study provided a comprehensive overview of various interventions and tools aimed at reducing AMU and AMR in the animal health sector by classifying the interventions into different major categories: (1) change in AMU practices, (2) change in the uptake of antimicrobial stewardship (AMS), (3) change in the development of AMR, (4) change in the knowledge of AMR and change in the knowledge of appropriate AMU and AMS practices, (5) change in attitudes and perceptions concerning AMU, AMR, and AMS, and (6) surveillance strategies. The review indicated that only one-fifth of the reviewed papers targeted developing countries. The review revealed that objective means of evaluating the interventions are not common, but self-reported subjective responses are. Specifically, financial aspects are not considered when interventions are evaluated. The authors assert that a full understanding of the interlinked global efforts toward evaluating interventions requires proportional coverage in developed and developing countries by using objective metrics and targeting financial aspects.

Conclusions

The theme One Health approach to AMR in food-producing animals attracted papers that evaluated the epidemiology and ecology of AMR including interventions taken to reduce it. The papers ranged from the baseline occurrence of AMR under low antibiotic selection pressure, AMR dissemination pathways such as animal manure, probiotics, and flies, AMU quantification and standardization, to the interventions taken to reduce AMR. The papers presented critical information needed to optimize AMU in food-producing animals and the roles of other factors in disseminating AMR.

Author contributions

GA: Writing – original draft, Writing – review & editing. KA: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. GA's research is supported by the U.S. Department of Agriculture, Agricultural Research Service. KA's research is supported by the CGIAR Initiative on One Health (https://www.cgiar.org/initiative/one-health/). KA thanks all funders who supported his research through their contributions to the CGIAR Trust Fund: https://www.cgiar.org/funders/.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Author disclaimer

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Agga, G. E., Cook, K. L., Netthisinghe, A. M. P., Gilfillen, R. A., Woosley, P. B., and Sistani, K. R. (2019). Persistence of antibiotic resistance genes in beef cattle backgrounding environment over two years after cessation of operation. *PloS One* 14, e0212510. doi: 10.1371/journal.pone.0212510

Agga, G. E., Couch, M., Parekh, R. R., Mahmoudi, F., Appala, K., Kasumba, J., et al. (2022a). Lagoon, anaerobic digestion, and composting of animal manure treatments impact on tetracycline resistance genes. *Antibiotics* 11, 391. doi: 10.3390/antibiotics11030391

Agga, G. E., Galloway, H. O., Appala, K., Mahmoudi, F., Kasumba, J., Loughrin, J. H., et al. (2023). Effect of continuous in-feed administration of tylosin to feedlot cattle on macrolide and tetracycline resistant enterococci in a randomized field trial. *Prev. Veterinary Med.* 215, 105930. doi: 10.1016/j.prevetmed.2023.105930

Agga, G. E., Galloway, H. O., Netthisinghe, A. M. P., Schmidt, J. W., and Arthur, T. M. (2022b). Tetracycline- resistant, third generation cephalosporin- resistant, and extended spectrum beta-lactamase- producing *Escherichia coli* in a beef cow-calf production system. *J. Food Prot*. 85, 1522–1530. doi: 10.4315/JFP-22–178

Agga, G. E., Schmidt, J. W., and Arthur, T. M. (2016a). Antimicrobial-resistant fecal bacteria from ceftiofur-treated and nonantimicrobial-treated comingled beef cows at a cow-calf operation. *Microb. Drug Resist.* 22, 598–608. doi: 10.1089/mdr.2015.0259

Agga, G. E., Schmidt, J. W., and Arthur, T. M. (2016b). Effects of in-feed chlortetracycline prophylaxis in beef cattle on animal health and antimicrobial-resistant *Escherichia coli*. *Appl. Environ. Microbiol.* 82, 7197–7204. doi: 10.1128/AEM.01928–16

Allel, K., Day, L., Hamilton, A., Lin, L., Furuya-Kanamori, L., Moore, C. E., et al. (2023). Global antimicrobial-resistance drivers: an ecological country-level study at the human animal interface. *Lancet Planetary Health* 7, e291–e303. doi: 10.1016/S2542-5196(23)00026-8

Cameron, A., and McAllister, T. A. (2019). Could probiotics be the panacea alternative to the use of antimicrobials in livestock diets? *Benef. Microbes* 10, 773–799. doi: 10.3920/bm2019.0059

CDC. (2019). *Antibiotic resistance threats in the United States 2019* (Atlanta, GA: U.S. Department of Health and Human Services, U.S. Centers for Disease control and Prevention (CDC). Available online at: https://www.cdc.gov/antimicrobial-resistance/data-research/threats/?CDC_AAref_Val=https://www.cdc.gov/drugresistance/Biggest-Threats.html (Accessed June 01, 2024).

FAO. (2016). *Drivers, dynamics and epidemiology of antimicrobial resistance in animal production*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Lancet, T. (2023). One Health: a call for ecological equity. *Lancet* 401, 169. doi: 10.1016/S0140–6736(23)00090–9

Mettenleiter, T. C., Markotter, W., Charron, D. F., Adisasmito, W. B., Almuhairi, S., Behravesh, C. B., et al. (2023). The one health high-level expert panel (OHHLEP). *One Health Outlook* 5, 18. doi: 10.1186/s42522–023-00085–2

Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 399, 629–655. doi: 10.1016/S0140–6736(21)02724–0

Robinson, T. P., Bu, D. P., Carrique-Mas, J., Fèvre, E. M., Gilbert, M., Grace, D., et al. (2016). Antibiotic resistance is the quintessential One Health issue. *Trans. R Soc. Trop. Med. Hyg* 110, 377–380. doi: 10.1093/trstmh/trw048

frontiers | Frontiers in Antibiotics

TYPE Original Research
PUBLISHED 03 November 2022
DOI 10.3389/frabi.2022.1052316

OPEN ACCESS

EDITED BY
Adrian Muwonge,
University of Edinburgh,
United Kingdom

REVIEWED BY
Faham Khamesipour,
Shahid Beheshti University of Medical Sciences, Iran
Md Latiful Bari,
University of Dhaka, Bangladesh

*CORRESPONDENCE
Getahun E. Agga
getahun.agga@usda.gov

†PRESENT ADDRESS
Hunter O. Galloway,
Williams Sausage Company, Inc.,
Union City, TN, United States

SPECIALTY SECTION
This article was submitted to Antibiotic Resistance, a section of the journal Frontiers in Antibiotics

RECEIVED 23 September 2022
ACCEPTED 20 October 2022
PUBLISHED 03 November 2022

CITATION
Agga GE, Galloway HO and Netthisinghe AMP (2022) Effects of age and pasture type on the concentration and prevalence of tetracycline and macrolide resistant *Enterococcus* species in beef cow-calf production system.
Front. Antibiot. 1:1052316.
doi: 10.3389/frabi.2022.1052316

Effects of age and pasture type on the concentration and prevalence of tetracycline and macrolide resistant *Enterococcus* species in beef cow-calf production system

Getahun E. Agga[1*], Hunter O. Galloway[2†] and Annesly M. P. Netthisinghe[2]

[1]Food Animal Environmental Systems Research Unit, Agricultural Research Service, United States Department of Agriculture, Bowling Green, KY, United States, [2]Department of Agriculture and Food Science, Western Kentucky University, Bowling Green, KY, United States

Enterococci are a normal flora of the gastrointestinal tracts of humans and animals. Enterococci can also cause life-threatening nosocomial infections. Antimicrobial-resistant *Enterococcus* species have been reported in the feedlot and dairy cattle productions and in meat and milk products, suggesting their foodborne importance. Cow-calf operations represent a significant segment in the beef production system by producing weaned calves. Weaned calves are brought into the feedlot to be finished for meat, and culled cows are also slaughtered for beef, primarily for ground beef products. Infection dynamics in the cow-calf operation can contribute to meat contamination. This study evaluated the effects of age and wheat grazing on the concentration and prevalence of a macrolide antibiotic erythromycin (ERY^r) and tetracycline (TET^r) resistant enterococci, associated resistance genes and species distribution in a cow-calf production system. In 2017 and 2018, 32 Angus breed cow-calf pairs were randomly assigned to feed on tall fescue or wheat pasture in two independent field experiments. During the grazing experiments of 2-3 weeks, fecal samples were collected weekly and cultured to enumerate, isolate and identify ERY^r, TET^r, and generic enterococci, using media supplemented with erythromycin, tetracycline or non-supplemented media, respectively. The two main species frequently associated with human illnesses, *Enterococcus faecium* and *E. faecalis*, were widely distributed in the cow-calf groups. Generic and TET^r- enterococci were prevalent (96-100% prevalence) and abundant (3.2-4.9 $\log_{10}$ CFU/g) in the cow-calf population; however, ERY^r enterococci were enumerable by direct plating only from a single cow despite being detected in at least 40% of the fecal samples after enrichment, showing their low abundance. TET- and ERY-resistance were mainly conferred by *tet*(M) and *erm*(B), respectively. Wheat grazing reduced the concentration of TET^r enterococci and modified enterococcal species and resistance gene distributions. Hence, it is necessary

to further investigate wheat grazing in cow-calf production as a potential strategy to mitigate antimicrobial resistance.

KEYWORDS

antimicrobial resistance, macrolide resistance, tetracycline resistance, *enterococcus*, cow-calf, beef cattle

Introduction

Enterococci are commensal bacteria colonizing the gastrointestinal tract (GIT) of healthy humans and animals, without causing intestinal problems. (USDA, 2012; Lebreton et al., 2014; Ahmed and Baptiste, 2018) Enterococci can also cause life threatening extraintestinal infections. (Arias and Murray, 2012; Lebreton et al., 2014; Ahmed and Baptiste, 2018) Consequently, antibiotic resistant enterococci are the leading cause of nosocomial infections in the United States. (Arias and Murray, 2012; Lebreton et al., 2014) *Enterococcus faecalis* and *E. faecium* are major species commonly associated with human infections. (Arias and Murray, 2012; Lebreton et al., 2014; Ahmed and Baptiste, 2018) Enterococci are ubiquitous; (Lebreton et al., 2014) people are colonized when exposed to contaminated environments from human and animal wastewater (Agga et al., 2015), contaminated drinking, and recreational water sources. (Lebreton et al., 2014; Cho et al., 2020; Agga et al., 2022a; Kaiser et al., 2022) Furthermore, enterococci have been reported from retail beef (Tyson et al., 2018), retail veal meat (Tate et al., 2021), and ground beef (Vikram et al., 2018; Schmidt et al., 2021) suggesting their foodborne implications. Antimicrobial resistance of enterococci have been extensively studied in feedlot cattle (Vikram et al., 2017; Davedow et al., 2020; Murray et al., 2022), dairy cows (Shipp and Dickson, 2012; Abdelfattah et al., 2021), and to a lesser extent in cull cows. (Pandit et al., 2021) However, such studies are rare in beef cow-calf (Agga et al., 2016) and backgrounding operations (Agga et al., 2019).

Beef cows are culled from the cow-calf operation and sold for beef production similarly to cull dairy cows with the majority of the beef used for ground beef production. While some female calves are selected as replacements for breeding stock, majority of calves are weaned and enter feedlots to be finished for beef production. Determining the level of antimicrobial resistant bacteria (ARB) in weaned calves prior to entering the feedlot is important. Antimicrobial resistant bacteria such as enterococci can contaminate beef carcasses during slaughter process and pose a significant public health risk. Establishing a baseline prevalence of ARB including enterococci in adult beef cows and beef claves is essential. Previous studies reported a decreasing trend in the prevalence of antimicrobial resistant fecal bacteria, mainly *E. coli*, as animals get older. (Hoyle et al., 2004; Gaire et al., 2021) However, studies assessing the impact of age on Gram-positive bacteria such as enterococci are scarce. Therefore, further understanding of the age effect will be useful to differentially target the adult cows and calves for the mitigation of antimicrobial resistance.

Livestock grazing of cover crops allows producers to gain an immediate economic benefit while reducing input costs. Cover crops provide higher quality forage for livestock as compared to typical native grass pastures. (Franzluebbers, 2007; Poffenbarger, 2010) In the southern Great Plains, it is typical to graze winter wheat fields to give stocker cattle high-quality forage. (Winterholler et al., 2008) Grazing of winter wheat cultivars until the joint stage has been reported to increase grain yield as compared to non-grazed winter wheat. (Redmon et al., 1995) However, no research investigated the impact of wheat grazing on the occurrence of ARB in beef cow-calf production systems in the southern plains. The impact of dual-purpose wheat grazing on grain yield and animal growth performance was reported in the previously published study. (Netthisinghe et al., 2020) In this paper, the impact of wheat grazing on the concentration and prevalence of tetracycline (TET^r)- and erythromycin (ERY^r)-resistant enterococci was compared with tall fescue grazing in a cow-calf production system.

Specific objectives of this study were to investigate the effect of grazing pasture type (wheat vs. tall fescue) and age (calf vs. cow) in beef cow-calf production system on the concentration and prevalence of TET^r and ERY^r, enterococcal species, and resistance gene distributions among the resistant strains. An additional objective was to determine the baseline level of antimicrobial resistant enterococci in weaned calves prior to entering the feedlot, and in the breeding cows that would transmit resistant bacteria to the beef calves or culled and used for beef production. Enterococci have been used as indicator organisms for Gram-positive bacteria in antimicrobial resistance (AMR) monitoring systems involving food animal production and animal products (Karp et al., 2017).

Tetracycline resistance was selected because of its abundance and widespread occurrence among various bacterial species and

environments (Roberts and Schwarz, 2016) which may be attributed to its highest level of sales for use in food-producing animals. (Tyson et al., 2018; Agga et al., 2022a) Erythromycin is a macrolide that has been investigated with respect to antimicrobial resistant enterococci in beef cattle production (Frye and Jackson, 2013) and retail beef. (Tyson et al., 2018) According to World Health Organization (WHO) categorization of antimicrobials, tetracyclines and macrolides are classified as highly important and highest priority critically important antimicrobial classes for human health, respectively. (Scott et al., 2019) TET^r in enterococci develops primarily through ribosomal protection or the efflux of the antibiotic. *tet*(M) and *tet*(L) are the most common tetracycline resistance (*tet*) genes encoding for ribosomal protection and efflux proteins in enterococci, respectively. (Frye and Jackson, 2013; Agga et al., 2022c) The most common acquired resistance mechanism for macrolides is target modification by erythromycin resistance methylase (*erm*) genes, primarily *erm*(B) (Frye and Jackson, 2013).

Materials and methods

Experimental design and sample collection

A randomized field trial consisting of two experiments was conducted at Western Kentucky University Agriculture Research and Education Complex in Bowling Green, KY during 2017 and 2018. The study protocol was approved by the Western Kentucky University's Institutional Animal Care and Use Committee (IACUC# 17-09). The study animals consisting of beef cows and calves close to weaning age were owned and managed by Western Kentucky University. Calves received clostridial vaccine against blackleg, viral respiratory vaccine, and pinkeye vaccine. No other antibiotics were given to the cows or the calves. Detailed description of the cow-calf experiments was previously published (Netthisinghe et al., 2020).

Briefly, in two independent experiments 16 Angus breed cow-calf pairs were equally randomized, blocked on the bodyweight of the calves, to graze on tall fescue or wheat pasture in 2017 and 2018. The cow–calf pairs grazed for three weeks from 21 March to 12 April 2017, or for two weeks from 14 March to 28 March 2018. For the 2017 experiment, fecal grabs or fecal swabs were collected rectally on 21 March (week 0), 28 March (week 1), 04 April (week 2) and 11 April 2017 (week 3). For the 2018 experiment, fecal samples were collected on 14 March (week 0), 21 March (week 1) and 28 March (week 2). Samples were kept on ice and transported to the lab and refrigerated until processed.

Enumeration and detection of generic-, TET^r- and ERY^r-enterococci

Samples were processed and cultured as described. (Agga et al., 2015; Agga et al., 2016; Agga et al., 2022c). Briefly, 10 g of fecal grabs were suspended in 90 mL of buffered peptone water (BPW; Becton, Dickson, and Company [BD], Franklin Lakes, NJ, USA) and homogenized in a laboratory blender. Fecal swabs were suspended in 5 mL BPW and homogenized by centrifugation. After an aliquot was taken for enumeration, the remaining BPW suspension was incubated at 25°C for 2 h, then at 42°C for 6 h and held at 4°C for secondary enrichment.

Generic-, ERY^r- and TET^r- enterococci were enumerated on Slanetz and Bartley medium (SBM) agar (Thermo Fisher Scientific, Waltham, MA), SBM plates supplemented with 8 mg/L erythromycin (SBM+ERY), and SBM plates supplemented with 16 mg/L tetracycline (SBM+TET), respectively. To determine prevalence, secondary enrichments were made by transferring 0.5 mL of BPW pre-enrichments to 2.5 mL of enterococcosel broth (ECB; BD), ECB supplemented with 16 mg/L tetracycline (ECB+TET) and ECB supplemented with 8 mg/L erythromycin (ECB+ERY). After incubation at 37°C for 18 to 24 h, ECB cultures were streaked onto SBM, SBM+TET and SBM+ERY plates and incubated overnight at 37°C.

Antibiotics used for selective isolation of resistant strains were obtained from Millipore Sigma (St. Louis, MO), and the Clinical Laboratories Standards Institute (CLSI) resistance breakpoint concentrations (CLSI, 2020) were used.

PCR confirmation, speciation, and detection of resistance genes

For all plate types, up to two presumptive colonies were inoculated into tryptic soy broth (TSB; BD) and incubated overnight at 37°C. After fresh aliquot was taken for DNA extraction, the remaining broth culture was stored at -20°C after adding 15% glycerol to each well. DNA was extracted from 10 ml of overnight culture by BAX lysis method following the manufacturer's instructions (DuPont Qualicon, Inc., Wilmington, DE). DNA lysates were used for PCR for confirmation of the genus *Enterococcus*, speciation, and detection of associated resistance genes.

Presumptive enterococci isolates were confirmed by genus specific PCR (Deasy et al., 2000), and *Enterococcus* species were identified by multiplex PCR (Jackson et al., 2004), using published primers and protocols (Supplementary Table 1). Phenotypically resistant strains were tested by PCR for the identification of resistance genes. TET^r *Enterococcus* isolates were tested for tetracycline resistance (*tet*) genes (Supplementary Table 2). ERY^r *Enterococcus* species were tested for macrolide, lincosamides and streptogramins (MLS$_B$)

resistance genes (Supplementary Table 3). PCR products were analyzed by capillary gel electrophoresis using the QIAxcel Fast Analysis system (Qiagen, Valencia, CA). All primers used in this study were obtained from Integrated DNA Technologies, Inc. (IDT; Coralville, IA). Representative gel images from capillary electrophoresis for enterococci genus confirmation and species identification, detection of tetracycline resistance genes, and macrolide resistance genes are depicted in Supplementary Figures 1–3, respectively.

Data analysis

Enumeration data were compared by animal age (calves vs. cows) and by treatment group (wheat vs tall fescue) using negative binomial regression, and marginal outputs were obtained as $\log_{10}$ colony forming units. The prevalence of bacteria and the resistance genes were compared by animal age and pasture type using logistic regression. For both regression analyses, cows and tall fescue served as reference groups. Pairwise contrasts were obtained after adjusting for multiple comparisons by Bonferroni method. Data was analyzed in STATA 16 (StataCorp, College Station, Texas); $P<0.05$ was considered statistically significant.

Results

Descriptive analysis

Of the 224 fecal samples, 128 were collected in 2017 and 96 were collected in 2018, equally distributed by animal age (calves and cows) and pasture type (tall fescue and wheat) for each year. However, some fecal samples were not sufficient for processing; this is reflected on the basis of individual bacterial strains presented in the tables. Except for the calves and the tall fescue group, concentration of TET^r *Enterococcus* spp. closely follows that of the total enterococci (generic) population. Similarly, TET^r *Enterococcus* spp. prevalence was also high (≥96%) closely following that of the generic population. On the other hand, ERY^r *Enterococcus* spp. was quantifiable only from a single animal although the prevalence was over 40% (Table 1).

Effects of age and pasture type on the concentrations and prevalence of generic-, ERY^r- and TET^r-*Enterococcus* species

Generic enterococci concentration did not significantly ($P>0.05$) differ by age or by pasture type (Figure 1A). Concentration of TET^r-*Enterococcus* spp. were significantly ($P=0.005$) lower in the calves than in the cows, with a greater age effect observed in the wheat group than in the tall fescue due to a significant ($P=0.008$) age by pasture type interaction (Figure 1B). ERY^r *Enterococcus* spp. was quantifiable at 2.7 logs from a single cow in the fescue group in 2018. The prevalence of generic-, TET^r-, and ERY^r- *Enterococcus* spp. did not significantly ($P>0.05$) differ by age or by pasture type (Figure 2).

Prevalence of *Enterococcus* species detected

Up to two isolates from each positive sample were PCR tested for species identification. Among generic enterococci isolates, seven *Enterococcus* spp. were identified dominated by *E. durans* (38%), followed by *E. mundtii* (22.5%), and *E. faecium* (21.5%), which together accounted for over 80% of the generic enterococci isolate population. Within age and treatment

TABLE 1 Concentrations and prevalence of generic- and antibiotic resistant- enterococci by age and treatment group in cow-calf production system.

Outcome	Concentration ($\log_{10}$/g)						
	Overall	Age group			Treatment group		
		Calves	Cows	*P*-value	Wheat	Fescue	*P*-value
Generic *Enterococcus* spp.	4.8	4.8	4.8	0.868	4.9	4.7	0.191
TET^r *Enterococcus* spp.	4.5	3.2	4.8	<0.001	4.7	4.1	0.032
	Prevalence (%)						
	Overall (n=202)	Calves (n=96)	Cows (n=106)	*P*-value	Wheat (n=101)	Fescue (n=101)	*P*-value
Generic enterococci	98.5	97.9	99.1	0.514	97.0	100	0.246*
TET^r enterococci	96.5	96.9	96.2	0.802	96.0	97.0	0.701
ERY^r enterococci	43.1	44.8	41.5	0.638	44.6	41.6	0.670

TET^r, tetracycline resistant; ERY^r, erythromycin resistant; *=Fisher's exact P-value; prevalence was calculated from the total number (n) of the fecal samples presented in parenthesis.

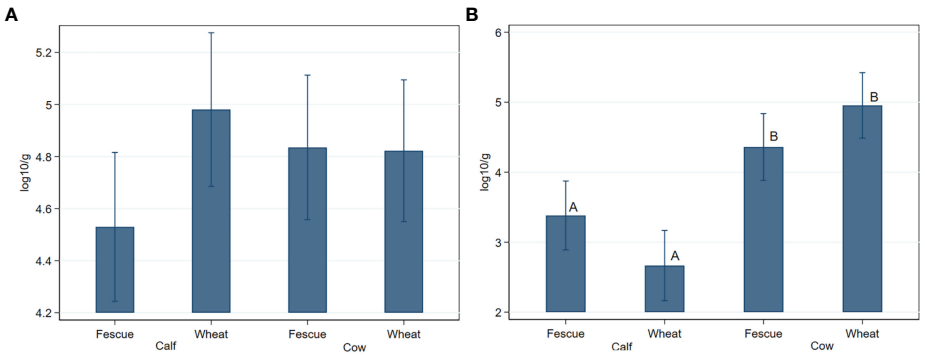


FIGURE 1
Fecal concentrations of generic- **(A)** and tetracycline resistant- **(B)** *Enterococcus* species in cow-calf production system. When shown, different letters on the bar graphs indicate statistically significant differences at $P < 0.05$. Bar graphs are presented as mean concentrations and their 95% confidence intervals.

groups, however, there was variation in terms of predominant spp. Six isolates could not be identified by the method used (Table 2). The proportion of *E. faecium* isolates obtained from cows that grazed on the wheat pasture was significantly (P=0.005) lower than in cows that grazed on tall fescue, with no significant difference in the calves. Proportions of *E. mundtii* isolates obtained from both cows and calves were significantly (P<0.001) lower in the wheat pasture than the tall fescue. The proportion of *E. durans* isolates obtained from both cows and calves grazed on wheat was about twice as much than that obtained from tall fescue (P<0.001). The *E. casseliflavus* isolates were all obtained from the calves (Fisher's exact test P=0.009).

TET^r enterococci isolates were identified into five spp. with *E. faecium* representing over half (53%) of the population,

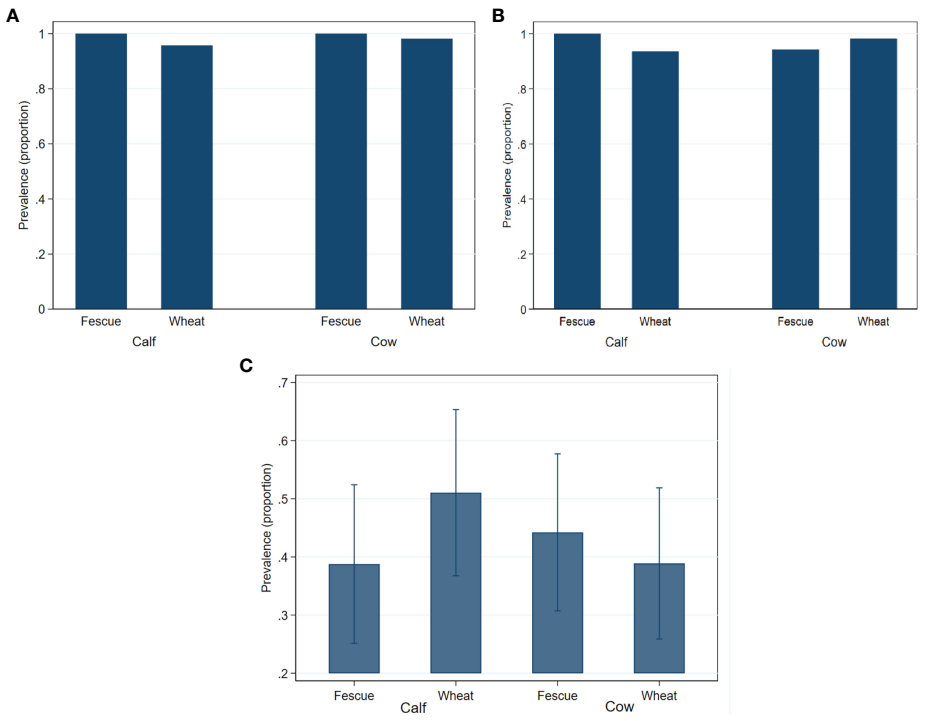


FIGURE 2
Fecal prevalence of generic- **(A)**, tetracycline resistant- **(B)** and erythromycin resistant- **(C)** *Enterococcus* species in cow-calf production system. Bar graphs are presented as mean prevalence values and their 95% confidence intervals.

TABLE 2 Prevalence of *Enterococcus* species among enterococci isolates identified from cow-calf populations, by media type.

Generic enterococci (n=302 isolates)					
Species	**Total (n=302)**	**Calves**		**Cows**	
		Fescue (n=65)	**Wheat (n=55)**	**Fescue (n=93)**	**Wheat (n=89)**
E. faecalis	10.9	5.8	11.3	8.8	16.6
E. faecium	21.5	13.8 ^{A}	23.6 ^{AB}	32.3 ^{B}	14.6 ^{A}
E. mundtii	22.5	39.9^{B}	11.0 ^{A}	31.2 ^{B}	7.8 ^{A}
E. casseliflavus	1.7	3.1	5.5	0	0
E. durans	37.8	26.0 ^{A}	49.2 ^{B}	27.0 ^{A}	50.5 ^{B}
E. gallinarum	0.3	0	0	0	1.1
E. hirae	3.3	3.1	1.8	2.2	5.6
Not identified	2.0	4.6	1.8	1.1	1.1
Tetracycline resistant enterococci (n=352 isolates)					
Species	Total (n=352)	Calves		Cows	
		Fescue (n=88)	Wheat (n=81)	Fescue (n=87)	Wheat (n=96)
E. faecalis	2.0	1.1	4.9	2.3	0
E. faecium	53.7	51.1	63.0	51.7	50.0
E. casseliflavus	1.7	2.3	0	1.1	3.1
E. durans	16.8	19.3^{AB}	9.9^{A}	9.2^{A}	27.1^{B}
E. hirae	25.0	23.9	21.0	35.6	19.8
Not identified	0.9	2.3	1.2	0	0
Erythromycin resistant enterococci (n= 111 isolates)					
Species	Total (n=111)	Calves		Cows	
		Fescue (n=16)	Wheat (n=30)	Fescue (n=33)	Wheat (n=32)
E. faecalis	22.5	62.5^{B}	3.3^{A}	21.2^{A}	21.9^{A}
E. faecium	21.6	12.5^{AB}	26.7^{AB}	36.4^{B}	6.3^{A}
E. casseliflavus	19.8	0	6.7^{A}	33.3^{B}	28.1^{AB}
E. durans	8.1	12.5	13.3	3.0	6.3
*E. hirae**	5.4	0	13.3	0	6.3
*E. avium**	7.2	0	10.0	0	15.6
E. asini	2.7	0	6.7	0	3.1
Not identified	12.6	12.5	20.0	6.1	12.5

Different superscripted letters indicate significant differences, where shown. *Fisher's exact test.

followed by *E. hirae* (25%), and *E. durans* (17%); the three spp. together accounted for over 95% of the TET^{r} enterococci population. Three TET^{r} enterococci isolates could not be identified into spp. by the method used (Table 2). Pasture type had age dependent effect (i.e., interaction) on the prevalence of *E. durans*: the prevalence was significantly (P=0.001) greater in the wheat group than the tall fescue group in the cows. Prevalence of the other spp. was not affected by age or treatment group (Table 2).

ERY^{r} enterococci were identified into seven spp.; 14 isolates identified by the method used. Top three spp. were *E. faecalis* (22.5%), *E. faecium* (21.6%) and *E. casseliflavus* (19.8%) accounting for approximately 64% of the population (Table 2). Pasture type had a significant (P= 0.002) age dependent effect on the prevalence of *E. faecalis*; prevalence of *E. faecalis* was greater in the calves that grazed on tall fescue than the remaining groups. Similarly, cows grazed on tall fescue had a significantly (P= 0.008) greater prevalence of *E. faecium* than cows grazed on wheat. Prevalence of *E. casseliflavus* isolates was significantly (P= 0.027) greater in the tall fescue cows than wheat calves; none was detected from tall fescue calves showing significant age effect (P= 0.003) and no significant pasture effect (P= 0.939). *E. hirae*, *E. avium* and *E. asini* were detected only in the wheat group (Table 2).

Effect of age and pasture type on the distribution of resistance genes

Seventy percent of TET^{r} enterococci isolates were positive for *tet*(M); *tet*(L) and *tet*(O) represent the remaining 30% (Table 3). One TET^{r} *Enterococcus* isolate was negative for all

the 11 *tet* genes tested. The proportion of isolates carrying *tet*(M) was significantly (P=0.001) greater among the calf isolates, regardless of pasture type, than the isolates obtained from cows that grazed on tall fescue. However, wheat grazing diluted the age effect by increasing the proportion of the isolates carrying *tet*(M) among the cows grazed on wheat, although the difference between the cows grazed on the two pasture types was not statistically different (Table 3). Calf TET^r enterococci isolates were almost three times (odds ratio =2.8; 95% confidence interval: 1.5-5.3) more likely to carry *tet*(M) compared to the cow isolates adjusted for pasture type and interaction. The prevalence of *tet*(L) was significantly (P<0.001) greater among the cows in the fescue group than the wheat groups; on the other hand, that of *tet*(O) was significantly (P=0.034) higher among the cows on the wheat group than calves in the tall fescue group (Table 3).

Overwhelming majority (84%) of ERY^r isolates carried *erm*(B). The prevalence of *erm*(B) was significantly (P=0.047) higher among the wheat isolates than the fescue group. On the other hand, the prevalence of *msr*(C) was significantly (P=0.006) higher in the fescue isolates than in the wheat isolates. While *mef*(A) gene was detected only in cows that grazed wheat, *erm*(Q) was detected only in the calves that grazed on tall fescue (Table 3). Two ERY^r isolates were not positive for the genes targeted.

Distribution of resistance genes by *Enterococcus* species

Among TET^r enterococci isolates, over 50% (range: 50-95%) of the isolates in all species but *E. hirae* carried *tet*(M). However, 85% of *E. hirae* isolates carried either *tet*(L) or *tet*(O). The two *tet*(S) positive isolates were *E. casseliflavus* (Table 4). While all minor *Enterococcus* spp. harbored *erm*(B), 10% of *E. faecalis*, and 39% of *E. faecium* isolates were positive for *msr*(C) (Table 4).

Discussion

In the adult beef cows, the concentration and prevalence of TET^r enterococci were closely similar to that of the generic enterococci population suggesting the widespread occurrence and abundance of TET^r enterococci population in beef cows. On the other hand, the prevalence of ERY^r enterococci was 42%. Erythromycin resistant enterococci was enumerable only from a single animal suggesting that ERY^r enterococci occur at a low concentration in the absence of or low antibiotic selection pressure under extensive animal production such as cow-calf operation. This phenomenon of low abundance (enumerable from only five animals) but high prevalence (69%) was previously reported from a beef cow population. (Agga et al., 2016) Unlike dairy cattle production, in cow-calf operation calves comingle with the cows until weaning, adult cows can potentially transmit ARB to the calves, as shown in the present study by similar prevalence of both TET^r- and ERY^r- enterococci in the calves and cows (Table 1).

Once in the feedlot, the level of resistant bacteria would increase (Call et al., 2008), with subsequent carcass contamination; ERY^r- enterococci were detected from 88% of colon fecal samples of beef cattle at slaughter. (Vikram et al., 2017) When culled cows are sold and processed, ground beef contamination can occur. ERY^r- enterococci were detected from 38% and 48% of organic and conventional retail ground beef claims, respectively. (Schmidt et al., 2021) A large retrospective longitudinal analysis of ground beef obtained from retail

TABLE 3 Effects of age and pasture type on the prevalence (%) of tetracycline- and macrolide- resistance genes among tetracycline- and erythromycin- resistant enterococci identified from the feces of cow-calf production system.

Tetracycline resistant enterococci (n=361 isolates)					
Gene	**Total (n=361)**	**Calves**		**Cows**	
		Fescue (n=93)	**Wheat (n=81)**	**Fescue (n=93)**	**Wheat (n=94)**
tet(M)	69.8	77.4^{B}	75.3^{B}	54.8^{A}	72.3^{AB}
tet(L)	19.1	18.3^{AB}	12.3^{A}	34.4^{B}	10.6^{A}
tet(O)	10.3	3.2^{A}	12.3^{AB}	10.8^{AB}	14.9^{B}
tet(S)	0.6	0	0	0	2.1
Erythromycin resistant enterococci (n=113 isolates)					
Gene	Total (n=113)	Calves		Cows	
		Fescue (n=11)	Wheat (n=34)	Fescue (n=34)	Wheat (n=34)
erm(B)	84.1	72.7	91.2	76.5	88.2
msr(C)	11.5	18.2	5.9	23.5	2.9
mef(A)	1.2	0	0	0	5.9
erm(Q)	0.9	9.1	0	0	0

Different superscripted letters indicate significant differences, where shown.

markets in the USA showed 92.7% enterococcal contamination (Tyson et al., 2018).

Although the prevalence of TET^r- and ERY^r- enterococci did not significantly differ, concentration of TET^r enterococci was significantly higher in the cows than in the calves. Several studies indicated that the level of ARB decreases with age of the animal. (Call et al., 2008; Gaire et al., 2021) Evaluating the age effect requires a longitudinal study of following a cohort of calves over time. Since our study was cross sectional, the study could not evaluate age effect within the cohorts of the calves. Rather, this study compared the level of ARB between the calf and cow populations that were kept together. Monitoring the status of ARB in beef calves and the breeding cows with periodic fecal sampling and testing would help answer the age effect and define the baseline level in calves prior to entering the feedlot. Furthermore, previous studies reporting age effect were conducted in *E. coli*. Therefore, our study can be used as a baseline for Gram-positive bacteria such as enterococci population in beef cattle production.

Wheat grazing at weaning tends to reduce the concentration of TET^r enterococci in the calves compared to cows grazing on wheat. The mechanisms and benefits to calves grazing on wheat need to be explored for the mitigation of AMR, especially because calves go to feedlot for beef production, with food safety implication. However, we previously reported from the same cow-calf population that wheat grazing had no effect on the prevalence and concentration of TET^r *E. coli* (Agga et al., 2022b) suggesting the differential effect of wheat grazing on Gram-positive and Gram-negative bacteria.

The top three *Enterococcus* species identified among the generic- (*E. drans, E. mundtii followed by E. faecium*), ERY^r- (*E. faecalis, E. faecium and E. casseliflavus*) and TET^r- (*E. faecium, E. hirae and E. durans*) isolates (Table 2) represent the most frequently identified species from the GIT of humans and animals (Aarestrup et al., 2002; Dec et al., 2019). These top six species (*E. faecium* was shared among the three media types; *E. durans* was shared between generic and TET^r isolates) are among the most frequently identified retail meat isolates (Tyson et al., 2018), signifying their foodborne importance (Murray et al., 2022). Among the ground beef isolates, ERY- (2.4% in *E. faecalis*, and 7.5% in *E. faecium* isolates) and TET- (23% in *E. faecalis*, and 25% in *E. faecium* isolates) resistance was observed relatively less frequently compared to other retail meat types (Tyson et al., 2018). The top species isolated from the cow-calf population are also among the species isolated from cattle feces and feedlot environments, and cow-calf operations. (USDA, 2012; Zaheer et al., 2020) Furthermore, TET and macrolide resistance were the most prevalent phenotypes in *E. hirae*, *E. faecalis*, and *E. faecium* isolates obtained from feedlot cattle. (Zaheer et al., 2020) The present study notes that TET^r- isolates were less diverse than generic and ERY^r isolates, with fewer number of species identified and dominated by three species which accounted for 95% of the total TET^r isolates (Table 2).

Although age modified the effect of wheat grazing on the species distribution, *E. casseliflavus* was the only species that was detected at a significantly higher prevalence in the calves than the cows. The reason behind this species differential is unknown, but *E. casseliflavus* is among the most commonly isolated species, together with *E. faecalis* and *E. faecium* (Aarestrup et al., 2002), from insects which aid in the spread of enterococcal species (Macovei and Zurek, 2006). Another significant finding of the current study is the potential of wheat grazing to modify the gut microbiota. Wheat grazing significantly reduced the prevalence of generic- and ERY^r- *E. faecium* (in the cows), generic *E. mundtii* (in both cows and calves), ERY^r *E. faecalis* (calves). On the other hand, wheat grazing significantly increased the prevalence of both generic- (doubled in the wheat group) and TET^r- *E. durans*; ERY^r- *E. hirae*, -*E. avium*, and -*E. asini* were detected only from the wheat group. The findings suggest that wheat grazing modifies the microbiota of the gut, either by increasing or decreasing the proportion of specific bacterial species. The mechanisms for this effect and potential adaptation of wheat grazing to mitigate the

TABLE 4 Prevalence (%) of tetracycline- and macrolide- resistance genes, respectively among phenotypically tetracycline- and erythromycin-resistant *Enterococcus* species obtained from the feces of beef cow-calf production.

	% Of tetracycline resistance genes					% Of erythromycin resistance genes			
Species	**No. of isolates**	***tet*(M)**	***tet*(L)**	***tet*(O)**	***tet*(S)**	**Species**	**No. of isolates**	***erm*(B)**	***msr*(C)**
E. faecalis	7	71.4	28.6	0	0	*E. faecalis*	20	90	10
E. faecium	179	95.0	4.5	0.6	0	*E. faecium*	23	60.9	39.1
E. casseliflavus	6	50.0	16.7	0	33.3	*E. casseliflavus*	22	100	0
E. durans	59	86.4	5.1	8.5	0	*E. durans*	9	100	0
E. hirae	86	15.1	54.7	30.2	0	*E. hirae*	6	83.3	0
						E. asini	3	100	0
						E. avium	8	100	0

A single erm(Q) positive isolate and the two mef(A) positive isolates were not identified to species.
One *E. hirae* isolate was negative for all resistance genes tested.

major enterococci species, *E. faecium* and *E. faecalis*, in beef calf production need to be further investigated.

Tetracycline- and macrolide- resistance of the enterococci isolates obtained from the cow-calf population were conferred exclusively by *tet*(M), *tet*(L) and *tet*(O), and *erm*(B) and *msr*(C), respectively as reported in the literature. (Frye and Jackson, 2013; Agga et al., 2022c) However, the distribution of the three *tet* genes is more diverse in the cows than in the calves, where *tet*(M) predominates. The age dependence for *tet*(M) in conferring TET resistance in enterococci indicates the unique role that *tet*(M) plays to counter antibiotic selection pressure in the calves which require antibiotic treatments than the breeding beef cows due to bacterial infections before weaning age. Beef cows on the other hand are less susceptible to infections due to less physiologic demand as well as less infection density, as opposed to dairy cattle, thus do not require much antibiotic treatment, and mass therapy is not common (Call et al., 2008; USDA, 2012). Much like the species distribution, wheat grazing affected the distribution of the *tet* and MLS_B genes by either increasing [*tet*(O), *erm*(B)] or decreasing [*tet*(L), *msr*(C)] their prevalence. Wheat grazing had a similar effect on resistance gene distribution among TET^r and third generation cephalosporin resistant *E. coli* isolates characterized from the same cow-calf populations (Agga et al., 2022b). These findings suggest that wheat grazing significantly affects the resistance gene distribution by favoring the expansion of bacterial population carrying certain genes, while suppressing bacterial population carrying other genes despite conferring the same phenotypic resistance.

Conclusions

The study detected erythromycin, a macrolide antibiotic categorized as high priority critically important for medical use, resistant *Enterococcus* species of significant public health importance in two-fifths of the fecal samples obtained from cow-calf production system. Tetracycline resistant *Enterococcus* species were abundant and widespread in the cow-calf populations. The study reported that wheat grazing, either alone or through its interaction with the age of the animal, affected the abundance of TET^r enterococci, enterococcal species and resistance gene distributions. Calves had a significantly lower abundance of tetracycline resistant enterococci, and *tet*(M) plays a major role in conferring tetracycline resistance among the calf isolates, with more diverse tetracycline resistance genes being detected in the cows. The study suggests that AMR can persist in food animal production systems with less antibiotic selective pressure such as cow-calf production. Further studies are needed to harness the dual benefit of wheat grazing to improve beef calf growth performance, and as a potential mitigation strategy to modify pathogenic and antimicrobial resistant bacteria pathogenic to humans such as enterococci prior to feedlot operation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Western Kentucky University's Institutional Animal Care and Use Committee (IACUC# 17-09).

Author contributions

GA conceptualized the study, acquired funding, led the investigation, analyzed the data and wrote the manuscript. HG was involved in conceptualization, funding acquisition and supervising animal management. AN was involved in conceptualization, funding acquisition and field study. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the U.S. Department of Agriculture, Agricultural Research Service (Project No. 5040-12630-006-00D) and through a USDA-WKU cooperative agreement (Project Number: 5040-12630-006-30-S).

Acknowledgments

We thank Rohan Parekh for technical support. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frabi.2022.1052316/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Representative gel images from capillary electrophoresis for enterococci genus confirmation and species identification from cow-calf production system. The molecular sizes are approximated base pairs (bp). Genus= genus specific gene marker; FL= *E. faecalis*; FM= *E. faecium*; DU= *E. durans*; CA= *E. casseliflavus;* GA= *E. gallinarum;* MU= *E. mundtii;* AV= *E. avium;* HI= *E. hirae;* AS= *E. asini.*

SUPPLEMENTARY FIGURE 2
Representative gel images from capillary electrophoresis for tetracycline resistance genes from enterococci isolated from cow-calf production system. The molecular sizes are approximated base pairs (bp).

SUPPLEMENTARY FIGURE 3
Representative gel images from capillary electrophoresis for macrolide resistance genes from enterococci isolated from cow-calf production system. The molecular sizes are approximated base pairs (bp).

SUPPLEMENTARY TABLE 1
Primers used for PCR speciation of enterococci isolates obtained from the feces of cows and pre-weaned calves in cow-calf herds (Jackson et al., 2004).

SUPPLEMENTARY TABLE 2
Primers used for PCR detection of tetracycline resistance *(tet)* genes from phenotypically tetracycline resistant *Enterococcus* species isolated from the feces of cows and pre-weaned calves in cow-calf herds.

SUPPLEMENTARY TABLE 3
Primers used for PCR detection of macrolide resistance genes from phenotypically erythromycin resistant *Enterococcus* species isolated from the feces of cows and pre-weaned calves in cow-calf herds.

References

Aarestrup, F. M., Butaye, P., and Witte, W. (2002). Nonhuman reservoirs of enterococci. In *The Enterococci* (Eds. M.S. Gilmore, D.B. Clewell, P. Courvalin, G.M. Dunny, B.E. Murray, L.B. Rice, et al.) doi: 10.1128/9781555817923.ch2

Abdelfattah, E. M., Ekong, P. S., Okello, E., Chamchoy, T., Karle, B. M., Black, R. A., et al. (2021). Epidemiology of antimicrobial resistance (AMR) on California dairies: descriptive and cluster analyses of AMR phenotype of fecal commensal bacteria isolated from adult cows. *PeerJ* 9, e11108. doi: 10.7717/peerj.11108

Agga, G. E., Arthur, T. M., Durso, L. M., Harhay, D. M., and Schmidt, J. W. (2015). Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. *PLos One* 10 (7), e0132586. doi: 10.1371/journal.pone.0132586

Agga, G. E., Cook, K. L., Netthisinghe, A. M. P., Gilfillen, R. A., Woosley, P. B., and Sistani, K. R. (2019). Persistence of antibiotic resistance genes in beef cattle backgrounding environment over two years after cessation of operation. *PLos One* 14 (2), e0212510. doi: 10.1371/journal.pone.0212510

Agga, G. E., Couch, M., Parekh, R. R., Mahmoudi, F., Appala, K., Kasumba, J., et al. (2022a). Lagoon, anaerobic digestion, and composting of animal manure treatments impact on tetracycline resistance genes. *Antibiotics* 11 (3), 391. doi: 10.3390/antibiotics11030391

Agga, G. E., Galloway, H. O., Netthisinghe, A. M. P., Schmidt, J. W., and Arthur, T. M. (2022b). Tetracycline- resistant, third generation cephalosporin- resistant, and extended spectrum beta-lactamase- producing *Escherichia coli* in a beef cow-calf production system. *J. Food Prot* 85 (11), 1522–1530. doi: 10.4315/JFP-22-178

Agga, G. E., Schmidt, J. W., and Arthur, T. M. (2016). Antimicrobial-resistant fecal bacteria from ceftiofur-treated and nonantimicrobial-treated comingled beef cows at a cow-calf operation. *Microb. Drug Resist.* 22 (7), 598–608. doi: 10.1089/mdr.2015.0259

Agga, G. E., Silva, P. J., and Martin, R. S. (2022c). Tetracycline and macrolide resistant *Enterococcus* species isolated from mink feces and feed samples in the united states. *Microbial. Drug Resistance* 28 (6), 734–743. doi: 10.1089/mdr.2021.0438

Ahmed, M. O., and Baptiste, K. E. (2018). Vancomycin-resistant enterococci: A review of antimicrobial resistance mechanisms and perspectives of human and animal health. *Microbial. Drug Resistance* 24 (5), 590–606. doi: 10.1089/mdr.2017.0147

Arias, C. A., and Murray, B. E. (2012). The rise of the *Enterococcus*: Beyond vancomycin resistance. *Nat. Rev. Microbiol.* 10 (4), 266–278. doi: 10.1038/nrmicro2761

Call, D. R., Davis, M. A., and Sawant, A. A. (2008). Antimicrobial resistance in beef and dairy cattle production. *Anim. Health Res. Rev.* 9 (2), 159–167. doi: 10.1017/S1466252308001515

Cho, S., Barrett, J. B., Frye, J. G., and Jackson, C. R. (2020). Antimicrobial resistance gene detection and plasmid typing among multidrug resistant enterococci isolated from freshwater environment. *Microorganisms* 8 (9), 1338. doi: 10.3390/microorganisms8091338

CLSI (2020). *Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100*. Wayne, PA: Clinical and Laboratory Standards Institute.

Davedow, T., Narvaez-Bravo, C., Zaheer, R., Sanderson, H., Rodas-Gonzalez, A., Klima, C., et al. (2020). Investigation of a reduction in tylosin on the prevalence of liver abscesses and antimicrobial resistance in enterococci in feedlot cattle. *Front. Veterinary Sci.* 7. doi: 10.3389/fvets.2020.00090

Deasy, B. M., Rea, M. C., Fitzgerald, G. F., Cogan, T. M., and Beresford, T. P. (2000). A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus*. *Syst. Appl. Microbiol.* 23 (4), 510–522. doi: 10.1016/S0723-2020(00)80025-9

Dec, M., Stepien-Pysniak, D., Gnat, S., Fratini, F., Urban-Chmiel, R., Cerri, D., et al. (2019). Antibiotic susceptibility and virulence genes in *Enterococcus i*solates from wild mammals living in Tuscany, Italy. *Microb. Drug Resist* 26 (5), 505–519. doi: 10.1089/mdr.2019.0052

Franzluebbers, A. J. (2007). Integrated crop–livestock systems in the southeastern USA. *Agron. J.* 99 (2), 361–372. doi: 10.2134/agronj2006.0076

Frye, J. G., and Jackson, C. R. (2013). Genetic mechanisms of antimicrobial resistance identified in *Salmonella enterica*, *Escherichia coli*, and *Enterococcus* spp. isolated from U.S. food animals. *Front. Microbiol.* 4. doi: 10.3389/fmicb.2013.00135

Gaire, T. N., Scott, H. M., Sellers, L., Nagaraja, T. G., and Volkova, V. V. (2021). Age dependence of antimicrobial resistance among fecal bacteria in animals: A scoping review. *Front. Vet. Sci.* 7. doi: 10.3389/fvets.2020.622495

Hoyle, D. V., Knight, H. I., Shaw, D. J., Hillman, K., Pearce, M. C., Low, J. C., et al. (2004). Acquisition and epidemiology of antibiotic-resistant *Escherichia coli* in a cohort of newborn calves. *J. Antimicrob. Chemother.* 53 (5), 867–871. doi: 10.1093/jac/dkh177

Jackson, C. R., Fedorka-Cray, P. J., and Barrett, J. B. (2004). Use of a genus-and species-specific multiplex PCR for identification of enterococci. *J. Clin. Microbiol.* 42 (8), 3558–3565. doi: 10.1128/JCM.42.8.3558-3565.2004

Kaiser, R. A., Polk, J. S., Datta, T., Parekh, R. R., and Agga, G. E. (2022). Occurrence of antibiotic resistant bacteria in urban karst groundwater systems. *Water* 14 (6), 960. doi: 10.3390/w14060960

Karp, B. E., Tate, H., Plumblee, J. R., Dessai, U., Whichard, J. M., Thacker, E. L., et al. (2017). National antimicrobial resistance monitoring system: Two decades of advancing public health through integrated surveillance of antimicrobial resistance. *Foodborne Pathog. Dis.* 14 (10), 545–557. doi: 10.1089/fpd.2017.2283

Lebreton, F., Willems, R. J. L., and Gilmore, M. S. (2014). "Enterococcus diversity, origins in nature, and gut colonization," in *Enterococci: From commensals to leading causes of drug resistant infection*. Eds. M. S. Gilmore, D. B. Clewell, Y. Ike and N. Shankar. (Boston: Massachusetts Eye and Ear Infirmary).

Macovei, L., and Zurek, L. (2006). Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. *Appl. Environ. Microbiol.* 72 (6), 4028–4035. doi: 10.1128/aem.00034-06

Murray, S. A., Holbert, A. C., Norman, K. N., Lawhon, S. D., Sawyer, J. E., and Scott, H. M. (2022). Effects of tylosin, a direct-fed microbial and feedlot pen environment on phenotypic resistance among enterococci isolated from beef cattle feces. *Antibiotics* 11 (1), 106. doi: 10.3390/antibiotics11010106

Netthisinghe, A., Galloway, H., DeGraves, F., Agga, G. E., and Sistani, K. (2020). Grain yield and beef cow–calf growth performance in dual-purpose and conventional grain wheat production systems and stockpiled tall fescue pasturing. *Agronomy* 10 (10), 1543. doi: 10.3390/agronomy10101543

Pandit, P. S., Williams, D. R., Rossitto, P., Adaska, J. M., Pereira, R., Lehenbauer, T. W., et al. (2021). Dairy management practices associated with multi-drug resistant fecal commensals and salmonella in cull cows: a machine learning approach. *PeerJ* 9, e11732. doi: 10.7717/peerj.11732

Poffenbarger, H. (2010). Ruminant grazing of cover crops: Effects on soil properties and agricultural production. *J. Natural Resour. Life Sci. Educ.* 39 (1), 49–39. doi: 10.4195/jnrlse.2010.0003se

Redmon, L. A., Horn, G. W., Krenzer, E. G.Jr., and Bernardo, D. J. (1995). A review of livestock grazing and wheat grain yield: Boom or bust? *Agron. J.* 87 (2), 137–147. doi: 10.2134/agronj1995.00021962008700020001x

Roberts, M. C., and Schwarz, S. (2016). Tetracycline and phenicol resistance genes and mechanisms: Importance for agriculture, the environment, and humans. *J. Environ. Qual.* 45 (2), 576–592. doi: 10.2134/jeq2015.04.0207

Schmidt, J. W., Vikram, A., Doster, E., Thomas, K., Weinroth, M. D., Parker, J., et al. (2021). Antimicrobial resistance in U.S. retail ground beef with and without label claims regarding antibiotic use. *J. Food Prot.* 84 (5), 827–842. doi: 10.4315/jfp-20-376

Scott, H. M., Acuff, G., Bergeron, G., Bourassa, M. W., Gill, J., Graham, D. W., et al. (2019). Critically important antibiotics: Criteria and approaches for measuring and reducing their use in food animal agriculture. *Ann. New York Acad. Sci.* 1441 (1), 8–16 doi: 10.1111/nyas.14058

Shipp, G. M., and Dickson, J. S. (2012). A longitudinal study of the establishment and proliferation of *Enterococcus* on a dairy farm. *Foodborne Pathog. Dis.* 9 (5), 425–430. doi: 10.1089/fpd.2011.0996

Tate, H., Li, C., Nyirabahizi, E., Tyson, G. H., Zhao, S., Rice-Trujillo, C., et al. (2021). A national antimicrobial resistance monitoring system survey of antimicrobial-resistant foodborne bacteria isolated from retail veal in the united states. *J. Food Prot.* 84 (10), 1749–1759. doi: 10.4315/jfp-21-005

Tyson, G. H., Nyirabahizi, E., Crarey, E., Kabera, C., Lam, C., Rice-Trujillo, C., et al. (2018). Prevalence and antimicrobial resistance of *Enterococci* isolated from retail meats in the united states 2002 to 2014. *Appl. Environ. Microbiol.* 84 (1), e01902–17. doi: 10.1128/AEM.01902-17

USDA (2012) *Beef 2007–08, antimicrobial drug use and antimicrobial resistance on U.S. cow-calf operations 2007–08* (Fort Collins, CO). Available at: https://www.aphis.usda.gov/animal_health/nahms/beefcowcalf/downloads/beef0708/Beef0708_ir_Antimicrobial_1.pdf (Accessed May 06, 2022). United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service, Veterinary Services, National Animal Health Monitoring System577.0212.

Vikram, A., Rovira, P., Agga, G. E., Arthur, T. M., Bosilevac, J. M., Wheeler, T. L., et al. (2017). Impact of "raised without antibiotics" beef cattle production practices on occurrences of antimicrobial resistance. *Appl. Environ. Microbiol.* 83 (22), e01682–e01617. doi: 10.1128/AEM.01682-17

Vikram, A., Miller, E., Arthur, T. M., Bosilevac, J. M., Wheeler, T. L., and Schmidt, J. W. (2018). Similar levels of antimicrobial resistance in U.S. food service ground beef products with and without a "raised without antibiotics" claim. *J. Food Prot.* 81 (12), 2007–2018. doi: 10.4315/0362-028x.Jfp-18-299

Winterholler, S., Lalman, D., Hudson, M., Ward, C., Krehbiel, C., and Horn, G. (2008). Performance, carcass characteristics, and economic analysis of calf-fed and wheat pasture yearling systems in the southern great plains. *Prof. Anim. Scientist* 24 (3), 232–238.

Zaheer, R., Cook, S. R., Barbieri, R., Goji, N., Cameron, A., Petkau, A., et al. (2020). Surveillance of enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a one-health continuum. *Sci. Rep.* 10 (1), 3937. doi: 10.1038/s41598-020-61002-5

frontiers | Frontiers in Antibiotics

TYPE Original Research
PUBLISHED 21 December 2022
DOI 10.3389/frabi.2022.1055507

OPEN ACCESS

EDITED BY
Kebede Amenu,
Addis Ababa University, Ethiopia

REVIEWED BY
Abebe Aseffa Negeri,
Ethiopian Public Health Institute, Ethiopia
Mashkoor Mohsin,
University of Agriculture, Faisalabad, Pakistan

*CORRESPONDENCE
Royford Magiri
roybundimag@gmail.com

SPECIALTY SECTION
This article was submitted to Antibiotic Resistance, a section of the journal Frontiers in Antibiotics

RECEIVED 27 September 2022
ACCEPTED 07 December 2022
PUBLISHED 21 December 2022

CITATION
Magiri R, Dissanayake C and Okello W (2022) Antimicrobial consumption in food animals in Fiji: Analysis of the 2017 to 2021 import data. *Front. Antibiot.* 1:1055507. doi: 10.3389/frabi.2022.1055507

Antimicrobial consumption in food animals in Fiji: Analysis of the 2017 to 2021 import data

Royford Magiri[1]*, Chaminda Dissanayake[2] and Walter Okello[3]

[1]Fiji National University, College of Agriculture, Fisheries and Forestry, Nausori, Fiji, [2]Animal Section, Biosecurity Authority of Fiji (BAF), Suva, Fiji, [3]Commonwealth Scientific and Industrial Research Organization, Black Mountain Science and Innovation Park, Acton, ACT, Australia

Introduction: Globally, the demand for animal protein for human consumption has beenQ7 Q6increasing at a faster rate in the last 5 to 10 decades resulting in increasedantimicrobial consumption in food producing animals. Antimicrobials arefrequently used as part of modern methods of animal production, which mayput more pressure on evolution of antibiotic resistant bacteria. Despite theserious negative effects on animal and human health that could result fromusing antibiotics, there are no assessment of antimicrobials consumed by thelivestock sector in Fiji as well as other Pacific Island Countries. The objective ofthis study was to quantify antimicrobials imported for consumption in foodanimals into Fiji from 2017 to 2021.

Methods: Data on imported antimicrobials, whichwere finished products, was obtained from Biosecurity Authority Fiji (BAF).Imported antimicrobials were then analyzed by antimicrobial class, andimportance to veterinary and human medicine.

Results: An average of 92.86 kg peryear (sd = 64.12) of antimicrobials as a net weight was imported into Fiji in the2017-2021 study period. The mean amount of imported active antimicrobialingredients after adjusting for animal biomass was 0.86 mg/kg (sd = 0.59). Fromthe total antimicrobial imports during the years 2017 to 2021, penicillins(69.72%) and tetracycline (15.95%) were the most imported antimicrobialclasses. For animal health 96.48% of the antimicrobial imports wereveterinary critically important antimicrobials. For human healthfluroquinolones, macrolides, aminoglycosides, and penicillins were theimported critically important antimicrobials.

Discussion: The study concluded that use ofantimicrobials in food producing animals is low but monitoring of antimicrobialconsumption and antimicrobial resistance was critical in Fiji due to overrelianceon critically important antimicrobials.

KEYWORDS

antimicrobial resistance, antimicrobial consumption, Fiji, animal biomass, imported antimicrobials, animal

Introduction

Antimicrobial resistance (AMR), which is considered a One Health problem as it occurs between humans, animals, plants, and the ecosystem, has emerged as one of the major global health threats (Prestinaci et al., 2015; Léger et al., 2021). AMR is linked to misuse (i.e., under or overuse) of antimicrobials in humans and animals. Antimicrobials are frequently utilized in food animals to promote growth and prevent and treat animal diseases (Schwarz et al., 2001; McEwen and Fedorka-Cray, 2002; Landers et al., 2012). The prudent use of antimicrobial agents in food producing animals is necessary to prevent the development and spread of antimicrobial resistance between animals and human (Anthony et al., 2001; Lekshmi et al., 2017; Aidara-Kane et al., 2018). However, indiscriminate use of antimicrobials in food producing animals leads to emergence of antimicrobial resistant microorganisms by way of natural selection and can result in decreased benefits gained from antimicrobial effectiveness over time (Cooper and Okello, 2021). Despite this challenge, no previous studies have been conducted on antimicrobial consumption (AMC) in human and animals in Fiji and the pacific. Antimicrobial resistant organisms of animal origin are transmitted to human *via* environment, consumption of animal food products and to animal health worker through direct contact with animals (Economou and Gousia, 2015; Founou et al., 2016; Graham et al., 2019). Human intestines may become colonized with animal-derived, drug-resistant bacteria like *Escherichia coli* and *Enterococcus* species (Phillips et al., 2004; Rousham et al., 2018). People who are frequently exposed, such as those who work in slaughterhouses, food establishments, and farms where animals are fed antibiotics, are more likely to develop resistance to *E. coli* than the general public (Van den Bogaard et al., 2001). There has been a noticeable surge in the appearance of resistant food pathogens such as *Salmonella* spp., *Campylobacter* spp., and other bacteria thought to be markers of AMR as a result of increased usage of antimicrobial drugs in food animals (Sanchez et al., 2002; Elhadidy et al., 2020). Furthermore, repeated exposure to low doses of antimicrobial drugs when used as growth-promoters or for prophylactic treatment in livestock production results in the development of ideal conditions for the emergence and spread of AMR organisms in animals (Chantziaras et al., 2014). To further exacerbate the problem of AMR in developing countries, consumption of antimicrobials in animals is set to increase exponentially over the coming decades particularly in low and middle income countries (Klein et al., 2018; Van Boeckel et al., 2019). Increased AMC in low and middle income countries is partly due to rising incomes resulting in increased demand for animal protein which necessitate the use of antimicrobials to increase livestock productivity (Rushton, 2015; Kirchhelle, 2018; Manyi-Loh et al., 2018).

The unprecedented increase in AMR has led to the development of a global strategy which includes monitoring of AMC in animals (Schar et al., 2018; Munkholm and Rubin, 2020). Monitoring of AMC enables detection of risk factors as well as understanding temporal association between AMC and AMR (Page and Gautier, 2012). Such analysis provides evidence for the development of policies for managing AMR both in human and animal health (Ferreira, 2017). Furthermore, some of the antimicrobials used in food producing animals are also used in humans to treat common infections hence development of resistance in animal has a great economic impact on human health (Magouras et al., 2017). At the global level, the World Organization for Animal Health (WOAH), founded as the Office International des Epizooties (OIE), has documented harmonized guidelines for AMC monitoring which includes sources of AMC data such as import data, sales, manufacturing, and farm use data (World Organisation for Animal Health, 2020a). Additionally, WOAH and the World Health Organization (WHO) have documented antimicrobial agents of veterinary and human health importance respectively (World Health Organization, 2019; World Organisation for Animal Health, 2021). Although, some countries have been collecting data on AMC, this has mostly been done in developed countries (Grave et al., 2010; Hosoi et al., 2013; Hillerton et al., 2017). Low and middle income countries face numerous challenges such as lack of data on antimicrobial use (AMU) mostly due to limited veterinary services (Tiseo et al., 2020).

Fiji is one of the Pacific Island countries in the Oceania region with the majority of the population depending on subsistence agriculture and keeps several livestock species such as cattle, chicken, sheep, and goat. The country has three hundred islands, but majority of the population lives in two main islands namely Viti Levu and Vanua Levu. Livestock keeping in Fiji is important as it is a source of income, protein, and weed control. According to the 2020 agricultural census, there were 119,691 cattle, 37,435 sheep, 143,853 goats, and 1,412, 901 chicken (Ministry of Agriculture, 2020). Despite the importance of livestock, there has been limited studies on animal diseases with brucellosis, and bovine tuberculosis being the most studied (Tukana et al., 2016; Borja et al., 2018). Additionally, the prevalence of AMR in food animals in Fiji remain unknown (Magiri et al., 2022). Lack of information on animal health issues in Fiji could be limited due to limited veterinary services; animal health providers have also been found to have limited knowledge on AMR (Khan et al., 2022a; Khan et al., 2022b).

The aim of this study is to address the gaps in understanding AMC in food animals in Fiji at the national level using antimicrobials imported between 2017 and 2021. The imported antimicrobials are described according to their antimicrobial class and their importance in veterinary and

human medicine. The findings can be useful for risk analysis and planning, evaluation of cost-effectiveness of initiatives to promote prudent antimicrobial usage, and development of strategies to reduce AMR.

Materials and methods

Data collection and characterization of imported antimicrobials

The data on imported antimicrobials between 2017 and 2021 was obtained, after seeking approval, directly from the official records of the Biosecurity Authority of Fiji (BAF). The BAF is a Public Enterprise under the Public Enterprises Act 2019 tasked with managing quarantine control at the Fiji border and provision of import and export inspection and certification. The Database of the imported antimicrobials for veterinary use contained name of importer, date of importation, active ingredients imported as finished products, package sizes, and antimicrobial chemical compound, and represents a tier 1 distribution system. All veterinary drugs imported into Fiji including antimicrobials have to be registered by BAF. Only the veterinary antimicrobials import data was obtained from BAF. The data was screened for quantity imported, recommendation for use in food animals, name of active ingredient, and concentration of active ingredient. Characterization of the extracted data was done using OIE list of antimicrobials of veterinary importance and the WHO list of antimicrobials of human health importance (World Health Organization, 2019; World Organisation for Animal Health, 2021). Also, the data was stored in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Animal biomass estimation

Animal biomass, which was the total number of food animals in Fiji in tons, was estimated from animal population, animal slaughter, quantity of meat produced, carcass weight, and live animal weight data with cattle, sheep, goats, pigs, and chicken being the major focus as they are the most consumed in Fiji. Regarding livestock population, the 2009 and 2020 agricultural census (Ministry of Agriculture, 2009; Ministry of Agriculture, 2020) was first used to estimate the annual population growth rate using the equation below.

$$r = (P_2/P_1)^{\frac{1}{y}} - 1 \quad (1)$$

Where r is annual growth rate of a particular livestock species, P_2 is the present livestock population (i.e., 2020) for a particular livestock species (e.g., cattle, sheep, goat, pigs, or chicken), P_1 is the past livestock population (i.e., 2009) livestock population for a particular livestock species, y is the number of years between the present and past years which was 11 years in this case. Number of animals slaughtered, and quantity of meat produced was obtained from Fiji meat industry report (Fiji meat industry board, 2016). However, number of chickens slaughtered, and quantity produced was obtained from FAOSTAT as this information was not available in the Fiji livestock industry report (Food and Agriculture Organization of the United Nations). Carcass weight was estimated by dividing total weight of animals slaughtered with total number of animals slaughtered whereas live weight was estimated by dividing carcass weight by conversion coefficient for a particular livestock species; cattle, sheep, goat, pig, and chicken conversion factors used in this study were 0.7, 0.47, 0.47, 0.78, and 0.7 respectively (Eurostat, 2009). The total animal biomass from 2017 to 2021 was calculated as described by the OIE (Góchez et al., 2019) except for cattle which was calculated by multiplying live weight with the cattle population due to lack of data on proportion of livestock slaughtered and quantity of meat for different age groups. More information on the animal biomass calculations can be found in the Supplementary Material.

Data analysis

To obtain the quantity of imported antimicrobials, the amount of each antimicrobial agent (chemical compound as declared in import permit) per package was calculated first, and the result subsequently multiplied by the number of packages imported to obtain the overall amount of antimicrobial agent, which was converted to kilograms as per the OIE recommendation (World Organisation for Animal Health, 2020b). Equation 2 was used to calculate the total amount (first as milligram then converted to grams) of antimicrobial agent in a container (e.g., bottles and syringes).

$$\begin{aligned} &Total\ amount\ of\ antimicrobial\ agent\ in\ a\ container\ (g) \\ &= \left(strength\ \left(\frac{mg}{ml}\right) \times container\ size\ (ml)\right)/1000 \end{aligned} \quad (2)$$

Where mg is milligram and ml is milliliter.

Afterwards, the content of the antimicrobial agent per package was calculated using Equation 3.

$$\begin{aligned} &Content\ of\ antimicrobial\ agent\ per\ package\ (g) \\ &= Total\ amount\ of\ antimicrobial\ agent\ in\ a\ container\ (g)\ x\ number\ of\ packs \end{aligned} \quad (3)$$

The number of packs were 4, 6, 10, 12, and 20. However, some importers occasionally imported single units.

Equation 4 was used to calculate the total amount of antimicrobial agent in a blister or a strip.

$$Content\ of\ antimicrobial\ agent\ per\ blister\ pack\ (g) = (strength\ per\ tablet\ (mg)\ x\ number\ of\ blisters\ x\ number\ of\ tablets\ in\ in\ each\ blister)/1000 \quad (4)$$

For antimicrobial agents that were reported using international units (UI) such as penicillin for intramuscular injection, conversion factors were used to convert this into mg/ml (World Organisation for Animal Health, 2020b). Equations 2 and 3 were then used to derive the content of antimicrobial agent per package. All weights of the imported active antimicrobial ingredients were expressed in kilograms except when adjusting for animal biomass which was done in milligrams. Tables S1, S2 in the Supplementary Materials show how the antimicrobial quantities for each antimicrobial agent was derived. The antimicrobials were mostly imported from Australia, New Zealand, India, and United Kingdom.

Antimicrobials used in food animals was adjusted for the relevant animal biomass by dividing antimicrobial agents imported in milligrams (mg) by the total animal biomass in kg (Góchez et al., 2019). The standard weight for sheep and goats used in this study for calculating their biomass was 37.5 kilograms (Galal, 2005). Trend analysis was done using Mann Kendall test in R Software (package = Kendall) to determine whether time series of the imported antimicrobials had an upward or downward monotonic trend (McLeod, 2022). However, the trend analysis is not the best form of presenting a data of a very short period. The hypothesis was that there was a trend in the imported antimicrobials. Apart from determining quantities of antimicrobials imported and their trend, antimicrobials of both veterinary and human importance were quantified between 2017 and 2021. Data analysis was done using R Software (R Core Team, 2022).

Results

A total of 464.31 kg of active antimicrobial agents (Table 1), which were all finished products, was imported to Fiji between 2017 and 2021 for use in food animals (mean = 92.86 kg per year, standard deviation (sd) = 64.12 kg per year). Notably, all antimicrobials for use in animals, were recorded by BAF at the point of entry. The annual quantities and antimicrobial classes imported over the study period is as shown in Table 1. We assumed that no antimicrobial that entered the country through Illegal route of importation which is usually a major problem in developing countries. The mean amount of imported antimicrobials after adjusting for animal biomass was 0.86 mg/kg (sd = 0.59). Additionally, the mean amount of imported antimicrobials after adjusting for animal biomass in 2017, 2018, 2019, and 2021 was 1.3 mg/kg, 1.3 mg/kg, 1.2 mg/kg, and 0.2 mg/kg respectively (Figure 1). The antimicrobial chemical compound names of the imported finished products included gentamycin sulphate, cephalothin sodium, cephazolin sodium,

TABLE 1 Annual quantities of antimicrobials imported in Fiji between 2017 and 2021.

Imported antimicrobial agents (class, sub-class)	Annual quantities of imported active antimicrobial agents (kg, %)					
	2017	2018	2019	2020	2021	Total
Aminoglycosides	0.29 (0.22)	0 (0)	0.16 (0.10)	0.14 (0.73)	1.39 (5.05)	1.98 (0.43)
Cephalosporins						
First-generation cephalosporin	0.83 (0.64)	0.1 (0.01)	0.43 (0.28)	0.08 (0.42)	0.60 (2.18)	1.94 (0.42)
Second-generation cephalosporin	0 (0)	0 (0)	0.01 (0.01)	0 (0)	0 (0)	0 (0)
Quinolones						
Fluoroquinolone	0.01 (0.01)	0.01 (0.01)	0.1 (0.07)	0 (0)	0.01 (0.04)	0.13 (0.03)
Lincosamides	14.25 (10.97)	0 (0)	0.04 (0.03)	0.03 (0.16)	0.05 (0.18)	14.36 (3.09)
Macrolides	0.02 (0.02)	28.75 (21.44)	0.01 (0.01)	0 (0)	0 (0)	28.78 (6.20)
Penicillins	103.16 (79.40)	101.84 (75.95)	94.33 (61.43)	13.63 (70.92)	10.78 (39.19)	323.73 (69.72)
Sulfonamides	1.85 (1.42)	0 (0)	1.35 (0.88)	3.29 (17.12)	12.57 (45.69)	19.06 (4.11)
Tetracyclines	9.49 (7.30)	3.48 (2.60)	57.06 (37.16)	2.06 (10.72)	1.97 (7.16)	74.06 (15.95)
Nitroimidazoles	0.03 (0.02)	0 (0)	0.08 (0.05)	0 (0)	0.15 (0.55)	0.26 (0.06)
Total	129.93 (27.98)	134.08 (28.87)	153.56 (33.07)	19.22 (4.13)	27.51 (5.92)	464.31 (100)

cefuroxime sodium, ciprofloxacin hydrochloride, norfloxacin, lincomycin hydrochloride monohydrate, erythromycin, penicillin G procaine, silver sulfadiazine, sulfamethoxazole, tetracycline hydrochloride, and metronidazole. Trend analysis revealed that there was no significant increasing or decreasing trend in the antimicrobials imported between 2017 and 2021 (test statistic: -0.20; p-value: 0.80).

A total of 13 antimicrobial active ingredients (namely gentamycin, cephalothin, cephazolin, cefuroxime, ciprofloxacin, norfloxacin, lincomycin, erythromycin, penicillin, sulfadiazine, sulfamethoxazole, tetracycline, and metronidazole belonging to nine antimicrobial classes (namely aminoglycosides, cephalosporins, quinolones, lincosamides, macrolides, penicillins, sulfonamides, tetracycline, and nitroimidazoles) was reported. Also, screening of the antimicrobial agents imported revealed that no nitrofuran was imported during the study period. Analysis of the imported antimicrobial agents between 2017 and 2021 revealed that 69.72% of the total imported antimicrobials within the study period were penicillins (Table 1). Another commonly imported antimicrobials were tetracyclines (15.95%); penicillins and tetracyclines comprised 85.64% of the total imported antimicrobials between 2017 and 2021.

Analysis of the imported antimicrobial agents according to animal health importance revealed that penicillins (72.30%) were the top veterinary critically important antimicrobials during the years 2017 to 2021 followed by tetracyclines (16.54%) (Table 2). Critically important antimicrobial agents in animal health are the limited agents available to treat serious infections in animals. The definition of clinically important antimicrobials is similar in animal health, but the serious infections include those from non-human sources. In human health, penicillins are regarded as critically important, high priority antimicrobials. Tetracycline was the second most imported veterinary critically important antimicrobial (16.54%) (Table 2). However, in human health, tetracycline is not regarded as a critically important antimicrobial. Other antimicrobial agents of both veterinary and medical critical importance imported in Fiji between 2017 and 2021 included fluoroquinolones and macrolides (Table 2). Results for highly important antimicrobial for animal use, revealed that linconsamides were the most imported (Table 2). No veterinary important antimicrobial was imported during the studied period.

Discussion

To the best of our knowledge, this is the first study in Fiji and within the broader Pacific Island countries to describe imported antimicrobial agents for food animals using international guidelines. Fiji imports all antimicrobials therefore this study was an important proxy for understanding AMC in animal health at the national level; obtaining data on AMU at the farm level or retail is challenging due to lack of records. The study also forms a baseline for analyzing future trends in AMC in food animals in Fiji and the Pacific.

The quantity of antimicrobials imported for use in food animals, adjusted for animal biomass, in Fiji was found to be 0.86 mg/kg on average compared to an average consumption of 237.72mg/kg in Oceania, Asia, and Far East, and a global average of 144.39 mg/kg antimicrobials in livestock (World Organisation for Animal Health, 2020b). Equally, in New Zealand, which is one of the countries in Oceania, AMC in food animals was found

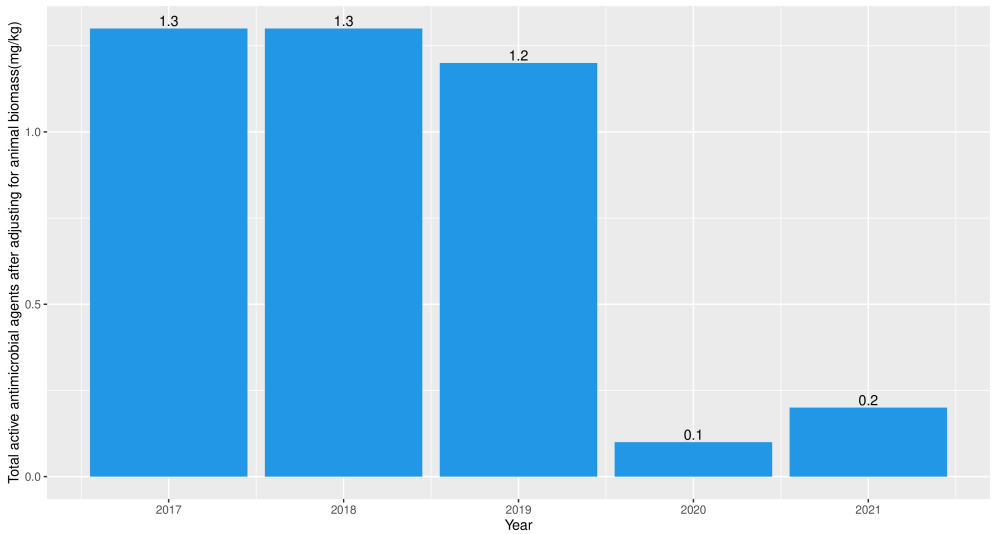


FIGURE 1
Antimicrobial import weight (mg) adjusted by animal biomass (kg) into Fiji between 2017 and 2021.

to be 9.4mg active ingredient/kg biomass (Hillerton et al., 2017). In Pakistan, AMC was found to be 10.05 mg/kg of the cumulative animal biomass, while in sub-Saharan Africa, it was found to be 5.24 ± 1.40 mg/population correction unit (Mouiche et al., 2020; Umair et al., 2022). Studies in Timor-Leste, which is a low and middle income country with a relatively similar agricultural system like Fiji, AMC in food animals was reported to be 0.55 mg/kg after adjusting for animal biomass (Ting et al., 2021).

The low consumption of antimicrobials in Fiji could be due to several factors such as low livestock population, relatively low occurrence of animal diseases, and less intensified livestock production systems. However, further studies are required in Fiji to determine the prevalence of animal diseases including farming practices especially AMU. A past study showed that farmers knowledge of AMR in Fiji is low (Khan et al., 2021; Khan et al., 2022a; Khan et al., 2022b). Another important observation on the quantities of imported antimicrobial agents, was the sharp decrease of imported antimicrobials in 2020 and 2021. The COVID-19 pandemic could be responsible for this decrease as Fiji relies on imported antimicrobials. This also shows how vulnerable Pacific Island Countries are to external shocks such as pandemics which may affect food security (Singh et al., 2022).

Analysis of the imported antimicrobial agents according to their importance in veterinary and human medicine, revealed that most antimicrobials imported for consumption in food animals are considered to be veterinary critically important; of the total antimicrobials imported for veterinary use between 2017 and 2021, 96.48% were veterinary critically important. This requires Fiji to judiciously use antimicrobials for food production to prevent a high risk of AMR occurrence which would render the antimicrobials ineffective and ultimately resulting in food insecurity. Furthermore, this study found that penicillins and tetracyclines are the most commonly imported antibiotics indicating overreliance on broad-spectrum antibiotics for treatment. Penicillins and tetracyclines are commonly used by farmers in developing countries due to their low cost and broad-spectrum antimicrobial activity (Beyene et al., 2015). Importation of fluroquinolone, which pose higher risk to public health regarding, and macrolides and penicillins, both of which pose limited risk to public health, need to be monitored in Fiji to prevent AMR occurrence in humans in Fiji. Monitoring for AMR is therefore a recommendation based on the study findings. A positive finding was that nitrofuran was not imported into Fiji during the study period. Several toxicological studies have revealed that nitrofuran drugs may have carcinogenic properties posing a major public health risk; use of nitrofurans in food animals has been banned by the European Union (McCalla, 1979; Antunes et al., 2006).

The study had limitations and challenges. First, data on AMC in the livestock sector in Fiji and the broader Pacific Island Countries is limited due to both the lack of comprehensive government level surveillance systems resulting from shortage of veterinarians and the reluctance of livestock industry (food animal producers and animal feed producers) to give the

TABLE 2 Total quantities (in kg) of antimicrobials imported in Fiji (2017-2021) according to animal and human health importance.

Imported antimicrobial agents (class, sub-class)	Total imported active antimicrobial agents according to veterinary importance (kg, %)		
	Veterinary critically important	Veterinary highly important	Veterinary important
Aminoglycosides[2]	1.98(0.44)	–	–
Cephalosporins			
First-generation cephalosporin	–	1.94(11.89)	
Second-generation cephalosporin	–	0.01(0.06)	
Quinolones			
Fluoroquinolones[1]	0.13(0.02)	–	
Lincosamides	–	14.36(88.05)	
Macrolides[1]	28.78(6.43)	–	–
Penicillins[2]	323.73(72.30)	–	–
Sulfonamides	19.06(4.26)	–	–
Tetracyclines	74.06(16.54)	–	–
Total	447.73(96.48)	16.31(3.52)	–

[1]Critically important, highest priority antimicrobial agent in human health.
[2]Critically important, high priority antimicrobial agent in human health.

comprehensive reports on antimicrobial consumption. In this study, data was from imported antimicrobials which represent a tier 1 distribution system. Imported antimicrobials data (tier 1 systems) may over or underestimate the actual quantities of antimicrobials consumed compared to data obtained from either retailers, veterinarians, or producers. However, farmers, veterinarians, retailers, and producers do not regularly keep data on AMU due to insufficient enforcement by regulatory authorities in Fiji (Magiri et al., 2022). Therefore, this study assumed that data on imported antimicrobials can be the best proxy for ascertaining quantities of antimicrobials consumed by food animals in Fiji nationally. Second, there was difficulty in obtaining parameters for estimating animal biomass (e.g., annual livestock population, number of livestock slaughtered, quantities of meat etc.). Livestock census in Fiji is done every ten years but the actual number of livestock per year is usually unavailable. This study mostly relied on country available data rather than FAOSTAT as these were deemed to be more reliable; FAOSTAT uses imputation methods to estimate number of livestock slaughtered and quantities of meat harvested. Additionally, the OIE estimation of AMC globally, relies on European parameters (e.g., standard weights) which could slightly overestimate animal biomass. Parameters that closely represented Fiji agricultural production systems was used in this study to enable accurate estimation of the animal biomass.

In conclusion, this study found that AMC in food animals is relatively low in Fiji possibly due to the subsistence nature of livestock production and low livestock population. However, overreliance on antimicrobials of last resort for livestock production as well as importation of antimicrobials of critical importance to human health warrant regular monitoring of AMU and AMR in Fiji for food security and protection of public health. The current Australia Centre for International Agricultural Research (ACIAR) funded AMR project is aimed at addressing some of the gaps in managing AMR in the region. The project is the first to adopt the One-Health approach to research into AMR in humans, animals and the environment in the Pacific region.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by CSIRO Health and Medical Human Research Ethics Committee (CHMHREC) approval 2020_113_RR as well as Fiji Human Health Research and Ethics Review Committee (FNHRERC number 25/2020).

Author contributions

Equal contribution: RM, CD, and WO contributed equally to this work. RM: Conceived the idea, analyzed the data, and edited manuscript. CD: Collected and analyzed the data. WO: Analyzed the data, edited the manuscript and provided resources for publication. All authors contributed to the article and approved the submitted version.

Funding

The study was funded by the Australian Centre for International Agricultural Research (ACIAR), project number LS/2019/119.

Acknowledgments

The authors acknowledge the research support and guidance provided by the Commonwealth Scientific and Industrial Research Organization to Fiji National University to sustainably enhance the management of AMR in Fiji. The authors acknowledge BAF for agreeing to provide the valuable data that underpinned this study. Also, the authors acknowledge ACIAR for funding this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frabi.2022.1055507/full#supplementary-material

References

Aidara-Kane, A., Angulo, F. J., Conly, J. M., Minato, Y., Silbergeld, E. K., McEwen, S. A., et al. (2018). World health organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. *Antimicrobl Resist.Infect. Control* 7 (1), 1–8. doi: 10.1186/s13756-017-0294-9

Anthony, F., Acar, J., Franklin, A., Gupta, R., Nicholls, T., Tamura, Y., et al. (2001). Antimicrobial resistance: responsible and prudent use of antimicrobial agents in veterinary medicine. *Rev. Sci. Tech. – Off. Int. Epizoot.* 20 (3), 829–848. doi: 10.20506/rst.20.3.1318

Antunes, P., Machado, J., and Peixe, L. (2006). Illegal use of nitrofurans in food animals: contribution to human salmonellosis? *Clin. Microbiol. Infect.* 2 (11), 1047–1049. doi: 10.1111/j.1469-0691.2006.01539.x

Beyene, T., Endalamaw, D., Tolossa, Y., and Feyisa, A. (2015). Evaluation of rational use of veterinary drugs especially antimicrobials and anthelmintics in bishoftu, central Ethiopia. *BMC Res. Notes* 8, 482. doi: 10.1186/s13104-015-1466-4

Borja, E., Borja, L. F., Prasad, R., Tunabuna, T., and Toribio, J-A.L.M.L. (2018). A retrospective study on bovine tuberculosis in cattle on Fiji: Study findings and stakeholder responses. *Front. Vet. Sci.* 5. doi: 10.3389/fvets.2018.00270

Chantziaras, I., Boyen, F., Callens, B., and Dewulf, J. (2014). Correlation between veterinary AMU and antimicrobial resistance in food-producing animals: A report on seven countries. *J. Antimicrob. Chemother.* 69, 827–834. doi: 10.1093/jac/dkt443

Cooper, B., and Okello, W. O. (2021). An economic lens to understanding antimicrobial resistance: disruptive cases to livestock and wastewater management in Australia. *Aust. J. Agric. Econ.* 65 (4), 900–917. doi: 10.1111/1467-8489.12450

Economou, V., and Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect. Drug Resist.* 8, 49–61. doi: 10.2147/IDR.S55778

Elhadidy, M., Ali, M. M., El-Shibiny, A., Miller, W. G., Elkhatib, W. F., Botteldoorn, N., et al. (2020). Antimicrobial resistance patterns and molecular resistance markers of campylobacter jejuni isolates from human diarrheal cases. *PloS One* 15 (1), e0227833. doi: 10.1371/journal.pone.0227833

Eurostat (2009) *Manual for the compilation of supply balance sheets for meat*. Available at: https://circabc.europa.eu/sd/a/90447c6f-5b7c-4b6f-87e9-27c5a7a5c923/ASA-TE-F-55%2520SBS%2520Manual%2520-%2520meat.doc (Accessed August 19, 2022). (2009).

Ferreira, P. J. (2017). Why antibiotic use data in animals needs to be collected and how this can be facilitated. *Front. Vet. Sci.* 4. doi: 10.3389/fvets.2017.00213

Fiji meat industry board (2016) *Annual report*. Available at: https://www.parliament.gov.fj/wp-content/uploads/2021/12/181-Fiji-Meat-Industry-Board-Annual-Report-2016-1.pdf (Accessed August 18, 2022).

Food and Agriculture Organization of the United Nations *Livestock primary in Fiji: Producing animals/slaughtered*. Available at: http://www.fao.org/faostat/en/#data/QL (Accessed August 18, 2022).

Founou, L. L., Founou, R. C., and Essack, S. Y. (2016). Antibiotic resistance in the food chain: a developing country-perspective. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.01881

Galal, S. (2005). Biodiversity in goats. *Small Rumin. Res.* 60 (1-2), 75–81. doi: 10.1016/j.smallrumres.2005.06.021

Góchez, D., Raicek, M., Pinto Ferreira, J., Jeannin, M., Moulin, G., and Erlacher-Vindel, E. (2019). OIE annual report on antimicrobial agents intended for use in animals: Methods used. *Front. Vet. Sci.* 6. doi: 10.3389/fvets.2019.00317

Graham, D. W., Bergeron, G., Bourassa, M. W., Dickson, J., Gomes, F., Howe, A., et al. (2019). Complexities in understanding antimicrobial resistance across domesticated animal, human, and environmental systems. *Ann. N. Y. Acad. Sci.* 1441 (1), 17–30. doi: 10.1111/nyas.14036

Grave, K., Torren-Edo, J., and Mackay, D. (2010). Comparison of the sales of veterinary antibacterial agents between 10 European countries. *J.Antimicrob. Chemother.* 65, 2037–2040. doi: 10.1093/jac/dkq247

Hillerton, J. E., Irvine, C. R., Bryan, M. A., Scott, D., and Merchant, S. C. (2017). Use of antimicrobials for animals in new Zealand, and in comparison with other countries. *N Z Vet. J.* 65 (2), 71–77. doi: 10.1080/00480169.2016.1171736

Hosoi, Y., Asai, T., Koike, R., and Tsuyuki, M. (2013). Use of veterinary antimicrobial agents from 2005 to 2010 in Japan. *Int. J.Antimicrob. Agents* 41, 489–490. doi: 10.1016/j.ijantimicag.2013.01.002

Khan, X., Lim, R. H. M., Rymer, C., and Ray, P. (2022a). Fijian Veterinarian and para-veterinarians' behavior, attitude and knowledge toward antimicrobial use and antimicrobial resistance: A qualitative study. *Front. Vet. Sci.* 9. doi: 10.3389/fvets.2022.898737

Khan, X., Lim, R. H. M., Rymer, C., and Ray, P. (2022b). Fijian Farmers' attitude and knowledge towards antimicrobial use and antimicrobial resistance in livestock production systems-a qualitative study. *Front. Vet. Sci.* 9. doi: 10.3389/fvets.2022.838457

Khan, X., Rymer, C., Ray, P., and Lim, R. (2021). Quantification of antimicrobial use in Fijian livestock farms. *One Health* 13, 1–8. doi: 10.1016/j.onehlt.2021.100326

Kirchhelle, C. (2018). Pharming animals: a global history of antibiotics in food production (1935–2017). *Palgrave Commun.* 4 (1), 1–13. doi: 10.1057/s41599-018-0152-2

Klein, E., Boeckel, T., Martinez, E., Pant, S., Gandra, S., Levin, S., et al. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U.S.A.* 115, 201717295. doi: 10.1073/pnas.171729511

Landers, T. F., Cohen, B., Wittum, T. E., and Larson, E. L. (2012). A review of antibiotic use in food animals: perspective, policy, and potential. *Public Health Rep.* 127 (1), 4–22. doi: 10.1177/003335491212700103

Léger, A., Lambraki, I., Graells, T., Cousins, M., Henriksson, P. J. G., Harbarth, S., et al. (2021). AMR-intervene: A social–ecological framework to capture the diversity of actions to tackle antimicrobial resistance from a one health perspective. *J. Antimicrob. Chemother.* 76, 1–21. doi: 10.1093/jac/dkaa394

Lekshmi, M., Ammini, P., Kumar, S., and Varela, M. F. (2017). The food production environment and the development of antimicrobial resistance in human pathogens of animal origin. *Microorganisms* 5(1), 11. doi: 10.3390/microorganisms5010011

Magiri, R., Gaundan, S., Choongo, K., Zindove, T., Bakare, A., Okyere, E., et al. (2022). Antimicrobial resistance management in pacific island countries: Current status, challenges, and strategic solutions. *Int. J. One Health* 8 (1), 1–8. doi: 10.14202/IJOH.2022.1-7

Magouras, I., Carmo, L. P., Stärk, K. D. C., and Schüpbach-Regula, G. (2017). Antimicrobial usage and - resistance in livestock: Where should we focus? *Front. Vet. Sci.* 4. doi: 10.3389/fvets.2017.00148

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules* 23 (4), 795. doi: 10.3390/molecules23040795

McCalla, D. R. (1979). "Nitrofurans," in *Mechanism of action of antibacterial agents*, vol. 5/1 . Ed. F. E. Hahn (Berlin: Springer). Antibiotics. doi: 10.1007/978-3-642-46403-4_11

McEwen, S. A., and Fedorka-Cray, P. J. (2002). AMU and resistance in animals. *Clin. Infect. Dis.* 34 (3), S93–S106. doi: 10.1086/340246

McLeod, A. (2022) *Kendall: Kendall rank correlation and Mann-Kendall trend test*. Available at: https://CRAN.R-project.org/package=Kendall.

Ministry of Agriculture (2009) *Fiji National agriculture census 2009*. Available at: http://www.fao.org/fileadmin/templates/ess/ess_test_folder/World_Census_Agriculture/Country_info_2010/Reports/Reports_3/FJI_ENG_REP_2009.pdf (Accessed August 18, 2022).

Ministry of Agriculture (2020) *Fiji Agriculture census,. volume 1: General table & descriptive analysis report*. Available at: https://www.agriculture.gov.fj/documents/census/VOLUMEI_DESCRIPTIVEANALYSISANDGENERALTABLEREPORT.pdf (Accessed August 18, 2022).

Mouiche, M. M. M., Moffo, F., Betsama, J. D. B., Mapiefou, N. P., Mbah, C. K., Mpouam, S. E., et al. (2020). Challenges of antimicrobial consumption surveillance in food-producing animals in sub-Saharan African countries: Patterns of antimicrobials imported in Cameroon from 2014 to 2019. *J. Glob Antimicrob. Resist.* 22, 771–778. doi: 10.1016/j.jgar.2020.06.021

Munkholm, L., and Rubin, O. (2020). The global governance of antimicrobial resistance: a cross-country study of alignment between the global action plan and national action plans. *Global. Health* 16, 109. doi: 10.1186/s12992-020-00639-3

Page, S. W., and Gautier, P. (2012). Use of antimicrobial agents in livestock. *Rev. Sci. Tech. – Off. Int. Epizoot.* 31 (1), 145–188 doi: 10.20506/rst.31.1.2106

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., et al. (2004). Does the use of antibiotics in food animals pose a risk to human health? a critical review of published data. *J. Antimicrob. Chemother.* 53 (1), 28–52. doi: 10.1093/jac/dkg483

Prestinaci, F., Pezzotti, P., and Pantosti, A. (2015). Antimicrobial resistance: A global multifaceted phenomenon. *Pathog. Glob. Health* 109 (7), 309–318. doi: 10.1179/2047773215Y.0000000030

R Core Team (2022). *R: A language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: https://www.R-project.org/.

Rousham, E. K., Unicomb, L., and Islam, M. A. (2018). Human, animal and environmental contributors to antibiotic resistance in low-resource settings: integrating behavioural, epidemiological and one health approaches. *Proc. R. Soc*

B. P. R. Soc B-Biol Sci. 285 (1876), 20180332. doi: 10.1098/rspb.2018.0332

Rushton, J. (2015). Anti-microbial use in animals: How to assess the trade-offs. *Zoonoses Public Health* 62 (1), 10–21. doi: 10.1111/zph.12193

Sanchez, M. X., Fluckey, W. M., Brashears, M. M., and McKEE, S. R. (2002). Microbial profile and antibiotic susceptibility of campylobacter spp. and salmonella spp. in broilers processed in air-chilled and immersion-chilled environments. *J. Food Prot.* 65 (6), 948–956. doi: 10.4315/0362-028x-65.6.948

Schar, D., Sommanustweechai, A., Laxminarayan, R., and Tangcharoensathien, V. (2018). Surveillance of antimicrobial consumption in animal production sectors of low- and middle-income countries: Optimizing use and addressing antimicrobial resistance. *PloS Med.* 15 (3), e1002521. doi: 10.1371/journal.pmed.1002521

Schwarz, S., Kehrenberg, C., and Walsh, T. (2001). Use of antimicrobial agents in veterinary medicine and food animal production. *Int. J. Antimicrob. Agents* 17 (6), 431–437. doi: 10.1016/S0924-8579(01)00297-7

Singh, R., Lal, S., Khan, M., Patel, A., Chand, R., and Jain, D. K. (2022). The COVID-19 experience in the Fiji islands: some lessons for crisis management for small island developing states of the pacific region and beyond. *N. Z. Econ Pap.* 56 (1), 67–72. doi: 10.1080/00779954.2020.1870534

Ting, S., Pereira, A., Alves, A., Fernandes, S., Soares, C., Soares, F. J., et al. (2021). Antimicrobial use in animals in timor-leste based on veterinary antimicrobial imports between 2016 and 2019. *Antibiotics* 10, (4), 426. doi: 10.3390/antibiotics10040426

Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P., and Van Boeckel, T. P. (2020). Global trends in antimicrobial use in food animals from 2017 to 2030. *Antibiotics (Basel)* 9, 918. doi: 10.3390/antibiotics9120918

Tukana, A., Hedlefs, R., and Gummow, B. (2016). *Brucella abortus* surveillance of cattle in Fiji, Papua new Guinea, Vanuatu, the Solomon islands and a case for active disease surveillance as a training tool. *Trop. Anim. Health Prod* 48, 1471–1481. doi: 10.1007/s11250-016-1120-8

Umair, M., Orubu, S., Zaman, M. H., Wirtz, V. J., and Mohsin, M. (2022). Veterinary consumption of highest priority critically important antimicrobials and various growth promoters based on import data in Pakistan. *PloS One* 17 (9), e0273821. doi: 10.1371/journal.pone.0273821

Van Boeckel, T. P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N. G., et al. (2019). Global trends in antimicrobial resistance in animals in low- and middle-income countries. *Science* 365 (6459), eaaw1944. doi: 10.1126/science.aaw1944

Van den Bogaard, A., London, N., Driessen, C., and Stobberingh, E. (2001). Antibiotic resistance of faecal escherichia coli in poultry, poultry farmers and poultry slaughterers. *J. Antimicrob. Chemother.* 47 (6), 763–771. doi: 10.1093/jac/47.6.763

World Health Organization (2019) *Critically important antimicrobials for human medicine 6th revision* (Geneva, Switzerland: World Health Organization). Available at: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf (Accessed August 18, 2022).

World Organisation for Animal Health (2020a) *Guidance for completing the OIE template for the collection of data on antimicrobial agents intended for use in animals*. Available at: https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/2020/ENG_AMUse_Guidance_Final_2020.pdf (Accessed August 18, 2022).

World Organisation for Animal Health (2020b) *OIE annual report on antimicrobial agents intended for use in animals. fourth report*. Available at: https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_FourthAnnual_Report_AMR.pdf (Accessed August 19, 2022).

World Organisation for Animal Health (2021) *OIE list of antimicrobial agents of veterinary importance*. Available at: https://www.woah.org/app/uploads/2021/06/a-oie-list-antimicrobials-june2021.pdf (Accessed August 18, 2022).

frontiers | Frontiers in Antibiotics

TYPE Original Research
PUBLISHED 23 March 2023
DOI 10.3389/frabi.2023.1116785

OPEN ACCESS

EDITED BY
Getahun E. Agga,
Food Animal Environmental Systems Research, Agricultural Research Service (USDA), United States

REVIEWED BY
Magdalena Popowska,
University of Warsaw, Poland
Lisa M. Durso,
Agricultural Research Service, United States Department of Agriculture, United States

*CORRESPONDENCE
Timothy P. Neher
tpneher@iastate.edu

SPECIALTY SECTION
This article was submitted to Antibiotic Resistance, a section of the journal Frontiers in Antibiotics

RECEIVED 05 December 2022
ACCEPTED 13 March 2023
PUBLISHED 23 March 2023

CITATION
Neher TP, Soupir ML, Andersen DS, O'Neill ML and Howe A (2023) Comparison of antibiotic resistance genes in swine manure storage pits of Iowa, USA. *Front. Antibiot.* 2:1116785. doi: 10.3389/frabi.2023.1116785

Comparison of antibiotic resistance genes in swine manure storage pits of Iowa, USA

Timothy P. Neher*, Michelle L. Soupir, Daniel S. Andersen, Maggie L. O'Neill and Adina Howe

Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA, United States

Antimicrobial resistance (AMR) can develop in deep-pit swine manure storage when bacteria are selectively pressured by unmetabolized antibiotics. Subsequent manure application on row crops is then a source of AMR into soil and downstream runoff water. Therefore, understanding the patterns of diverse antibiotic resistance genes (ARGs) in manure among different farms is important for both interpreting the results of the detection of these genes from previous studies and for the use of these genes as bioindicators of manure borne antibiotic resistance in the environment. Previous studies of manure-associated ARGs are based on limited samples of manures. To better understand the distribution of ARGs between manures, we characterized manures from 48 geographically independent swine farms across Iowa. The objectives of this study were to characterize the distribution of ARGs among these manures and to evaluate what factors in manure management may influence the presence of ARGs in manures. Our analysis included quantification of two commonly found ARGs in swine manure, *ermB* and *tetM*. Additionally, we characterized a broader suite of 31 ARGs which allowed for simultaneous assays of the presence or absence of multiple genes. We found the company integrator had a significant effect on both *ermB* ($P=0.0007$) and *tetM* gene concentrations ($P=0.0425$). Our broad analysis on ARG profiles found that the *tet(36)* gene was broadly present in swine manures, followed by the detection of *tetT*, *tetM*, *erm(35)*, *ermF*, *ermB*, *str*, *aadD*, and *intI3* in samples from 14 farms. Finally, we provide a comparison of methods to detect ARGs in manures, specifically comparing conventional and high-throughput qPCR and discuss their role in ARG environmental monitoring efforts. Results of this study provide insight into commonalities of ARG presence in manure holding pits and provide supporting evidence that company integrator decisions may impact ARG concentrations.

KEYWORDS

antimicrobial resistance, livestock management, production system, integrator, manure storage, swine manure, qPCR (quantitative PCR), high-throughput qPCR

Introduction

Large-scale swine production and growing demand for pork has resulted in the consequent increased production of swine manures (OECD and Food and Agriculture Organization of the United Nations, 2021). Manures are a reservoir for unmetabolized antibiotics and antibiotic resistant bacteria (Marti et al., 2014; Mu et al., 2015; He et al., 2020; Lima et al., 2020; Howe and Soupir, 2021). The enrichment of antibiotics in manure originates from the use of antibiotic administration to therapeutically and sub-therapeutically control, prevent, and treat disease (Klein et al., 2018). In the United States, more than two million kilograms, or 39% of medically important antibiotics intended for use in food-producing animals, were used in swine production in 2019 (Center for Veterinary Medicine, 2020). Much of the administered antibiotic is unmetabolized and remains in the animal tissue or excreted with manure (Elmund et al., 1971; Bacanlı and Başaran, 2019). Excess manure and associated antibiotic residues are often retained in deep pit storage structures until field application as fertilizer (Elmund et al., 1971; Zhang et al., 2017). Manure can remain in storage structures for more than a year, between intervals of land application (IADNR, 2022). Within these deep pits, there is continuous interaction between antibiotics and bacteria, which can lead to the development and/or enrichment of antibiotic resistance, both by genetic mutation and horizontal gene transfer (Chee-Sanford et al., 2009; Zhao et al., 2019; He et al., 2020). Generally, manure has been identified as a potential hotspot for the accumulation and dissemination of antibiotic resistance to the environment.

Diverse antibiotic resistant genes (ARGs) associated with medically important classes of antibiotics have been observed in swine manure bacteria. Swine manure associated ARGs include tetracyclines (*tet*), macrolides (*erm*, *msr*, *mef*), lincosamides (*lnu*, *lin*), aminoglycosides (*aac*, *aad*, *aph*, *str*), sulfonamides (*sul1*, *sul2*), amphenicols (*cpr*, *cml*, *floR*), and fluoroquinolones (*qnr*), ranked by total mass distributed in the US. (Fang et al., 2018; Center for Veterinary Medicine, 2020; Checcucci et al., 2020). The most commonly detected ARG determinants in swine manure encode resistance to tetracyclines (*tet*), sulfonamides (*sul*), and macrolides (*erm*) (Chen et al., 2007; Whitehead and Cotta, 2013; Li et al., 2019). A number of these ARGs have been detected within environments adjacent to animal production or manure application and are attributed to manure management practices (Wang et al., 2020), supporting the theory that manure-borne antibiotics and subsequent antimicrobial resistance contribute to the overall resistome in environmental soil and water (Wellington et al., 2013; Checcucci et al., 2020; Zhou et al., 2020).

To understand the risk of AMR from swine manure, broad and effective surveillance methods are necessary. Ideally, these methods would be sensitive and specific to swine-specific AMR risks, such as ARGs or pathogens. Unfortunately, the ARGs that are associated with swine manures are also detected in other animal production where similar antibiotics are used (Zalewska et al., 2021). Furthermore, ARGs and antibiotic resistant bacteria are naturally occurring in the environment (Martínez, 2012; Van Goethem et al., 2018), making it necessary to distinguish antibiotic resistance determinants derived from swine production to those that are found in the natural environment (Allen et al., 2010; Meyers et al., 2020). Additionally, swine manures themselves can vary significantly in the suite of ARGs that are characteristic of their microbial communities (Xue et al., 2021; Shui et al., 2022). We have a limited understanding of this variation among manures because most studies of swine-associated ARGs have been focused on demonstrating an enrichment of ARGs in a small sample of a single farm or a small number of manure samples (Li et al., 2019; Wen et al., 2019; Yang et al., 2020; Xue et al., 2021).

We focused on swine manures originating from the state of Iowa, which is the highest swine producing state in the United States, where there are more than 5,400 swine farms (IPPA, 2012). The rationale for selecting a state-wide sampling was based on accessibility to samples within a similar time period and also our expectation that we would observe high variability in swine production systems and company integrators within regional samples. Swine farms can vary in specialized production systems such as wean-finish or grow-finish, and company integrators that manage supplies like weaners, feed, and medication (Cooper, 2018). It is yet unclear how these variables may influence resulting AMR in stored manure.

In this study, we expand our knowledge of the presence of antibiotic resistant determinants in swine manure by providing a broad comparison of ARGs among manures from 48 farms. We aimed to quantify the presence of ARGs that have been demonstrated to be consistently enriched in swine manures, *tetM* and *ermB* (Whitehead and Cotta, 2013; Wen et al., 2019; Alt et al., 2021) and also characterized the presence of diverse resistance genes associated with other antibiotics and with swine manure, including aminoglycoside, carbapenem, lincosamide, phenicol, and sulfonamide resistance (Table 1). Our justification for the gene selection is that these genes are associated with the most sold antibiotics in swine production (Center for Veterinary Medicine, 2020). Additionally, a parallel study of the manures from these farms measured high levels of tetracyclines and macrolides (Congilosi et al., 2022). Our objective of this study was to better understand ARG representation across multiple swine sources in a similar region and to assess the variability of ARGs in swine manure and their usefulness as broad bioindicators of manure influence. Concurrently with evaluating ARGs among farm manures, we assessed the differences in farm management: production system (wean-finish or grow-finish) and company integrator (integrator 1 or integrator 2). Understanding the distribution of these genes under varying farm management conditions will help us better understand whether broad management factors influence the concentrations of manure-associated ARGs in swine manure from deep pit storage structures.

Materials and methods

Sample collection

A total of 48 swine farms were sampled from across the state of Iowa in the summer of 2020. At each farm, a single representative

TABLE 1 Antibiotic resistance genes observed in previous studies in soil and water influenced by swine manure.

Antibiotic class of resistance	Antibiotic Resistance Gene	Studies reported
Aminoglycoside	*aadD, aada2, str*	(Chen et al., 2017; Han et al., 2018; Liu et al., 2019)
Carbapenem	*blaPSE, blaOXA10*	(Han et al., 2018; Cheng et al., 2020; Radu et al., 2021)
Lincosamide	*lnuA, lnuB*	(Han et al., 2018; Cheng et al., 2020)
Macrolide	*erm(35), erm(36), ermB, ermC, ermF, ermQ, ermT*	(Chen et al., 2017; Peng et al., 2017; Han et al., 2018; Liu et al., 2019; Lopatto et al., 2019; Wen et al., 2019; Meyers et al., 2020; Radu et al., 2021)
Mobile Genetic Element	*intI1, intI2, intI3, intI1F165*	(Chen et al., 2019; Lopatto et al., 2019; Meyers et al., 2020)
Phenicol	*floR, cmlA1, cmlA5*	(Chen et al., 2017; Liu et al., 2019)
Sulfonamide	*sul1, sul2*	(Peng et al., 2017; Chen et al., 2019; Liu et al., 2019; Lopatto et al., 2019; Meyers et al., 2020; Radu et al., 2021)
Tetracycline	*tet(36), tetA, tetL, tetM, tetO, tetT, tetW, tetX*	(Chen et al., 2017; Chen et al., 2019; Peng et al., 2017; Han et al., 2018; Liu et al., 2019; Wen et al., 2019; Meyers et al., 2020; Radu et al., 2021)

manure sample was collected from deep pit storage structures. Specific locations of the farms are not disclosed due to privacy restrictions, but all farms are within the state of Iowa and are geographically independent of each other. Samples were collected at the edge of the pits through a manure pump out *via* dipping the sample from the top six inches of the manure surface. All farms were deep pit barn facilities where pigs were either grow-finish (GF) or wean-finish (WF) pigs raised on a slatted floor. Pigs were fed commercial production diets consisting primarily of corn, soybean meal, and distillers grains with percentages fed varying by growth stage and price of different feed ingredients. After collection, manure was stored at -20°C for one month until further processing. Each manure sample was subsampled in triplicate prior to DNA extraction. Each farm included was categorized based on originating integrator and production system. Specifically, these categories were company integrator: Integrator 1 (n=24) or integrator 2 (n=24); production system: wean-finish (n=34), or grow-finish (n=14). Ethical review and approval was not required for the study on animals in accordance with the local legislation and institutional requirements. This work was conducted in collaboration with local swine growers who made all animal decisions regarding health and well-being and allowed the collection of manure at their site.

DNA extraction

The DNA extraction procedure followed protocols from the MagAttract PowerSoil DNA EP Kit (Qiagen) and an epMotion 5075 automated robot for extraction (Eppendorf). Samples of 0.25 grams wet weight of liquid swine manure were used for DNA extraction. Each manure was sub-sampled into three replicates ("farm replicates"). For each farm replicate, we performed three DNA extractions, resulting in three technical extraction replicates ("extraction technical replicates") for each farm replicate manure sample. The resulting DNA was cleaned using a DNA Clean and Concentrator kit (Zymo Research). Subsequent DNA concentrations were measured with the Quant-it dsDNA Assay Kit, high sensitivity (Thermo Fisher Scientific). The DNA samples were stored at -80°C until further use.

Conventional qPCR quantification (quantification of concentrations of *tetM* and *ermB*)

Conventional qPCR assays were performed on a CFX96 Touch Real-Time PCR Detection System (BioRad) and measured in triplicate using primers targeting the 16S rRNA gene, *ermB* gene, and *tetM* gene (Supplementary Table 1). Genes were quantified in all 48 swine manure samples. The DNA template was diluted (1:10) to optimize qPCR detection, to minimize inhibitors, and increase primer efficiency to a target gene. The limit of quantification was determined for each gene using oligonucleotide standards. Standard curves ranged from 10^7 to 10^1 copies, and all samples measured above the limit of quantification. Outliers in the triplicate were omitted if above 1.5 times the standard deviation in the average of the three values. Efficiencies calculated by standard curves ranged from 82.2 to 100.6% and all R^2 values were above 0.98 (Supplementary Table 2). All reported absolute abundance (copies/gram) are reported in gene copies per gram of wet weight of manure and were calculated by the equation:

$$= \frac{x\ copies}{reaction} * \frac{100\mu L\ final\ volume}{\frac{2\mu L}{reaction}} * dilution\ factor\ 10 * \left(\frac{1}{0.25g\ manure}\right)$$

High-throughput qPCR (presence absence of ARGs)

Extracted DNA was analyzed for a wide host of ARGs encoding resistance to a broad spectrum of antibiotics used in swine production; *str*, *aadD*, *aadA2* (Aminoglycosides), *ermB*, *ermC*, *ermF*, *ermQ*, *ermT*, *erm(35)*, *erm(36)* (Macrolides), *sul2*, *sul1* (Sulfonamides), *tetA*, *tetL*, *tetM*, *tetO*, *tetT*, *tetW*, *tetX*, *tet(36)* (Tetracyclines), *blaPSE*, *blaOXA10* (Carbapenems), *lnuC*, *lnuA*

(Lincosamides), *cmlA5*, *cmlA1*, *floR* (Phenicols), *intI3*, *intI2*, *intI1F165*, and *intI1* (Integrons). The high-throughput qPCR primers used for the analysis are originally described in Stedtfeld et al. (2018), Supplementary Table 1. The high-throughput qPCR assay was performed on the Biomark Fluorescent machine in the 96x96 primer target layout. Each assay was performed in triplicate. The template DNA was diluted in a 1:500 dilution for optimal performance on the Biomark machine and to decrease potential inhibitor effects. Samples reading a cycle threshold value greater than 30 were omitted from further analysis. Cycle threshold detections greater than 30 were assumed to be non-detected. Verification of the high-throughput qPCR machine performance is supported with internal standards for standard curve development of 16S rRNA, *ermB*, *ermF*, *sul2*, *tetM*, and *tetW* genes. Each internal standard gene amplified successfully with efficiencies ranging from 80.0 to 104.2% (Supplementary Table 3).

Quality control

In order to be deemed a successful amplification, we required that the conserved total bacteria gene 16S rRNA was detected in each manure sample. Additionally, we required that detection was observed for each gene in 2 out of 3 farm manure replicates and 2 out of 3 technical extraction replicate detections for each sample.

Statistical analysis

All statistical analyses were performed using R version 4.0.3. The quantified *ermB* and *tetM* gene concentrations (copies/gram wet weight) were log10 transformed to fit a normal distribution. Normality was confirmed with visual inspection of histograms and Q-Q Plots. The linear regression models were fit using the lme4 package (Bates et al., 2015). The two gene responses were analyzed separately. The integrator and production system were treated as fixed effects. Gene concentrations of subsampled triplicates from one representative manure sample per farm were averaged before model building. Model performance was evaluated using the Performance package (Lüdecke et al., 2021) (Supplementary Table 4).

The R package emmeans (Lenth, 2021) was used for calculating the estimated marginal means from the verified models and making pairwise comparisons of fixed effects. All pairwise comparisons were made with a 95% confidence level ($P<0.05$) and P-values were adjusted using Tukey's method for multiple comparisons. The main effects refer to the overall effect of the variable while ignoring, or averaging over, the levels of the other predictor variable. The main and interaction effects of each model were analyzed using ANOVA and type-III error.

Results

Conventional qPCR gene quantification

The number of gene copies of *tetM* and *ermB* were quantified in DNA extracted from all manures using targeted amplification of these genes. Additionally, gene copies of the 16S rRNA gene, a phylogenetic marker present in all bacteria, were estimated and used for normalizing total bacterial counts among manure comparisons. Overall, there was a large range of detection of both genes across all 48 farms (Figure 1); the absolute gene concentrations of *ermB* ranged from $2.20x10^4$ copies $gram^{-1}$ to $1.53x10^8$ copies $gram^{-1}$ and *tetM* ranged from $1.33x10^5$ copies $gram^{-1}$ to $2.23x10^8$ copies $gram^{-1}$. The limit of quantification for each individual qPCR plate are reported in Supplementary Table 2. The concentrations of *ermB* and *tetM* were significantly different across the 48 manure samples (ANOVA, $P< 0.0001$) (Supplementary Table 5), and a general trend was observed that *tetM* and *ermB* concentrations increased with concentrations of 16S rRNA genes.

The company integrator had a significant main effect on observed *ermB* absolute gene concentrations based on the overall ANOVA with type-III error ($P=0.0007$) (Supplementary Table 6). The mean concentration of *ermB* in manures associated with integrator 2 manure was 15% greater than manures from integrator 1. Integrator 2 had an *ermB* estimated marginal mean of $4.8x10^6$ copies/gram compared to integrator 1 with $6.5x10^5$ copies/gram. This result exists when *ermB* was normalized to 16S rRNA ($P=0.0020$) (Supplementary Figure 1 and Supplementary Table 7). Likewise, there is evidence that the integrator had a significant effect on *tetM* concentrations ($P=0.0425$), with *tetM* also being enriched in integrator 2 relative to integrator 1 (Figure 2). However, this result is non-significant when *tetM* was normalized to 16S rRNA ($P= 0.3670$). The production system had no significant main effect on *ermB* or *tetM* gene concentrations or relative abundance to 16S rRNA (Supplementary Figures 2, 3). Additionally, there was no significant interaction between the two fixed effects in both the absolute copy number model and the 16S rRNA normalized model for each gene (Supplementary Tables 6, 7).

HT-qPCR gene survey

In addition to quantification of *tetM* and *ermB* in manures, we also evaluated the presence of 31 ARGs listed in Table 1 and the 16S rRNA gene in manures using methods similar to those previously described (Stedtfeld et al., 2018) to leverage the ability to assay numerous genes simultaneously with high-throughput qPCR (HT-qPCR). Each internal standard gene of 16S rRNA, *ermB*, *ermF*, *sul2*, *tetM*, and *tetW* were amplified successfully with efficiencies ranging from 80-104% (Supplementary Table 3). However, while all 48 manures were evaluated against these 32 genes, in total, we detected 22 unique ARGs in 14 independent farm manure samples (Figure 3). In 34 manures, we were unable to amplify the 16S rRNA gene with HT-qPCR assays and thus these samples were removed from further analysis. Within successfully amplified samples, the most frequently detected ARG in manure was *tet (36)*, which was detected in all 14 manures. The second most detected ARG was *tetT* at 93% detection, followed by *erm(35)* at 78.6% detection. Genes encoding resistance to tetracycline, *tetT*, *tetM*, and *tet(36)*, were present in 13/14, 8/14, and 14/14 farm manure samples, respectively. The macrolide resistance gene class,

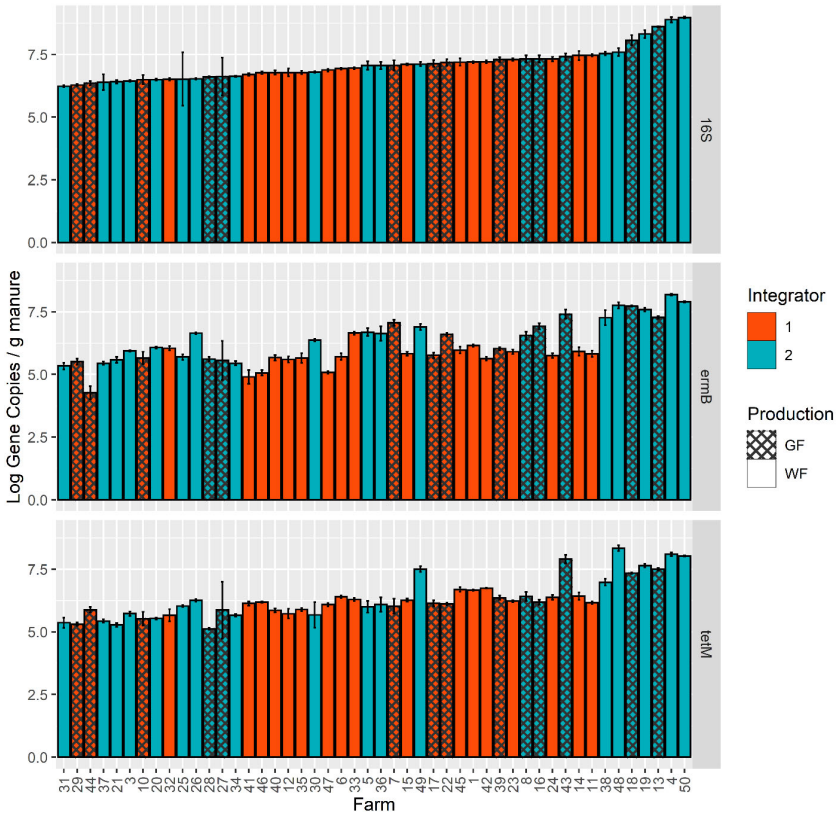


FIGURE 1
Absolute gene copies/g (wet weight) of 16S rRNA, *ermB*, and *tetM* as measured by qPCR assays for 48 farm manure samples. Samples are ordered by lowest to highest mean concentrations for the 16S rRNA gene. Colors indicate the different company integrators, and the hash marks denote the growth stage (production of the farm, GF (Grow-Finish) and WF (Wean-Finish)).

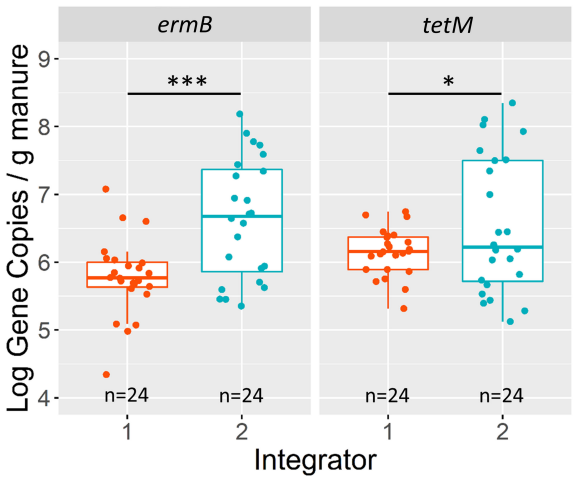


FIGURE 2
Log10 gene copies/g (wet weight) of *ermB* and *tetM* grouped by company integrator. Asterisks above boxplots signify p-values (alpha = 0.05) based on results of the linear model (not significant [ns] p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.00001). Interquartile ranges are indicated by boxes and the upper 25% and lower 25% are indicated by whiskers. The number of farms (n) are labelled on the x-axis.

erm, had the second most detected antibiotic resistance genes with *erm(35)*, *ermF* and, *ermB* detected in 11/14, 10/14, and 7/14, respectively. There was no detection of *blaPSE*, *blaOXA10*, *cmlA5*, *cmlA1*, *floR*, *lnuA*, *erm(36)*, *tetL*, and *tetA* in any of the manure samples.

Based on the detection of ARGs, we have developed recommendations of the most commonly detected ARGs in Iowa swine manures (Table 2). Importantly, we also identify the ARGs that were not strongly present in manure holding pits, and these ARGs include *tetL*, *tetA*, *erm(36)*, *floR*, *cmlA5*, *cmlA1*, *blaPSE*, and *blaOXA10* (no detection), *sul1*, *inti1*, *and inti1F165*, (7.1%), *aadA2* (14.2%), *inti2* and *sul2* (21.4%). In general, we observed that the two main resistance mechanisms of ARGs present in the manures studied were associated with target protection and target alteration.

Discussion

Many previous studies have characterized ARGs in swine manures (Whitehead and Cotta, 2013; Yang et al., 2020; Howe and Soupir, 2021) but are limited in the numbers of manure from different farms represented in a single study. To help understand the broad presence of ARGs in swine manures, this study identified patterns in diverse manures from 48 geographically independent farms. These farms represented variations in company integrator

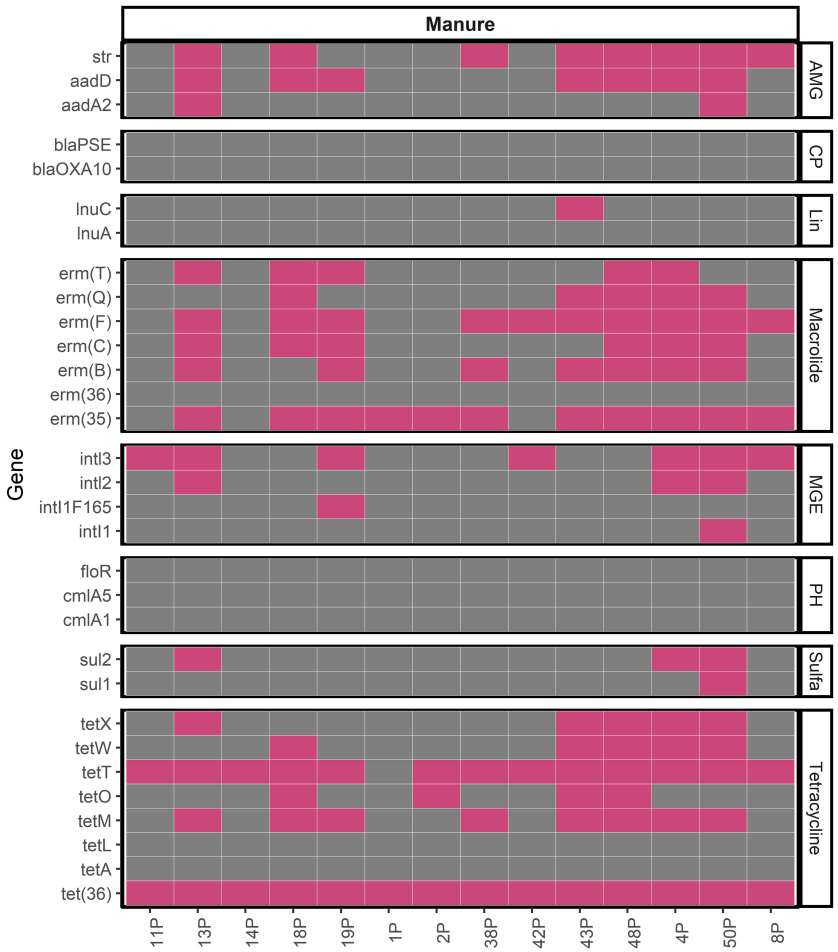


FIGURE 3
Presence (Pink) and absence (Grey) for ARGs in manure samples for which amplification of 16S rRNA gene was observed. Aminoglycoside (AMG), Carbapenem (CP), Lincosamide (Lin), Macrolide, Mobile Genetic Element (MGE), Phenicol (PH), Sulfonamide (Sulfa), Tetracycline.

and production system, thus providing an opportunity to assess generalized management factors. The ARGs selected for characterization in this study were based on previous research in environmental monitoring, and these genes have been previously detected in manure, manure amended soil, and in the downstream waters of agricultural land (Berendonk et al., 2015; Chen et al., 2019; Lima et al., 2020; Neher et al., 2020; Zhang et al., 2021). While we know these genes have been enriched in association with manures in experimental studies, observations of their abundances in environmental samples may not be able to be linked to a manure reference. In other words, in environmental monitoring, it is unknown if abundances observed of these genes are substantial. Understanding the distribution of these genes in manures will help us frame their observed abundances in the environment. While we acknowledge that a study of 48 regional farms is far from comprehensive, we believe that this study fills an important data gap on ARG bioindicators from broad manures within a single comparative study.

In our evaluation of ARGs as bioindicators for swine manure, we used two approaches on select genes. Our rationale for leveraging both these methods was to balance our abilities to accurately quantify relevant ARGs to understand the distribution of their presence in diverse manures while also providing a broad survey of multiple ARGs. The first method we used was conventional gene amplification with qPCR, which is an absolute quantification method using known standard concentrations to estimate specific gene concentrations within manures. To survey a broad range of genes, we also used a second method, which is a relative quantification method on a HT-qPCR platform. This method has recently been used by numerous studies (Muurinen et al., 2021; Fernanda et al., 2022; Flater et al., 2022; Kasuga et al., 2022; Mware et al., 2022; Samanta et al., 2022) because it allows for simultaneous presence/absence detection of numerous genes (Stedfeld). HT-qPCR is also limiting in the volume of each reaction (6.7×10^{-3} µL vs 2 µL in conventional qPCR), which directly influences its detection limits. Thus, these amplification methods, conventional qPCR and HT-qPCR, are complements, the former allowing for more sensitive quantification of a limited number of ARGs and the latter broad detection of numerous ARGs simultaneously. As with any amplification method for

TABLE 2 Ranked recommendations of ARGs for detection of AMR in swine manure holding pits, based on both detection of 16S rRNA genes and specified ARG.

Gene	Percent Detection	Drug Class	Resistance Mechanism
tet(36)	100	Tetracycline	Target protection
tetT	92.9	Tetracycline	Target protection
erm(35)	78.6	Macrolide	Target alteration
ermF	71.4	Macrolide	Target alteration
tetM	57.1	Tetracycline	Target protection
str	57.1	Aminoglycoside	Inactivation
ermB	50	Macrolide	Target alteration
aadD	50	Aminoglycoside	Inactivation
intl3	50	Integrase	N/A
ermC	42.9	Macrolide	Target alteration
ermQ	35.7	Macrolide	Target alteration
ermT	35.7	Macrolide	Target alteration
tetW	35.7	Tetracycline	Target protection
tetX	35.7	Tetracycline	Inactivation
tetO	28.6	Tetracycline	Target protection
sul2	21.4	Sulfonamide	Target replacement
intl2	21.4	Integrase	N/A
aadA2	14.2	Aminoglycoside	Inactivation
intI1F165	7.1	Integrase	N/A
intI1	7.1	Integrase	N/A
sul1	7.1	Sulfonamide	Target replacement
lnuC	7.1	Lincosamide	Inactivation
lnuA	1.6	Lincosamide	Inactivation
tetL	0	Tetracycline	Efflux
tetA	0	Tetracycline	Efflux
erm(36)	0	Macrolide	Target alteration
floR	0	Phenicol	Efflux
cmlA5	0	Phenicol	Efflux
cmlA1	0	Phenicol	Efflux
blaPSE	0	Carbapenem	Inactivation
blaOXA10	0	Carbapenem	Inactivation

The percent detection is the proportion of 14 manure samples with concurrent positive detection of 16S rRNA gene. The antibiotic resistance genes analyzed in this study in 14 swine manures from Iowa farms.
N/A, Not Applicable.

manure samples, both methods will be influenced and likely disproportionately by the sample complexity of manures, where inhibitors (which vary among manure samples) may prevent adequate amplification (Sidstedt et al., 2020; Waseem et al., 2020; Park et al., 2021). We provide a comparison of these methods to target ARGs in our swine manure samples below.

Concentrations of *ermB* and *tetM* in swine manure pits (conventional qPCR)

Consistent with previous observations of the association and enrichment of *ermB* and *tetM* genes with manures (Whitehead and Cotta, 2013; Joy et al., 2014; Zalewska et al., 2021) and

adjacent soils and waters (Peng et al., 2017; Zhang et al., 2021), we detected these genes in all 48 manures in this study. The concentrations measured in our study were consistent with those detected in manure holding pits measured in other studies (Mackie et al., 2006; Joy et al., 2013; Hall et al., 2020; Alt et al., 2021) and also demonstrate the wide variations of ARGs that can be observed within manures, with variations up to three-fold. The wide ranges of measured *ermB* and *tetM* in these manures may be caused by covariates in manure holding pits that have yet unknown implications on ARG concentrations after long-term exposure such as concentrations of heavy metals, manure pit additives, or changes in chemical properties such as pH or organic substrates (Hölzel et al., 2012; He et al., 2020). While it is clear that these ARGs are consistently observed between swine manures, it is less clear what the implications are of the magnitude and variability of these gene concentrations (*ermB* and *tetM* varying between $2.20x10^4$ and $1.53x10^8$ copies/gram in our samples). We speculate that the concentration of ARGs may be associated with the time spent in storage, with manure sampled right at defecation presumably containing different concentrations of ARGs than in manure stored for up to six months (Joy et al., 2014). Future studies of the relationship between these gene concentrations and to risks antibiotic resistance are much needed (Gullberg et al., 2011; Hughes and Andersson, 2017), and the results of this study provide some insight the variability of these concentrations in varying manures.

The abundances of these genes also followed observable patterns based on their farm of origin. We observed significant differences of *ermB* and *tetM* gene concentrations among farms with different company integrators, with both genes consistently largest in the same integrator. Integrators generally manage piglet source, feedstock, and veterinary practices (Tsoulouhas and Vukina, 1999; McBride and Key, 2003; Reimer, 2006). Our observations that different integrators have different concentrations of these genes suggest that these management decisions may affect ARG concentrations in manures (Lu et al., 2017; Ghanbari et al., 2019; Cheng et al., 2021). We did not observe any significant differences in *tetM* or *ermB* in association to the production system, or whether manure originated from wean or grow-finished pigs. This finding is consistent with previous studies who investigated the differences of ARGs in swine from the same farm over time and found that similar genes were consistently observed among samples from different stages in the production process (Petrin et al., 2019) and also at similar concentrations (Wen et al., 2019). Our results combined with these previous studies suggest that despite higher quantities of antibiotics administered to younger weanling pigs than mature growers (Dunlop et al., 1998; Dewey et al., 1999), the concentrations of these ARGs in manure do not change significantly. Overall, our results also indicate that the integrator is a larger source of variation among these genes than production stage and highlight the opportunity to engage in AMR stewardship towards integrators in partnership with farms (Hayes, 2022; Mitchell et al., 2022).

Potential ARG indicators in swine manure pits (HT-qPCR)

We also studied the detection of other ARGs to expand this study beyond *ermB* and *tetM* by leveraging high-throughput qPCR (HT-qPCR) methods which allow simultaneous testing of multiple gene probes. ARG targets were selected based on published primers (Stedtfeld et al., 2018) of ARGs previously observed to be present in swine manures (Table 1). Between manures, the tetracycline resistance gene class was the most prevalently detected in our samples, which is consistent with its wide use in swine production (Center for Veterinary Medicine, 2020). Likewise, the macrolide resistance gene class, *erm*, had the second most detected antibiotic resistance genes and is consistent with previous literature (Whitehead and Cotta, 2013; Joy et al., 2014). For instance, a study by Wen et al. (2019) studied nine ARGs at 18 different swine farms and found *tetO* as the predominant gene in manure and *tetQ*, *tetW*, *ermB*, and *ermF* were identified as having the highest risk of spread to the soil and water environment through manure application. Moreover, a study by Mu et al. (2015) took manure samples right after defecation from swine in nine feedlots in China finding *oqxB* (plasmic mediated quinolone) as the highest detected ARG followed by *sul1*, *sul2*, *tetO*, *tetM*, and *ermB*. Surprisingly, *sul1* and *sul2* were only detected 11.1% and 23% respectively, in the manure storage pits from the current study, suggesting a temporal shift in ARG presence between fresh manure and stored manure. Finally, a study of manure from three swine farms in China measured 28 tetracycline resistance genes and reported detection of 22 with the most common genes *tetA*, *tetL*, *tetM*, and *tetG* (Zhu et al., 2013), whereas in the current study, *tetA* and *tetL* were not detected in any of the 14 farms. These variations in detected classes of ARGs among studies and farms are speculated to be caused by differing antibiotic treatments, legacy resistance in piglets passed down by the maternal gut (Pärnänen et al., 2018), and co-selection of resistance genes (Looft et al., 2012).

Compared to conventional qPCR, fewer detections of ARGs were observed on HT-qPCR, most likely due to a combination of both the significantly reduced reaction volume (and thus lower limit of quantification) and presence of inhibitors (Funes-Huacca et al., 2011; Sandberg et al., 2018; Luo et al., 2021; Keenum et al., 2022). Specifically, we observed *ermB* and *tetM* gene detection in 100% of manure samples with conventional qPCR but 50% with HT-qPCR. To better understand these results, we compared the lower limit of quantification for *ermB* and *tetM* for traditional qPCR and HT-qPCR and found that traditional qPCR was 63 (*ermB*) and 94 (*tetM*) times more sensitive than HT-qPCR (Supplementary Tables 2, 3), suggesting that limit of quantification contributed to the inconsistency among ARG detections. Additionally, the DNA for the HT-qPCR assays were diluted 500:1 to balance measuring high 16S-rRNA gene copies, enabling the detection of low concentration ARGs, and reducing inhibitor effects. We conclude that the combination of diluting DNA and the HT-qPCR's significantly reduced reaction volume contributed to the inconsistent detection of ARGs. This observation should be considered in selecting

monitoring methods for ARG detection in future studies. Although HT-qPCR is not as robust as conventional qPCR, the advantages of this method are its ability to simultaneously measure multiple gene targets, use of much less reagent per sample, and significantly reduced labor. We recommend that HT-qPCR be used to screen the presence or absence of diverse ARG targets in environmental samples, and conventional qPCR be used for more rigorous quantification.

While *ermB* and *tetM* were inconsistently detected with HT-qPCR methods, there were specific genes that were broadly present using this method. Specifically, the *tet36* and *erm35* genes, encoding resistance to tetracyclines and macrolides respectively, were detected more frequently with the HT-qPCR than their counterpart *tetM* and *ermB*. This suggests that *tet36* and *erm35* are consistently associated with swine manure and able to be detected with current high throughput methods. The *tet36* gene was first discovered in swine manure pits, and is yet unclear whether it is enriched or persists in the environment upon manure application (Whittle et al., 2003; Kang et al., 2018; He et al., 2019). Less is known about the *erm35* gene, except that it was detected in poultry manure with metagenomics (Błażejewska et al., 2022; Wang and Chai, 2022). The *erm35* gene may have potential as a swine indicator since it was detected so frequently with HT-qPCR in the current study. One major difference between the two sets of genes is their association with mobile genetic elements (MGEs) where *ermB* and *tetM* are highly associated with MGEs while *erm35* and *tet36* are not (Zhang et al., 2022). MGEs are associated with the mobility of ARGs, which may be a significant variable for the dissemination of the gene after manure application. The class-3 MGE *inti3* was present in half of the manure samples tested in the ARG survey, and this is significant as this gene has the potential for horizontal gene transfer (Martínez et al., 2015). We highlight these genes *tet36* and *erm35* as potential targets for swine manure borne resistance.

Conclusions

Overall, this study justifies the continued use of macrolide and tetracycline resistant ARGs as broad indicators of swine manure-borne resistance due to their presence in diverse manure samples. The observation of the concentrations of these genes in manures helps us to interpret whether abundances of these genes in the environment are substantial. Additionally, results of this study also highlight variations of using different methods to detect genes and their variability across ARGs. Due to the observed variation of ARGs in diverse manures, future studies should aim to characterize not only antibiotic residues, but also physiochemical properties of the manure to analyze for specific correlations that can explain this variability. We also provide supporting evidence that company integrator decisions may impact ARG concentrations, and we recommend future multidisciplinary studies to determine which company decisions may cause these observed differences.

The development of AMR bioindicators of manure impact is greatly needed for standardizing studies and for use in routine environmental monitoring (He et al., 2020; Howe and Soupir, 2021). This study provides support that standardized monitoring is likely but requires further evidence in development methods in gene selection and gene quantification. An ideal swine manure associated bioindicator should be commonly found in swine manure at the time of manure application and also specific to swine manure and not detected in natural environments. Often, the selection of ARGs are based on previous detection of ARGs, and our results justify the selection of these genes on broad manure samples. However, we also suggest that other genes within the tetracycline and erythromycin resistant classes may complement these genes and be more suitable for high-throughput methods. For detection of AMR impact in complex environments, like manures, it is likely that a single ARG will not be sufficient and methods that can detect and quantify multiple genes simultaneously provide opportunity for increased sensitivity and specificity of detection for monitoring efforts.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical review and approval was not required for the animal study because it is not needed in accordance with the local legislation and institutional requirements. This work was conducted in collaboration with local swine growers who made all animal decisions regarding health and well-being and allowed the collection of manure at their site. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

TN: Investigation, validation, data curation, formal analysis, writing – original draft, visualization. MS: Writing – review & editing. DA: Resources, writing – review & editing. MO: Investigation, writing - review & editing. AH: Conceptualization, methodology, resources, writing – review & editing, supervision, funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This project was partially supported by AFRI food safety grant no. 2018-67017-27629 from the USDA National Institute of Food and Agriculture and partially supported by AFRI food safety grant no. 2021-68015-33495 from the USDA National Institute of Food and Agriculture.

Acknowledgments

The authors would like to thank JiJY Thanwalee for assistance with laboratory methods and data analyses, and we thank the Iowa State Statistical Consulting Service for their guidance on the statistical analysis.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frabi.2023.1116785/full#supplementary-material

References

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259. doi: 10.1038/nrmicro2312

Alt, L. M., Iverson, A. N., Soupir, M. L., Moorman, T. B., and Howe, A. (2021). Antibiotic resistance gene dissipation in soil microcosms amended with antibiotics and swine manure. *J. Environ. Qual.* 50, 911–922. doi: 10.1002/jeq2.20240

Bacanlı, M., and Başaran, N. (2019). Importance of antibiotic residues in animal food. *Food Chem. Toxicol.* 125, 462–466. doi: 10.1016/j.fct.2019.01.033

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models Usinglme4. *J. Stat. Software* 67, 1–48. doi: 10.18637/jss.v067.i01

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., et al. (2015). Tackling antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol.* 13, 310–317. doi: 10.1038/nrmicro3439

Błażejewska, A., Zalewska, M., Grudniak, A., and Popowska, M. (2022). A comprehensive study of the microbiome, resistome, and physical and chemical characteristics of chicken waste from intensive farms. *Biomolecules* 12, 1132. doi: 10.3390/biom12081132

Center for Veterinary Medicine (2020) *FDA 2019 report on antimicrobial sales for food-producing animals*. Available at: https://www.fda.gov/animal-veterinary/cvm-updates/fda-releases-annual-summary-report-antimicrobials-sold-or-distributed-2019-use-food-producing (Accessed September 13, 2021).

Checcucci, A., Trevisi, P., Luise, D., Modesto, M., Blasioli, S. , Braschi, I., et al. (2020). Exploring the animal waste resistome: The spread of antimicrobial resistance genes through the use of livestock manure. *Front. Microbiol.* 11, 1416. doi: 10.3389/fmicb.2020.01416

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y.-F., Yannarell, A. C., et al. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *J. Environ. Qual.* 38, 1086–1108. doi: 10.2134/jeq2008.0128

Chen, J., Yu, Z., Michel, F. C.Jr., Wittum, T., and Morrison, M. (2007). Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin b in livestock manure and manure management systems. *Appl. Environ. Microbiol.* 73, 4407–4416. doi: 10.1128/AEM.02799-06

Chen, Q.-L., An, X.-L., Li, H., Zhu, Y.-G., Su, J.-Q., and Cui, L. (2017). Do manure-borne or indigenous soil microorganisms influence the spread of antibiotic resistance genes in manured soil? *Soil Biol. Biochem.* 114, 229–237. doi: 10.1016/j.soilbio.2017.07.022

Chen, Z., Zhang, W., Yang, L., Stedtfeld, R. D., Peng, A., Gu, C., et al. (2019). Antibiotic resistance genes and bacterial communities in cornfield and pasture soils receiving swine and dairy manures. *Environ. pollut.* 248, 947–957. doi: 10.1016/j.envpol.2019.02.093

Cheng, D., Liu, Y., Shehata, E., Feng, Y., Lin, H., Xue, J., et al. (2021). In-feed antibiotic use changed the behaviors of oxytetracycline, sulfamerazine, and ciprofloxacin and related antibiotic resistance genes during swine manure composting. *J. Hazard. Mater.* 402, 123710. doi: 10.1016/j.jhazmat.2020.123710

Cheng, J., Tang, X., and Liu, C. (2020). Occurrence and distribution of antibiotic resistance genes in various rural environmental media. *Environ. Sci. pollut. Res. Int.* 27, 29191–29203. doi: 10.1007/s11356-020-09287-x

Congilosi, J. L., Wallace, J. S., Neher, T. P., Howe, A., Soupir, M. L., and Aga, D. S. (2022). Co-Occurrence of antimicrobials and metals as potential drivers of antimicrobial resistance in swine farms. *Front. Environ. Sci. Eng. China* 10. doi: 10.3389/fenvs.2022.1018739

Cooper, O. (2018) *How integration works on one US pig farm. farmers weekly*. Available at: https://www.fwi.co.uk/livestock/pigs/how-integration-work-on-one-us-pig-farm (Accessed September 20, 2022).

Dewey, C. E., Cox, B. D., Straw, B. E., Bush, E. J., and Hurd, S. (1999). Use of antimicrobials in swine feeds in the united states. *J. Swine Health Production* 7, 19–25.

Dunlop, R. H., McEwen, S. A., Meek, A. H., Friendship, R. A., Clarke, R. C., and Black, W. D. (1998). Antimicrobial drug use and related management practices among Ontario swine producers. *Can. Vet. J.* 39, 87–96.

Elmund, G. K., Morrison, S. M., Grant, D. W., and Nevins, S. M. (1971). Role of excreted chlortetracycline in modifying the decomposition process in feedlot waste. *Bull. Environ. Contam. Toxicol.* 6, 129–132. doi: 10.1007/BF01540093

Fang, H., Han, L., Zhang, H., Long, Z., Cai, L., and Yu, Y. (2018). Dissemination of antibiotic resistance genes and human pathogenic bacteria from a pig feedlot to the surrounding stream and agricultural soils. *J. Hazard. Mater.* 357, 53–62. doi: 10.1016/j.jhazmat.2018.05.066

Fernanda, P. A., Liu, S., Yuan, T., Ramalingam, B., Lu, J. , and Sekar, R. (2022). Diversity and abundance of antibiotic resistance genes and their relationship with nutrients and land use of the inflow rivers of taihu lake. *Front. Microbiol.* 13, 1009297. doi: 10.3389/fmicb.2022.1009297

Flater, J. S., Alt, L. M., Soupir, M., Moorman, T. B., and Howe, A. (2022). Prairie strips' effect on transport of antimicrobial resistance indicators in poultry litter. *J. Environ. Qual.* 51, 260–271. doi: 10.1002/jeq2.20333

Funes-Huacca, M. E., Opel, K., Thompson, R., and McCord, B. R. (2011). A comparison of the effects of PCR inhibition in quantitative PCR and forensic STR analysis. *Electrophoresis* 32, 1084–1089. doi: 10.1002/elps.201000584

Ghanbari, M., Klose, V., Crispie, F., and Cotter, P. D. (2019). The dynamics of the antibiotic resistome in the feces of freshly weaned pigs following therapeutic administration of oxytetracycline. *Sci. Rep.* 9, 4062. doi: 10.1038/s41598-019-40496-8

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., et al. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PloS Pathog.* 7, e1002158. doi: 10.1371/journal.ppat.1002158

Hall, M. C., Mware, N. A., Gilley, J. E., Bartelt-Hunt, S. L., Snow, D. D., Schmidt, A. M., et al. (2020). Influence of setback distance on antibiotics and antibiotic resistance genes in runoff and soil following the land application of swine manure slurry. *Environ. Sci. Technol.* 54, 4800–4809. doi: 10.1021/acs.est.9b04834

Han, X.-M., Hu, H.-W., Chen, Q.-L., Yang, L.-Y., Li, H.-L., Zhu, Y.-G., et al. (2018). Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. *Soil Biol. Biochem.* 126, 91–102. doi: 10.1016/j.soilbio.2018.08.018

Hayes, J. F. (2022). Fighting back against antimicrobial resistance with comprehensive policy and education: A narrative review. *Antibiotics (Basel)* 11, 644. doi: 10.3390/antibiotics11050644

He, L.-Y., He, L.-K., Liu, Y.-S., Zhang, M., Zhao, J.-L., Zhang, Q.-Q., et al. (2019). Microbial diversity and antibiotic resistome in swine farm environments. *Sci. Total Environ.* 685, 197–207. doi: 10.1016/j.scitotenv.2019.05.369

He, Y., Yuan, Q., Mathieu, J., Stadler, L., Senehi, N., Sun, R., et al. (2020). Antibiotic resistance genes from livestock waste: occurrence, dissemination, and treatment. *NPJ Clean Water* 3, 4. doi: 10.1038/s41545-020-0051-0

Hölzel, C. S., Müller, C., Harms, K. S., Mikolajewski, S., Schäfer, S., Schwaiger, K., et al. (2012). Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environ. Res.* 113, 21–27. doi: 10.1016/j.envres.2012.01.002

Howe, A. C., and Soupir, M. L. (2021). Antimicrobial resistance in integrated agroecosystems: State of the science and future opportunities. *J. Environ. Qual.* 50, 1255–1265. doi: 10.1002/jeq2.20289

Hughes, D., and Andersson, D. I. (2017). Environmental and genetic modulation of the phenotypic expression of antibiotic resistance. *FEMS Microbiol. Rev.* 41, 374–391. doi: 10.1093/femsre/fux004

IADNR (2022) *Confinements. iowa.gov*. Available at: https://www.iowadnr.gov/Environmental-Protection/Animal-Feeding-Operations/Confinements (Accessed July 13, 2022).

IPPA (2012) *Iowa Pork facts. Iowa pork*. Available at: https://www.iowapork.org/news-from-the-iowa-pork-producers-association/iowa-pork-facts (Accessed September 29, 2022).

Joy, S. R., Bartelt-Hunt, S. L., Snow, D. D., Gilley, J. E., Woodbury, B. L., Parker, D. B., et al. (2013). Fate and transport of antimicrobials and antimicrobial resistance genes in soil and runoff following land application of swine manure slurry. *Environ. Sci. Technol.* 47, 12081–12088. doi: 10.1021/es4026358

Joy, S. R., Li, X., Snow, D. D., Gilley, J. E., Woodbury, B., and Bartelt-Hunt, S. L. (2014). Fate of antimicrobials and antimicrobial resistance genes in simulated swine manure storage. *Sci. Total Environ.* 481, 69–74. doi: 10.1016/j.scitotenv.2014.02.027

Kang, Y., Li, Q., Yin, Z., Shen, M., Zhao, H., Bai, Y., et al. (2018). High diversity and abundance of cultivable tetracycline-resistant bacteria in soil following pig manure application. *Sci. Rep.* 8, 1489. doi: 10.1038/s41598-018-20050-8

Kasuga, I., Nagasawa, K., Suzuki, M., Kurisu, F., and Furumai, H. (2022). High-throughput screening of antimicrobial resistance genes and their association with class 1 integrons in urban rivers in japan. *Front. Environ. Sci. Eng. China* 10. doi: 10.3389/fenvs.2022.825372

Keenum, I., Liguori, K., Calarco, J., Davis, B. C., Milligan, E., Harwood, V. J., et al. (2022). A framework for standardized qPCR-targets and protocols for quantifying antibiotic resistance in surface water, recycled water and wastewater. *Crit. Rev. Environ. Sci. Technol.* 52, 1–25. doi: 10.1080/10643389.2021.2024739

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., et al. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U. S. A.* 115, E3463–E3470. doi: 10.1073/pnas.1717295115

Lenth, R. V. (2021) *Emmeans: Estimated marginal means, aka least-squares means*. Available at: https://CRAN.R-project.org/package=emmeans (Accessed January 15, 2022).

Li, N., Zhu, C., Liu, C., Zhang, X., Ding, J., Zandi, P., et al. (2019). The persistence of antimicrobial resistance and related environmental factors in abandoned and working swine feedlots. *Environ. pollut.* 255, 113116. doi: 10.1016/j.envpol.2019.113116

Lima, T., Domingues, S., and Da Silva, G. J. (2020). Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. *Vet. Sci. China* 7, 110. doi: 10.3390/vetsci7030110

Liu, Z., Klümper, U., Shi, L., Ye, L., and Li, M. (2019). From pig breeding environment to subsequently produced pork: Comparative analysis of antibiotic resistance genes and bacterial community composition. *Front. Microbiol.* 10, 43. doi: 10.3389/fmicb.2019.00043

Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci. U. S. A.* 109, 1691–1696. doi: 10.1073/pnas.1120238109

Lopatto, E., Choi, J., Colina, A., Ma, L., Howe, A., and Hinsa-Leasure, S. (2019). Characterizing the soil microbiome and quantifying antibiotic resistance gene dynamics in agricultural soil following swine CAFO manure application. *PloS One* 14, e0220770. doi: 10.1371/journal.pone.0220770

Lu, X.-M., Li, W.-F., and Li, C.-B. (2017). Characterization and quantification of antibiotic resistance genes in manure of piglets and adult pigs fed on different diets. *Environ. pollut.* 229, 102–110. doi: 10.1016/j.envpol.2017.05.080

Lüdecke, D., Ben-Shachar, M., Patil, I., Waggoner, P., and Makowski, D. (2021). Performance: An r package for assessment, comparison and testing of statistical models. *J. Open Source Software* 6, 3139. doi: 10.21105/joss.03139

Luo, L., Feng, J., Xue, R., Ma, J., Lou, L., He, J., et al. (2021). The insufficient extraction of DNA from swine manures may underestimate the abundance of antibiotic resistance genes as well as ignore their potential hosts. *J. Environ. Manage.* 278, 111587. doi: 10.1016/j.jenvman.2020.111587

Mackie, R. I., Koike, S., Krapac, I., Chee-Sanford, J., Maxwell, S., and Aminov, R. I. (2006). Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. *Anim. Biotechnol.* 17, 157–176. doi: 10.1080/10495390600956953

Marti, R., Tien, Y.-C., Murray, R., Scott, A., Sabourin, L., and Topp, E. (2014). Safely coupling livestock and crop production systems: How rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? *Appl. Environ. Microbiol.* 80, 3258–3265. doi: 10.1128/aem.00231-14

Martínez, J. L. (2012). Natural antibiotic resistance and contamination by antibiotic resistance determinants: the two ages in the evolution of resistance to antimicrobials. *Front. Microbiol.* 3, 1. doi: 10.3389/fmicb.2012.00001

Martínez, J. L., Coque, T. M., and Baquero, F. (2015). What is a resistance gene? ranking risk in resistomes. *Nat. Rev. Microbiol.* 13, 116–123. doi: 10.1038/nrmicro3399

McBride, W. D., and Key, N. (2003). *Economic and structural relationships in U.S. hog production* (USDA-ERS Agricultural Economic Report No. 818). doi: 10.2139/ssrn.758464

Meyers, M. A., Durso, L. M., Gilley, J. E., Waldrip, H. M., Castleberry, L., and Millmier-Schmidt, A. (2020). Antibiotic resistance gene profile changes in cropland soil after manure application and rainfall. *J. Environ. Qual.* 49, 754–761. doi: 10.1002/jeq2.20060

Mitchell, J., O'Neill, A. J., and King, R. (2022). Creating a framework to align antimicrobial resistance (AMR) research with the global guidance: a viewpoint. *J. Antimicrob. Chemother.* 77, dkac205. doi: 10.1093/jac/dkac205

Mu, Q., Li, J., Sun, Y., Mao, D., Wang, Q., and Luo, Y. (2015). Occurrence of sulfonamide-, tetracycline-, plasmid-mediated quinolone- and macrolide-resistance genes in livestock feedlots in northern China. *Environ. Sci. pollut. Res. Int.* 22, 6932–6940. doi: 10.1007/s11356-014-3905-5

Muurinen, J., Richert, J., Wickware, C. L., Richert, B., and Johnson, T. A. (2021). Swine growth promotion with antibiotics or alternatives can increase antibiotic resistance gene mobility potential. *Sci. Rep.* 11, 5485. doi: 10.1038/s41598-021-84759-9

Mware, N. A., Hall, M. C., Rajendran, S., Gilley, J. E., Schmidt, A. M., Bartelt-Hunt, S. L., et al. (2022). Resistome and mobilome in surface runoff from manured soil as affected by setback distance. *J. Hazard. Mater.* 429, 128278. doi: 10.1016/j.jhazmat.2022.128278

Neher, T. P., Ma, L., Moorman, T. B., Howe, A., and Soupir, M. L. (2020). Seasonal variations in export of antibiotic resistance genes and bacteria in runoff from an agricultural watershed in Iowa. *Sci. Total Environ.* 738, 140224. doi: 10.1016/j.scitotenv.2020.140224

OECD and Food and Agriculture Organization of the United Nations (2021). *OECD-FAO agricultural outlook 2021-2030* (OECD Publishing, Paris: OECD).

Park, S., Rana, A., Sung, W., and Munir, M. (2021). Competitiveness of quantitative polymerase chain reaction (qPCR) and droplet digital polymerase chain reaction (ddPCR) technologies, with a particular focus on detection of antibiotic resistance genes (ARGs). *Appl. Microbiol.* 1, 426–444. doi: 10.3390/applmicrobiol1030028

Pärnänen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D. G. J., et al. (2018). Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nat. Commun.* 9, 3891. doi: 10.1038/s41467-018-06393-w

Peng, S., Feng, Y., Wang, Y., Guo, X., Chu, H., and Lin, X. (2017). Prevalence of antibiotic resistance genes in soils after continually applied with different manure for 30 years. *J. Hazard. Mater.* 340, 16–25. doi: 10.1016/j.jhazmat.2017.06.059

Petrin, S., Patuzzi, I., Di Cesare, A., Tiengo, A., Sette, G., Biancotto, G., et al. (2019). Evaluation and quantification of antimicrobial residues and antimicrobial resistance genes in two Italian swine farms. *Environ. pollut.* 255, 113183. doi: 10.1016/j.envpol.2019.113183

Radu, E., Woegerbauer, M., Rab, G., Oismüller, M., Strauss, P., Hufnagl, P., et al. (2021). Resilience of agricultural soils to antibiotic resistance genes introduced by agricultural management practices. *Sci. Total Environ.* 756, 143699. doi: 10.1016/j.scitotenv.2020.143699

Reimer, J. J. (2006). Vertical integration in the pork industry. *Am. J. Agric. Econ.* 88, 234–248. doi: 10.1111/j.1467-8276.2006.00850.x

Samanta, P., Horn, H., and Saravia, F. (2022). Removal of diverse and abundant ARGs by MF-NF process from pig manure and digestate. *Membranes* 12, 661. doi: 10.3390/membranes12070661

Sandberg, K. D., Ishii, S., and LaPara, T. M. (2018). A microfluidic quantitative polymerase chain reaction method for the simultaneous analysis of dozens of antibiotic resistance and heavy metal resistance genes. *Environ. Sci. Technol. Lett.* 5, 20–25. doi: 10.1021/acs.estlett.7b00552

Shui, J., Tuo, H., Liu, J., Zhang, X., Feng, J., Feng, Y., et al. (2022). Insights into the fates of plasmids and antimicrobial resistance genes during swine manure treatment and related factors based on plasmidome and metagenome analyses. *Environ. Sci. pollut. Res. Int* 29, 69037–69047. doi: 10.1007/s11356-022-20574-7

Sidstedt, M., Rådström, P., and Hedman, J. (2020). PCR inhibition in qPCR, dPCR and MPS-mechanisms and solutions. *Anal. Bioanal. Chem.* 412, 2009–2023. doi: 10.1007/s00216-020-02490-2

Stedtfeld, R. D., Guo, X., Stedtfeld, T. M., Sheng, H., Williams, M. R., Hauschild, K., et al. (2018). Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. *FEMS Microbiol. Ecol.* 94, fiy130. doi: 10.1093/femsec/fiy130

Tsoulouhas, T., and Vukina, T. (1999). Integrator contracts with many agents and bankruptcy. *Am. J. Agric. Econ.* 81, 61–74. doi: 10.2307/1244450

Van Goethem, M. W., Pierneef, R., Bezuidt, O. K. I., Van De Peer, Y., Cowan, D. A., and Makhalanyane, T. P. (2018). A reservoir of 'historical' antibiotic resistance genes in remote pristine Antarctic soils. *Microbiome* 6, 40. doi: 10.1186/s40168-018-0424-5

Wang, L., and Chai, B. (2022). Fate of antibiotic resistance genes and changes in bacterial community with increasing breeding scale of layer manure. *Front. Microbiol.* 13, 857046. doi: 10.3389/fmicb.2022.857046

Wang, F., Han, W., Chen, S., Dong, W., Qiao, M., Hu, C., et al. (2020). Fifteen-year application of manure and chemical fertilizers differently impacts soil ARGs and

microbial community structure. *Front. Microbiol.* 11, 62. doi: 10.3389/fmicb.2020.00062

Waseem, H., Saleem ur Rehman, H., Ali, J., Iqbal, M. J., and Ali, M. I. (2020). "Global trends in ARGs measured by HT-qPCR platforms," in *Antibiotics and antimicrobial resistance genes in the environment*. Ed. M. Z. Hashmi (Elsevier), 206–222.

Wellington, E. M. H., Boxall, A. B., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., et al. (2013). The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect. Dis.* 13, 155–165. doi: 10.1016/S1473-3099(12)70317-1

Wen, X., Mi, J., Wang, Y., Ma, B., Zou, Y., Liao, X., et al. (2019). Occurrence and contamination profiles of antibiotic resistance genes from swine manure to receiving environments in guangdong province southern China. *Ecotoxicol. Environ. Saf.* 173, 96–102. doi: 10.1016/j.ecoenv.2019.02.023

Whitehead, T. R., and Cotta, M. A. (2013). Stored swine manure and swine faeces as reservoirs of antibiotic resistance genes. *Lett. Appl. Microbiol.* 56, 264–267. doi: 10.1111/lam.12043

Whittle, G., Whitehead, T. R., Hamburger, N., Shoemaker, N. B., Cotta, M. A., and Salyers, A. A. (2003). Identification of a new ribosomal protection type of tetracycline resistance gene, tet(36), from swine manure pits. *Appl. Environ. Microbiol.* 69, 4151–4158. doi: 10.1128/AEM.69.7.4151-4158.2003

Xue, J., Wu, J., Hu, Y., Sha, C., Yao, S., Li, P., et al. (2021). Occurrence of heavy metals, antibiotics, and antibiotic resistance genes in different kinds of land-applied manure in China. *Environ. Sci. pollut. Res. Int.* 28, 40011–40021. doi: 10.1007/s11356-021-13307-9

Yang, F., Han, B., Gu, Y., and Zhang, K. (2020). Swine liquid manure: a hotspot of mobile genetic elements and antibiotic resistance genes. *Sci. Rep.* 10, 15037. doi: 10.1038/s41598-020-72149-6

Zalewska, M., Błażejewska, A., Czapko, A. , and Popowska, M. (2021). Antibiotics and antibiotic resistance genes in animal manure - consequences of its application in agriculture. *Front. Microbiol.* 12, 610656. doi: 10.3389/fmicb.2021.610656

Zhang, B., Tian, H., Lu, C., Dangal, S. R. S., Yang, J., and Pan, S. (2017). Global manure nitrogen production and application in cropland during 1860–2014: a 5 arcmin gridded global dataset for earth system modeling. *Earth System Sci. Data* 9, 667. doi: 10.5194/essd-9-667-2017

Zhang, K., Xin, R., Zhao, Z., Li, W., Wang, Y., Wang, Q., et al. (2021). Mobile genetic elements are the major driver of high antibiotic resistance genes abundance in the upper reaches of huaihe river basin. *J. Hazard. Mater.* 401, 123271. doi: 10.1016/j.jhazmat.2020.123271

Zhang, Z., Zhang, Q., Wang, T., Xu, N., Lu, T., Hong, W., et al. (2022). Assessment of global health risk of antibiotic resistance genes. *Nat. Commun.* 13, 1553. doi: 10.1038/s41467-022-29283-8

Zhao, X., Wang, J., Zhu, L., and Wang, J. (2019). Field-based evidence for enrichment of antibiotic resistance genes and mobile genetic elements in manure-amended vegetable soils. *Sci. Total Environ.* 654, 906–913. doi: 10.1016/j.scitotenv.2018.10.446

Zhou, X., Wang, J., Lu, C., Liao, Q., Gudda, F. O., and Ling, W. (2020). Antibiotics in animal manure and manure-based fertilizers: Occurrence and ecological risk assessment. *Chemosphere* 255, 127006. doi: 10.1016/j.chemosphere.2020.127006

Zhu, Y.-G., Johnson, T. A., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R. D., et al. (2013). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. U. S. A.* 110, 3435–3440. doi: 10.1073/pnas.1222743110

frontiers | Frontiers in Antibiotics

TYPE Original Research
PUBLISHED 15 June 2023
DOI 10.3389/frabi.2023.1162636

OPEN ACCESS

EDITED BY
Getahun E. Agga,
Food Animal Environmental Systems Research, United States Department of Agriculture (USDA), United States

REVIEWED BY
Mohd Nasir Mohd Desa,
Putra Malaysia University, Malaysia
Atmika Paudel,
Hokkaido University, Japan

*CORRESPONDENCE
Yvonne Agersø
DKYVAG@chr-hansen.com

RECEIVED 09 February 2023
ACCEPTED 11 April 2023
PUBLISHED 15 June 2023

CITATION
Nøhr-Meldgaard K, Struve C, Ingmer H, Koza A, Al-Nakeeb K and Agersø Y (2023) Antimicrobial susceptibility testing and tentative epidemiological cut-off values for *Lactobacillaceae* family species intended for ingestion.
Front. Antibiot. 2:1162636.
doi: 10.3389/frabi.2023.1162636

Antimicrobial susceptibility testing and tentative epidemiological cut-off values for *Lactobacillaceae* family species intended for ingestion

Katrine Nøhr-Meldgaard[1,2], Carsten Struve[1], Hanne Ingmer[2], Anna Koza[1], Kosai Al-Nakeeb[1] and Yvonne Agersø[1,2]*

[1]Research & Development, Chr. Hansen A/S, Hørsholm, Denmark, [2]Department of Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg, Denmark

Introduction: In this work, 170 strains covering 13 species from the *Lactobacillaceae* family were analyzed to determine minimal inhibitory concentration (MIC) distributions to nine antimicrobial agents, and genes potentially conferring resistance. This allows a proposal of tentative Epidemiological Cut-Offs (ECOFFs) that follows the phylogeny for interpretation of resistance in the 13 species.

Methods: The 170 strains originated from different sources, geographical areas, and time periods. MICs for nine antibiotics were determined according to the ISO 10932 standard for lactobacillia and by a modified CLSI-method for *Leuconostoc* and *Pediococcus* which ensured sufficient growth. The strains were whole genome sequenced, subtyped by core genome analysis, and assessed for the presence of antibiotic resistance genes using the ResFinder and NCBI AMRFinder databases.

Results and discussion: The data provide evidence that antimicrobial susceptibility follows phylogeny instead of fermentation pattern and accordingly, tentative ECOFFs were defined. For some species the tentative ECOFFs for specific antibiotics are above the cut-off values set by the European Food Safety Authority (EFSA) which are primarily defined according to fermentation pattern or at genus level. The increased tolerance for specific antibiotics observed for some species was evaluated to be innate, as only for one strain phenotypic resistance was found to be related to an acquired resistance gene. In general, more data are needed to define ECOFFs and since the number of isolates available for industrial relevant bacterial species are often limited compared to clinically relevant species, it is important; 1) that strains are unambiguously defined at species level and subtyped through core genome analysis, 2) MIC determination are performed by use of a standardized method to define species-specific MIC distributions and 3) that known antimicrobial resistance genes are determined in whole genome sequences to support the MIC determinations.

KEYWORDS

antibiotic, epidemiological cut-offs, tentative ECOFFs, intrinsic resistance, antibiotic resistance, lactic acid bacteria

Introduction

Antibiotic resistant organisms are present in all environments and both pathogenic and non-pathogenic bacteria encode antibiotic resistance genes (Allen et al., 2010). When non-pathogenic bacteria are included in food and feed cultures, it is a requirement that they are free of acquired antibiotic resistance genes as these may be transferred to pathogenic bacteria potentially compromising antimicrobial therapy (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018). Intrinsic (innate) antibiotic resistance is, however, not considered a safety concern, as it is conserved within specific species and spread clonally rather than horizontally. The major intrinsic mechanisms are absence of the antibiotic target, mutations conferring a low affinity or permeability or intrinsic genes e.g. encoding an efflux mechanism (EFSA, 2005; Cox and Wright, 2013; EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018; Nøhr-Meldgaard et al., 2021).

To reduce the risk of transmissible antibiotic resistance genes from food and feed, the European Food Safety Authority (EFSA) provides antimicrobial microbiological cut-off values, for nine antimicrobial compounds, which are considered as highly or critically important for treatment of infections in humans (World Health Organisation (WHO), 2018). The cut-off values are a pragmatic tool for differentiating between resistant and susceptible bacterial strains within a population (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018). The current EFSA cut-off values are defined based on published minimal inhibitory concentration (MIC) data of industrially relevant species. However, much of the data have been generated using different methods (broth microdilution, Etest, disk diffusion and agar dilution method) and test conditions, either because the studies were performed before the ISO 10932 standard on determination of MIC for lactic acid bacteria (LAB) was published or because the proposed test conditions, such as using cation-adjusted Mueller-Hinton broth (CAMHB) with lysed horse blood for *Leuconostoc* and *Pediococcus*, does not provide the optimal growth conditions compared to the LAB susceptibility test medium (LSM) (Klare et al., 2005; International Organization for Standardization, 2010; Clinical and Laboratory Standards Institute (CLSI), 2016). Furthermore, the amount of MIC data on industrially relevant bacterial species are limited and not enough to define epidemiological cut-offs (ECOFFs), which require data from at least five separate laboratories, at least 15 values from each laboratory and at least 100 MIC values in the wild-type distribution (European Committe on Antimicrobial Susceptibility Testing (EUCAST), 2021). Due to the limited amount of MIC data on LAB, the current cut-off values for the *Lactobacillus* genus are defined primarily according to fermentation pattern e.g., obligate homofermentative, facultative heterofermentative and obligate heterofermentative, and for *Leuconostoc* and *Pediococcus* cut-off values are only defined at genus level. This is not optimal as the recommendation from EUCAST is to define cut-off values at species level, which is also supported by previous studies on industrially relevant bacterial species (Agersø et al., 2018; EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018; European Committe on Antimicrobial Susceptibility Testing (EUCAST), 2021). Therefore, more antimicrobial susceptibility data for industrially relevant species are needed.

Traditionally, *Lactobacillus* species have been characterized based on the type of sugars fermented and the fermentation product formed and grouped as either obligate homofermentative, facultative heterofermentative or obligate heterofermentative (Salvetti et al., 2012). However, recent studies have shown that this division of *Lactobacillus* species is obsolete as it does not follow phylogeny and in 2020, a major taxonomic revision of the *Lactobacillus* genus was performed, which resulted in the splitting of the *Lactobacillus* genus into 25 genera and the inclusion of the *Leuconostoc* genera in the *Lactobacillaceae* family, which already included *Pediococcus* (Salvetti et al., 2012; Zheng et al., 2015; Duar et al., 2017; Zheng et al., 2020). As a consequence of the taxonomic revision, the MIC of species belonging to different genera, such as *Lentilactobacillus parabuchneri and Limosilactobacillus fermentum* should be evaluated using the same cut-off values, namely the *Lactobacillus* obligate heterofermentative cut-off values (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018). This illustrates the need for updated microbiological cut-off values for *Lactobacillaceae* that follows phylogeny instead of fermentation patterns.

Leuconostoc species are important for the production of fermented dairy products (Cardamone et al., 2011) and the majority of published microbiological susceptibility data are on the industrially relevant species *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*. However, several different methods and test conditions have been used, wherefore data generated using standardized test conditions are needed (Swenson et al., 1990; Katla et al., 2001; Casado Muñoz M del et al., 2014; Basbülbül et al., 2015; Jeong and Lee, 2015; Flórez et al., 2016). Recently, the *L. pseudomesenteroides* species were divided into two species, namely *L. pseudomesenteroides* and the novel *Leuconostoc falkenbergense* species (Wu and Gu, 2021). However, *L. falkenbergense* and *L. pseudomesenteroides* are more closely related to each other than to other *Leuconostoc* species including *L. mesenteroides* (Wu and Gu, 2021).

Strains of the species *P. acidilactici* and *P. pentosaceus* are frequently used for cheese production, but are also used as probiotics, and meat and vegetables fermentations as they produce characteristic flavor and improve hygienic quality and extend shelf life due to the production of bacteriocins (Stiles, 1996; Holzapfel et al., 1998; Beresford et al., 2001). Due to their important role in fermentation, most of the published antimicrobial susceptibility data for *Pediococcus* are for the *P. acidilactici* and *P. pentosaceus* species; however, different methods and test conditions have been used which can affect the MIC values (Swenson et al., 1990; Danielsen et al., 2007; Klare et al., 2007; Muñoz-Atienza et al., 2013).

In the present study, tentative ECOFFs will be defined for 13 LAB species and evaluated against the currently available EFSA cut-off values which are primarily defined according to fermentation pattern or at genus level. Our results show that cut-off values should be based on phylogenetic relatedness rather than fermentation pattern and at

species rather than genus level. This will improve the interpretation criteria for antimicrobial susceptibility for these species.

Materials and methods

Bacterial strains

One hundred and seventy strains, including the specific type strains, belonging to 13 species were included in the study (Table S1). The strains were obtained from Chr. Hansen's Culture collection (CHCC), where they were stored at -80°C. The strains cover different geographic areas, sources and timepoints (Table S1).

Genomic DNA extractions, library preparation and QC for *de novo* short read (Illumina) whole genome sequencing

Genomic DNA for *de novo* short read WGS was extracted from bacterial cell pellets harvested from 1 mL of overnight culture normalized to OD_{600} = 1. Clean Blood & Tissue DNA Kit (NACBT-D0384) (Clean NA, The Netherlands) was used and manufactures protocol was modified. The extraction method was automated and performed on Biomek i5 liquid handler (Beckman Coulter, USA). Modifications to the manufactures protocol: cell pellets were resuspended in 200 µL of pre-lysis buffer (PBS, 20 mg/mL lysozyme, 50 U/mutanolysin, 100 mg/mL RNase A) instead of the Tissue Lysis buffer supplied in the kit.

Genomic libraries were generated for most of the strains using modified Kapa Hyper Plus Library Preparation Kit (Roche, Switzerland) on Biomek i5 Liquid Handler (Beckman Coulter, USA). 150 ng of genomic DNA diluted in 15 µL EB buffer (Tris-Cl, pH 8.0) was used in the half-volume reaction mixes for fragmentation, end-repair/A-tailing, ligation, and final amplification. 0.1 mM conditioning solution was added to fragmentation mix and fragmentation time was optimized to 10 minutes. 5 µL of 1 µM Kapa Dual-Indexed adapter (Roche, Switzerland) was used during adapter ligation step. 10 µL of the adapter-modified DNA fragments were enriched by 8-cycle PCR. Clean NGS beads (Clean NA, The Netherlands) were used for two post-ligation and two post-amplification clean-ups to purify fragments at average size between 450 to 550 bp.

For about 15 of the strains, genomic libraries were generated using NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® with NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA Set 1), (New England Biolabs Inc., USA) on Biomek i5 Liquid Handler (Beckman Coulter, USA). 200 ng of genomic DNA diluted in 15 µL EB buffer (Tris-Cl, pH 8.0) was used in the half-volume reaction mixes for fragmentation, end-repair/A-tailing, ligation, and final amplification. Fragmentation time was optimized to 8 minutes. 5 µL of 2.5 µM NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA Set 1), (New England Biolabs Inc., USA) was used during adapter ligation step. 10 µL of the adapter-modified DNA fragments were enriched by 9-cycle PCR. Clean NGS beads (Clean NA, The Netherlands) were used for double-sided post ligation size selection and one post-amplification clean-up to purify fragments at average size between 450 to 550 bp.

Concentration of genomic DNA and dsDNA libraries were measured by QubitFlex® Fluorimeter using Qubit dsDNA Broad range and Qubit 1x dsDNA HS assays (Thermo Fisher Scientific, USA), respectively. Average dsDNA library size distribution was determined using the Agilent HS NGS Fragment (1-6000 bp) kit on the Agilent Fragment Analyzer (Agilent Technologies, USA). Libraries were normalized and pooled in the normalization buffer (10 mM Tris-Cl, pH 8.0, 0.05% Tween 20) to the final concentration of 10 nM.

For most of the strains, denaturated in 0.2N NaOH, 10 pM pool of libraries in 600 µL ice-cold HT1 buffer was loaded onto the flow cell provided in the MiSeq Reagent kit v3 (600 cycles) and sequenced on a MiSeq platform (Illumina Inc., San Diego, USA) with a paired-end protocol and read lengths of 301 nt.

For about 15 of the strains, denaturated in 0.2N NaOH, 1 pM pool of libraries in 1300 µL ice-cold HT1 buffer was loaded onto the flow cell provided in the NextSeq Reagent Mid Output (300 cycles) and sequenced on a NextSeq platform (Illumina, USA) with a paired-end protocol and read lengths of 151 nt.

Genome assembly

All processing of the short reads was done in either CLC Genomics Server version 20.0.5 or CLC Genomics Workbench version 20.0.5.

The short reads were mapped with default parameters to the reference sequence of the phage Phi X 174 using the tool "Map reads to reference". Unmapped reads from the mapping were trimmed for quality using the PHRED score 23 as the threshold and with the non-default parameter of discarding reads that were less than 50 base pairs long using the tool "Trim Sequences".

The trimmed reads were *de novo* assembled with default parameters except for the minimum contig length which was set to 350 base pairs using the tool "*De Novo* Assembly". Afterwards, a decontamination step was performed where contigs with low depth of coverage were removed using a custom plugin written by Qiagen. The decontamination step first removes all contigs where the average depth of coverage is below 15X and afterwards removes all contigs where the depth of coverage is below 25% of the median average depth of coverage for the entire genome assembly.

Gene calling of the filtered contigs was done with Prodigal version 2.6.3 using the default parameters. Finally, the genome assemblies with annotated genes were functionally annotated with BLAST against a local annotation database using a custom plugin written by Qiagen.

Species identification

Species identification was done in an automated flow by either blasting of the WGS against 16S, rpoA sequences of type strain, or

average nucleotide identity in CLC Genomics Workbench version 20 (Qiagen Bioinformatics, Aarhus, Denmark). The species identification was further confirmed using core genome analysis. In brief, the genomes, either fully assembled or contigs were annotated by Prokka, which annotates genomes through the use of different tools including Prodigal (coding sequences), RNAmmer (Ribosomal RNA genes), Aragorn (Transfer RNA genes), SignalP (Signal leader peptides) and Infernal (Non-coding RNA) (Seemann, 2014). Prokka annotation is a requirement for using Roary, since the.gff file (file containing sequences and annotations) provided by Prokka is used by Roary to create a multi-FASTA alignment of all the core genes (Page et al., 2015). Roary was set to perform nucleotide alignment using MAFFT and a BLASTP percentage identity between 80-100%, depending on species (Katoh, 2002). FastTree was used to produce an approximately-maximum-likelihood phylogenetic tree from the core gene alignment file, which was visualized by MEGA X (Price et al., 2009; Price et al., 2010; Kumar et al., 2018).

Antimicrobial susceptibility testing

The MIC of nine antimicrobial agents was determined by use of broth microdilution, where the MIC is the lowest concentration of the antimicrobial that inhibits bacterial growth (Adimpong et al., 2012). All species were tested in LSM medium, which consist of 10% Iso-Sensitest (IST) broth and 90% MRS (De Man, Rogosa, Sharpe) medium both from Oxoid.

For the *Lactobacillus* species and species formerly belonging to the *Lactobacillus* genus, the strains were tested as recommended by the ISO 10932 standard (International Organization for Standardization, 2010), *P. acidilactici* was tested by use of the CLSI method (LSM media, 35°C, aerobic with film), while *P. pentosaceus* was tested by the use of a modified CLSI method (LSM, 30°C, aerobic with a lid). *L. mesenteroides, L. falkenbergense* and *L. pseudomesenteroides* were also tested by use of a modified CLSI method (LSM, 30°C, aerobic with film). MIC was read at both 20 and 24 hours for the *Pediococcus* genus and at 24 and 48 hours for the *Leuconostoc* genus.

L. plantarum ATCC 14917 and *L. paracasei* ATCC 334 were included for quality control using quality control ranges reported in the ISO 10932 standard (International Organization for Standardization, 2010). For 10 out of 40 *Leuconostoc* strains (3 media batches) the quality control strain *L. plantarum* exhibited ampicillin and clindamycin MIC one 2-fold below the accepted range, however when the quality control strain *L. paracasei* was tested with the same medium batch it was within the accepted range.

All tests were performed in duplicates in a customized Sensititre panel from Thermo Fisher Scientific. Nine antimicrobial agents are included in the customized Sensititre panel: ampicillin 0.03-16 mg/L, chloramphenicol 0.5-54 mg/L, clindamycin 0.03-32 mg/L, erythromycin 0.015-16 mg/L, gentamycin 0.25-128 mg/L, kanamycin 1-1024 mg/L, streptomycin 1-256 mg/L, tetracycline 0.12-64 mg/L and vancomycin 0.12-16 mg/L. Retesting was performed if the duplicates varied more than one 2-fold dilution for one or more antimicrobial agents. The results were accepted if they varied by three or fewer two-fold concentrations as previously described being within the technical variation for MIC broth dilution methods (Clinical and Laboratory Standards Institute (CLSI), 2018).

If the MIC value differed one 2-fold between the duplicates, the highest MIC was reported. All strains were streaked on blood agar plates to ensure that the samples were pure.

To compare the results from the customized Sensititre panel and the discontinued VetMIC panels Lact-1 and Lact-2 (SVA, Uppsala, Sweden), MIC data from 2012-2019 was compared for 25 strains on both MIC panels using the same method.

Epidemiological cut-off values for differentiation of susceptible (wildtype) and resistant (non-wildtype) populations

For each species-antimicrobial combination, MIC distributions were determined and from this tentative ECOFFs were defined together with MIC_{50} and MIC_{90} (MICs inhibiting 50% and 90% of the strains, respectively). ECOFFs is defined according to guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Turnidge et al., 2006; European Committe on Antimicrobial Susceptibility Testing (EUCAST), 2021), which state that the population with MIC at or below the ECOFF are susceptible (wildtype) and therefore also devoid of acquired resistance mechanisms and/or mutations leading to resistance (European Committe on Antimicrobial Susceptibility Testing (EUCAST), 2021).

Moreover, according to EUCAST the intrinsic (or wildtype) population is also characterized by the absence of acquired resistance mechanisms and/or mutations leading to resistance (European Committe on Antimicrobial Susceptibility Testing (EUCAST), 2021). The data were also evaluated with the interpretation criteria defined by EFSA for *Bacillus* ssp. (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018).

Detection of known antimicrobial resistance genes and comparison with phenotype

The presence of genes with identity to known antimicrobial resistance genes, in all the strain genomes, was assessed using ResFinder (Zankari et al., 2012) (nucleotide) and NCBI AMRFinderPlus (Feldgarden et al., 2019) (amino acid). Both databases were downloaded and imported into CLC Genomics Workbench 20.0.5. ResFinder was imported on 20 April 2021 and AMRFinderPlus on 27 April 2021. The assembled contigs of each strain were joined using the join function in CLC. The joined contigs were screened for resistance genes against the Resfinder database using BLASTn with a minimum word size of 11 and maximum E-value of 1.0E-10 and AMRFinderPlus using BLASTn with a minimum word size of 3 and a maximum E-value of 1.0E-50.

EFSA require that sequences with at least 80% identity and 70% coverage to known antimicrobial resistance genes should be

reported. In the case two or more fragments covering less than 70% length of the subject sequence with at least 80% identity to the same antimicrobial resistance gene are detected these should be reported, and it should be checked whether the full gene is present (European Food Safety Authority, 2021). The same criteria were used in this study.

Results and discussion

Comparison of MIC measured by VetMIC and Sensititre panels

The ISO 10932 standard on antimicrobial susceptibility testing of industrially used species suggest using VetMIC panels (SVA, Uppsala, Sweden) for MIC determination (International Organization for Standardization, 2010). However, as VetMIC panels have been discontinued by the provider alternative panels need to be evaluated. Therefore, MIC for 25 strains covering nine of the 13 species included in the study were measured using the VetMIC and the customized Sensititre panels (Table S2) to ensure comparable results are obtained. The MIC for specific strain-antimicrobial agents combinations varied less than three 2-fold dilutions for the VetMIC and Sensititre panels, which is described as the technical variation acceptable for the broth microdilution method (Clinical and Laboratory Standards Institute (CLSI), 2018). Therefore, the results obtained from the two panels are comparable when the strains are tested with the same conditions and the customized Sensititre panels can replace the VetMIC panels.

Included strains and grouping based on phylogenetic relatedness

In the present study, 170 strains belonging to 13 species, including the type strains were obtained from Chr. Hansen's Culture collection. The strains were epidemiologically unrelated and have been isolated from different geographic areas, sources and timepoints (Table S1). The criteria for including the specific species were 1) the current microbiological cut-offs are only defined at genus level (*Pediococcus* and *Leuconostoc*) or 2) the current microbiological cut-offs are defined based on fermentation groups and novel genera have been defined due to the recent *Lactobacillaceae* taxonomic revision (*Lactobacillus, Lactilactobacillus, Lentilactobacillus, Ligilactobacillus, Limosilactobacillus*) (Zheng et al., 2020). The included *Lactobacillus* species (*L. delbrueckii, L. gasseri, L. paragasseri, L. helveticus*) were chosen as a broad representation of the *Lactobacillus* genus (Zheng et al., 2020).

Core genome analysis was performed for each species to ensure that the included strains were phylogenetically different and based on this, 32 strains were excluded, which resulted in 170 strains included in the study.

Furthermore, core genome analysis of the type strains from the 13 included species was performed (Figure 1) to determine whether some of the species are so closely related that combined tentative ECOFFs can be defined and to verify that phylogeny and fermentation patterns is not related. The analysis shows that the phylogenetic grouping does not follow the fermentation pattern for *Lactobacillus* species and species previously belonging to the *Lactobacillus* genus, which is in agreement with previous studies (Zheng et al., 2015; Zheng et al., 2020) (Figure 1). This supports that *Lactobacillaceae* tentative ECOFFs should be defined according to phylogeny instead of fermentation patterns. Species specific tentative ECOFFs will therefore be defined for all the included *Lactobacillus, Lactilactobacillus, Lentilactobacillus, Ligilactobacillus* and *Limosilactobacillus* species, expect the phylogenetically closely related species *Lactobacillus gasseri* and *Lactobacillus paragasseri* (Tanizawa et al., 2018; Zheng et al., 2020) (Figure 1) for which the MIC distributions for the eight examined agents were overlapping.

For *Leuconostoc*, EFSA have defined microbiological cut-off values at genus level (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018). Two *Leuconostoc* species, *L. mesenteroides* and *L. pseudomesenteroides* was initially included in the present study; however, recently, the *L. pseudomesenteroides* species was divided into two species: *L. pseudomesenteroides* and the novel species *L. falkenbergense* (Wu and Gu, 2021). Core genome analysis revealed that all but two of the included *L. pseudomesentoides* strains belong to the *L. falkenbergense* species (Figure 2). As *L. falkenbergense* and *L. pseudomesenteroides* are very closely related both based on 16S rRNA sequence (Wu and Gu, 2021) and core genome analysis (Figure 1), tentative ECOFFs will be defined for the *L. falkebergense*/*L. pseudomesenteroides* group while tentative ECOFFs will be defined individually for *L. mesenteroides*.

Overall, the strains were epidemiologically unrelated and genetically diverse, so the strain collection displays a good representation of most of the included species, although the

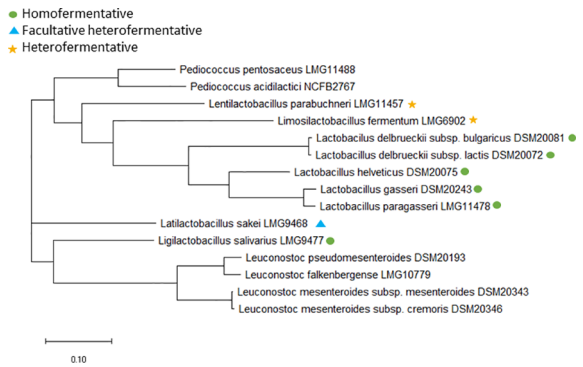


FIGURE 1
Core genome phylogenetic tree based on 65 core genes for the type strains of the included species. *Lactobacillus, Limosilactobacillus, Lentilactobacillus, Lactilactobacillus and Ligilactobacillus* species are either marked with a green dot if obligate homofermentative, a blue triangle if facultative heterofermentative or a yellow star if obligate heterofermentative. NCBI accession no.: LMG11488, SAMN33225762; NCFB2767, SAMN33225763; LMG11457, SAMN33225768; LMG6902, SAMN33225764; DSM20081, SAMN33225756; DSM20072, CP022988.1; DSM20075, SAMN33225755; DSM20243, SAMN33225757; LMG11478, SAMN33225767; LMG9468, SAMN33225759; LMG9477, SAMN33225758; DSM20193, SAMN33225760; LMG10779, SAMN33225766; DSM20343, SAMN33225765; DSM20346; SAMN33225761.

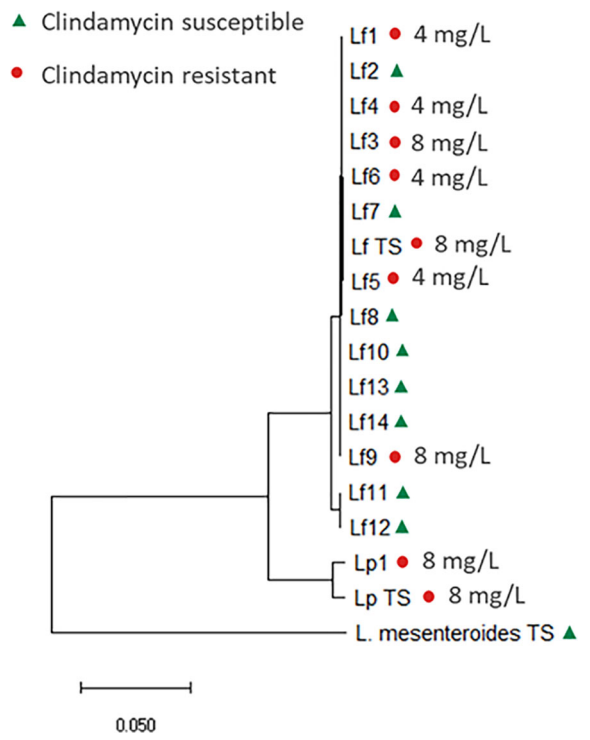


FIGURE 2
Core genome phylogenetic tree based on 592 core genes, including the 15 *L. falkenbergense* strains, named Lf1-14 and Lf TS (type strain, LMG10779) and the two *L. pseudomesenteroides* strains named Lp1 and Lp TS (type strain, DSM20193). The tree is rooted with the *L. mesenteroides* type strain (DSM20346). Clindamycin susceptible strains are marked with a triangle (green) and resistant strains with a dot (red).

number of isolates were limited. Another limitation is that the MIC analysis was performed in only one laboratory and not in several, the ECOFFs defined in this study are therefore tentative.

Comparison of MIC

Obligate homofermentative

The MIC range of the four homofermentative *Lactobacillus* species (*L. delbrueckii, L. gasseri/paragasseri, L. helveticus*) was compared to the *Lactobacillus* obligate homofermentative microbiological cut-off values provided by EFSA (Table 1) (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018; Zheng et al., 2020). Overall, these species exhibit different MIC distributions for all nine tested antimicrobial agents illustrating the need for tentative ECOFFs that follow phylogeny (Table 1).

None of the *Lactobacillus* species (*L. gasseri/paragasseri, L. helveticus, L. delbrueckii*) exhibit vancomycin MIC above the *Lactobacillus* obligate homofermentative cut-off value of 2 mg/L, in accordance with previous findings (Delgado et al., 2005; Zhang et al., 2018).

It is generally reported in the scientific literature that *Lactobacillus* spp. exhibits a high tolerance towards aminoglycosides and especially kanamycin as an intrinsic property of the genus (Danielsen and Wind, 2003; Mathur and Singh, 2005; Mayrhofer et al., 2010; Nawaz et al., 2011; Adimpong et al., 2012). In the present study, both *L. gasseri/paragasseri* and *L. delbrueckii* exhibit a kanamycin MIC range up to 128 mg/L (8-128 mg/L and ≤ 1-128 mg/L, respectively) and most of the population showed MICs above the EFSA cut-off value at 16 mg/L. This is in accordance with previous studies using broth microdilution method and test conditions as recommended in the ISO 10932 standard (International Organization for Standardization, 2010; Mayrhofer et al., 2010; Nawaz et al., 2011) (Table 1). Based on the included strains, *L. delbrueckii* subsp. *lactis* exhibit one 2-fold dilution higher kanamycin MIC range than the *L. delbrueckii* subsp. *bulgaricus* strains, but both subspecies exhibit a broad kanamycin MIC range. Furthermore, the *L. delbrueckii* subsp. *bulgaricus* type strain exhibit kanamycin MIC of 64 mg/L, while *L. delbrueckii* subsp. *lactis* type strain exhibit kanamycin MIC of 4 mg/L. This indicates that reduced kanamycin susceptibility is not only related to a specific subspecies; however, more strains belonging to the two subspecies need to be examined to evaluate this.

In contrast, *L. helveticus* exhibit a kanamycin MIC range of 8-32 mg/L, suggesting that innate tolerance to kanamycin is species specific and tentative ECOFFs should be defined according to phylogeny. Furthermore, *L. helveticus* exhibit streptomycin (and gentamycin) MIC values markedly below the current cut-off at 16 mg/L, as previously shown (Klare et al., 2007) showing that aminoglycoside susceptibility differ within species belonging to the *Lactobacillus* genus and obligate homofermentative species.

The current erythromycin EFSA cut-off is 1 mg/L, which is two-four 2-fold dilutions higher than the observed MIC distributions for the four *Lactobacillus* species (Table 1), in accordance with previous findings (Klare et al., 2007; Nawaz et al., 2011; EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018). This illustrates that the current EFSA cut-off values also can be too high for specific species and should be adjusted to divide the wild-type population from strains potentially coding for acquired resistance genes.

Facultative heterofermentative

As recommended by EFSA, the MIC ranges of *L. sakei* and the homofermentative *Ligilactobacillus salivarius* species were compared to the *Lactobacillus* facultative heterofermentative microbiological cut-off values (Table 2) (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018; Zheng et al., 2020).

Previous studies have shown that *L. salivarius* exhibits elevated kanamycin MIC (Nawaz et al., 2011; Adimpong et al., 2012; Stefańska et al., 2021), which is also observed in the present study, where 92% of the *L. salivarius* population exhibit kanamycin MICs above the current cut-off (64 mg/L), with a MIC range of 64-512 mg/L (Table 2). Since the whole population exhibit an elevated kanamycin MIC range it can be considered as an inherent trait of the species and the kanamycin tentative ECOFFs should be adjusted to reflect this. In contrast, *L. sakei* exhibit a lower kanamycin MIC range of 8-32 mg/L.

TABLE 1 MIC distribution and tentative ECOFFs for nine antimicrobial agents for *Lactobacillus* obligate homofermentative species.

Antimicrobial agent	Species	Distribution (%) of MICs																	Tentative ECOFF	MIC50	MIC90
		0.0075	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024			
Ampicillin	*L. delbrueckii* (28)		18		39	36	7													0.25	0.06	0.21
	L. gasseri (7)/*L. paragasseri* (9)					12	50	38												0.5	0.25	0.5
	Lactobacillus helveticus (7)						14	16												0.5	0.5	0.5
Chloramphenicol	*L. delbrueckii* (28)								4	18	75	4								8	4	4
	L. gasseri (7)/*L. paragasseri* (9)										38	62								8	8	8
	Lactobacillus helveticus (7)										100									4	4	4
Clindamycin	*L. delbrueckii* (28)		21		50	21	4	4												0.5	0.06	0.12
	L. gasseri (7)/*L. paragasseri* (9)						19	13	6	6	31	25								8	4	8
	Lactobacillus helveticus (7)							29	14	57										2	2	2
Erythromycin	*L. delbrueckii* (28)	21		29	39	11														0.12	0.03	0.06
	L. gasseri (7)/*L. paragasseri* (9)				31	56	13													0.25	0.12	0.12
	Lactobacillus helveticus (7)			29	71															0.06	0.06	0.06
Gentamycin	*L. delbrueckii* (28)					4		14	25	25	18	14								8	2	8
	L. gasseri (7)/*L. paragasseri* (9)									56	38	6								8	4	4
	Lactobacillus helveticus (7)							29	43	29										2	1	2
Kanamycin	*L. delbrueckii* (28)							7		7	18	11	25	4	18	11				128	8	32
	L. gasseri (7)/*L. paragasseri* (9)											13	6	31	38	13				128	64	128
	Lactobacillus helveticus (7)											57	29	14						32	8	32
Streptomycin	*L. delbrueckii* (28)							4		14	11	14	25	4						32	8	32
	L. gasseri (7)/*L. paragasseri* (9)										31	56	6	6						32	8	16
	Lactobacillus helveticus (7)							43		57										2	2	2
Tetracycline	*L. delbrueckii* (28)						7	4	32	50	7									4	2	4
	L. gasseri (7)/*L. paragasseri* (9)									13	69	19								8	4	8
	Lactobacillus helveticus (7)									43	57									4	4	4
Vancomycin	*L. delbrueckii* (28)						21	75	4											1	0.5	1
	L. gasseri (7)/*L. paragasseri* (9)								81	19										2	1	2
	Lactobacillus helveticus (7)								86	14										2	1	2

MIC is compared to the Lactobacillus obligate homofermentative microbiological cut-off values by EFSA (vertical line). The white area shows the tested concentration of the specific antimicrobials and the grey area shows the concentration of the specific antimicrobials not tested.

10.3389/frabi.2023.1162636

TABLE 2 MIC distribution and tentative ECOFFs for nine antimicrobial agents for *Ligilactobacillus salivarius and Lactilactobacillus sakei*.

Antimicrobial	Species	Distributions (%) of MICs																	Tentative ECOFF	MIC50	MIC90
		0.0075	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024			
Ampicillin	*L. salivarius* (12)						8	92												0.5	0.5	0.5
	L. sakei (10)								10	50	40									4	2	4
Chloramphenicol	*L. salivarius* (12)									17	50	33								8	4	8
	L. sakei (10)										100									4	4	4
Clindamycin	*L. salivarius* (12)		25		8		42	17	8											1	0.25	0.5
	L. sakei (10)		20		10	10		20	20	20										2	0.5	2
Erythromycin	*L. salivarius* (12)					8	17	75												0.5	0.5	0.5
	L. sakei (10)						80	20												0.5	0.25	0.5
Gentamycin	*L. salivarius* (12)									8	17	50	25							16	8	16
	L. sakei (10)										40	50	1							16	8	16
Kanamycin	*L. salivarius* (12)														8	33	25	33		512	256	512
	L. sakei (10)											20	30	50						32	32	32
Streptomycin	*L. salivarius* (12)												8	8	50	33				128	64	128
	L. sakei (10)													10	30	60				128	128	128
Tetracycline	*L. salivarius* (12)								17	25	50	8								8	4	8
	L. sakei (10)									10	80					10				4	4	4
Vancomycin	*L. salivarius* (12)													100						>16	>16	>16
	L. sakei (10)													100						>16	>16	>16

MIC is compared to the Lactobacillus facultative heterofermentative microbiological cut-off values by EFSA (vertical line). The white area shows the tested concentration of the specific antimicrobial and the grey area shows the concentration of the specific antimicrobial not tested.

10.3389/frabi.2023.1162636

For ampicillin and clindamycin, the examined *L. salivarius* strains exhibit ampicillin and clindamycin MIC distributions two or three 2-fold dilutions below the current cut-off, suggesting the need for adjusting the cut-off values for these two antimicrobial agents.

Both *L. salivarius* and *L. sakei* are resistant to vancomycin (MIC >16 mg/L), which previously have been reported for several species of LAB (Swenson et al., 1990; Klare et al., 2007; Muñoz-Atienza et al., 2013; Flórez et al., 2016; Zhang et al., 2018). This is related to the presence of D-Ala-D-lactate in the peptidoglycan of these species rather than a D-Ala-D-Ala dipeptide (Flórez et al., 2016).

Of the tested *L. sakei* strains, 60% were found to exhibit streptomycin MIC values of 128 mg/L, which is above the current cut-off of 64 mg/L, indicating that the cut-off should be adjusted.

One *L. sakei* strain (Accession number JANRGY000000000) showed a tetracycline MIC value above 64 mg/L, which is more than four 2-fold dilutions above the rest of the population, which showed a MIC distribution below the EFSA cut-off value (Table 2). Genomic analysis revealed that the strain encodes a ribosomal protection *tet*(M) gene with 100% nucleotide identity and 100% coverage to a gene from *Staphylococcus aureus* (accession number FN433596) and also a truncated variant of a gene with high identity (99.55% nucleotide identity and 81% coverage) to *tet*(L) gene from a *Bacillus* sp. plasmid encoding a an MFS efflux resistance pump (accession number HM235948). A previous study has reported a *L. sakei* strain encoding both a chromosomally located transposon-associated *tet*(M) gene (accession number EF605269) and a plasmid-carried *tet*(L) gene (accession number EF605268), with high identity to a plasmid-encoded *tet*(L) gene from *Paenibacillus larvae* (Murray and Aronstein, 2006; Ammor et al., 2008). The *tet*(M) and *tet*(L) encoded by the *L. sakei* strain (Accession number JANRGY000000000) in the present study are surrounded by genes both originating from EF605269, EF605268 and a *L. sakei* plasmid (CP025207) (Figure S1), suggesting it have been acquired (Davray et al., 2021).

Obligate heterofermentative

For the two heterofermentative species (*Lentilactobacillus parabuchneri* and *Limosilactobacillus fermentum*), the MIC ranges are compared to the EFSA microbiological cut-off values for *Lactobacillus* obligate heterofermentative (Table S3). The two species exhibit different MIC distributions toward the tested antimicrobial agents, which was expected as they belong to different genera, again supporting the need for defining cut-off values that follows phylogeny rather than fermentation pattern.

All the tested *L. parabuchneri* strains exhibit tetracycline MIC above the current cut-off value at 8 mg/L, with a MIC range of 16-64 mg/L (Table S3), in accordance with previous findings (Nawaz et al., 2011). A previous study has found that the species belonging to the novel *Lentilactobacillus* genus all exhibit tetracycline MIC above the EFSA cut-off of 8 mg/L, suggesting that the EFSA recommended tetracycline cut-off value for *L. buchneri* at 128 mg/L is also applicable to all the species belonging to *Lentilactobacillus;* however, more data on the individual species are needed to conclude this (Feichtinger et al., 2016; EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018).

Even though studies have shown that some *Lactobacillus* species exhibit a high tolerance toward aminoglycosides (Turnidge et al., 2006; Price et al., 2009; Price et al., 2010; Zankari et al., 2012; Kumar et al., 2018), both *L. parabucneri* and *L. fermentum* exhibit gentamycin MIC two-four 2-fold dilutions below the current cut-off of 16 mg/L, in accordance with previous studies (Klare et al., 2007; Nawaz et al., 2011) again illustrating that aminoglycoside resistance pattern is species specific.

All the tested *L. fermentum* strains exhibit chloramphenicol MIC above the current cut-off at 4 mg/L, which is in accordance with previous findings (Klein, 2011).

Pediococcus

MIC values were measured for strains belonging to *P. acidilactici* and *P. pentosaceus* (Table 3). CLSI recommend using CAMHB with lysed horse blood when assessing antimicrobial susceptibility for *Pediococcus*. However, as a study has shown that LSM provide better growth of *Pediococcus*, LSM was used in the present study. Furthermore, CLSI recommend reading MIC between 20-24 hours to ensure good growth, however, a standardized MIC reading time point is preferable to correctly compare MIC values. In this study, the MIC was read both after 20 and 24 hours incubation and all the included strains were found to show adequate growth in the control wells at 20 hours. Furthermore, the MIC values did not increase more than one 2-fold between the 20 hours and 24 hours reading. We therefore recommend recording MIC at 20 hours for *Pediococcus* species, since adequate growth in the control wells was observed for alle the tested strains at this timepoint and further growth could potentially lead to overestimation of the MIC values.

For both *P. acidilactici* and *P. pentosaceus*, trailing endpoints were observed for tetracycline, which are defined as a gradual fading of growth over two-three wells. This phenomenon have been described for Gram-positive cocci when tested against bacteriostatic antimicrobial agents such as tetracycline (EUCAST, 2022). The tetracycline MIC was determined as the first well with significant growth inhibition compared to the control wells as recommended by EUCAST (2022).

Overall, *P. acidilactici* and *P. pentosaceus* (Table 3) exhibit similar MIC distributions for the tested antimicrobial agents. The MIC ranges for chloramphenicol, kanamycin, streptomycin, and tetracycline were found to be one-two 2-fold dilutions higher than the current microbiological cut-offs provided by EFSA (Table 3), which could be explained by the different methods and test conditions used to measure MIC for *Pediococcus* and that the LSM medium provide better growth of *Pediococcus* compared to CAMHB with lysed horse blood (Swenson et al., 1990; Tankovic et al., 1993; Klare et al., 2005; Rojo-Bezares et al., 2006; Danielsen et al., 2007; Klare et al., 2007; Muñoz-Atienza et al., 2013; Basbülbül et al., 2015). This supports the need for standardized methods and test conditions when measuring MIC for defining tentative ECOFFs.

TABLE 3 MIC distribution and tentative ECOFFs for nine antimicrobial agents for the *Pediococcus* species.

Antimicrobial	Species	Distribution (%) of MICs																		Tentative ECOFF	MIC50	MIC90
		0.0075	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024			
Ampicillin	*P. acidilactici* (21)								5	48	48									4	2	4
	P. pentosaceus (21)									10	90									4	4	4
Chloramphenicol	*P. acidilactici* (21)										100									4	4	4
	P. pentosaceus (21)									38	57	5								8	4	4
Clindamycin	*P. acidilactici* (21)		81		19															0.06	≤0.03	0.06
	P. pentosaceus (21)		72		14	14														0.12	≤0.03	0.12
Erythromycin	*P. acidilactici* (21)				5	43	47	5												0.5	0.25	0.25
	P. pentosaceus (21)					62	33	5												0.5	0.12	0.25
Gentamycin	*P. acidilactici* (21)									9	81	10								8	4	4
	P. pentosaceus (21)								9	29	43	14	5							16	4	8
Kanamycin	*P. acidilactici* (21)													5	19	76				128	128	128
	P. pentosaceus (21)											5		14	48	33				128	64	128
Streptomycin	*P. acidilactici* (21)													14	86					64	64	64
	P. pentosaceus (21)													10	57	33				128	64	128
Tetracycline	*P. acidilactici* (21)											57	43							16	8	16
	P. pentosaceus (21)											52	48							16	8	16
Vancomycin	*P. acidilactici* (21)													100							>16	>16
	P. pentosaceus (21)													100							>16	>16

MIC is compared to the Pediococcus microbiological cut-off values by EFSA (vertical line).The white area shows the tested concentration of the specific antimicrobials and the grey area shows the concentration of the specific antimicrobials not tested.

10.3389/frabi.2023.1162636

Leuconostoc

MIC was measured for strains belonging to *L. falkebergense*/*L. pseudomesenteroides* and *L. mesenteroides* (Table 4). CLSI recommend reading MIC between 24 and 48 hours for the *Leuconostoc* genus and in the present study, MIC was therefore read both at 24 and 48 hours (Clinical and Laboratory Standards Institute (CLSI), 2016). Overall, the *Leuconostoc* strains showed adequate growth at 24 hours, except for one *L. falkenbergense* strain and one *L. mesenteroides* strain, which showed limited growth at 24 hours; therefore, incubation for 48 hours was required for these two strains. For the remaining strains the MIC increased no more than two 2-fold dilutions between the 24 and 48 hours reading, and the population MIC range only increased one 2-fold dilution for most of the tested antimicrobial agents (chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline). Based on the results in the present study, MIC recording at 24 hours is recommended, since most of the strains showed adequate growth at this timepoint. However, in cases where poor growth is observed for specific strains it is recommended to incubate for 48 hours to obtain the correct MIC values.

In the present study, the chloramphenicol, clindamycin and kanamycin MIC range was higher than the current cut-offs provided by EFSA (Table 4), which could be due to the difference in test conditions in the present study and previous published data (Swenson et al., 1990; Casado Muñoz M del et al., 2014; Jeong and Lee, 2015; Flórez et al., 2016).

Overall, the MIC distributions for *L. falkebergense*/*L. pseudomesenteroides* and *L. mesenteroides* were similar, expect for clindamycin.

The clindamycin MIC distribution for the *L. falkebergense*/*L. pseudomesenteroides* group was found to be divided into two subgroups with either clindamycin MIC at or below the current cut-off value of 1 mg/L (≤0.03-1 mg/L) and strains with MICs above (4-8 mg/L), respectively. The type strains of both species showed clindamycin MIC values of 8 mg/L, suggesting that decreased clindamycin susceptibility is an inherent trait of both species originating before species differentiation. In agreement, strains with clindamycin MIC above the current cut-off value were scattered throughout the phylogenetic tree (Figure 2) but the trait appears to have been lost from specific strains. Genome comparisons of clindamycin resistant and susceptible strains did not identify any evidence of acquired genes that could explain the resistance, supporting that decreased clindamycin susceptibility is intrinsic for the *L. falkebergense*/*L. pseudomesenteroides* group. A gene encoding a protein with 51.8% similarity to LsaA of *E. faecalis* has been suggested to be involved in the clindamycin resistance observed for the *L. pseudomesenteroides* type strain (Salvetti et al., 2021). However, this gene was found in all 17 strains included in the present study including strains with clindamycin MIC values below the EFSA cut-off value. Furthermore, whereas the intact 1,448 bp gene was present in some strains with low clindamycin MIC values, all *L. falkenbergense* strains with clindamycin MIC values above the EFSA cut-off value were found to encode a truncated 333 bp pseudogene due to a premature stop codon. Accordingly, the *lsaA*-like gene cannot explain the decreased clindamycin susceptibility. As there are no indications that the decreased clindamycin susceptibility commonly observed in strains of the *L. falkebergense*/*L. pseudomesenteroides* group is related to acquired genes, this can be considered as an inherent trait of the species and the clindamycin ECOFF should be adjusted to reflect this (Table 4).

Detection of known antibiotic resistance genes

For all strains included in the study, the presence of genes with identity to known antimicrobial resistance genes was assessed using the curated databases ResFinder (Zankari et al., 2012) (nucleotide level) and NCBI AMRFinderPlus (Feldgarden et al., 2019) (amino acid level).

Out of the 170 included strains, correlation between phenotypic and genotypic resistance was only observed for one *L. sakei* strain (Accession number JANRGY000000000), which exhibit highly elevated tetracycline MIC compared to the wild-type population (Table 1) and encodes acquired tetracycline resistance genes (Figure S1) as described above.

In the remaining strains, no antibiotic resistance genes were detected using the EFSA cut-offs (% identity and coverage above 80% and 70%, respectively) (European Food Safety Authority, 2021). This supports that the decreased antimicrobial susceptibility observed in some of the species is an innate tolerance to specific antimicrobial agents. Innate tolerance or intrinsic resistance does not normally spread horizontally between bacteria but spreads clonally and is often seen as a common trait within a bacterial species or subpopulation which share a common evolutionary history (Cox and Wright, 2013).

Conclusions

ECOFFs are a useful tool to differentiate susceptible and resistant strains within species, however MIC data on species level determined using a standardized method need to be available. In the present study, we were able to show that antimicrobial susceptibility for the *Lactobacilliaceae* family follows phylogeny and tentative ECOFFs were defined accordingly. Furthermore, the data shows that several of the current cut-offs defined by EFSA are either too high or too low for specific species and that several of the species exhibit intrinsic resistance towards specific antimicrobial agents, e.g., *L. pseudomesenteroides/falkenbergense* toward clindamycin and *L. salivarius* toward kanamycin. Furthermore, correlation between phenotypic resistance and presence of known antibiotic resistance genes was observed for one *L. sakei* strain out of the 170 included strains. Therefore, it is important that future tentative ECOFFs are defined based on phylogeny and that more data become available to define ECOFFs. When defining tentative ECOFFs for industrially

TABLE 4 MIC distribution and tentative ECOFFs for nine antimicrobial agents for the *Leuconostoc* species.

	Species	Distribution (%) of MICs																	Tentative ECOFF	MIC50	MIC90
		0.0075	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024			
Ampicillin	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)					12	6	35	47											1	0.5	1
	L. mesenteroides (26)					15	19	23	27	15										2	0.5	2
Chloramphenicol	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)									12	70	18								8	4	8
	L. mesenteroides (26)								15	46	39									4	2	4
Clindamycin	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)		23		6	12			6		23	29								8	4	8
	L. mesenteroides (26)		73		27															0.06	≤0.03	0.06
Erythromycin	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)			6	23	65	6													0.25	0.12	0.12
	L. mesenteroides (26)			27	38	31	4													0.25	0.06	0.12
Gentamycin	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)					18		41	23	18										2	0.5	2
	L. mesenteroides (26)					69		23	8											1	≤0.25	0.5
Kanamycin	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)							12			6	35	23	23						32	8	32
	L. mesenteroides (26)							39		23	12	8	15	4						32	2	16
Streptomycin	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)							6			18	41	18	18						32	8	32
	L. mesenteroides (26)							30		31		12	23	4						32	2	16
Tetracycline	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)				6		6	18	47	18	6									4	1	2
	L. mesenteroides (26)						8	34	35	19	4									4	1	2
Vancomycin	*L. falkebergense*(15)/*L. pseudomesenteroides*(2)													100							>16	>16
	L. mesenteroides (26)													100							>16	>16

MIC is compared to the Leuconostoc microbial cut-off values by EFSA (vertical line). The white area shows the testedconcentration of the specific antimicrobials and the grey area shows the concentration of the specific antimicrobials not tested.

10.3389/frabi.2023.1162636

relevant bacterial species the number of isolates available are often limited compared to clinically important species. It is therefore important; 1) that strains are unambiguously defined at species level and subtyped to support a diverse strain collection e.g., through core genome analysis, 2) MIC population studies are performed by use of a standardized method to define species-specific MIC distributions and 3) that the presence of known antimicrobial resistance genes are searched for to support the MIC distributions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, SAMN33225755-SAMN33225768, https://www.ncbi.nlm.nih.gov/genbank/, JANRGY000000000.

Author contributions

KN-M produced the data, wrote the manuscript, made figures, tables, performed the analysis, and was involved in developing the concept and the method. CS was involved in developing the concept, guiding the analysis, discussion, review and editing. HI was involved in developing the concept, discussion, review, and editing. YA was involved in conceiving the idea, developing, and guiding the concept, analysis, design, discussion, review and editing. AK and KA-N did the sequencing and generation of genome assemblies. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by Innovation Fund Denmark (Grant no. 9065-00029B) as well as internal funding at Chr. Hansen A/S.

Acknowledgments

We would like to thank Technician Anna Minor Nielsen for guidance and good advice on the susceptibility testing.

Conflict of interest

Authors KN-M, CA, AK, KA-N and YA are employees of Chr. Hansen A/S, a company that produces strains for plant protection, animal, and human health as well as for the food and dairy industry. Authors AK, KA-N and YA are share-holders in Chr. Hansen A/S.

The remaining author HI declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that the study received internal funding from Chr. Hansen A/S. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frabi.2023.1162636/full#supplementary-material

References

Adimpong, D. B., Nielsen, D. S., Sørensen, K. I., Derkx, P. M. F., and Jespersen, L. (2012). Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. *BMC Microbiol.* 12, 10–12. doi: 10.1186/1471-2180-12-75

Agersø, Y., Stuer-Lauridsen, B., Bjerre, K., Jensen, M. G., Johansen, E., Bennedsen, M., et al. (2018). Antimicrobial susceptibility testing and tentative epidemiological cutoff values for five bacillus species relevant for use as animal feed additives or for plant protection. *Appl. Environ. Microbiol.* 84, 1–12. doi: 10.1128/AEM.01108-18

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259. doi: 10.1038/nrmicro2312

Ammor, M. S., Gueimonde, M., Danielsen, M., Zagorec, M., Van Hoek, A. H. A. M., De Los Reyes-Gavilán, C. G., et al. (2008). Two different tetracycline resistance mechanisms, plasmid-carried tet(L) and chromosomally located transposon-associated tet(M), coexist in lactobacillus sakei rits 9. *Appl. Environ. Microbiol.* 74, 1394–1401. doi: 10.1128/AEM.01463-07

Basbülbül, G., Özteber, M., and Biyik, H. H. (2015). Antibiotic resistance in lactic acid bacteria isolated from fermented dairy products and boza. *J. Microbiol. Biotechnol. Food Sci.* 04, 513–517. doi: 10.15414/jmbfs.2015.4.6.513-517

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., and Cogan, T. M. (2001). Recent advances in cheese microbiology. *Int. Dairy J.* 11, 259–274. doi: 10.1016/S0958-6946(01)00056-5

Cardamone, L., Quiberoni, A., Mercanti, D. J., Fornasari, M. E., Reinheimer, J. A., and Guglielmotti, D. M. (2011). Adventitious dairy leuconostoc strains with interesting technological and biological properties useful for adjunct starters. *Dairy Sci. Technol.* 91, 457–470. doi: 10.1007/s13594-011-0022-9

Casado Muñoz M del, C., Benomar, N., Lerma, L. L., Gálvez, A., and Abriouel, H. (2014). Antibiotic resistance of lactobacillus pentosus and leuconostoc pseudomesenteroides isolated from naturally-fermented aloreña table olives throughout fermentation process. *Int. J. Food Microbiol.* 172, 110–118. doi: 10.1016/j.ijfoodmicro.2013.11.025

Clinical and Laboratory Standards Institute (CLSI) (2016). "Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria," in *CLSI guideline M45, 3rd ed*(Pennaylvania, USA), ISBN: .

Clinical and Laboratory Standards Institute (CLSI) (2018). "Development of in vitro susceptibility testing criteria and quality control parameters; approved guideline," in *Guideline M23, 5th Edition*(Pennsylvania, USA), ISBN: .

Cox, G., and Wright, G. D. (2013). Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. *Int. J. Med. Microbiol.* 303, 287–292. doi: 10.1016/j.ijmm.2013.02.009

Danielsen, M., Simpson, P. J., O'Connor, E. B., Ross, R. P., and Stanton, C. (2007). Susceptibility of pediococcus spp. to antimicrobial agents. *J. Appl. Microbiol.* 102, 384–389. doi: 10.1111/j.1365-2672.2006.03097.x

Danielsen, M., and Wind, A. (2003). Susceptibility of lactobacillus spp. to antimicrobial agents. *Int. J. Food Microbiol.* 82, 1–11. doi: 10.1016/S0168-1605(02)00254-4

Davray, D., Deo, D., and Kulkarni, R. (2021). Plasmids encode niche-specific traits in lactobacillaceae. *Microb. Genomics* 7. doi: 10.1099/mgen.0.000472

Delgado, S., Flórez, A. B., and Mayo, B. (2005). Antibiotic susceptibility of lactobacillus and bifidobacterium species from the human gastrointestinal tract. *Curr. Microbiol.* 50, 202–207. doi: 10.1007/s00284-004-4431-3

Duar, R. M., Lin, X. B., Zheng, J., Martino, M. E., Grenier, T., Pérez-Muñoz, M. E., et al. (2017). Lifestyles in transition: evolution and natural history of the genus lactobacillus. *FEMS Microbiol. Rev.* 41, S27–S48. doi: 10.1093/femsre/fux030

EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA J.* 16, 1–24. doi: 10.2903/j.efsa.2018.5206

EUCAST. (2022). EUCAST reading guide for broth microdilution (version 4.0). Available at: https://www.eucast.org/ast_of_bacteria/mic_determination.

European Committe on Antimicrobial Susceptibility Testing (EUCAST). (2021). *MIC distributions and the setting of epidemiological cut-off (ECOFF) values SOP 10.2.* doi: 10.2903/j.efsa.2021.6506

European Food Safety Authority. (2021). EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain. *EFSA J.* 19.

Feichtinger, M., Mayrhofer, S., Kneifel, W., and Domig, K. J. (2016). Tetracycline resistance patterns of lactobacillus buchneri group strains. *J. Food Prot* 79, 1741–1747. doi: 10.4315/0362-028X.JFP-15-577

Feldgarden, M., Brover, V., Haft, D. H., Prasad, A. B., Slotta, D. J., Tolstoy, I., et al. (2019). Validating the AMRFINder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. *Antimicrob. Agents Chemother.* 63. doi: 10.1128/AAC.00483-19

Flórez, A. B., Campedelli, I., Delgado, S., Alegría, Á, Salvetti, E., Felis, G. E., et al. (2016). Antibiotic susceptibility profiles of dairy leuconostoc, analysis of the genetic basis of atypical resistances and transfer of genes *in vitro* and in a food matrix. *PloS One* 11, 1–20. doi: 10.1371/journal.pone.0145203

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U., and Huis In'T Veld, J. H. J. (1998). Overview of gut flora and probiotics. *Int. J. Food Microbiol.* 41, 85–101. doi: 10.1016/S0168-1605(98)00044-0

International Organization for Standardization. (2010). Milk and milk products — determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and nonenterococcal lactic acid bacteria (LAB)ISO 10932.

Jeong, D. W., and Lee, J. H. (2015). Antibiotic resistance, hemolysis and biogenic amine production assessments of leuconostoc and weissella isolates for kimchi starter development. *LWT - Food Sci. Technol.* 64, 1078–1084. doi: 10.1016/j.lwt.2015.07.031

Katla, A. K., Kruse, H., Johnsen, G., and Herikstad, H. (2001). Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. *Int. J. Food Microbiol.* 67, 147–152. doi: 10.1016/S0168-1605(00)00522-5

Katoh, K. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3059–3066. doi: 10.1093/nar/gkf436

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., et al. (2005). Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. *Appl. Environ. Microbiol.* 71, 8982–8986. doi: 10.1128/AEM.71.12.8982-8986.2005

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., et al. (2007). Antimicrobial susceptibilities of lactobacillus, pediococcus and lactococcus human isolates and cultures intended for probiotic or nutritional use. *J. Antimicrob. Chemother.* 59, 900–912. doi: 10.1093/jac/dkm035

Klein, G. (2011). Antibiotic resistance and molecular characterization of probiotic and clinical lactobacillus strains in relation to safety aspects of probiotics. *Foodborne Pathog. Dis.* 8, 267–281. doi: 10.1089/fpd.2010.0672

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Mathur, S., and Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria - a review. *Int. J. Food Microbiol.* 105, 281–295. doi: 10.1016/j.ijfoodmicro.2005.03.008

Mayrhofer, S., van Hoek, A. H. A. M., Mair, C., Huys, G., Aarts, H. J. M., Kneifel, W., et al. (2010). Antibiotic susceptibility of members of the lactobacillus acidophilus group using broth microdilution and molecular identification of their resistance determinants. *Int. J. Food Microbiol.* 144, 81–87. doi: 10.1016/j.ijfoodmicro.2010.08.024

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2005). Opinion of the Scientific Panel on additives and products or substances used in animal feed (FEEDAP) on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. *EFSA J.3.* doi: 10.2903/j.efsa.2005.223

Muñoz-Atienza, E., Gómez-Sala, B., Araújo, C., Campanero, C., Del Campo, R., Hernández, P. E., et al. (2013). Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. *BMC Microbiol.* 13. doi: 10.1186/1471-2180-13-15

Murray, K. D., and Aronstein, K. A. (2006). Oxytetracycline-resistance in the honey bee pathogen paenibacillus larvae is encoded on novel plasmid pMA67. *J. Apic Res.* 45, 207–214. doi: 10.1080/00218839.2006.11101349

Nøhr-Meldgaard, K., Struve, C., Ingmer, H., and Agersø, Y. (2021). The tetracycline resistance gene, tet(W) in bifidobacterium animalis subsp. lactis follows phylogeny and differs from tet(W) in other species. *Front. Microbiol.* 12, 1–12. doi: 10.3389/fmicb.2021.658943

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J. E., et al. (2011). Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. *Curr. Microbiol.* 62, 1081–1089. doi: 10.1007/s00284-010-9856-2

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Price, M. N., Dehal, P. S., and Arkin, A. P. (2009). Fasttree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* 26, 1641–1650. doi: 10.1093/molbev/msp077

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - approximately maximum-likelihood trees for large alignments. *PloS One* 5. doi: 10.1371/journal.pone.0009490

Rojo-Bezares, B., Sáenz, Y., Poeta, P., Zarazaga, M., Ruiz-Larrea, F., and Torres, C. (2006). Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. *Int. J. Food Microbiol.* 111, 234–240. doi: 10.1016/j.ijfoodmicro.2006.06.007

Salvetti, E., Campedelli, I., Larini, I., Conedera, G., and Torriani, S. (2021). Exploring antibiotic resistance diversity in leuconostoc spp. by a genome-based approach: focus on the lsaA gene. *Microorganisms* 9, 491. doi: 10.3390/microorganisms9030491

Salvetti, E., Torriani, S., and Felis, G. E. (2012). The genus lactobacillus: a taxonomic update. *Probiot Antimicrob. Proteins* 4, 217–226. doi: 10.1007/s12602-012-9117-8

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Stefańska, I., Kwiecień, E., Jóźwiak-Piasecka, K., Garbowska, M., Binek, M., and Rzewuska, M. (2021). Antimicrobial susceptibility of lactic acid bacteria strains of potential use as feed additives - the basic safety and usefulness criterion. *Front. Vet. Sci.* 8, 1–11. doi: 10.3389/fvets.2021.687071

Stiles, M. E. (1996). Biopreservation by lactic acid bacteria. antonie van Leeuwenhoek. *Int. J. Gen. Mol. Microbiol.* 70, 331–345. doi: 10.1007/BF00395940

Swenson, J. M., Facklam, R. R., and Thornsberry, C. (1990). Antimicrobial susceptibility of vancomycin-resistant leuconostoc, pediococcus, and lactobacillus species. *Antimicrob. Agents Chemother.* 34, 543–549. doi: 10.1128/AAC.34.4.543

Tanizawa, Y., Tada, I., Kobayashi, H., Endo, A., Maeno, S., Toyoda, A., et al. (2018). Lactobacillus paragasseri sp. nov., a sister taxon of lactobacillus gasseri, based on whole-genome sequence analyses. *Int. J. Syst. Evol. Microbiol.* 68, 3512–3517. doi: 10.1099/ijsem.0.003020

Tankovic, J., Leclercq, R., and Duval, J. (1993). Antimicrobial susceptibility of pediococcus spp. and genetic basis of macrolide resistance in pediococcus acidilactici HM3020. *Antimicrob. Agents Chemother.* 37, 789–792. doi: 10.1128/AAC.37.4.789

Turnidge, J., Kahlmeter, G., and Kronvall, G. (2006). Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clin. Microbiol. Infect.* 12, 418–425. doi: 10.1111/j.1469-0691.2006.01377.x

World Health Organisation. (WHO) (2018). *Critically important antimicrobials for human medicine.*

Wu, Y., and Gu, C. T. (2021). Leuconostoc falkenbergense sp. nov., isolated from a lactic culture, fermentating string beans and traditional yogurt. *Int. J. Syst. Evol. Microbiol.* 71, 1–8. doi: 10.1099/ijsem.0.004602

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, S., Oh, J. H., Alexander, L. M., Özçam, M., and van Pijkeren, J. P. (2018). D-Alanyl-D-alanine ligase as a broad-host-range counterselection marker in vancomycin-resistant lactic acid bacteria. *J. Bact.* 200, e00607–e00617. doi: 10.1128/JB.00607-17

Zheng, J., Ruan, L., Sun, M., and Gänzle, M. (2015). A genomic view of lactobacilli and pediococci demonstrates that phylogeny matches ecology and physiology. *Appl. Environ. Microbiol.* 81, 7233–7243. doi: 10.1128/AEM.02116-15

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., et al. (2020). A taxonomic note on the genus lactobacillus: description of 23 novel genera, emended description of the genus lactobacillus beijerinck 1901, and union of lactobacillaceae and leuconostocaceae. *Int. J. Syst. Evol. Microbiol.* 70, 2782–2858. doi: 10.1099/ijsem.0.004107

frontiers | Frontiers in Antibiotics

TYPE Original Research
PUBLISHED 14 August 2023
DOI 10.3389/frabi.2023.1176817

OPEN ACCESS

EDITED BY
Getahun E. Agga,
Agricultural Research Service (USDA),
United States

REVIEWED BY
Michael Apley,
Kansas State University, United States
Patrick John Gorden,
Iowa State University, United States
Terry Lehenbauer,
University of California, Davis, United States

*CORRESPONDENCE
Zhengyu Lu
zl426@cornell.edu

RECEIVED 28 February 2023
ACCEPTED 25 July 2023
PUBLISHED 14 August 2023

CITATION
Lu Z, Bulut E, Nydam DV
and Ivanek R (2023) Standardization and
evaluation of indicators for quantifying
antimicrobial use on U.S. dairy farms.
Front. Antibiot. 2:1176817.
doi: 10.3389/frabi.2023.1176817

Standardization and evaluation of indicators for quantifying antimicrobial use on U.S. dairy farms

Zhengyu Lu[1]*, Ece Bulut[1], Daryl V. Nydam[2] and Renata Ivanek[1]

[1]Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, United States, [2]Department of Public and Ecosystem Health, College of Veterinary Medicine, Cornell University, Ithaca, NY, United States

Antimicrobial resistance (AMR) is a global One Health threat. A portion of AMR development can be attributed to antimicrobial use (AMU) in animals, including dairy cattle. Quantifying AMU on U.S. dairy farms is necessary to inform antimicrobial stewardship strategies and help evaluate the relationship between AMU and AMR. Many AMU indicators have been proposed for quantifying AMU in dairy cattle. However, these indicators are difficult to interpret and compare because they differ in the type of data used, the calculation approach, and the definitions of variables and parameters used in the calculation. Therefore, we selected 16 indicators (count-based, mass-based, and dose-based) applicable for quantifying AMU on U.S. dairy farms. We systematized the indicators by standardizing their variables and parameters to improve their interchangeability, interpretation, and comparability. We scored indicators against six data-driven criteria (assessing their accuracy, data and effort needs, and level of privacy concern) and five stewardship-driven criteria (assessing their ability to capture trends and inform antimicrobial stewardship). The derived standardized indicators will aid farmers and veterinarians in selecting suitable indicators based on data availability and stewardship needs on a farm. The comparison of indicators revealed a trade-off requiring farmers to balance the granularity of data necessary for an accurate indicator and effort to collect the data, and a trade-off relevant to farmers interested in data sharing to inform stewardship because more accurate indicators are typically based on more sensitive information. Indicators with better accuracy tended to score better in stewardship criteria. Overall, two dose-based indicators, estimating the number of treatments and administered doses, scored best in accuracy and stewardship. Conversely, two count-based indicators, estimating the length of AMU, and a mass-based indicator, estimating the mass of administered antimicrobials, performed best in the effort and privacy criteria. These findings are expected to benefit One Health by aiding the uptake of farm-level AMU indicators by U.S. dairy farms.

KEYWORDS

dairy cattle, antimicrobial use, antimicrobial stewardship, indicators, privacy

1 Introduction

Antimicrobial resistance (AMR) is a serious One Health concern threatening not just human, animal, and environmental health but also agricultural production and the economy (WHO, 2021). In 2019 alone, the global human health burden associated with bacterial AMR was an estimated 1.27 million deaths (Murray et al., 2022). By 2050, approximately ten million people could die from AMR annually (O'Neill, 2016). The mechanism of AMR emergence and spread is complex, but antimicrobial use (AMU) in food producing animals, dairy cattle included, is considered to contribute to the One Health burden associated with AMR (Marshall and Levy, 2011; Hoelzer et al., 2017; Ma et al., 2021).

The U.S. is one of the top countries in the world with respect to the size of the national dairy cattle population (FAOSTAT). According to the FDA, in the U.S. in 2020, medically important antimicrobials for use in cattle (beef and dairy cattle combined because data were not available for these two different production categories separately) accounted for 41% of the total sales of antimicrobials for use in food animals (FDA, 2021). On dairy farms, antimicrobials are used to treat bacterial infections, such as mastitis in lactating cows and respiratory disease in calves (Llanos-Soto et al., 2021; Casseri et al., 2022). Studies have suggested variable levels of association between the level of AMU on dairy farms and the emergence of AMR in the commensals and pathogens of dairy cattle (Snow et al., 2012; Duse et al., 2015; Gonggrijp et al., 2016; Hordijk et al., 2019). However, conclusive evidence that AMU in dairy farms leads to AMR infections that cause extended illnesses or deaths in dairy cattle is still lacking, implying the presence of multiple factors influencing the epidemiology of AMR diseases (de Verdier et al., 2012; Cummings et al., 2013; Owen et al., 2017; Bokma et al., 2020), as well as exposing the lack of quantitative data to allow causal inference (Cummings et al., 2013; Owen et al., 2017). Therefore, gathering quantitative data about AMU is a crucial step to understanding the relationship between AMU and the development of AMR (MacFadden et al., 2016) and informing antimicrobial stewardship (Redding et al., 2019; Schrag et al., 2020c; Cheng et al., 2022; Fonseca et al., 2022).

Scientists and governments worldwide have proposed different indicators to quantify AMU in cattle (Redding et al., 2019; Brault et al., 2019a; Schrag et al., 2020a; Cheng et al., 2022; Fonseca et al., 2022). An indicator is usually calculated using a division equation with different combinations of animal, antimicrobial, and temporal information as the numerator and denominator. Consequently, each indicator has a different focus, granularity, interpretation, and data requirements (Brault et al., 2019a; Schrag et al., 2020b). For example, an indicator that uses the mass of the active substance administered as the numerator and the population of animals at risk as the denominator (mg/100 cattle-at-risk) is easy to calculate but may be misleading because it does not consider the animal body mass and antimicrobial potency and dosage differences (Brault et al., 2019a). For mass-based indicators, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) defined the population correction unit (PCU) to adjust antimicrobial sales data, where PCU is the product of the number of animals in the population and animal body mass at treatment (European Medicines Agency, 2019). The U.S. equivalent of the PCU denominator is the target animal biomass (TAB) developed by FDA, which also adjusts antimicrobial sales data (FDA Center for Veterinary Medicine).

Antimicrobial use can be quantified by several dose-based indicators as well, which are calculated by using different dose definitions and were developed by various research and government groups. Timmerman et al. adopted the used daily dose (UDD), which means the administered dose per day per kilogram of animal body mass (Timmerman et al., 2006). Jensen et al. defined the animal daily dose (ADD), which means the average maintenance dose for treatment in a specific species (Jensen et al., 2004). To harmonize and better monitor antimicrobial sales data in EU countries, ESVAC developed the defined daily dose (DDDvet) and the defined course dose (DCDvet) for animals, which provide standard parameters to adjust AMU data for different antimicrobials and animal species (European Medicines Agency, 2015). In addition, Schrag et al. defined the concepts of the standard regimen (SReg), which means the use of an antimicrobial product for a disease event in an animal and implicitly accounts for dose, therapy length, and the number of administrations (Schrag et al., 2020a; Schrag et al., 2020b). Schrag et al. used the counts and grouping of SRegs to quantify AMU (Schrag et al., 2020b; Schrag et al., 2022). While indicators that quantify AMU in terms of the applied course doses and regimens differ, the two terms, 'course' and 'regimen', refer to the same concept (defined above for regimen).

Countries like the Netherlands and Denmark have implemented national AMU surveillance systems that quantify AMU based on their national DDDvet (Kasabova et al., 2019; Moura et al., 2022). However, there is still a need for a national unified or recommended indicator(s) to quantify AMU on U.S. dairy farms. Many farms in the U.S. have yet to use different indicators to evaluate AMU, contributing to a poor understanding of AMU and its role in the emergence of AMR and missing the opportunity to inform antimicrobial stewardship (de Campos et al., 2021). Also, a review from 2022 reported that many U.S. dairy producers rely on their experience to make treatment decisions without veterinary input (Ruegg, 2022). Due to the inconsistent definitions of indicators for quantifying on-farm AMU, veterinarians and farmers need more guidance in selecting suitable indicators for quantifying and adjusting their AMU (Gozdzielewska et al., 2020; Moore et al., 2021; Ruegg, 2022). Quantification of AMU contributes to reducing costs of excess antimicrobials while keeping healthy dairy cattle, which is the primary motivation for dairy farmers to adjust AMU (Gozdzielewska et al., 2020). In addition, farmers are also interested in knowing how their AMU compares to other farms (Casseri et al., 2022). However, comparing their AMU with other farms requires sharing AMU data, which may raise privacy concerns. At the national level, detailed and accurate on-farm AMU data are the cornerstone of a national AMU monitoring system and provide support for developing interventions (Sanders et al., 2020).

The objectives of this study were to: (i) standardize published indicators for monitoring farm-level AMU in dairy cattle by

standardizing their underlying variables and parameters and (ii) compare AMU indicators based on their data needs and effort, level of privacy concerns, and ability to capture trends and inform antimicrobial stewardship on the U.S. dairy farms. This information will provide guidelines for a more intuitive comparison and selection of AMU indicators by farmers and veterinarians, which can drive meaningful antimicrobial stewardship decisions on dairy farms and help evaluate the relationship between AMU and AMR.

2 Method

2.1 Indicator selection

We conducted a literature review to identify existing indicators that can quantify AMU on U.S. dairy farms. A total of 16 indicators were selected, and we categorized them into three groups: count-based (five), mass-based (two), and dose-based (nine).

The selected count-based indicators were all from Schrag et al.'s studies, which were the number of therapeutic events (*nTE*), number of standard regimens (*nREG*), antimicrobial regimen to therapy ratio (*RT-ratio*), number of regimen time frame days (*nRTFD*), and total length of all therapies (*nDOT*) (Schrag et al., 2020b; Schrag et al., 2022). These five indicators contain neither the total mass of antimicrobial administered nor the dose information in the calculation. Instead, the numerators are the number of therapeutic events, regimens, or days. The denominators for all five indicators are either the number of animals (*nTE*, *nREG*, *nRFTD*, and *nDOT*) or the number of therapeutic events (*RT-ratio*). Some of the information contained in the count-based indicators overlaps with the information contained in the dose-based indicators, but they are not identical. Because the count-based indicators don't depend on the availability of globally accepted standard dose-related parameters needed for calculating the administrated dose (e.g., the defined daily dose (DDD)), they are simpler to calculate and interpret. Also, they are more robust since they are not affected by changes or variability in standard dose-related parameters over time and across farms.

The selected mass-based indicators were *mg/100 cattle-at-risk* (referred to as "*mg/100 animals-at-risk*" in our study) and *mg/TAB*. The *mg/100 cattle-at-risk* indicator is the easiest to calculate and interpret (Brault et al., 2019a). In this study, we used an adaptation of the FDA's definition of *mg/TAB* for quantifying farm-level AMU that is otherwise applicable only to the national-level AMU. This was achieved by replacing in the calculation the national antimicrobial sales data with the farm-level AMU data and by using the farm-level specific animal body mass instead of the national standard animal body mass (FDA Center for Veterinary Medicine). We did not consider the EU indicator with the PCU denominator because the *mg/TAB* indicator has the same principle and is more suitable for the U.S. farming settings.

Most (nine) of the selected 16 indicators fall into the dose-based group. Specifically, we selected the number of study defined daily doses (*nDDDp*), the number of standard defined daily doses (*nDDDv*), the number of study defined course doses (*nDCDp*), and the number of standard defined course doses (*nDCDv*) from Schrag et al.'s study (Schrag et al., 2020b). Additionally, we selected indicators that combine the treatment frequency with the used daily dose (TF_{UDD}) or standard defined daily dose (TF_{DDD}) from Kasabova et al.'s study (Kasabova et al., 2019). Also, we selected the indicators from Brault et al.'s study quantifying the number of animal daily doses per 100 treated animals that use the individual animal AMU and body mass information (*nADD(kg$_a$)/100 treated animals*) or use the average animal AMU and body mass information (*nADD(kg$_m$)/100 treated animals*) (Brault et al., 2019a). Finally, we selected the number of Canadian-defined daily doses per 1,000 animal days at risk (*nDDDv/1,000 animal days-at-risk*) proposed by the Canadian Government (Canadian Integrated Program for Antimicrobial Resistance Surveillance).

2.2 Parameter standardization

The definitions of terms (variables and parameters) appearing in the equations for calculating the original AMU indicators are inconsistent. For example, the numerators in TF_{UDD}, *ADD*-based indicators, and *nDCDp* describe the amount of antimicrobial used, and the unit in all three is mg of an active substance. However, the numerators in these three indicators are defined differently: as "the amount of active substance for every active compound" in TF_{UDD}, "the quantity of active substance in mg administered" in *ADD*-based indicators, and "substance specific total milligrams" in *nDCDp* (Brault et al., 2019a; Kasabova et al., 2019; Schrag et al., 2020b). The subtle differences in definitions can cause confusion (Moore et al., 2021).

In addition, the indicators often use different methods to estimate animal body mass on a farm, and the body mass information often does not include the animal production category (e.g., unweaned calf, weaned calf, pregnant heifer, lactation #1). For example, Kasabova et al. estimated the animal body mass by rearranging the formula (equation (2) in Kasabova et al.) for calculation of the used daily dose (UDD), i.e., by dividing the mass of the administered active substance by the product of the number of treated animals, the recommended UDD, and treatment days (Kasabova et al., 2019); while Brault et al. used the mean animal body mass of animals on a feedlot at the time of exposure to any antimicrobial (Brault et al., 2019a); and Schrag et al. used the assumed animal body mass of 680 kg that is based on a prior study on the U.S. dairy farms (Schrag et al., 2020b).

To address these inconsistencies, we redefined variables and parameters based on the equations for each of the 16 selected indicators and expressed them in a standardized way. This included assigning identical definitions to the numerators with the same meaning and describing body mass variables/parameters in a way that the distinctions among them are obvious. We grouped all terms appearing in the indicator equations into: (i) data collected per treatment (C); (ii) composite records of collected data for each individual administrated treatment (*a*) or regimen (*R*) (CR); (iii) data collected periodically (e.g., weekly) (P); (iv) 'farm standard', a constant value obtained from a one-time calculation or approximation for a specific farm (FS); (v) 'general standard', a

constant value available from the literature (GS); and (vi) the derived terms (D). The terms (i)-(v) represent the 'primary data' required for the calculation of indicators that need to be assembled by a farmer/veterinarian (Table 1), while the 'derived terms' in (vi) are calculated from the collected/identified primary data or other calculated terms and they are presented as an intermediate step for ease of indicator calculation and comparison (Table 2). Additionally, we categorized all terms (primary data and derived terms) into three categories based on the fundamental requirements for estimating an AMU indicator: antimicrobial, animal, and time. We have standardized definitions of terms while maintaining their original meaning so that the identical components in calculation can be easily identified across all indicators. This also achieved interchangeability between indicators. For example, the definition of the animal body mass now is the same for *mg/TAB*, *nDDDp*, *nDCDp*, TF_{UDD}, and $nADD(kg_m)/100$ *treated animals*, which is farm-specific average body mass for the production category of the treated animal. Therefore, users can use the same body mass data for these five indicators.

For all AMU indicators, the time period T of data recording for periodic calculation of an AMU indicator is user defined (e.g., month, quarter, year). We summarized the definitions, notations, and sources of primary data and derived terms used in calculating 16 AMU indicators in Tables 1, 2. In Table 1, we included specific terms as subscripts to describe AMU: treatment indication, production category, active substance, and route of administration. These terms are crucial for accurate calculations and meaningful comparisons and interpretation of indicators, and will be helpful in evaluating implications of AMU (e.g., when comparing intramammary vs parenteral therapies with the count-, mass- and dose-based indicators and evaluating implications of these therapies for AMR). We showed how these terms are used in calculating AMU indicators in Table 3. For transparency, the new and original definitions of the terms are shown side-by-side in Supplementary Table S1. Definitions of main abbreviations and acronyms used in derivation and evaluation of antimicrobial use indicators are provided in alphabetical order in Supplementary Table S2. We also created a simplified dataset for a hypothetical dairy farm, and we illustrated step by step how to obtain all values listed in Tables 1–3, which respectively include primary data, derived terms, and the indicators for quantifying AMU on the farm (Supplementary Data).

2.3 Indicators' comparison

To assess the data needs and interchangeability of indicators, we cross-tabulated the 16 indicators and the data/terms needed for their calculation. Additionally, to compare the 16 indicators, ZL, EB, and RI established six data requirement- and five stewardship information-driven criteria for evaluation (Table 4). The data requirements focused on data needs and evaluated the level of detail provided by an indicator about (i) the actual animals treated (Animal information, ani); (ii) antimicrobials used (Exposure data, ed); (iii) ability to detect extra-label use (Extra-label use, el); (iv) the ease of implementation (Ease of data recording and calculation, edr);

TABLE 1 Definition of primary data (variable and standard parameter terms) required for calculation of farm-level antimicrobial drug use indicators.

Category	Notation	Definition (unit)	Type[1]	Source
Animal	i	Individual animal identification number on a farm f for an animal that was treated with an antimicrobial product (animal)	C	NA[2]
	d	Specific treatment indication/disease syndrome (treatment indication)	C	Adapted from (Schrag et al., 2020a)
	p	Specific production category of a treated animal at the time of antimicrobial product administration (production category)	C	NA
	$n_{wk,p}$	Number of animals of a given production category (p) present on a farm f in a given week (wk) (animal)	P	Adapted from (Schrag et al., 2020a)
	w_i	Body mass of an individually treated animal at the time of antimicrobial product administration (can be measured or estimated from animal age at the time of treatment using growth charts) (kg)	C	Adapted from (Kasabova et al., 2019)
	$w_{f,p}$	Farm f specific average body mass (or farm-specific standard body mass) for the production category p of a treated animal at the time of antimicrobial product administration. Can be obtained from historical farm records or by measuring a representative subset of animals (kg)	FS	Adapted from (Brault et al., 2019a)
	w_p	Standard average body mass for the production category p of a treated animal at the time of drug product administration (kg)[3]	GS	(Canadian Integrated Program for Antimicrobial Resistance Surveillance; European Medicines Agency, 2023)
Antimicrobial	s	Specific administrated active substance (s) (active substance)	C	NA
	r	Specific route of antimicrobial product administration (administration route)	C	NA
	m_s	Mass of an active substance (s) in a single administration of an antimicrobial product (listed on the product label) (mg)	GS	NA

(Continued)

TABLE 1 Continued

Category	Notation	Definition (unit)	Type[1]	Source
	m_{s_i}	Mass of an active substance (s) actually administrated in a single administration of an antimicrobial product, including for extra-label use. Recorded only if different from the mass (m_s) listed on the product label (mg)	C	NA
	C_R	Prescribed number of antimicrobial product administrations as part of a single regimen (administration)	GS/FS	NA
	c_{R_i}	The actual number of antimicrobial product administrations as part of a regimen administrated to animal i. Recorded only if different from the general/farm standard (c_R) for the regimen (administration)	C	NA
	AD_i	The actual dose (m_i/w_i) of an active substance (s) in a single antimicrobial administration for a therapeutic purpose targeting a single disease event (d) in an individual animal (i) (mg active substance/kg animal)	C	Adapted from (Brault et al., 2019a; Schrag et al., 2020a)
	AD_m	Prescribed or mean dose of an active substance (s) in a single antimicrobial administration for a therapeutic purpose targeting a single disease event (d) in an individual animal (i) (mg active substance/kg animal)	GS/FS	Adapted from (Brault et al., 2019a; Schrag et al., 2020a)
	DDDv	Standard defined daily dose by the European Surveillance of Veterinary Antimicrobial Consumption or Government of Canada (mg active substance/kg animal/day)[4]	GS	(Canadian Integrated Program for Antimicrobial Resistance Surveillance; Defined daily doses for animals (DDDvet) and defined course doses for animals (DCDvet))
	DCDv	Standard defined course dose proposed by European Surveillance of Veterinary Antimicrobial Consumption or Government of Canada (mg active substance/kg animal/course)[4]	GS	(Canadian Integrated Program for Antimicrobial Resistance Surveillance; Defined daily doses for animals (DDDvet) and defined course doses for animals (DCDvet))
	a	Single administration: Antimicrobial product administered at a single restraining event to an individual animal (i). Dataset associated with each individual administration: a = $\{i, t, r, s, m, d, p, w\}$(administration)	CR	Adapted from (Schrag et al., 2020a)
	R	Standard regimen (course): Recorded antimicrobial product administration(s) for a therapeutic purpose targeting a single disease event (d) in an individual animal (i). Multiple administrations in an animal (a_i) are counted as part of a single regimen when product administrations are consecutive, never resulting in a time gap between administrations of greater than the pre-determined administration interval of 5 days. Dataset associated with each individual administrated regimen: $R = \{i, t_{first}, t_{last}, r, s, m, d, p, w, c_R, int, adjF\}$ (regimen)	CR	Adapted from (Schrag et al., 2020b)
Time	t	The date of an individual single administration of an antimicrobial product to an individual animal (i) at a single restraining event. In the case of a regimen, t_{first} and t_{last} denote the first and last day of the regimen (date)	C	NA
	int	Interval between administrations within a single regimen that is less than 24h (day)	GS	(Schrag et al., 2020b)
	adjF	Adjustment factor for long-acting antimicrobial products, for which single administration provides > 1 day of therapy. Can be the time interval between administrations or the estimated duration of antimicrobial effect (unitless)	GS	Adapted from (Schrag et al., 2020b)
	ADR	Average days at risk: an average number of days individual animals of production category p are present on farm f (days)[5]	GS/FS	Adapted from (Canadian Integrated Program for Antimicrobial Resistance Surveillance)

[1]Term types: C, collected per treatment; P, collected periodically (e.g., weekly); FS, farm standard (obtained from a one-time calculation or approximation for a specific farm); GS, general standard (available from the literature); CR, composite data for each individual administrated treatment (a) or regimen (R).
[2]NA, not applicable.
[3]Examples of general standard body mass values available from the literature: From Schrag et al.'s paper, lactating cow w_p=680kg (Schrag et al., 2020b); From the FDA: livestock dairy cows w_p=635.03 kg (FDA, 2022); From the European ESVAC standard: Veal calves w_p=80kg, dairy cattle w_p=500kg, meat cattle w_p=500kg (European Medicines Agency, 2023).
[4]Currently defined for pigs, cattle and poultry. For example, DDDv and DCDv for oral route Amoxicillin in cattle are 20 mg/kg and 81 mg/kg, respectively; DDDv and DCDv for oral route Ampicillin in cattle are 29 mg/kg and 123 mg/kg, respectively (Defined daily doses for animals (DDDvet) and defined course doses for animals (DCDvet)).
[5]Examples of the general standard average length of stay in a production category available from the literature: e.g., unweaned calves= 2 months; heifers= 13 months (Lang, 2017).

TABLE 2 Definition of derived terms required for calculation of farm-level antimicrobial drug use indicators[1].

Category	Notation	Definition (unit)	Equation	Source
Animal	D	List of all treatment indications (diseases; d) treated with antimicrobial products on a farm f during a period of time T (categorical)[3]	$D = \{digestive,\ mastitis, \ldots\}$	NA[2]
	P	List of all animal production categories (p) present on a farm f during a period of time T (categorical)[3]	$P = \{calf,\ parity\ 1,\ \ldots\}$	NA
	n_{wk}	Number of animals of any production category present on a farm f in a given week (wk) (animal)	$\sum_{p \in P} n_{wk,p}$	Adapted from (Schrag et al., 2020a)
	$\overline{N_p}$	Average number of animals of a given production category (p) on a farm f (or average farm inventory of a given production category (p)) during a time period T (animal)	$\frac{\sum_{wk=1}^{tw} n_{wk,p}}{tw}$, where tw=floor(T/7)	Adapted from (Schrag et al., 2020a)
	K	Total number of animals on a farm f *ever* treated with an antimicrobial product during a time period T. Can be calculated overall (K), or subset for a specific production category (p), active substance (s), route of administration (r), disease (d), or their combination (animal)	$\#(i_{R \geq 1}) \vert t \in \{1, .. T\}$	NA
Antimicrobial	S	List of all active substances (s) administered on a farm f during a period of time T (categorical)[3]	$S = \{Tetracycline,\ Sulfonamide,\ \ldots\}$	NA
	RA	List of routes of antimicrobial product administration (categorical)[3]	$RA = \{intramuscular,\ subcutaneous, \ldots\}$	NA
	a_T	Total number of all single antimicrobial administrations (a) administered on a farm f during a period of time T. Can be calculated overall (a_T), or subset for a specific production category (p), active substance (s), route of administration (r), disease (d), or their combination (administration)	$\#(a)\ \ \vert t \in \{1, .. T\}$	
	R_T	Total number of all standard regimens (R) administered on a farm f during a period of time T. Can be calculated overall (R_T), or subset for a specific production category (p), active substance (s), route of administration (r), disease (d), or their combination. (regimen)	$\#(R_d) \vert t \in \{1, .. T\}$	Adapted from (Schrag et al., 2020b)
	m_R	Total mass of an active substance (s) over all administrations (c) administrated as part of a specific single regimen in an individual animal (i) (mg)	$c_R m_s$ or $\sum_{a_i=1}^{c_{R_i}} m_{s_i}$	Adapted from (Schrag et al., 2020a)
	$\overline{m_R}$	Mean mass of an active substance (s) over all instances of application of a specific regimen administrated during a period of time T (m_R) (mg)	$\frac{\sum_{R_{s,r}=1}^{R_T s,r} m_{R_{s,r}}}{R_T}$	NA
	$m_{p,s}$	Total mass of an active substance (s) used in an animal production category (p) on farm f during a period of time T (mg)	$\sum_{a_{p,s}=1}^{a_{T_{p,s}}} m_{a_{p,s}}$	NA
	m_p	Total mass of all active substances used in an animal production category (p) on farm f during a period of time T (mg)	$\sum_{s \in S} m_{p,s}$	NA
	ADD_i	Actual daily dose for an active substance (s) in a single antimicrobial administration for a therapeutic purpose targeting a single disease event (d) in an individual animal (i) (mg active substance/kg animal/day)	$\frac{AD_i}{adjF}$ or $\frac{AD_i}{int}$	Adapted from (Brault et al., 2019a)
	ADD_m	Prescribed or mean daily dose for an active substance (s) in a single antimicrobial administration for a therapeutic purpose targeting a single disease event (d) in an individual animal (i) (mg active substance/kg animal/day)	$\frac{AD_m}{adjF}$ or $\frac{AD_m}{int}$	Adapted from (Brault et al., 2019a)
	UDD	Median (preferred) or mean[4] of actual used daily doses administered per day as part of a regimen per actual kg of animal body mass at the time of treatment (w_R) on farm f during a time period T (mg active substance/kg animal/day)	$median(\frac{m_R}{w_R \times \mathrm{DOT}})$ or $\frac{\sum_{R=1}^{R_T} \frac{m_R}{w_R \times \mathrm{DOT}}}{R_T}$, where $w_R = w_i$at tfirst of R	Adapted from (Kasabova

(Continued)

TABLE 2 Continued

Category	Notation	Definition (unit)	Equation	Source
				et al., 2019)
	DDDp	Study-defined daily dose that is specific for the population under study (mg active substance/kg animal/day)	$\frac{\overline{m_R}}{\mathrm{DOT}} \times \frac{1}{w_{f,p}}$	Adapted from (Schrag et al., 2020b)
	DCDp	Study-defined course dose that is specific for the population under study (mg active substance/kg animal/course)	$\frac{\overline{m_R}}{w_{f,p}}$	Adapted from (Schrag et al., 2020b)
Time	*DOT*	Duration of treatment. Depending on antimicrobial product used, *DOT* is expressed as: *cDOT*: Count of calendar days on which treatment was administered as part of a single regimen, used for antimicrobials administered in intervals ≤1 day; *aDOT*: Adjusted length of therapy for a single regimen used for a long-acting antimicrobial product or product administered in intervals > 1 day. (day)	$\mathrm{DOT} = \{\mathrm{cDOT}, \mathrm{aDOT}\}\ cDOT = \begin{cases} c \times int, & int < 1\ day \\ c, & int = 1\ day \end{cases}\ aDOT = c \times adjF$	Adapted (Schrag et al., 2020b)
	TE	Total number of therapeutic events among treated animals. Each therapeutic event is identified by grouping regimens in an individual animal by date of administration so that regimens within 7 days are part of the same treatment event (event)	NA	(Schrag et al., 2022)
	cfl_R	The number of calendar days between the first and last administration of a regimen to an animal (*i*) (day)	$t_{last}R - t_{first}R$	Adapted from (Schrag et al., 2020a)

[1]Data collected for calculation of antimicrobial use indicators on a farm f are defined in Table 1. The time period T of data recording for periodic calculation of an antimicrobial use indicator is user defined (e.g., month, quarter, year).
[2]NA, not applicable.
[3]Examples for levels of categorical variables listed in the set are for illustration purposes.
[4]Median is preferable when the distribution of applied UDDs is skewed, however, the mean is acceptable and is easier to calculate on a farm.

(v) standard parameter use (Standard parameters, sp); and (vi) the potential for privacy concerns regarding sharing of indicators or data used for their calculation (Privacy concerns, pc). Among these data requirements, the criteria ani, ed, and el relate to the accuracy of the AMU measurement, which we call accuracy criteria. Here, the term accuracy is defined in terms of granularity and exactness. Granularity means the level of actual and detailed information an indicator will include, and exactness is used to represent the absence of standard parameters (FS or GS) in the indicator calculation. Therefore, in this study, we evaluate the indicators' procedural accuracy, which describes their capacity or potential to capture the true application of an antimicrobial, rather than their field accuracy when these indicators are applied in a farm setting. The procedural accuracy is necessary but not sufficient to achieve the field accuracy as an indicator with great granularity and exactness may be inaccurate in a field application, for example, because of incorrectly recorded or missing data. The involved effort and privacy were represented by edr and pc criteria, respectively. The stewardship information criteria assessed the type of inference available from an indicator to inform AMU stewardship. These criteria evaluated whether an indicator can (i) monitor AMU in specific animal groups (Trends over time regarding treated animals, tt); (ii) track changes in the population at risk (Trends over time regarding population at risk, tp); (iii) track changes in the proportion of sick animals treated (Trends over time regarding treatment effort, tte); and track changes in the antimicrobial exposure in terms of (iv) antimicrobial substance (texam) and (v) length of treatments (texle). The characteristics and relationship of terms in the formula for calculation of the indicators were the most important factors for the score. ZL and EB independently scored the indicators with scores 1 (worst) – 5 (best) for each of the 11 criteria and discussed any differences in scores with RI until a consensus was reached. We considered the 5-point scale to be sufficient to distinguish the performance of each indicator in each criterion. Supplementary Tables S3, S4 provide a detailed rationale for each score for the data- and stewardship-driven criteria, respectively. To interpret scores, we compared indicator scores with respect to (i) accuracy (ani, ed, and el), (ii) effort (edr), (iii) privacy (pc), and (iv) stewardship (tt, tp, tte, texam, and texle overall and individually). Due to the absence of established weights that different criteria should be given in comparisons, all criteria were given the same weight, and multiple criteria for accuracy and stewardship were averaged. The averaging process for accuracy and stewardship criteria ensured that the four criteria groups ((i)-(iv)) had the same scale, which was necessary to allow their direct and fair comparison. To aid interpretation, indicator scores for the four criteria groups were visually evaluated using a spider plot.

TABLE 3 Formulas for calculation of antimicrobial drug use indicators[1].

Group	Indicator	Definition	Equation[2]	Reference
Count-based	*nTE*	Number of therapeutic events per animal of a given production category (p) on farm f during a time period T. (therapeutic events/animal)	$\frac{TE}{\overline{N_p}}$	Adapted from (Schrag et al., 2022)
	nREG	Number of regimens per animal of a given production category (p) on farm f during a time period T. (regimens/animal)	$\frac{R_T}{\overline{N_p}}$	Adapted from (Schrag et al., 2020b)
	RT-ratio	Antimicrobial regimen to therapy ratio (RT-ratio), calculated by dividing the number of antimicrobial regimens by the number of therapeutic events. (regimens/therapeutic event)	$\frac{nREG}{nTE}$	(Schrag et al., 2022)
	nRTFD	Regimen time frame days (RTFD) per animal of a given production category (p) on farm f during a time period T. Numerator is estimated as the sum of cfl_R (days/animal)	$\frac{\sum_{R=1}^{R_T} cfl_R}{\overline{N_p}}$	Adapted from (Schrag et al., 2020b)
	nDOT	Total length of all therapies in days per animal of a given production category (p) on farm f during a time period T. (days/animal)	$\frac{R_T \times DOT}{\overline{N_p}}$	Adapted from (Schrag et al., 2020b)
Mass-based	*mg/TAB*	Total mass of all active substances used per animal biomass of a given production category (p) treated with these active substances on farm f during a time period T. (mg active substance/kg animal)	$\frac{m_p}{w_{f,p} \times \overline{N_p}}$	Adapted from (European Medicines Agency, 2023; FDA Center for Veterinary Medicine)
	mg/100 animals-at-risk	Total mass of all active substances used per 100 animals-at-risk of a given production category (p) on farm f during a time period T. (mg active substance/animal)	$\frac{m_p}{\overline{N_p}} \times 100$	(Brault et al., 2019a)
Dose-based	*nDDDp*	Number of study-defined daily doses per animal of a given production category (p) for the farm f during a time period T. (doses/animal)	$\frac{\frac{m_{p,s}}{DDDp \times w_{f,p}}}{\overline{N_p}}$	Adapted from (Schrag et al., 2020b)
	nDDDv	Number of the standard defined daily doses per animal of a given production category (p) on farm f during a time period T. (doses/animal)[3]	$\frac{\frac{m_{p,s}}{DDDv \times w_p}}{\overline{N_p}}$	(Schrag et al., 2020b)
	TF_{UDD}	Treatment frequency per animal of a given production category (p) on farm f based on the median (preferred) or mean Used Daily Dose for a drug product with active substance s during a time period T. (doses/animal)	$\frac{m_{p,s}}{UDD \times w_{f,p} \times \overline{N_p}}$	(Kasabova et al., 2019)
	TF_{DDD}	Treatment frequency per animal of a given production category (p) on farm f based on standard (EU) defined daily doses for a drug product with active substance s during a time period T. (doses/animal)[3]	$\frac{m_{p,s}}{DDDv \times w_p \times \overline{N_p}}$	(Kasabova et al., 2019)
	nADD (kg_a)/100 treated animals	Number of actual individually administered daily doses per 100 treated animals of a given production category (p) on farm f during the time period T. Estimated by accounting for the actual administered dose and the actual body mass (kg) of treated animals. Can be interpreted as: how many days on average 100 animals on farm f were treated during a time period T. (doses/animal)	$\sum_{i=1}^{R_T} \frac{m_{s,i}}{w_i \times ADD_i} \times \frac{100}{K_p}$	(Brault et al., 2019a)
	nADD (kg_m)/100 treated animals	Number of prescribed or individually administered mean daily doses per 100 treated animals of a given production category (p) on farm f during the time period T. Estimated by accounting for the standard administered dose and the mean body mass (kg) of treated animals. Can be interpreted as: how many days on average 100 animals on the farm f were treated during a time period T. (doses/animal)	$\frac{m_{p,s}}{w_{f,p} \times ADD_m} \times \frac{100}{K_p}$	Adapted from (Brault et al., 2019a)
	nDDDv/1,000 animal days-at-risk	Number of Canadian-defined daily dose per 1,000 animal-days-at-risk of a given production category (p) on farm f during a time period T. (doses/animal-days-at-risk)	$\frac{m_{p,s}/DDDv}{ADR \times w_p \times \overline{N_p}} \times 1000$	(Canadian Integrated Program for Antimicrobial Resistance Surveillance)
	nDCDp	Number of study-defined course doses per animal of a given production category (p) for the farm f during a time period T. (courses/animal)	$\frac{\frac{m_{p,s}}{DCDp \times w_{f,p}}}{\overline{N_p}}$	Adapted from (Schrag et al., 2020b)
	nDCDv	Number of standard defined course doses per animal of a given production category (p) on farm f during a time period T. (courses/animal)	$\frac{\frac{m_{p,s}}{DCDv \times w_p}}{\overline{N_p}}$	(Schrag et al., 2020b)

[1]Equations are illustrated for estimating indicators for a single active substance except for *mg/TAB* and *mg/100 animals-at-risk*, which by definition represent the use of all administrated active substances. Additionally, equations illustrate the estimation of indicators for a given animal production category.

[2]Terms in the equations are defined in Tables 1, 2.

[3]If *nDDDv* and TF_{DDD} use the same DDDv (Standard defined daily dose by the European Surveillance of Veterinary Antimicrobial Consumption or Government of Canada (mg active substance/kg animal/day)), they will result in identical values of nDDDv and TF_{DDD} indicators.

TABLE 4 Definition of criteria used for scoring antimicrobial drug use indicators.

Group	Criteria (abbreviation)	Definition
Data requirement	Animal Information (ani)	Use of the actual number of treated or total animals and the actual animal body mass for an individual animal in an indicator calculation
	Exposure data (ed)	Use of the actual amount (mass or dose) of antimicrobial administrated in the treatment of an individual animal in an indicator calculation
	Extra-label use (el)	Ability to account for the extra label use (i.e., antimicrobial use per kg animal, treatment interval, or treatment protocol that is not in accordance with the approved labeling)
	Standard parameters (sp)	Use of standard parameters for animal body mass and/or dose
	Privacy concerns (pc)	The level of privacy concerns associated with sharing data used for the calculation of the indicator or sharing the indicator value itself
	Ease of data recording and calculation (edr)	The ease of recording or obtaining data for calculation of an indicator and/or the complexity of involved calculations
Stewardship information	Trends over time regarding treated animals (tt)	Provides information about changes in specific groups of animals receiving an antimicrobial treatment (in terms of individual characteristics (body mass (*w*), production category (*p*), treatment indication (*d*))
	Trends over time regarding population at risk (tp)	Accounts for changes in the population at risk of antimicrobial treatment in a herd (through $\overline{N_p}$) or number of treated animals (through *Kp*)
	Trends over time regarding treatment effort (tte)	Provides information about the proportion of diseased animals in a herd that are receiving treatment
	Trends over time regarding exposure: antimicrobial substance (texam)	Provides information about changes in exposure to a specific antimicrobial substance (in terms of the amount (mass or dose) of the antimicrobial substance used in a herd or production category).
	Trends over time regarding exposure: length (texle)	Provides information about changes in the total length of antimicrobial treatments (through *DOT*)

3 Results

3.1 Required parameters and standardization

In the standardization of terms used in the calculation of AMU indicators, we focused on the mass of active substances, animal population, animal body mass, and treatment days (Tables 1, 2) because these terms are the essential components of most indicators (Table 3). The standardization of active substance mass, which always appears in the numerator of the AMU indicator, resulted in four types of this parameter that are directly used for the calculation of the indicators: the total mass of an active substance administered for an individual animal in one regimen (m_R), the mean mass of an active substance over all regimens ($\overline{m_R}$) recorded during a defined period of time *T*, the total mass of an active substance used in an animal production category ($m_{p,s}$) during a defined period of time *T*, and the total mass of all active substances used in an animal production category (m_p) over a defined period of time *T*. Parameters m_R, $\overline{m_R}$, and $m_{p,s}$ are calculated separately for each active substance used on a farm. This allows tracking the use of individual drug classes and calculation of AMU indicators for individual active substances. The only exceptions are the mass-based indicators which use m_p and thus calculate the total mass of all active substances combined, masking differences in the AMU across drug classes and the related indications for their use. Two kinds of the animal population parameters appeared in the denominators of indicators: the average number of animals in a production category ($\overline{N_p}$) and the total number of treated animals in a production category (K_p). The two *ADD*-based indicators use the K_p parameter, while all other indicators except *RT-ratio* use $\overline{N_p}$ (Table 3).

Standardization of animal body mass resulted in three types of data: the measured or estimated body mass of an individual treated animal (w_i), the average body mass for animals of the treated animal production category on a specific farm ($w_{f,p}$), and standard average body mass for the treated animal production category (w_p). We adapted the definition of duration of treatment (*DOT*) from Schrag et al.'s study to standardize the treatment time information in the indicators (Schrag et al., 2020b). For drugs with administration intervals ≤ 24 hours, the *DOT* is the count of calendar days of treatment (Table 2). For drugs with administration intervals greater than 24 hours, the *DOT* is the multiplication of the number of administrations and the time interval between administrations or the estimated duration of effect (Table 2).

A cross-tabulation of AMU indicators and their standardized terms showed that TF_{UDD} required the most variables/parameters for calculation while *nREG* and *mg/100 animals-at-risk* required the least (Table 5). Dose-based indicators tended to require more variables/parameters than count-based and mass-based indicators because they included dose and additional animal information. Some of the indicators provide flexibility regarding the required data accuracy (specifically regarding terms describing animal body mass). In Table 5, we show this by using superscripts "*a*" and '*b*" when there are two options for a variable/parameter, with the superscript "*a*" indicating the preferred, more accurate option but which also requires more detailed data. For indicators *mg/TAB*, *nDDDp*, TF_{UDD}, *nADD*(kg_m)*/100 treated animals*, and *nDCDp*, the

TABLE 5 Cross-tabulation of the required collected primary data and derived terms (variables and standard parameters) for estimating antimicrobial drug use indicators[1] .

Indicator	Antimicrobial: Primary data (Derived term)									Animal: Primary data (Derived term)					Time: Primary data (Derived term)		
	m,c_R (m_R)	m,c_R,R ($\overline{m_R}$)	m,p,s ($m_{p,s}$)	m,p (m_p)	R (R_T)	m,w (AD_i)	AD_m	$DDDv$	$DCDv$	$n_{wk,p}$($\overline{N_p}$)	i_R (K_p)	w_i	$w_{f,p}$	w_p	t (cfl_R)	$c,int/adjF$ (DOT)	ADR
nTE					×					×					×		
nREG					×					×							
RT-ratio					×										×		
nRTFD					×					×					×		
nDOT					×					×						×	
mg/TAB				×						×			×[a]	×[b]			
mg/100 animals-at-risk				×						×							
nDDDp		×	×							×			×[a]	×[b]		×	
nDDDv			×					×		×				×			
TF_{UDD}	×[a]	×[b]	×		×					×		×[2]	×[a]	×[b]		×	
TF_{DDD}			×					×		×				×			
nADD(kga)/100 treated animals	×[a]	×[b]			×	×[a]	×[a]				×	×				×	
nADD(kgm)/100 treated animals			×				×				×		×[a]	×[b]		×	
nDDDv/1,000 animal days-at-risk			×					×		×				×			×
nDCDp		×	×							×			×[a]	×[b]			
nDCDv			×						×	×				×			

[1]Superscript letters "a" and "b" are used when there are two choices for a variable in the calculation of an indicator, where "a" indicates the preferred more accurate choice (according to the indicator's definition), and "b" indicates the acceptable alternative.
[2]When calculating the Used Daily Dose (UDD) explained in Table 2, w_i is the preferred choice but $w_{f,p}$ is also acceptable.

preferred option is $w_{f,p}$ because it represents the farm-specific average animal body mass. However, users who prefer a less time-consuming option for estimating $w_{f,p}$, have privacy concerns regarding the animal body mass records or do not have the data available can use one of the available general standards, such as the FDA-estimated standard body mass of 635.03kg for dairy cows (FDA, 2022). When calculating *ADD*-based indicators and TF_{UDD}, the preferred option for the numerator is m_R, but $\overline{m_R}$ is also acceptable.

The most used terms $m_{p,s}$ and $\overline{N_p}$ appeared in eight and thirteen indicators, respectively. Mass-based indicators use m_p as the numerator because they do not account for the mass of individual active substances. Count-based indicators require R_T (sum of all standard regimens over a defined period of time T) because they are based on standard regimens and need to account for all administrated regimens to quantify AMU. Only *ADD*-based indicators require individual dose information for calculation. The indicators *nDDDv*, TF_{DDD}, *nDCDv*, and *nDDDv/1,000 animal days-at-risk* need ESVAC and Canada-defined standard daily dose and course dose, which can be replaced with the U.S.-specific standard doses when they become available. The number of calendar days between the first and last administration in a regimen (cfl_R) is required for the calculation of *nRTFD*, knowing the start dates (t) of regimen treatments for individual animals is necessary for the calculation of *nTE* and, therefore, also for *RT-ratio*, while the average days at risk (*ADR*) is needed for *nDDDv/1,000 animal days-at-risk*.

In addition to providing a visual comparison of the required primary data and derived terms for each indicator, Table 5 provides the basis for creating education materials to guide farmers and veterinarians in selecting suitable indicators based on the data they have available. For example, if a farm only records the total amount of an active substance used and only has an average animal body mass at the animal production level rather than at an individual

10.3389/frabi.2023.1176817

animal level, *nADD(kg_m)/100 treated animals* is an applicable indicator, but TF_{UDD} and *nADD(kg_a)/100 treated animals* cannot be calculated. Two options of parameters in the same category, e.g., $w_{f,p}$ and w_p, applicable to some indicators, add flexibility and simplicity. For example, if a farm does not have data to infer the farm-specific average animal body mass, they can use the FDA-estimated standard animal body mass to calculate *nADD(kg_m)/100 treated* animals. Furthermore, a user can refer to Table 5 to plan data collection based on the AMU indicator(s) they want to use in the future.

3.2 Scoring

Heatmaps in Figures 1, 2 show scores for the 16 AMU indicators against each individual data requirement- and stewardship information-driven criteria, respectively. A spider plot in Figure 3 shows how the 16 indicators compare to each other with respect to the effort, privacy, and average scores in accuracy and stewardship criteria. Dose-based indicators generally scored better than count-based and mass-based indicators when accuracy criteria were considered (i.e., ani, ed, and el) (Figure 1). Among dose-based indicators, *nADD(kg_a)/100 treated animals*, TF_{UDD}, *nDDDp*, *nDCDp*, and *nADD(kg_m)/100 treated animals* have higher accuracy because they include farm-specific animal body mass and administered dose information. For example, *nADD(kg_a)/100 treated animals* scored "5" for the three accuracy criteria because it includes actual body mass and dose for each individual treated animal, and consequently, can detect extra-label use. Indicators that use standard dose (*nDDDv*, TF_{DDD}, *nDDDv/1,000 animal days-at-risk*, and *nDCDv*) or do not include dose information (*nTE*, *nREG*, *RT-ratio*, *nRTFD*, *nDOT*, *mg/TAB*, and *mg/100 animals-at-risk*) cannot detect extra-label use. TF_{UDD}, which scored "4" in the ed (Exposure data) criterion, does not require the actual dose for each animal but uses the median/mean used daily dose (UDD) administered for an animal production category. Mass-based indicators (*mg/TAB* and *mg/100 animals-at-risk*) capture information about the mass of antimicrobial used but score low ("2") in the ed criterion demonstrating the limited value of antimicrobial mass alone in characterizing antimicrobial exposure.

The scores for Privacy concerns (pc) and Ease of data recording and calculation (edr or effort criterion) were similar to each other and negatively correlated with the accuracy criteria (Figure 3). Indicators with better scores in accuracy criteria (ani, ed, and el) performed poorly in pc and edr. Accurate indicators need farm-level actual information on AMU and require more data. Farmers may have privacy concerns about whether they should record actual animal and dose information and use that granular information in the calculation of indicators, and it takes time to record data needed for all equation terms. In contrast, indicators that include standard animal body mass or defined dose are less accurate but, with a few exceptions, score better in pc and edr because they use existing standard values instead of farm-specific data, which eases the process of recording data and alleviates privacy concerns since they reveal less about the farm practices or herd health. Indicators with standard parameters do not require a prior calculation for the dose terms, such as *UDD* and ADD_i, which eases their calculation.

Based on the scores for data requirements (Figure 1), *nADD (kg_m)/100 treated animals* generally performs well in all criteria (score range: 3-4). On the other hand, TF_{UDD} and *nADD(kg_a)/100 treated animals* have the highest accuracy, while *nRTFD*, *nDOT*, and mass-based indicators perform well in pc and edr.

	Criteria					
Indicator	Animal Information (ani)	Exposure data (ed)	Extra label use (el)	Standardized parameters (sp)	Privacy concerns (pc)	Ease of data recording and calculation (edr)
nTE	2	3	4	1	2	2
nREG	2	3	4	1	2	2
RT-ratio	1	3	4	1	2	2
nRTFD	2	1	1	1	5	4
nDOT	2	1	1	1	5	4
mg/TAB	4	2	1	4	4	4
mg/100 animals-at-risk	1	2	1	1	4	5
nDDDp	4	4	4	3	3	2
nDDDv	3	3	2	5	4	4
TF_{UDD}	5	4	5	1	1	2
TF_{DDD}	3	3	2	5	4	4
nADD(kg_a)/ 100 treated animals	5	5	5	2	1	1
nADD(kg_m)/ 100 treated animals	4	4	3	4	3	3
nDDDv/1,000 animal days-at-risk	3	3	2	5	4	3
nDCDp	4	4	4	3	3	2
nDCDv	3	3	2	5	4	4

FIGURE 1
Heatmap showing antimicrobial drug use indicator scores based on the data requirement criteria (1=worst, 5=best). Colors range from white (worst) to dark green (best). Criteria are defined in Table 4, and each score is explained in Supplementary Table S3.

10.3389/frabi.2023.1176817

	Criteria				
Indicator	Trends over time regarding treated animals (tt)	Trends over time regarding population at risk (tp)	Trends over time regarding treatment effort (tte)	Trends over time regarding exposure: antimicrobial substance (texam)	Trends over time regarding exposure: length (texle)
nTE	3	5	4	3	3
nREG	3	5	3	4	3
RT-ratio	3	2	5	5	3
nRTFD	1	4	2	1	5
nDOT	1	4	2	1	5
mg/TAB	4	4	1	2	1
mg/100 animals-at-risk	1	4	1	2	1
nDDDp	4	4	3	4	4
nDDDv	2	4	1	3	2
TF_{UDD}	5	5	3	5	4
TF_{DDD}	2	4	1	3	2
nADD(kg_a)/ 100 treated animals	5	4	3	5	4
nADD(kg_m)/ 100 treated animals	4	4	3	4	3
nDDDv/1,000 animal days-at-risk	2	4	1	3	2
nDCDp	4	4	3	4	3
nDCDv	2	4	1	3	2

FIGURE 2
Heatmap showing antimicrobial drug use indicator scores based on the stewardship-driven criteria (1=worst, 5=best). Colors range from white (worst) to dark green (best). Criteria are defined in Table 4, and each score is explained in Supplementary Table S4.

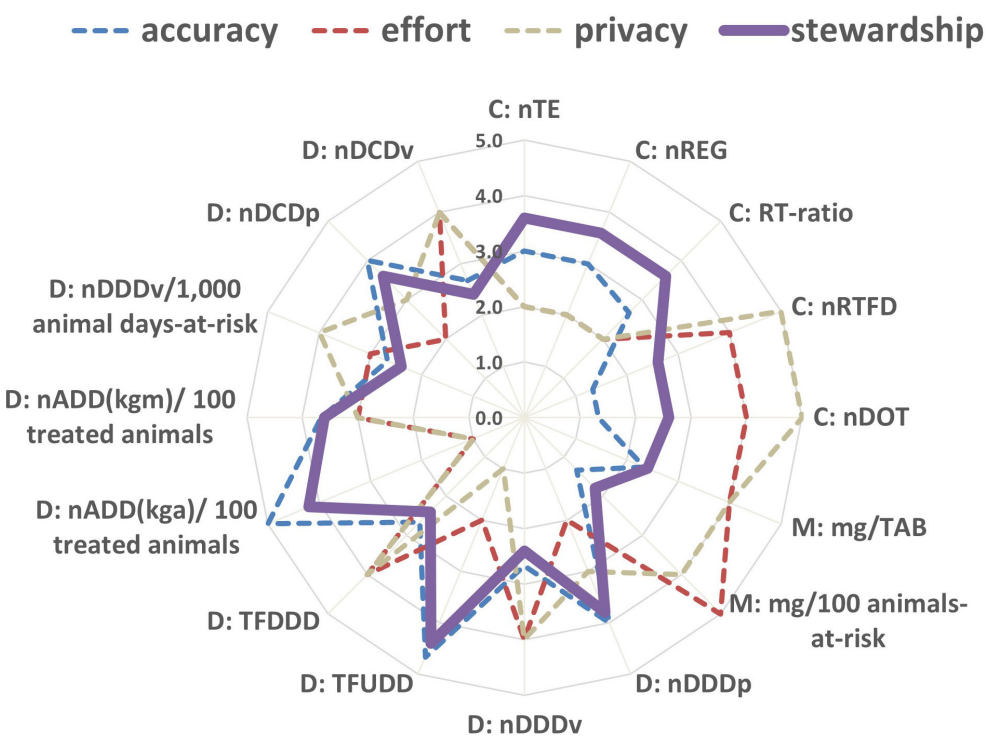


FIGURE 3
Spider plot showing antimicrobial drug use indicator scores with respect to accuracy (average), effort, privacy and stewardship (average) criteria. Notations 'C:', 'M:' and 'D:' respectively represent the count-based, mass -based, and dose-based indicators defined in Table 3.

As for the stewardship-driven criteria, all indicators except for *RT-ratio* have a term describing the treated or total animal population in an animal production category that changes over time, so they score well in the population trend criterion (tp) (Figure 2). The highest scores for *nTE*, *nREG*, and TF_{UDD} are because they account for both the treated animal and total animal population.

Scores for criteria describing the trends regarding the treated animals (tt) and the antimicrobial substance exposure (texam) showed similar patterns (Figure 2) because indicators that use farm-level specific animal information tend to use the farm-specific dose information; that is, the standard animal body mass and defined dose usually appear simultaneously in indicators. Indicators *nDDDp*, TF_{UDD}, *nDCDp*, *nADD(kg$_a$)/100 treated animals*, and *nADD(kg$_m$)/100 treated animals* perform well in these two criteria because they use farm-specific animal body mass and doses, which allows observation of potential changes over time. Since TF_{UDD} and *nADD(kg$_a$)/100 treated animals* use individual animal-level information, they get the best scores in these two criteria. However, standard parameters negatively influence the indicators' ability to monitor trends because they are constant and cannot reflect the changes in animal body mass and/or dose.

A key goal for an antimicrobial stewardship program is to promote shorter durations of antimicrobial therapy when clinically appropriate (Yarrington and Moehring, 2019). The length of antimicrobial treatments differs over time based on many factors, such as the type and severity of the diseases, and the type and effectiveness of the antimicrobial drugs. Therefore, the ability to follow trends in the length of the received antimicrobial treatment (texle) is essential to understanding herd health and informing antimicrobial stewardship. The length of the received antimicrobial treatment (*DOT*) is directly implemented in *nDOT*, *nDDDp*, TF_{UDD}, and the two *ADD*-based indicators and is accounted for via regimens in *nTE*, *nREG*, *RT-ratio*, and *nRTFD*, resulting in their overall better scores in texle (Figure 2). For example, *nDOT* scored a "5" in the texle criterion because it directly provides information on the length of AMU exposure, while it received a poor score ("1") in the Exposure data (ed) criterion, in the data requirement category, since it captures no information about the actual amount (mass or dose) of antimicrobial administrated in the treatment of an individual animal (according to the definition of the ed criterion in Table 4).The remaining indicators not mentioned in this paragraph cannot track treatment length, which results in their poor scores.

Indicators *nTE*, *nREG*, *RT-ratio*, TF_{UDD}, *nDDDp*, *nDCDp* and the two *nADD*-based indicators either use the population of treated animals or the regimen information for treated animals in the calculation, which strengthens them with respect to the criterion that evaluates the ability to track treatment effort (tte) (Figure 2). *RT-ratio* shows the number of antimicrobial regimens by the number of therapeutic events. As such, this indicator proxies the extent of antimicrobial administration per treated disease event, which reflects the level of preference to use antimicrobials to treat disease, and thus it performs best in tte. Only *nTE*, *nREG*, and TF_{UDD} indicators account for treated animals and the total population size and track AMU for a given population category, thus can, to a limited degree, reflect the disease pressure in the herd.

Indicators TF_{UDD} and *nADD(kg$_a$)/100 treated animals* have the best performance considering the stewardship-driven criteria, as they can track changes in several aspects relevant to antimicrobial stewardship (Figure 2). Indicators with better scores in accuracy tended to have better scores in stewardship (Figure 3). *RT-ratio* is a unique indicator; although it does not include animal information, it can show the level of preference for AMU to treat disease events and help explore the feasibility of treating disease with less or no antimicrobials. *nRTFD*, *nDOT*, mass-based indicators, and dose-based indicators using standard parameters are relatively less ideal indicators to inform stewardship because they either lack important animal and antimicrobial information or use standard parameters that cannot capture temporal trends.

4 Discussion

Dairy farmers are under pressure to reduce AMU in animals to contribute to the fight against AMR within the One Health initiatives. A number of indicators for tracking farm-level AMU have been published, but they are challenging to interpret and compare (Sanders et al., 2020). Therefore, this study aimed to standardize and evaluate multiple existing AMU indicators based on their accuracy, data needs and effort required, privacy concerns, and ability to inform antimicrobial stewardship in order to aid their uptake on U.S. dairy farms. Our main findings are that: (i) standardized variables/parameters in the AMU indicators allow interchangeable and simultaneous calculation of indicators, (ii) accuracy vs. effort and accuracy vs. privacy trade-offs characterize the evaluated AMU indicators, and (iii) the evaluated AMU indicators can only partially inform antimicrobial stewardship. These findings are discussed in the following paragraphs.

4.1 Standardized variables and parameters allow interchangeable and simultaneous calculation of AMU indicators

We standardized and streamlined the calculation of existing AMU indicators, contributing to a better understanding, fair comparison, and easier interpretation of AMU indicators. The availability of these derived standardized indicators is expected to aid their use for on-farm AMU monitoring (Sanders et al., 2020). There have been calls for harmonization and clarification of AMU indicators and their calculations (Monitoring and Evaluation of the Global Action Plan on Antimicrobial Resistance; Sanders et al., 2020; Umair et al., 2021). For example, Umair et al. raised a concern that different AMU measurement metrics create difficulties in comparing AMU data from different sources, which stressed the urgency for developing a globally harmonized AMU measurement system (Umair et al., 2021). Interchangeability between standardized indicators will aid interpretation and allow for more flexibility in their use in

monitoring AMU. To our knowledge, the standardization of equations and underlying terms for 16 indicators was an unparalleled undertaking for AMU in animals that may provide a template for future AMU indicator harmonization efforts in other animal species and production categories. The simultaneous calculation of multiple indicators provides a more holistic view of AMU on a farm since no single indicator can comprehensively represent all aspects of antimicrobial decisions, which require nuanced, complex clinical decision-making, and dynamic changes over the course of therapy (Yarrington and Moehring, 2019). For example, some veterinarians perceived that *ADD-based* indicators are less intuitive than the treatment duration (Redding et al., 2020). Also, some standard parameters, such as those for the animal body mass, and the dose and duration of treatment, may differ significantly from the actual situation and thus limit the accuracy of indicators calculated from them. Specifically, the recommended doses of drugs containing the same active substance and used for the same animal body mass can vary considerably between countries and even within countries. Therefore, indicators with actual and updated information instead of standard parameters have better operational accuracy (i.e., better capacity to accurately reflect a farm's AMU) (Collineau et al., 2017; Mills et al., 2018). However, future research is needed to evaluate the 16 standardized AMU indicators in a field study. Additionally, research is needed to develop a dashboard for simultaneous visualization of multiple AMU indicators and their trends, and an assessment of the experience of farmers and veterinarians using it (Taber et al., 2021).

4.2 Evaluated AMU indicators are characterized by the accuracy vs. effort and accuracy vs. privacy trade-offs

Accurate indicators for quantifying AMU in dairy cattle are critical for monitoring trends in animal exposure to antimicrobial drugs over time. Accuracy is necessary, though not sufficient, for the indicator's utility in informing antimicrobial stewardship (Figure 3). Also, in the long run, accurate indicators will enable researchers to examine potential associations between AMU and the emergence of AMR determinants and resistant bacteria in cattle (Brault et al., 2019b). Among the 16 AMU indicators evaluated in this study, we consider TF_{UDD} and *nADD(kg$_a$)/100 treated animals* to be the most accurate. However, their accuracy comes with a price in terms of the increased effort required for data collection (Figure 3); *nADD(kg$_a$)/100 treated animals* requires data about the dose and body mass for each treated animal, the collection of which is not currently feasible on all farms (Brault et al., 2019a). The data needed for these indicators could be simplified (Table 5), reducing the required effort, but at a loss of accuracy and utility for informing antimicrobial stewardship. Thus, a farmer will need to balance accuracy and effort in calculating AMU indicators if their goal is to maximize the private benefits from the AMU indicators for their farm.

A recent survey revealed that 69.3% of dairy farmers in the northeastern U.S. would be interested in knowing how AMU on their farm compares to the use of antimicrobials on other comparable dairy farms (Casseri et al., 2022). This suggests that farmers are not just interested in learning-by-doing (through private use of AMU indicators for their farms) but also learning from their social networks, which would require some form of data sharing (ignoring the possibility of free-riding). For shared AMU indicators to be useful to other farmers in the social network, the values of AMU indicators would need to be accompanied by information that describes the context in terms of farm characteristics and practices (e.g., farm size, number of animals and their production categories, herd health and management, AMU data used for calculation of AMU indicators, and even any evidence of AMR on the farm). Such assembled shared database of AMU indicators and contextual data would provide unprecedented opportunities for data-driven innovation of antimicrobial stewardship in dairy farming. However, the requirement for sharing contextual data with AMU indicators, particularly for more accurate indicators that are based on granular antimicrobial and animal data, may raise concerns, such as regarding potential reputational damages, misuse of data, unauthorized use of data, loss of business advantage, or legal liabilities (Wiseman et al., 2019; Linsner et al., 2021). Thus, sharing AMU data among farmers may raise privacy concerns, creating a trade-off between indicator accuracy (utility) and privacy implications. The conflict between privacy and utility is a well-known concept in research about data sharing (Wirth et al., 2021). Accordingly, sharing accurate indicators would require limitations in the amount/type of contextual data shared to provide privacy, while data sharing that protects privacy would decrease the utility of the data (Wirth et al., 2021). To alleviate privacy concerns, several data sharing techniques have been suggested, from combining, perturbing, removing, or summarizing the data in a way that maintains confidentiality, to algorithms for differential privacy and federated learning (Ritchie, 2011; Qian et al., 2022). Based on the privacy concern and accuracy criteria evaluated in this study, farmers may elect to share *nDDDp* or *nDCDp* indicators. These indicators are limited in answering some questions on stewardship, but do not require individual animal information for calculation. Regarding data sharing, recently emerged initiatives work towards setting principles on data privacy, storage, collection, ownership and processing in the agriculture systems globally and in the U.S (Farm Progress; Data Privacy and Use White Paper), as well as providing educational training for farmers so that farmers can advance their skills on intelligent systems and protect their data (Kaur et al., 2022). A recent pilot project in the U.S. has demonstrated the willingness of swine farms to share AMU data (Davies and Singer, 2020). A century-old National Cooperative Dairy Herd Improvement Program (NCDHIP) provides a roadmap on how to develop a system for cooperative data governance and sharing of AMU and AMR data in the dairy sector (Hutchins and Hueth, 2023).

4.3 Evaluated indicators can only partially inform antimicrobial stewardship

Antimicrobial stewardship is defined as finding an optimal approach to sustaining animal health, welfare and production, minimizing selection for AMR and preserving antimicrobial efficacy through conscientious oversight (Apley et al., 2018; American Veterinary Medical Association; Antimicrobial Stewardship Guidelines). This may involve reductions in antimicrobials for animals who do not require them or increases for those who need them; notably, a responsible antimicrobial stewardship program cannot, and should not, strive towards "zero" AMU when measuring over large populations of animals (Yarrington and Moehring, 2019). Unfortunately, based on the information that makes up the components of AMU indicators, which focus on the antimicrobials rather than disease information, these indicators cannot capture the nature of the diseases, the accuracy of diagnosis, or the stage of the disease when treated. However, while stewardship has to include knowledge of what disease is being treated and for what purpose, these indicators contain information regarding some aspects of antimicrobial stewardship. In our study, all but one (*RT-ratio*) evaluated AMU indicator can account for changes in the population at risk. Several indicators (*mg/TAB*, *nDDDp*, TF_{UDD}, *nADD*(kg_a)*/100 treated animals*, *nADD*(kg_m)*/100 treated animals*, and *nDCDp*) can monitor changes over time in the body mass of treated animals. All evaluated indicators capture the production category of treated animals. As such, these indicators can inform stewardship since body mass of treated animals, the at-risk population, and animal production category information can potentially be used for comparisons of AMU among animal groups or to track changes in the same group over time (Canadian Integrated Program for Antimicrobial Resistance Surveillance; Brault et al., 2019b). A few indicators (*RT-ratio*, *nTE*, *nREG*) track antimicrobial treatments, and as such can directly inform antimicrobial stewardship, especially when used along with collected antimicrobial amount data (Schrag et al., 2022). This way, AMU can be associated with specific diseases, and actionable insights can be gained about the necessity of AMU. For example, Schrag et al. proposed recording therapeutic events as a proxy for disease incidence on dairy farms. As a result, they showed the frequency of AMU per treatment event on each farm and were able to identify farms with a high AMU (per treatment event); such farms may be interested in learning about stewardship practices on farms with a low AMU per treatment event (Schrag et al., 2022). Additionally, several indicators (*mg/TAB*, *mg/100 animals-at-risk*, *nDDDp*, *nDDDv*, TF_{UDD}, TF_{DDD}, *nADD*(kg_a)*/100 treated animals*, *nADD*(kg_m)*/100 treated animals*, *nDDDvC/1,000 animal days at risk* and *nDCDv*) can monitor changes in the amount of antimicrobial administered (either in terms of mass or dose). Mass-based indicators provide an intuitive interpretation of AMU, are relatively easy to record, and are frequently used for surveillance (Merle and Meyer-Kühling, 2019; Köper et al., 2020; Tiseo et al., 2020). Also, they are suitable for tracking AMU in specific target populations (e.g., same animal species and production type), and focusing on the same active substance and administration route (Collineau et al., 2017). However, mass-based indicators are confounded by drug potency (Jensen et al., 2004; Brault et al., 2019a). Specifically, if we compare the use of two antimicrobial products with different potency, the product with higher potency will have a lower mass of antimicrobial consumption, but that does not necessarily represent a more judicious antimicrobial use. Therefore, when mass-based indicators are used to compare the AMU of drugs with different potency, this comparison can be misleading (Brault et al., 2019a). In addition, drug potency reflected by dose and duration of treatment is necessary to compare treatment effectiveness and selection pressure, which is not available with mass-based indicators (Chauvin et al., 2001). On the other hand, dose-based indicators reflect how antimicrobial drugs are actually used in animals and consequently are better indicators for informing antimicrobial stewardship efforts (Bright-Ponte, 2020). Brault et al. effectively demonstrated the contrast of using mass- and dose-based metrics in a specific case of macrolide use on beef cattle feedlots, where the use of the mass-based metric resulted in the interpretation of less macrolide use than if the dose-based metric was used (Brault et al., 2019b). Their results demonstrated that mass-based indicators should be used in conjunction with dose-based indicators for creating effective stewardship strategies, especially for macrolides and other medically important antimicrobials with relatively low dose/kg rates (More, 2019). This also demonstrates the value of standardized AMU indicators resulting from this study and the value of simultaneous calculation and visualization of multiple AMU indicators discussed above.

The length of therapeutic effect is important to consider in planning stewardship efforts (More, 2019) and to study the association between antimicrobial exposure in animals and subsequent potential selection of AMR organisms in humans, animals or the environment. In our study, the texle criterion focused on indicators' ability to provide information on the duration of treatment (*DOT*). The DOT was also used to indirectly evaluate the duration of antimicrobial effect (DOE), which is the period that antimicrobials remain active in an animal's body. As the DOE of some antimicrobials in animals had not been established, using indicators that utilize DOT can serve to indirectly reflect the DOE of different types and doses of antimicrobials (Brault et al., 2019a). The higher scoring indicators were identified among the count-based (*nRTFD* and *nDOT*) and dose-based indicators (*nDDDp*, TF_{UDD}, and *nADD* (kg_a)*/100 treated animals*). This is unsurprising, since regimens and doses intrinsically account for *DOT* in calculation. However, high scoring indicators in this criterion (texle) should be interpreted carefully since *DOT* is affected by how the dose and/or regimen data are collected. Use of standard doses (*DDDv* and *DCDv*) or standard time intervals (*int* and *adjF*), that are taken directly from treatment protocols or prescriptions, are unable to capture deviations from the protocol or prescription (Schrag et al., 2020b). Furthermore, for long-acting antimicrobials, the actual DOE is not always clear (Brault et al., 2019a). Consequently, these indicators may provide an imprecise information about the length of antimicrobial selective pressure.

It is known that AMU exerts selective pressure on commensal microflora and pathogens, increasing the risk of recovery of AMR microorganisms from treated animals during or after the treatment (Catry et al., 2016; European Centre for Disease Prevention and Control (ECDC) et al., 2017; Lhermie et al., 2017). However, as mentioned above, none of the 16 evaluated indicators can quantify antimicrobial selective pressure. Administration of a single drug leads to selective pressure and the potential development of resistance or cross-resistance. Higher doses of antimicrobials and long treatment times intensify the selective pressure (Raymond, 2019). The antimicrobial administration routes, such as oral administration and intravenous injection routes, have different selective pressure effects on resistance (Zhang et al., 2013). Thus, the antimicrobial drug type, dose, treatment time, and administration route can all shape selective pressure and influence the AMR risk. For example, Volkova et al. successfully established a mathematical model to explore the effect of pharmacokinetics and biodegradation of parenterally administered ceftiofur on the dynamics of ceftiofur-resistant commensal enteric *Escherichia coli* in cattle (Volkova et al., 2012). Future research could be directed at building similar models of the relationship between AMU and selective pressure and the AMR risk level on a farm and scaling them up for use as a novel AMU indicator.

While indicators like nTE and RT-ratio can track the treatment effort on a farm, none of the 16 evaluated indicators can reflect the true disease burden (in terms of disease incidence and prevalence) on a farm. That is because these indicators primarily focus on antimicrobial treatments but do not combine AMU with information about disease occurrence in individual animals. Information about the true disease occurrence in an animal would help avoid misuse of antimicrobials and reduce AMU in healthy individuals, and expose the absence of AMU in situations when treatment is indicated (thus protect animal welfare); therefore, information about true disease occurrence would improve antimicrobial stewardship (Scott, 2013; Nielsen et al., 2021). Repeated testing of all or even a representative sample of animals on a farm to determine the true disease occurrence over time for multiple diseases clearly is not feasible. However, advances in precision livestock farming that uses sensors and other technologies to gather data about every animal on a farm and use that data to optimize herd health management and for early disease detection, are expected to fill that gap (Monteiro et al., 2021). These technologies should be investigated as a source of information about the true disease incidence to improve novel AMU indicators and contribute to antimicrobial stewardship.

In this study, we reviewed the literature and selected for standardization and comparison 16 internationally known AMU indicators suitable for monitoring AMU on U.S. dairy farms. In the future, the same standardization and evaluation approach could be applied to other AMU indicators that may have been missed in the current study. Additionally, future studies should apply the derived standardized AMU indicators to data from multiple farms to evaluate their field accuracy and practical utility and to statistically compare indicators to improve understanding of the best approach to using multiple indicators simultaneously. In the absence of established weights for the criteria used in indicator scoring, all criteria were given the same weights, which affected our conclusions. Future research with stakeholders is necessary to determine whether these criteria should have different weights. We acknowledge that AMU indicator scores are authors', and, thus, by definition, subjective. However, because scoring involved comparisons of the elements of equations (i.e., presence or absence of terms or information in the formula), the room for subjective interpretation of AMU indicators was limited, allowing us to consider the approach sufficiently objective for their scoring.

5 Conclusion

Standardizing the definitions and formula of the AMU indicators will facilitate their uptake by farmers and veterinarians while enabling their interchangeability and fair comparison of AMU indicators across farms. Accuracy and data availability are the first factors to consider, particularly because accuracy is necessary to inform stewardship and for analyses of the relationship between AMU and AMR. At the same time, privacy considerations are also crucial for farmers interested in learning stewardship from their social network, because farmers may be reluctant to share indicators based on detailed AMU information. Overall, according to the criteria established in this study, two dose-based indicators (TF_{UDD} and *nADD(kg$_a$)/100 treated animals*) scored best in accuracy and ability to inform stewardship, while two count-based indicators (*nRTFD* and *nDOT*) and a mass-based indicator (*mg/100 animals-at-risk*) performed best in the effort and privacy criteria.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

ZL finished the literature review and drafted the first version of the manuscript. ZL, EB and RI built the standardization and scoring approaches together. EB and RI gave their initial comments on the first manuscript version. ZL, EB, DN, and RI contributed to subsequent revisions and approved the final version.

Funding

This research was funded by the National Institute of Food and Agriculture, USDA, Hatch under Accession number 7000433; as

well as Multistate Research Funds accession number 1016738 awarded to RI.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Author disclaimer

Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of USDA.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frabi.2023.1176817/full#supplementary-material

References

Ag data: its value, who owns it and where's it going? [Internet]. Farm Progress. Available at: https://www.farmprogress.com/cotton/ag-data-its-value-who-owns-it-and-where-s-it-going-.

American Veterinary Medical Association. Antimicrobial stewardship definition and core principles [Internet]. [cited 2023 Feb 27]. Available at: https://www.avma.org/resources-tools/avma-policies/antimicrobial-stewardship-definition-and-core-principles.

Antimicrobial Stewardship Guidelines [Internet]. [cited 2023 Feb 27]. Available at: http://www.aabp.org/resources/AABP_Guidelines/.

Apley, M. D., Godden, S., Hope, K. J., and Schrag, N. F. D. (2018). Antimicrobial use monitoring: A useful tool, or a disciplinary stick? *Am. Assoc. Bovine Practitioners Conf. Proc.* 51 (2), 91–95. doi: 10.21423/aabppro20183115

Bokma, J., Boone, R., Deprez, P., and Pardon, B. (2020). Short communication: Herd-level analysis of antimicrobial use and mortality in veal calves: Do herds with low usage face higher mortality? *J. Dairy Science* 103 (1), 909–914. doi: 10.3168/jds.2019-16764

Brault, S. A., Hannon, S. J., Gow, S. P., Otto, S. J. G., Booker, C. W., and Morley, P. S. (2019a). Calculation of antimicrobial use indicators in beef feedlots—Effects of choice of metric and standardized values. *Front. Vet. Sci.* 6, 330. doi: 10.3389/fvets.2019.00330

Brault, S. A., Hannon, S. J., Gow, S. P., Warr, B. N., Withell, J., Song, J., et al. (2019b). Antimicrobial use on 36 beef feedlots in Western Canada: 2008–2012. *Front. Veterinary Sci.* doi: 10.3389/fvets.2019.00329

Bright-Ponte, S. J. (2020). Antimicrobial use data collection in animal agriculture. *Zoonoses Public Health* 67 (S1), 1–5. doi: 10.1111/zph.12771

Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2018: Design and Methods. Available at: https://www.Canada.ca/content/dam/phac-aspc/documents/services/surveillance/canadian-integrated-program-antimicrobial-resistance-surveillance-cipars/cipars-reports/2018-annual-report-design-methods/2018-annual-report-design-methods.pdf.

Casseri, E., Bulut, E., Llanos Soto, S., Wemette, M., Stout, A., Greiner Safi, A., et al. (2022). Understanding Antibiotic Resistance as a Perceived Threat towards Dairy Cattle through Beliefs and Practices: A Survey-Based Study of Dairy Farmers. *Antibiotics.* 11 (8), 997. doi: 10.3390/antibiotics11080997

Catry, B., Dewulf, J., Maes, D., Pardon, B., Callens, B., Vanrobaeys, M., et al. (2016). Effect of antimicrobial consumption and production type on antibacterial resistance in the bovine respiratory and digestive tract. *PloS One* 11 (1), e0146488. doi: 10.1371/journal.pone.0146488

Chauvin, C., Madec, F., Guillemot, D., and Sanders, P. (2001). The crucial question of standardisation when measuring drug consumption. *Veterinary Res.* 32 (6), 533–543. doi: 10.1051/vetres:2001145

Cheng, T. Y., Almeida, B. G., Pempek, J. A., Masterson, M. A., and Habing, G. G. (2022). The use of common antimicrobial agents in US veal calves. *Zoonoses Public Health* 69 (4), 359–369. doi: 10.1111/zph.12928

Collineau, L., Belloc, C., Stärk, K. D. C., Hémonic, A., Postma, M., Dewulf, J., et al. (2017). Guidance on the selection of appropriate indicators for quantification of antimicrobial usage in humans and animals. *Zoonoses Public Health* 64 (3), 165–184. doi: 10.1111/zph.12298

Cummings, K. J., Perkins, G. A., Khatibzadeh, S. M., Warnick, L. D., and Altier, C. (2013). Antimicrobial resistance trends among salmonella isolates obtained from dairy cattle in the Northeastern United States, 2004–2011. *Foodborne Pathog. Dis.* 10 (4), 353–361. doi: 10.1089/fpd.2012.1285

Data Privacy and Use White Paper [Internet]. [cited 2023 Jan 25]. Available at: https://s3.amazonaws.com/aggateway_public/AgGatewayWeb/WorkingGroups/Committees/DataPrivacySecurityCommittee/2017-03-31%20Data%20Privacy%20and%20Use%20White%20Paper%20-%201.2.pdf.

Davies, P. R., and Singer, R. S. (2020). Antimicrobial use in wean to market pigs in the United States assessed via voluntary sharing of proprietary data. *Zoonoses Public Health* 67 (S1), 6–21. doi: 10.1111/zph.12760

Defined daily doses for animals (DDDvet) and defined course doses for animals (DCDvet) [Internet]. [cited 2023 Jan 23]. Available at: https://www.ema.europa.eu/en/documents/other/defined-daily-doses-animals-dddvet-defined-course-doses-animals-dcdvet-european-surveillance_en.pdf.

de Campos, J. L., Kates, A., Steinberger, A., Sethi, A., Suen, G., Shutske, J., et al. (2021). Quantification of antimicrobial usage in adult cows and preweaned calves on 40 large Wisconsin dairy farms using dose-based and mass-based metrics. *J. Dairy Science* 104 (4), 4727–4745. doi: 10.3168/jds.2020-19315

de Verdier, K., Nyman, A., Greko, C., and Bengtsson, B. (2012). Antimicrobial resistance and virulence factors in Escherichia coli from swedish dairy calves. *Acta Veterinaria Scandinavica* 54 (1), 2. doi: 10.1186/1751-0147-54-2

Duse, A., Waller, K. P., Emanuelson, U., Unnerstad, H. E., Persson, Y., and Bengtsson, B. (2015). Risk factors for antimicrobial resistance in fecal Escherichia coli from preweaned dairy calves. *J. Dairy Science* 98 (1), 500–516. doi: 10.3168/jds.2014-8432

European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA). (2017) *ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals | EFSA*. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4872.

European Medicines Agency. (2023) *Revised ESVAC reflection paper on collecting data*. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/revised-european-surveillance-veterinary-antimicrobial-consumption-esvac-reflection-paper-collecting_en.pdf.

European Medicines Agency. (2015). *Principles on assignment of defined daily dose for animals (DDDvet) and defined course dose for animals (DCDvet)*. (London, United Kingdom: EMA/224954/2016).

European Medicines Agency. (2019). *Sales of veterinary antimicrobial agents in 31 European countries in 2017*. Available at: https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2017_en.pdf.

FAOSTAT. Available at: https://www.fao.org/faostat/en/#data/TCL.

FDA. (2021). *2020 Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals*. Available at: https://www.fda.gov/media/154820/download.

FDA. (2022). *Antimicrobial Use and Resistance in Animal Agriculture in the United States 2016-2019 Summary Report*. Available at: https://www.fda.gov/media/159544/download

FDA Center for Veterinary Medicine. *FDA's Proposed Method for Adjusting Data on Antimicrobials Sold or Distributed for Use in Food-Producing Animals, Using a Biomass Denominator*. Available at: https://www.fda.gov/files/animal%20&%20veterinary/

published/FDA%E2%80%99s-Proposed-Method-for-Adjusting-Data-on-Antimicrobials-Sold-or-Distributed-for-Use-in-Food-Producing-Animals-Using-a-Biomass-Denominator–Technical-Paper.pdf.

Fonseca, M., Heider, L. C., Léger, D., Mcclure, J. T., Rizzo, D., Dufour, S., et al. (2022). Canadian dairy network for antimicrobial stewardship and resistance (CaDNetASR): An on-farm surveillance system. *Front. Veterinary Sci.* 8. doi: 10.3389/fvets.2021.799622

Gonggrijp, M. A., Santman-Berends, I. M. G. A., Heuvelink, A. E., Buter, G. J., van Schaik, G., Hage, J. J., et al. (2016). Prevalence and risk factors for extended-spectrum β-lactamase- and AmpC-producing Escherichia coli in dairy farms. *J. Dairy Science* 99 (11), 9001–9013. doi: 10.3168/jds.2016-11134

Gozdzielewska, L., King, C., Flowers, P., Mellor, D., Dunlop, P., and Price, L. (2020). Scoping review of approaches for improving antimicrobial stewardship in livestock farmers and veterinarians. *Prev. Veterinary Med.* 180, 105025. doi: 10.1016/j.prevetmed.2020.105025

Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., and Coukell, A. (2017). Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? *BMC Vet. Res.* 13 (1), 211. doi: 10.1186/s12917-017-1131-3

Hordijk, J., Fischer, E. A. J., van Werven, T., Sietsma, S., Van Gompel, L., Timmerman, A. J., et al. (2019). Dynamics of faecal shedding of ESBL- or AmpC-producing Escherichia coli on dairy farms. *J. Antimicrobial Chemotherapy* 74 (6), 1531–1538. doi: 10.1093/jac/dkz035

Hutchins, J., and Hueth, B. (2023). 100 years of data sovereignty: cooperative data governance and innovation in US dairy. *Appl. Economic Perspect. Policy.* 1–26.

Jensen, V. F., Jacobsen, E., and Bager, F. (2004). Veterinary antimicrobial-usage statistics based on standardized measures of dosage. *Prev. Veterinary Med.* 64 (2), 201–215. doi: 10.1016/j.prevetmed.2004.04.001

Kasabova, S., Hartmann, M., Werner, N., Käsbohrer, A., and Kreienbrock, L. (2019). Used Daily Dose vs. Defined Daily Dose—Contrasting Two Different Methods to Measure Antibiotic Consumption at the Farm Level. *Front. Veterinary Sci.* 6. doi: 10.3389/fvets.2019.00116

Kaur, J., Hazrati Fard, S. M., Amiri-Zarandi, M., and Dara, R. (2022). Protecting farmers' data privacy and confidentiality: Recommendations and considerations. *Front. Sustain. Food Syst.* 6. doi: 10.3389/fsufs.2022.903230

Köper, L. M., Bode, C., Bender, A., Reimer, I., Heberer, T., and Wallmann, J. (2020). Eight years of sales surveillance of antimicrobials for veterinary use in Germany—What are the perceptions? *PloS One* 15 (8), e0237459. doi: 10.1371/journal.pone.0237459

Lang, R. (2017). The lifecycle of the dairy cow. *Medium.*

Lhermie, G., Dupouy, V., El Garch, F., Ravinet, N., Toutain, P. L., Bousquet-Mélou, A., et al. (2017). Impact of low and high doses of marbofloxacin on the selection of resistant enterobacteriaceae in the commensal gut flora of young cattle: Discussion of data from 2 study populations. *Foodborne Pathog. Dis.* 14 (3), 152–159. doi: 10.1089/fpd.2016.2176

Linsner, S., Kuntke, F., Steinbrink, E., Franken, J., and Reuter, C. (2021). The role of privacy in digitalization – analyzing perspectives of German farmers. *Proc. Privacy Enhancing Technologies* 2021, 334–350. doi: 10.2478/popets-2021-0050

Llanos-Soto, S. G., Vezeau, N., Wemette, M., Bulut, E., Greiner Safi, A., Moroni, P., et al. (2021). Survey of perceptions and attitudes of an international group of veterinarians regarding antibiotic use and resistance on dairy cattle farms. *Prev. Veterinary Med.* 188, 105253. doi: 10.1016/j.prevetmed.2020.105253

Ma, F., Xu, S., Tang, Z., Li, Z., and Zhang, L. (2021). Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety Health* 3 (1), 32–38. doi: 10.1016/j.bsheal.2020.09.004

MacFadden, D. R., Fisman, D., Andre, J., Ara, Y., Majumder, M. S., Bogoch, I. I., et al. (2016). A platform for monitoring regional antimicrobial resistance, using online data sources: ResistanceOpen. *J. Infect. Diseases* 214 (suppl_4), S393–S398. doi: 10.1093/infdis/jiw343

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: Impacts on human health. *Clin. Microbiol. Rev.* 24 (4), 718–733. doi: 10.1128/CMR.00002-11

Merle, R., and Meyer-Kühling, B. (2019). Sales data as a measure of antibiotics usage: Concepts, examples and discussion of influencing factors. *Vet. Med. Sci.* 6 (1), 154–163. doi: 10.1002/vms3.205

Mills, H. L., Turner, A., Morgans, L., Massey, J., Schubert, H., Rees, G., et al. (2018). Evaluation of metrics for benchmarking antimicrobial use in the UK dairy industry. *Veterinary Rec.* 182 (13), 379–379. doi: 10.1136/vr.104701

Monitoring and Evaluation of the Global Action Plan on Antimicrobial Resistance (AMR): Regional Expert Consultation on Monitoring and Evaluation of AMR Interventions. Available at: https://www.paho.org/hq/dmdocuments/2017/2017-cha-monit-eval-gapar-meeting-report.pdf.

Monteiro, A., Santos, S., and Gonçalves, P. (2021). Precision agriculture for crop and livestock farming—Brief review. *Animals.* 11 (8), 2345. doi: 10.3390/ani11082345

Moore, D. A., McConnel, C. S., Busch, R., and Sischo, W. M. (2021). Dairy veterinarians' perceptions and experts' opinions regarding implementation of antimicrobial stewardship on dairy farms in the western United States. *J. Am. Veterinary Med. Assoc.* 258 (5), 515–526. doi: 10.2460/javma.258.5.515

More, J. B. (2019). What are the best metrics to monitor antibiotic use? *Veterinary Rec.* 184 (7), 226–226. doi: 10.1136/vr.l1675

Moura, P., Sanders, P., Heederik, D., Van Geijlswijk, I. M., and Niza-Ribeiro, J. (2022). Use of a new antimicrobial consumption monitoring system (Vet-AMNet): Application to Dutch dairy sector over a 9-year period. *Front. Veterinary Sci.* 9. doi: 10.3389/fvets.2022.984771

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 399 (10325), 629–655. doi: 10.1016/S0140-6736(21)02724-0

Nielsen, S. S., Houe, H., Denwood, M., Nielsen, L. R., Forkman, B., Otten, N. D., et al. (2021). Application of methods to assess animal welfare and suffering caused by infectious diseases in cattle and swine populations. *Anim. (Basel)* 11 (11), 3017. doi: 10.3390/ani11113017

O'Neill, J. (2016). *Tackling drug-resistant infections globally: final report and recommendations* (Government of the United Kingdom). Available at: https://apo.org.au/node/63983.

Owen, J. R., Noyes, N., Young, A. E., Prince, D. J., Blanchard, P. C., Lehenbauer, T. W., et al. (2017). Whole-genome sequencing and concordance between antimicrobial susceptibility genotypes and phenotypes of bacterial isolates associated with bovine respiratory disease. *G3 Genes|Genomes|Genetics* 7 (9), 3059–3071. doi: 10.1534/g3.117.1137

Qian, C., Liu, Y., Barnett-Neefs, C., Salgia, S., Serbetci, O., Adalja, A., et al. (2022). A perspective on data sharing in digital food safety systems. *Crit. Rev. Food Sci. Nutr.* 0 (0), 1–17. doi: 10.1080/10408398.2022.2103086

Raymond, B. (2019). Five rules for resistance management in the antibiotic apocalypse, a road map for integrated microbial management. *Evol. Appl.* 12 (6), 1079–1091. doi: 10.1111/eva.12808

Redding, L. E., Bender, J., and Baker, L. (2019). Quantification of antibiotic use on dairy farms in Pennsylvania. *J. Dairy Sci.* 102 (2), 1494–507.

Redding, L. E., Muller, B. M., and Szymczak, J. E. (2020). Small and large animal veterinarian perceptions of antimicrobial use metrics for hospital-based stewardship in the United States. *Front. Veterinary Sci.* 7. doi: 10.3389/fvets.2020.00582

Ritchie, F. (2011). *Disclosure control for regression outputs*. Available at: https://wiserd.ac.uk/wp-content/uploads/WISERD_WDR_005.pdf.

Ruegg, P. L. (2022). Realities, challenges and benefits of antimicrobial stewardship in dairy practice in the United States. *Microorganisms.* 10 (8), 1626. doi: 10.3390/microorganisms10081626

Sanders, P., Vanderhaeghen, W., Fertner, M., Fuchs, K., Obritzhauser, W., Agunos, A., et al. (2020). Monitoring of farm-level antimicrobial use to guide stewardship: Overview of existing systems and analysis of key components and processes. *Front. Vet. Sci.* 7, 540. doi: 10.3389/fvets.2020.00540

Schrag, N. F. D., Apley, M. D., Godden, S. M., Lubbers, B. V., and Singer, R. S. (2020a). Antimicrobial use quantification in adult dairy cows – Part 1 – Standardized regimens as a method for describing antimicrobial use. *Zoonoses Public Health* 67 (S1), 51–68. doi: 10.1111/zph.12766

Schrag, N. F. D., Apley, M. D., Godden, S. M., Singer, R. S., and Lubbers, B. V. (2020b). Antimicrobial use quantification in adult dairy cows – Part 2 – Developing a foundation for pharmacoepidemiology by comparing measurement methods. *Zoonoses Public Health* 67 (S1), 69–81. doi: 10.1111/zph.12772

Schrag, N. F. D., Godden, S. M., Apley, M. D., Singer, R. S., and Lubbers, B. V. (2020c). Antimicrobial use quantification in adult dairy cows – Part 3 – Use measured by standardized regimens and grams on 29 dairies in the United States. *Zoonoses Public Health* 67 (S1), 82–93. doi: 10.1111/zph.12773

Schrag, N. F. D., Godden, S. M., Singer, R. S., Lombard, J. E., Wenz, J. R., Amrine, D. E., et al. (2022). Improving farm-level antimicrobial stewardship benchmarks by reporting antimicrobial use within the context of both the magnitude of disease pressure and the outcome of therapy. *Front. Veterinary Sci.* 9. doi: 10.3389/fvets.2022.1022557

Scott, P. R. (2013). The challenges to improve farm animal welfare in the United Kingdom by reducing disease incidence with greater veterinary involvement on farm. *Animals.* 3 (3), 629–646. doi: 10.3390/ani3030629

Snow, L. C., Warner, R. G., Cheney, T., Wearing, H., Stokes, M., Harris, K., et al. (2012). Risk factors associated with extended spectrum beta-lactamase Escherichia coli (CTX-M) on dairy farms in North West England and North Wales. *Prev. Veterinary Med.* 106 (3), 225–234. doi: 10.1016/j.prevetmed.2012.03.009

Taber, P., Weir, C., Butler, J. M., Graber, C. J., Jones, M. M., Madaras-Kelly, K., et al. (2021). Social dynamics of a population-level dashboard for antimicrobial stewardship: A qualitative analysis. *Am. J. Infection Control* 49 (7), 862–867. doi: 10.1016/j.ajic.2021.01.015

Timmerman, T., Dewulf, J., Catry, B., Feyen, B., Opsomer, G., de Kruif, A., et al. (2006). Quantification and evaluation of antimicrobial drug use in group treatments for fattening pigs in Belgium. *Prev. Veterinary Med.* 74 (4), 251–263. doi: 10.1016/j.prevetmed.2005.10.003

Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P., and Van Boeckel, T. P. (2020). Global trends in antimicrobial use in food animals from 2017 to 2030. *Antibiotics (Basel)* 9 (12), 918. doi: 10.3390/antibiotics9120918

Umair, M., Mohsin, M., Sönksen, U. W., Walsh, T. R., Kreienbrock, L., and Laxminarayan, R. (2021). Measuring antimicrobial use needs global harmonization. *Glob Chall* 5 (10), 2100017. doi: 10.1002/gch2.202100017

Volkova, V. V., Lanzas, C., Lu, Z., and Gröhn, Y. T. (2012). Mathematical model of plasmid-mediated resistance to ceftiofur in commensal enteric escherichia coli of cattle. *PloS One* 7 (5), e36738. doi: 10.1371/journal.pone.0036738

WHO (2021). *Antimicrobial resistance*. Available at: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.

Wirth, F. N., Meurers, T., Johns, M., and Prasser, F. (2021). Privacy-preserving data sharing infrastructures for medical research: systematization and comparison. *BMC Med. Inf. Decision Making* 21 (1), 242. doi: 10.1186/s12911-021-01602-x

Wiseman, L., Sanderson, J., Zhang, A., and Jakku, E. (2019). Farmers and their data: An examination of farmers' reluctance to share their data through the lens of the laws impacting smart farming. *NJAS - Wageningen J. Life Sci.* 90–91, 100301. doi: 10.1016/j.njas.2019.04.007

Yarrington, M. E., and Moehring, R. W. (2019). Basic, advanced, and novel metrics to guide antibiotic use assessments. *Curr. Treat Options Infect. Dis.* 11 (2), 145–160. doi: 10.1007/s40506-019-00188-3

Zhang, L., Huang, Y., Zhou, Y., Buckley, T., and Wang, H. H. (2013). Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota. *Antimicrob. Agents Chemother.* 57 (8), 3659–3666. doi: 10.1128/AAC.00670-13

frontiers | Frontiers in Antibiotics

TYPE Review
PUBLISHED 31 August 2023
DOI 10.3389/frabi.2023.1233698

OPEN ACCESS

EDITED BY
Kebede Amenu,
Addis Ababa University, Ethiopia

REVIEWED BY
Laura Montoro-Dasi,
Universidad Cardenal Herrera-CEU, CEU Universities, Spain
Indranil Samanta,
West Bengal University of Animal and Fishery Sciences, India

*CORRESPONDENCE
Abel B. Ekiri
ab.ekiri@surrey.ac.uk

RECEIVED 02 June 2023
ACCEPTED 27 July 2023
PUBLISHED 31 August 2023

CITATION
Jacobsen ABJE, Ogden J and Ekiri AB (2023) Antimicrobial resistance interventions in the animal sector: scoping review.
Front. Antibiot. 2:1233698.
doi: 10.3389/frabi.2023.1233698

Antimicrobial resistance interventions in the animal sector: scoping review

Alice B. J. E. Jacobsen[1,2], Jane Ogden[2] and Abel B. Ekiri[1*]

[1]Department of Comparative Biomedical Sciences, School of Veterinary Medicine, University of Surrey, Guildford, United Kingdom, [2]Department of Psychological Sciences, School of Psychology, University of Surrey, Guildford, United Kingdom

Animals are considered key contributors to the development and spread of antimicrobial resistance (AMR). However, little is known about the existing AMR interventions in the animal sector. This scoping review examines the existing evidence on AMR interventions aimed at livestock, animal health professionals (AHPs), and farmers, while reviewing their impact, limitations, gaps, and lessons for future use. The scoping review was conducted following guidelines from the PRISMA-ScR checklist. The databases, Web of Science, Scopus, PubMed, and international organisations' websites (WHO, FAO, WOAH) were searched for articles reporting interventions targeting livestock, farmers, and AHPs. Interventions were categorised based on seven pre-defined primary measures including: change in antimicrobial use (AMU) practices; change in the uptake of antimicrobial stewardship (AMS); change in development of AMR; change in knowledge of appropriate AMU practices, AMR, and AMS; change in attitudes and perceptions concerning AMU, AMR, and AMS; and surveillance strategies. In total, ninety three sources were included: 66 studies, 20 reports, and 7 webpages. The reviewed interventions focused mostly on AMU practices (22/90), AMS uptake (8/90), and reduction of bacterial or resistant strains (30/90). Changes in knowledge (14/90) and attitude (1/90) were less frequently assessed and were often implicit. Most interventions were conducted within a select country (83/90) and 7/90 were at a global level. Only 19% (16/83) of interventions were implemented in low- and middle-income countries (LMICs) and most were at herd level with many self-reporting changes. Most of the interventions that focused on surveillance strategies (30/83) were implemented in high-income countries (62/83). Only one study investigated the financial implications of the intervention. The study findings provide an overview of existing AMR interventions and insights into the gaps which can be addressed to guide future interventions and research. A focus on developing, implementing and evaluating interventions in LMICs coupled with the use of objective outcome measures (e.g., measurable outcomes vs. self-reporting) will improve our understanding of the impact of interventions in these settings. Finally, assessing the financial benefits of interventions is necessary to inform feasibility and to encourage uptake of interventions aimed at reducing AMR in the animal health sector.

KEYWORDS

antimicrobial resistance, interventions, antimicrobial stewardship, antimicrobial usage, behavioural science, One Health, animal

1 Introduction

Antimicrobial resistance (AMR) is a critical issue for both human and animal health (O'Neill, 2016). Globally, an estimated 1.27 million human deaths in 2019 were attributed to AMR (Murray et al., 2022). This is predicted to rise to 10 million in 2050 if no action is taken (O'Neill, 2016). AMR-attributed deaths in humans have been linked to the transfer of AMR- bacteria and AMR genes from animals to humans (Rhouma et al., 2022). The antimicrobials considered of high priority and essential for humans, Highest Priority Critically Important Antimicrobials (HPCIAs), are often used to treat resistant infections in animals (World Health Organization, 2018). The continued use of critically important antimicrobials (CIAs) in animals poses the risk of developing AMR, and onward transmission of CIA-resistant bacteria to humans which can reduce the effectiveness of the available CIAs (Tang et al., 2017). Other sources of AMR bacteria are community and hospital-acquired infections that can develop after misuse or overuse of antimicrobials in humans, lack of sanitation and diagnostics (e.g., lack of sensitivity testing and toilet/hand washing facility), and failure to use appropriate infection control measures in hospitals (World Health Organization, 2019). AMR infections can also be acquired from contaminated environments (World Health Organization, 2019; Stanton et al., 2022).

Despite success in reducing antimicrobial usage (AMU) in some countries (Lam et al., 2017; DANMAP, 2022), AMU is anticipated to rise around the world. Antimicrobial consumption in food-producing animals is projected to reach over 100,000 tonnes per annum by 2030, a 67% increase since 2015, with an estimated 99,502 tonnes used in 2020 (Van Boeckel et al., 2015; Tiseo et al., 2020; Mulchandaniid et al., 2023). In the United Kingdom alone, the use of CIAs on pig farms doubled from 2015 to 2019. The use of aminoglycosides, deemed critically important, rose from 2.607 to 5.957 mg per kilogram of body weight in pigs (Mahase, 2021). In the United States of America, 54% of antimicrobials used for livestock are CIAs. After a reduction in the use of CIAs by 27% from 2009 to 2017, this rose again by 8% from 5.6 million kilograms in 2017 to 6.0 million kilograms of antibiotic active ingredient in 2020 (US Food and Drug Administration Center for Veterinary Medicine, 2020).

The problem with AMR is that it knows no country boundaries, so reducing it globally is essential (Ruckert et al., 2020). At the global and country levels, there are varying efforts and interventions to preserve the repertoire of antimicrobials available for human use and to reduce the development and spread of AMR. The quadripartite, consisting of the World Health Organisation, Food, and Agriculture Organisation of the United Nations (FAO), World Organisation for Animal Health (WOAH), and United Nations Environment Programme (UNEP) calls for a reduction in antimicrobial usage (AMU) and AMR while enhancing antimicrobial stewardship (AMS), within the human medical and animal industry (FAO UNEP WHO and WOAH, 2022). This will mean enhanced research and understanding, One Health collaboration, and implementation of action plans to ensure best practices globally (FAO UNEP WHO and WOAH, 2022).

Working across sectors to reduce AMU and the development and transmission of AMR is essential to reduce the increasing burden and mortality attributed to AMR. Within human medicine, most AMR interventions are implemented in high income countries, with only, an estimated, 1 – 2% focusing on low- and middle-income countries (LMICs) (Cox et al., 2017). A similar trend likely occurs in the animal health sector. In high income countries such as The Netherlands and Denmark, interventions at the national levels have contributed to reductions in AMU in the animal health sector. For example, The Netherlands implemented a strategy aimed primarily at farmers based on the RESET mindset (rules and regulation, education, and information, social pressure, economics, and tools) to reduce AMU and the use of HPCIAs (Lam et al., 2017). This included obligatory aspects such as transitioning from the use of HPCIAs to less critical antimicrobials, having a registered herd veterinarian to discuss herd health with, and voluntary aspects such as lectures and study groups for farmers and animal health professionals (AHPs). This intervention resulted in a reduction in AMU of 47% between 2009 to 2015 (Lam et al., 2017). In Denmark, since 1995 DANMAP, the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, has monitored both AMU (grouped by antimicrobial class) by farmers, AHPs, and human medical professionals and AMR in both animals and humans. Coupled with other initiatives, DANMAP has led to an overall reduction in prescriptions and use of HPCIAs within all livestock sectors and eradication of HPCIA use within pig production. The monitoring of AMR of indicator bacterial isolates has shown a fluctuating trend for different bacteria. Full sensitivity to antimicrobials increased in *E.coli* isolated from broilers during 2014 – 2019, but the upward trend was not the same for pigs and cattle (DANMAP, 2022).

The burden of AMR is unevenly distributed across the globe. In 2019, high income countries had nearly 50% fewer deaths attributed to AMR (13.0 out of 100,000 deaths) compared to Africa which had the highest rate globally (23.7 out of 100,000 deaths), nearly 1.5 times higher than the global average (16.4 out of 100,000 deaths) (Murray et al., 2022). With a rising middle class and growing population in LMICs, there is increased demand to intensify food production which can lead to higher levels of AMU to sustain the high level of production (Manyi-Loh et al., 2018). The increased intensive farming in LMICs highlights the importance of identifying viable solutions to reduce AMU in livestock production.

Abbreviations: AHP, Animal Health Professional; AmpC, AmpC Beta-lactamases; AMR, Antimicrobial Resistance; AMS, Antimicrobial Stewardship; AMU, Antimicrobial Use; CIA, Critically important antimicrobials; DANMAP, Danish Integrated Antimicrobial Resistance Monitoring and Research Programme; *E. coli, Escherichia coli;* EFSA, European Food Safety Authority; ESBL, Extended Spectrum Beta-Lactamase; ESVAC, European Surveillance of Veterinary Antimicrobial Consumption; EU, European Union; FAO, Food and Agriculture Organization; HPCIA, Highest Priority Critically Important Antimicrobials; LMICs, Low- and middle-income countries; NARMS, National Antimicrobial Resistance Monitoring SystemSulfa; TMP, Sulfadiazine and Trimethoprim; UNEP, United Nations Environment Programme; Vet-LIRN, Veterinary Laboratory Investigation and Response Network; WHO, World Health Organization; WOAH, World Organisation of Animal Health.

There is limited data on existing interventions on AMR, AMS, and AMU in the animal health sector, particularly in LMICs. A few studies attempted to explore these aspects, but the scope was narrow. The aspects investigated in previous review studies included AMS in AHPs (Gozdzielewska et al., 2020), resistance genes within broiler production (Becker et al., 2021), and levels of transmission of AMR to humans after AMU in animals (Tang et al., 2017). An understanding of existing interventions focused on reducing AMU and AMR and increasing AMS within AHPs, farmers, and livestock, globally and in LMICs, is important. To address this gap, we have undertaken a scoping review with the intent of providing a broad overview and categorisation of interventions about AMR in the animal health sector within the last decade. The scoping review provides an overview map of existing evidence on AMR interventions in the animal health sector, and the related impact, current gaps, and limitations. Findings from the review can be used to inform and shape new interventions and to tailor future research on AMR interventions.

2 Methods

2.1 Study approach

A scoping review was conducted following the PRISMA-ScR checklist (Tricco et al., 2018). The focus of the review was AMR interventions in the animal health sector, specifically interventions aimed at reducing inappropriate AMU, increasing/enhancing uptake of AMS, and/or reducing the risk of development and spread of AMR. The groups of interest to which the AMR interventions were applied included AHPs (veterinarians and para-veterinarians), farmers, and livestock (poultry, cattle, goats, sheep, swine, and aquaculture).

2.2 Data sources

The databases PUBMED, Scopus, and Web of Science were searched (Appendix 1). The websites of the World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), and World Organisation for Animal Health (WOAH), were also searched. Backward citation tracking was performed on articles including reviews that were otherwise excluded.

2.3 Search strategy, inclusion, and exclusion criteria

A combination of words relating to Africa, America, Animal, Antibiotic, Antimicrobial, Asia, Australia, Bacteria, Environment, Europe, Farmer, Intervention, North, Para-veterinarian, South, Surveillance, Veterinarian, and Veterinary was used for the article search (Appendix 1).

No limits were set on study design, the language was set as English and only papers published from the 1st of January 2013 through 31st of December 2022 were included. Reviews were excluded but were accessed for citations. Studies focusing on interventions at various levels were included: global, continent, country, regional (area within a country), or small-scale (multiple or singular herds or farms). Studies were excluded if they solely focused on human, environmental, or human and environmental aspects. Single cross-sectional studies, focusing on opinions or current practices only, and reports with a focus on providing singular time point surveillance data with no intervention action were excluded.

2.4 Study screening

The article search was performed in January 2023. Titles and abstracts of identified records were screened against the above inclusion and exclusion criteria. Eligible records were exported to Mendeley and then screened by the author (AJ). Articles that met the inclusion and exclusion criteria were included in the review. References from the full-text searches of articles deemed relevant were also screened against the inclusion and exclusion criteria and included in the review if relevant.

2.5 Data extraction

For each article, the following data were extracted into an excel file: author, year of publication, country where activity was implemented, level of intervention (small scale, regional, national, continental, international), study design, study population (AHPs, farmers, or livestock), results relevant to the primary intervention outcome measures (Figure 1, Appendix 1), outcomes of the intervention, impact of the interventions, strengths, and limitations. Extraction was performed by one reviewer (AJ) for all eligible articles and a second reviewer (AE) evaluated a subset of 20% of the extracted articles.

2.6 Synthesis and reporting results

A key aim of this study was to characterise the reported AMR interventions in the animal health sector, which focused on either livestock or AHPs or farmers. For the purpose of this study, interventions were grouped into seven categories based on primary outcome measures: 1) change in AMU practices of animal health professionals (AHPs) and farmers, 2) change in the uptake of AMS by AHPs and farmers, 3) change in development and/or spread/distribution of AMR, 4) change in knowledge of appropriate AMU practices, AMR and AMS, 5) change in attitudes and perceptions concerning AMU, AMR, and AMS, 6) surveillance strategies (with a focus on animals and either both or one of the following: environment, and/or humans), and 7) Other. For each of the above seven categories, primary and secondary outcome measures were defined (Figure 1). Details of defined primary and secondary measures are provided in Appendix 2. For purposes of interpretation, the reported interventions were also categorised by level of the geographical area covered as follows: small-scale (e.g.,

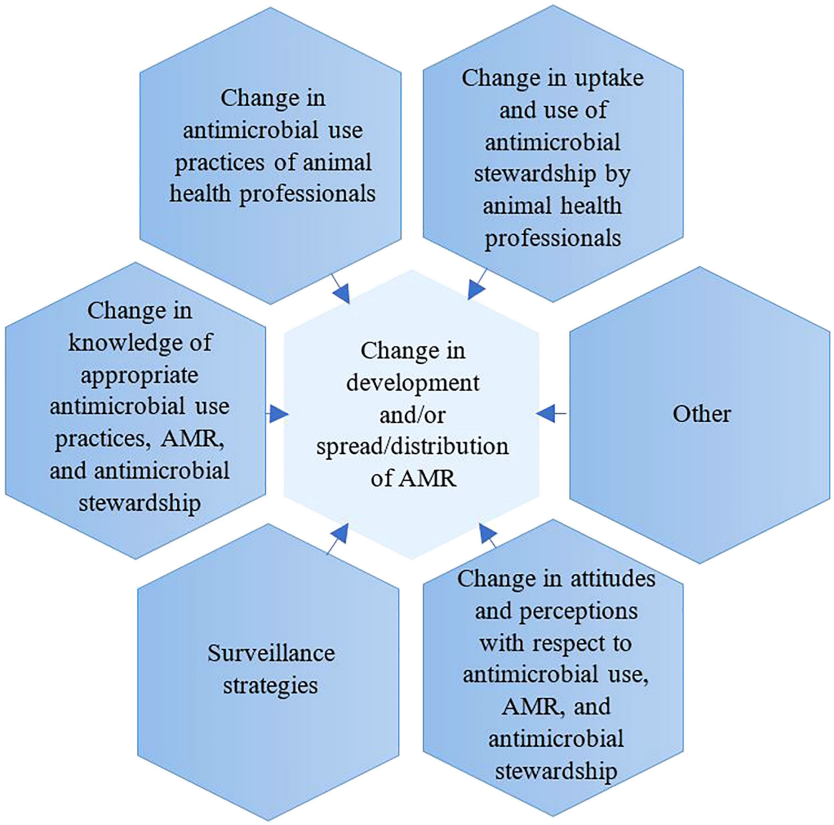


FIGURE 1
Primary outcome measures.

(i.e., singular or multiple herds or farms), regional (region within a country), national (country-level), continental (across an entire continent), and international (across continents) interventions. Due to this review article being a scoping review, and the variety of study designs, a light touch study design assessment was performed.

2.7 Study design appraisal

For intervention studies, a light touch quality review of the study design and subjectivity of outcome measurements was performed within the research group (AJ & JO) by asking the questions: (1) what design was used for the intervention? and (2) were the outcome measurements subjective or objective? Interventions were evaluated on a six-point scale based on the above two questions. For question 1, a maximum of four points could be obtained for study design: use of randomisation with control group (4 points), control group with no randomisation (3 points), two time points at which the intervention was measured (pre and post intervention) and with no control group (2 points), description of intervention without use of pre-and post-intervention measurement and no control group (1 point). For question 2, a maximum of two points could be obtained: including objective outcome measurements (2 points) or only subjective outcome measurements (1 point). No points were obtained if outcomes were not included. Outcome measurements were considered objective if were directly measurable (e.g., AMU, AMR genes, bacterial strains) and considered subjective if there was potential bias in reporting by participants (e.g., self-reporting of change). The interventions were split into 3 categories, high, medium, and low quality, based on the combined points (maximum of six points): high = 5 or 6 points, medium = 3 or 4 points, or low = 1 or 2 points. Surveillance reports were not included in this intervention design appraisal.

3 Findings

The database search identified 10069 articles, including duplicates, for inclusion. After title and abstract screening, 59 articles were deemed to fall within the inclusion criteria (Figure 2). Among the 59 articles duplicates were checked for and none were found. Of the 59 articles, 57 were successfully retrieved and 2 that were not available from online databases were accessed through intra-library loans. Six of the 59 articles were reviews and were therefore excluded resulting in 53 eligible articles. Through citation search, 28 additional sources were found (13 articles, 10 reports, and 5 webpages). International Organisation websites were searched for interventions that fit within the scope of the primary outcome measures and 10 reports and 2 webpages were included. In

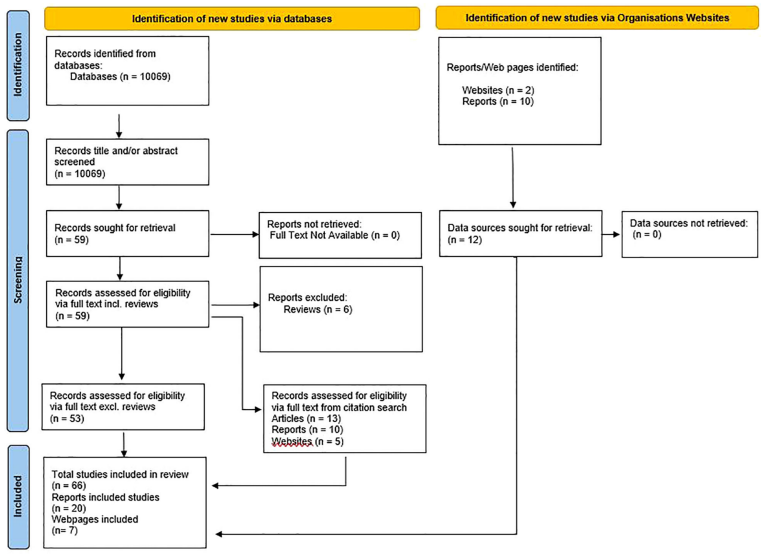


FIGURE 2
Prisma-ScR Flowchart.

total 93 sources were included – 66 articles, 20 reports, and 7 webpages (Figure 2).

The ninety-three studies and reports resulted in 90 interventions (66 articles, 20 reports, and 7 webpages. The distribution of primary outcome measures for interventions assessed in this study was broad (Figure 3), with some overlap between measures. The reported interventions focused mostly on surveillance strategies (30/90), change in development and/or spread of AMR (30/90), change in AMU practices (22/90), change in knowledge (14/90), and change in the uptake of AMS (8/90). Studies reporting change in attitude and perceptions were nearly non-existent (1/90). Six (6/90) sources were categorised as 'Other'.

The interventions were implemented at various levels including small-scale (singular or multiple herds or farms), regional (region within a country), national (country-level), continental (across an entire continent), and international (across continents). Most interventions were implemented on a small scale (51/90) or national/country level (24/90), with fewer on an international (across continents) (7/90), continental (4/90), or regional level (area within a country) (4/90).

National interventions mostly took place in countries in Europe (14/24), North America (5/24), and Asia (5/24), whereas all continental interventions took place in Europe (4/4). Small-scale interventions were mostly implemented in countries in Europe (23/51), North America (14/51) and Asia (8/51). Of the country level studies, 16/83 were performed in LMICs (international studies are excluded from the denominator).

Intervention design appraisal was performed on 62/90 of the included studies. Of these, 25/62 were categorised as high quality, 32/62 were of medium, and 5/62 of low quality (Tables 1–7). The distribution of studies based on design quality in LMICs was high (2/13), medium (9/13), and low (2/13).

Throughout the key findings described in the sub-sections below, studies were highlighted as examples to illustrate the main themes of the outcomes. Further information on all the included studies can be found in Tables 1–7.

3.1 Change in AMU practices of AHPs and farmers

Change in AMU practices of AHPs was reported in 22 interventions across 24 studies (Table 1). The most frequent aspects measured were reduction of volume/weight of AMU at

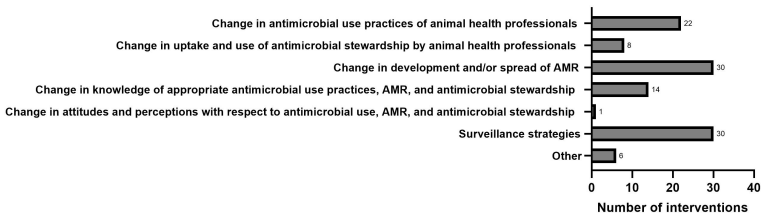


FIGURE 3
Interventions grouped by primary outcome measurements. Interventions are counted in more than one group if they incorporated more than one primary outcome measurement.

TABLE 1 Change in antimicrobial use practices of animal health professionals.

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Continental	The European Parliament and The Council of the European Union, 2022	Regulation (EU) 2019/6 on veterinary medicinal products and repealing Directive 2001/82/EC.	Farmers, AHP	Area	Ban on preventative AMU to groups or via food, reinforce AMU for growth promotion is banned.	Highlights the need for methods to reduce AMU.	Not voluntary - easier to incentive/ create repercussions for non-compliance.	Only used within the EU.	Europe	N/A
National	Agunos et al., 2017, Agunos et al., 2018	Canadian Integrated Program for Antimicrobial Resistance Surveillance.	AHP, Broilers, Turkey	Area	Between 2003 - 2015, ceftiofur resistant *Salmonella* decreased by 7 % at farm level. Ceftiofur resistant *E. coli* decreased by 16%, 11%, 8 %, in farm, abattoir and retail samples respectively.	Reduction in ceftiofur resistant *Salmonella* and *E. coli*. Increase in *E. coli* resistance to gentamycin and S/ TMP.	Long time frame, all testing performed at national reference lab.	No detailed risk factor analysis	Canadian	Medium
National	Bradley et al., 2017	Same intervention as Breen et al 2017. National mastitis control scheme: AHDB Dairy Mastitis Control Plan (DMCP) (includes surveillance and actions).	AHP, Farmers & Cattle	Area, DDx	Multiple outcomes around AMU and AMS incl. 400 AHP and Farmers trained. 40% reduction in intramammary lactating cow use. 20 % reduction in clinic mastitis rates.	Increased training and knowledge for farmers and AHPs, reduced AMU for dairy cattle.	No evidence presented.	Letter in journal, no evidence or evaluation.	United Kingdom	Medium
National	Breen et al., 2017	Same intervention as Bradley et al 2017. National mastitis control scheme: AHDB Dairy Mastitis Control Plan (DMCP) (includes surveillance and actions).	AHP, Farmers & Cattle	Area, DDx	Reduction from Total DDD of 14.59 to 6.99 in 600 dairy cattle herd.	Reduction of AMU in Dairy herd.	Easy to follow control plan, using parameters that are already evaluated as reference.	Single herd example.	United Kingdom	Medium
National	Dupont et al., 2017	Yellow card scheme. Same intervention as Jensen et al., 2014.	AHP, farmers, swine	Area	38.4 - 56.2 % reduction mg active ingredients/pig/day (37.2 - 53.6 % reduction in ADDs/100pigs/day). Biggest perceived factors: Vaccines increased; herd medication decreased	AMU reduction in both high and low usage herds.	National programme (large sample size, even with exclusions).	Factors only measured in herds with >10 % antimicrobial reduction.	Denmark	Medium
National	Jensen et al., 2014	Yellow card scheme. Same intervention as Dupont et al., 2017.	AHP, farmers, swine	HPCIA	27 % (weaner) and 53 % (finisher) reduction ADDD25 per pig produced of	Reduction of AMU HPCIA.	National programme (large sample size,	Short time frame compared to Dupont et al., 2017	Denmark	Medium

(Continued)

10.3389/frabi.2023.1233698

TABLE 1 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
					macrolides and pleuromutilins 2009 - 2011		even with exclusions).			
National	Moura et al., 2022	Vet-AMNet.	Livestock AHPs Farmers	Area, HPCIA	Reduction of 70.8% kg in AMU.	Reduced AMU nationally in Netherlands for dairy cattle.	National project - both voluntary and legislation.	Article looking to validate system, not outcomes.	Netherlands	Medium
National	Phu et al., 2021	ViParc (Education of small-scale broiler Farmers, antimicrobial replacement products, designated project veterinarian for herds).	Farmers, Broilers	Herd	66% decrease in AMU (p=0.002) from a baseline of 343.4 Animal Daily Doses per 1,000 chicken-days.	Reduction in AMU while decreasing mortality and increasing body weight of broilers.	Simple illustration of educational intervention.	Not evaluated on intensive farming. Only applicable for small scale farms.	Vietnam	Medium
Regional	Millar et al., 2022	Regulation restricting use of HPCIAs in animals.	Farmer, AHP, cattle	HPCIA	HPCIA reduction from 14,258 - 21,528 Canadian Defined Course Doses for cattle (DCDbovCA) /month to a range of 1,494 - 4,707 DCDbovCA/month (Sales data).	Significant reduction of HPCIA on dairy herds.	Large sample size.	Sales data does not show actual usage. No reflection on potential increase in other AMU.	Quebec, Canada	Medium
Small Scale	Becker et al., 2020	Outdoor Veal: 1) Transported directly to farm with no intermingling, 2) Vaccination against pneumonia and 3-week quarantine, 3) Raised in hutches with max 10 cattle.	AHP, cattle	Herd	Treatment intensity defined daily dose method (TIDDD) in days per animal year was 5.3-fold lower (5.9±6.5 vs. 31.5 ±27.4 days per animal year; p<0.001) than control group.	AMU reduced significantly in outdoor veal calf herds.	Reduced AMU, mortality, and no compromise to animal health.	Requires flat land and access to isolation transport and hutches. No mention of costs.	Switzerland	High
Small Scale	Collineau et al., 2017	Herd specific intervention plans.	Farmer, Swine	Herd	Median 47% reduction in AMU (treatment incidents), without increased mortality. No correlation to type or number of interventions.	Reduction of AMU across countries and intervention types.	Big variation in net profit post intervention.	Personalised intervention: time and economy considerations need for broader scale.	Belgium, Germany, France, Sweden.	Medium
Small Scale	Dorado-García, Dohmen, et al., 2015	1) AMU reduction 2) Improving personnel and farm hygiene 3) Change animal contact structures.	Farmer, Swine	Herd	See *Table 3: Change in development and/or spread/distribution of AMR.*				Netherlands	Medium
Small scale	Gerber et al., 2021	17 interventions from three groups 1) Udder 2)	Farmer, Cattle	Herd, DDx, HPCIA	AMU reduced by an udder or uterine health strategy (p < 0.04), including	Uterine and udder strategies saw a	Gave Farmers choice in intervention.	Intervention vs. control group	Switzerland	High

(Continued)

10.3389/frabi.2023.1233698

TABLE 1 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
		Uterine health 3) Calf health. Farmers had to pick at least one.			HPCIA for udder strategies (p = 0.05). Calf health interventions no reduction.	reduction in AMU and HPCIA while calf strategies did not.		vastly different in breed, herd size and milk yield (selection bias across both).		
Small scale	Gomez et al., 2021	Management modification, health training and algorithm.	Farmer, Cattle	DDx	AMU reduced from 85% to 18% of diarrhoea calves being treated with AM, with mortality and diarrhoea incidence staying the same.	AMU reduced significantly in outdoor veal calf herds.	Included education – this exists beyond intervention frame.	Control was prior to intervention, not concurrent.	Ontario, Canada	Medium
Small scale	Gomez et al., 2017	Two algorithms looking at calf diarrhoea and AMU incidence rate in herd.	Farmer, Cattle	DDx	Cumulative Incidence Risk (CIR) of antimicrobial treatment rates 80% lower after implementation.	Use of the algorithm reduced incidence of AMU.	Good sample size, replicated. Algorithm can be used elsewhere.	The two algorithms did not run concurrently.	Ontario, Canada	Medium
Small scale	Kuipers et al., 2016	Actively guided use of antibiotics, biannual meetings with project members and a veterinarian, AMU feedback reports.	Farmers, cattle	Herd, DDx, Area	ADDD reduced earlier in study for guided group than control (n=2) groups & less overall use. Mean guided = 5.45. Control groups = 6.34 & 5.63. Variation between herds decreased.	Reduction in AMU and differences both pre-post study but also in control groups.	Case control study, taking trends in time into account.	Role of herd health and veterinarian not analysed as a factor.	Netherlands	High
Small Scale	Morgans et al., 2021	Facilitated farmer action groups.	Farmers, cattle	HPCIA	See *Table 4: Change in knowledge of appropriate AMU practices, AMR, and AMS*				United Kingdom	Medium
Small scale	Pempek et al., 2022	AMS training for farmers in two parts: didactic presentations, calf-side training, and veterinarian feedback.	Farmers, Cattle	Herd	Increased knowledge in post intervention test compared to pre-test and control group (CG) (p= 0.05). Correct identification of cases 50% (73/146) of the cases vs. 14.3% (9/63) in GC (p= 0.002). (Increased later in intervention compared to earlier also (p< 0.001). 50 % decrease in AMU compared to CG.	Increased understanding of AMS, increased correct identification of cases' need for AMU, and decrease in AMU	Holistic approach. "Test" to evaluate increased understanding, rather than farmer perception.	Cannot randomly allocate farms. Bias due to farms with interest in AMS/AMR. Small sample size.	Ohio, USA	High
Small Scale	Postma et al., 2017	Same intervention as Rojo-Gimeno et al., 2016. Herd specific intervention plans (including herd management, biosecurity, vaccination strategy, anti-helminthic therapy, and AMU).	Swine, Farmers	Herd, HPCIA	Decrease of 52% in AMU (from birth till slaughter) and 32% for breeding animals, based on treatment incidences. Ceftiofur long-acting AMU in sucklers reduced 83%.	Decrease in AMU and HPCIA.	Looked at AMU for different age groups. Personalised interventions that work for individual herds.	Veterinarians reluctant to provide information on curative AMU.	Belgium	Medium

(Continued)

10.3389/frabi.2023.1233698

TABLE 1 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Small scale	Raasch et al., 2020	Herd specific intervention plans (includes biosecurity, vaccination, changes of feeding schemes or drinking water quality, health and welfare care, stable climate and zootechnical measures).	Farmers, Swine	Herd, HPCIA	93% median compliance of participants. Median 35% reduction in AMU treatment in % of expected lifespan. Reduction from 35 % to 16 % ($p < 0.001$). HPCIA reduced 69% polymyxin $p < 0.001$. Tetracycline 49% ($p = 0.01$).	Decrease in AMU and HPCIA.	Looked at AMU for different age groups and DDx. Personalised interventions that work for individual herds.	Control herd also changes over a year period, therefore own control. External factors impact over a year. Farmer with AMR interest participate.	Belgium Germany France Sweden	Medium
Small Scale	Rojo-Gimeno et al., 2016	Same intervention as Postma et al., 2017. Herd specific intervention plans (including herd management, biosecurity, vaccination strategy, anti-helminthic therapy, and AMU).	Farmers, swine	Herd	Median reduction of 7.68 euro/sow/year spent on AMU prophylaxis.	Increased net profit while reducing AMU.	Profit focused, economic trade off important for farmers.	Little data on antimicrobial groups and usage of these. No follow up post intervention, veterinarians reluctant to provide curative AMU data.	Belgium	Medium
Small Scale	Schreuder et al., 2022	Health plan and improved biosecurity personalised, multiple intervention cycle.	Farmers, broilers	Herd	A number of farms did not use any antimicrobial after intervention cycle 1 ($n = 4$) and 2 ($n=5$). Mean days of treatment pre-post intervention cycles did not change in any country.	Reduction in AMU on some farms in Cyprus but mean days of treatment stayed the same in all countries.	Some comparison and reflection between countries, impact of personalisation varies.	Only descriptive statistics for AMU.	Netherlands, Greece, Cyprus	Medium
Small Scale	Speksnijder et al., 2017	Animal Health Planning Program.	Farmers, Cattle	Herd	DDDA of antimicrobial - 19% vs. 14% in control group after 1 year.	No significant difference (intervention vs. control) in AMU reduction ($P = 0.498$).	Farm selection was from group with higher AMU load "signalling zone". Good reflection on needing real world reduction, potentially reducing participant bias.	AMU was measured via prescription not usage, does not account for wastage/ stockpiling. Participating bias if interested in AMU/AMR.	Nederlands	High
Small scale	Toya et al., 2022	Intervention in 3 parts: 1) awareness of AMR, 2) consent for diagnosis and treatment, and 3) Reduce AMU.	Farmers, cattle	Herd	DDD/slaughter pig 43% post intervention of what it was pre intervention (910.2 vs. 397). Non-intervention farms 146.2% (531 vs. 777).	Decrease for all indicators on intervention farms, while there was an increase on control farms.	Study had both a control group and pre-post intervention assessments.	Small sample size, bias due to voluntary participation.	Japan	High

(Continued)

10.3389/frabi.2023.1233698

TABLE 1 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Small Scale	Turner et al., 2018	Farmer meetings, herd health planning meeting, change of treatment protocols, no HPCIAs without prescriptions.	Farmers, swine	HPCIA	HPCIA on farms reduced from 7.2 ADD (41%) to none.	Complete cessation of HPCIA use on farms.	Multifaceted approach.	All farms under care of one veterinary practice.	United Kingdom	High

AMU, Antimicrobial use.
AMR, Antimicrobial resistance.
AMS, Antimicrobial stewardship.
AHP, Animal Health professional.
ADD, Animal daily dose.
ADDD, Animal defined daily doses.
DDD, Defined daily dose.
DDDA, Yearly moving average total antimicrobial use.
E. coli, Escherichia coli.
Herd, Reduction of volume/weight of AMU on herd level.
DDx, Reduction of volume of AMU for a specific diagnosis.
Area, Reduction of volume of AMU for livestock animals on regional, national, or continental level.
HPCIA, Reduction in volume of use of Critically Important Antimicrobials for Human Medicine.
Sulfa/TMP, Sulfadiazine/Trimethoprim.

herd level (12/22), reduction in the volume of HPCIAs used (9/22), reduction of volume of AMU for a specific diagnosis (5/22), and reduction of volume of AMU for livestock animals at regional, national, or continental level (5/22). Nine of the twenty-two (9/22) interventions that assessed change in AMU practices were aimed at AHPs and 20/22 were aimed at farmers. Most of the interventions in this category were implemented in Europe (15/22), followed by North America (5/22) and Asia (2/22). The geographical coverage of the interventions varied from continental (1/22), national (3/22), and regional (1/22) to small scale (17/22). The quality of the intervention studies design was considered high (7/24) and medium (17/24).

The following subsections further describe the interventions in which change in AMU practices was assessed, and the related impacts, limitations, and gaps.

3.1.1 Reductions in AMU at herd level

Herd-specific interventions showed a reduction in AMU. These interventions focussed on implementation of farmers and AHP education, increased health and welfare care (e.g., stable climate, management), biosecurity (external and/or internal), and vaccine strategy (Collineau et al., 2017; Speksnijder et al., 2017; Raasch et al., 2020; Gerber et al., 2021; Gomez et al., 2021; Phu et al., 2021; Pempek et al., 2022; Schreuder et al., 2022). The targeted study groups included cattle farmers, swine farmers, broiler farmers, and AHPs (Collineau et al., 2017; Speksnijder et al., 2017; Raasch et al., 2020; Gerber et al., 2021; Gomez et al., 2021; Phu et al., 2021; Pempek et al., 2022; Schreuder et al., 2022).

As a first example of herd-specific interventions, Raasch et al. (2020) measured the reduction of AMU and HPCIAs among a swine farmer population in Belgium, Germany, France, and Sweden. A significant median reduction of AMU of 35% was reported. After the intervention was implemented, the duration for which pigs were treated reduced from 25% of their expected lifespan (200 days) to 16% (Raasch et al., 2020). The authors reported a compliance rate of 93% with the intervention plans by the target population. The strengths of this intervention were customised interventions for each herd and a broken-down assessment of AMU by diagnosis and age group. However, no control group was used on the basis that this group could change over the year (Raasch et al., 2020). This means it was not possible to adjust results for external factors that otherwise might be seen in a control group.

In a second example, reduction in AMU at the herd level was assessed for cattle farmers in Ohio, USA. A 50% reduction in AMU for calves was accomplished through didactic presentations, calf-side training, and veterinarian feedback for farmers. There was also an increased understanding of AMS and higher correct identification of cases in need of AMU (50%, 73/146) vs. the control group (14.3%, 9/63) (p=0.002) (Rojo-Gimeno et al., 2016; Pempek et al., 2022). This intervention allowed for an integrated approach looking at both AMU but also testing farmer knowledge and not relying on self-reporting. The observed weaknesses in that study were that the control and test groups were not randomly allocated, and both were presumed to have an increased interest in

TABLE 2 Change in uptake and use of antimicrobial stewardship by animal health professionals.

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Continental	The European Parliament and The Council of the European Union, 2022	Regulation (EU) 2019/6 on veterinary medicinal products and repealing Directive 2001/82/EC.	Farmers, AHP	Px	See Table 1: Change in antimicrobial use practices of animal health professionals.				Europe	N/A
National	Dupont et al., 2017	Yellow card scheme.	AHP, farmers, swine	Px, Other	See Table 1: Change in antimicrobial use practices of animal health professionals.				Denmark	Medium
Regional	Abdelfattah et al., 2022	Senate Bill 27 - Prescription required for medically important antimicrobials.	Farmers, AHP	Diagnostic, Other	Self-reported: 29.4% changed disease management, 26.8 % report using antimicrobial preventative alternatives.	Reported increases in preventative alternatives and changed disease prevention/ management.	Law bill, not voluntary.	Low response rate & most likely only those interested in AMS responded. Did not report factors within categories or actual AMU changes.	California, USA	Medium
Regional	Rees et al., 2021	The Arwain Vet Cymru Project - Veterinary Prescribing Champions (VPC) (incl. webinars, workshops, discussion).	AHP	Px, guidelines	See Table 4: Change in knowledge of appropriate AMU practices, AMR, and AMS.				Wales, United Kingdom	Low
Small Scale	Morgans et al., 2021	Facilitated farmer action groups.	Farmers, cattle	Other	See Table 4: Change in knowledge of appropriate AMU practices, AMR, and AMS.				United Kingdom	Medium
Small Scale	Musoke et al., 2020	One Health training - knowledge on AMR, sanitation (case studies, group discussions).	AHP, MHP	Px, guidelines, diagnostics, Other	Of health professionals (%) reported improved: handwashing (57.3 %), guideline use (52.9 %), treatment based on diagnostics (44.1%) + reduction in unnecessary AMU (51.3 %).	Improved practices and knowledge of AMS.	One Health approach, part of already existing structure.	Low participation of AHP compared to MHP. Self-reporting of improvement.	Uganda	Low
Small scale	Pempek et al., 2022	AMS training for farmers in two parts: didactic presentations, calf-side training, and veterinarian feedback.	Farmers, Cattle	Other	See Table 1: Change in antimicrobial use practices of animal health professionals.				Ohio, USA	High
Small Scale	Roulette et al., 2017	Knowledge and innovations for: 1) prudent AMU (tape measures & dosage charts (calculate weight for more accurate dosage), 2) pasteurization milk (thermometers) to reduce resistant E. coli.	Farmers	Other	See Table 4: Change in knowledge of appropriate AMU practices, AMR, and AMS.				Tanzania	Medium

AMU, Antimicrobial use;
AMR, Antimicrobial resistance;
AMS, Antimicrobial stewardship;
AHP, Animal Health professional;
MHP, Medical Health Professional;
Px, Change in prescribing habits (define the prescribing habits for which change will be measured).
Guidelines, Increased adherence to guidelines.
Diagnostics, Increase in frequency of use of diagnostics e.g., sensitivity testing.

10.3389/frabi.2023.1233698

TABLE 3 Change in development and/or spread/distribution of AMR.

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
National	Agunos et al., 2017, Agunos et al., 2018	Canadian Integrated Program for Antimicrobial Resistance Surveillance.	AHP, Broilers, Turkey	B strain, R strain, Area	See *Table 1: Change in antimicrobial use practices of animal health professionals.*				Canada	Medium
National	Hiki et al., 2015	Voluntary reduction of ceftiofur in hatcheries.	Broilers	R strain	Reduction of cephalosporin resistant *E. coli* from 16.8% (27/161) to 4.6% (6/131) (p=0.001).	Reduction of cephalosporin resistant *E. coli* after ceftiofur reduction.	Assessed multiple resistance genes.	Longitudinal study. No control group to assess for confounding. No assessments of mortality or animal health.	Japan	Medium
Small scale	Bahrndorff et al., 2013	Fly screen placement on broiler houses, which remove 95% of fly population.	Broilers	B strain	Reduction in prevalence of *Campylobacter spp* in flocks from 41.4 % to 10.3 % ($p < 0.001$). (Control house reduced from 41.8 % to 36 % ($p = 0.454$).	Reduction in prevalence of *Campylobacter spp* among flocks.	4-year data set, use of a control.	No data collected on poultry disease levels or end meat product.	Denmark	Medium
Small Scale	Brusa et al., 2019	Reducing Shiga toxin gene in hide samples, washing abattoir pens using 1) electrolytically generated hypochlorous acid, and 2) chlorinated water, electrolytically generated hypochlorous acid, and isochlor.	Cattle	R strain	1) Pre - post intervention 96.6% vs. 16.6% positive samples ($p < 0.001$) 2) 9.4 times less risk of positive sample post intervention ($p = 0.003$).	Reduction of Shiga toxin in samples post intervention.	Used steers from high intensity breed farms to increase probability of high bacterial load.	High chlorine levels might not be allowed in some countries. Small sample size.	Argentina	Medium
Small Scale	Ceccarelli et al., 2017	Aviguard (probiotic) given to two chick groups: infectious and susceptible.	Broilers	R strain	Excretion: 1.17 CFU/g faeces (infectious and susceptible chicks) vs. control 5.68 CFU/g ($p < 0.001$).	Statistically significant reduction in transmission + excretion of ESBL- *E. coli.*	Tested every day. Multiple scenarios used.	Only tested for 13 days after ESBL-*E.coli* given.	Netherlands	High
Small Scale	Chinwe et al., 2014	No antibiotic feed additives given and assessed for *E. coli.*	Broilers	R strain, B strain	*E. coli* in cloacal swabs was 11% lower (17%) in flocks with no antimicrobial feed additives. Higher number of susceptible *E. coli* isolates across all antimicrobials assessed.	Less prevalence of *E. coli* in cloacal samples and resistant *E. coli* isolates.	Trialled in LMIC farm environment.	The reported data on carriage is not clear/ confusing.	Nigeria	High
Small Scale	Cicconi-Hogan et al., 2014	Organic certification.	Cattle	R strain	Methicillin resistance coagulase negative staphylococci in 2 % of organic and 5 % of conventional bulk tank milk, MRSA in 0.3 % organic.	Reduced prevalence of methicillin resistance coagulase negative staphylococci in organic bulk tank milk.	Provides data on AMR specific (MRSA) information.	Few farms from each area, very general overview. Only Farmers with interest (already low levels).	USA	High
Small Scale	Dame-Korevaar,	Competitive exclusion: 1) fermented intestinal bacteria	Broiler, chicks	R strain	Challenge on day 0 CEP + SYN no effect. Challenge day 5 CEP + SYN prevented CTX-	Competitive exclusion reduced	Trialled different challenge	No data collected on poultry disease levels or end meat product.	Netherlands	High

(Continued)

10.3389/frabi.2023.1233698

TABLE 3 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
	Fischer, et al., 2020	(CEP), 2) selection of pre- and probiotics (SYN).			M-1-*E.coli*. excretion (up to -1.60 log10cfu/g), and caecal content (up to -2.80 log10cfu/g).	prevalence of CTX-M-1-*E.coli*.	points - day 0 and 5.			
Small Scale	Dame-Korevaar, Kers, et al., 2020	Competitive exclusion in semi-field conditions.	Broiler	R strain	0/200 broilers CTX-M-1-*E. coli* positive on day 21 vs. Control 187/200 positive.	Competitive exclusion reduced prevalence of CTX-M-1-*E.coli*.	Detailed microbiota composition.	Performed with stringent biosecurity, outcome might vary by field environment.	Netherlands	High
Small Scale	Dorado-García, Dohmen, et al., 2015	Intervention with multiple steps: 1) reduce AMU, 2) improving personnel and farm hygiene, and 3) change animal contact structures.	Farmer, Swine	R strain	44% decrease in AMU (DDDA/Y) and decrease of MRSA positive farms from 31 to 29.	Reduced AMU and MRSA positive isolates, with a correlation to avoiding teeth clipping and keeping sows in stable groups.	Assessed multiple factors and retested over 4 intervals of intervention.	Pooled samples in testing, short time frame for MRSA.	Netherlands	Medium
Small Scale	Dorado-García, Graveland, et al., 2015	Two intervention groups: 1) Reducing AMU with protocol (RAB) and 2) RAB + Cleaning and disinfection (CD). Testing for MRSA.	Cattle	R strain, environment	2 - 3 times higher level of MRSA in veal cattle in Control & RAB-CD than RAB at 12 weeks in both cycles of intervention (p value = 0.5 and < 0.01, 1st and 2nd cycle respectively). Human nasal samples not statistically significant, and environmental samples were negatively impacted by CD.	Statistically significant lower levels of MRSA in RAB cattle intervention population but not human workers.	Multiple areas of swabbing within populations to see if reduction in cattle reflects in human workers.	Different sample techniques in first and second cycle. Only 12 weeks of intervention.	Netherlands	High
Small Scale	Hao et al., 2013	Slightly acidic water for *E. coli* and *Salmonella* reduction (pH 5.0 - 6.5) with chlorine concentration (300mg/L).	Broilers	B strain, Environment	Number *E. coli* and *Salmonella* positive swabs reduced by 16%.	Reduction in presence of *E.coli* in broiler house.	Swabbing large range of area.	Intervention not feasible on cage floor.	China	Medium
Small Scale	Haskell et al., 2018	RWA for MRSA.	Poultry, Cattle, swine	Food	15.7% of conventional raw meat samples contain MRSA, 0% of RWA turkey or chicken contained MRSA. However, increased level of MSSA.	No MRSA isolates from RWA turkey and chicken retail meat.	Intervention had high success rate for MRSA even with some MSSA prevalence.	Only tested RWA turkey and chicken, small sample size. Does not reflect worker contamination in relation to MRSA prevalence in herd. RWA comes with ethical issues.	Utah, USA	High
Small Scale	Kannan et al., 2019	Dietary brown seaweed +/- spray with chlorinated water.	Goat	B strain	Spray wash reduced aerobic plate count of *E.coli* on skin (3.65 vs. 4.30 log10CFU cm^2). Rumen *E.coli* count reduced with seaweed diet ($p < 0.05$).	Both seaweed and spray resulted in reduction of *E.coli*.	Range of samples used. Seaweed good food source.	Small sample size, no information to determine if rumen *E. coli* translates to less AMR or AMU.	Georgia, USA	High
Small Scale	Kassem et al., 2017	Organic certification.	Broilers	R strain	Lower presence of ciprofloxacin, erythromycin, and tylosin resistance ($p <$ 0.05) in faecal *Campylobacter* samples.	Reduced presence in *Campylobacter*	Showed differences between	Only three organic farms investigated and one	Ohio, USA	High

(Continued)

10.3389/frabi.2023.1233698

TABLE 3 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
						resistance genes on some farms.	management, and biosecurity.	farm varied widely from other two farms.		
Small Scale	Kempf et al., 2017	Organic certification.	Swine	B strain, R strain	No significant difference in *Campylobacter* in conventional vs. organic in France and Sweden. France: 43/58 (74%); 43/56 (77%) and Sweden: 24/36 (67%); 20/36 (56%). Erythromycin resistance in conventional vs. organic in France: 62 (50%) and 25 (18%).	No significant differences between *Campylobacter* spp but there were differences in AMR gene prevalence.	Illustrates how interventions might work in some countries but not in others.	Organic definition differs depending on country/ continent.	France, Sweden	High
Small Scale	Lee et al., 2017	Sanitation and education intervention about cleaning milking equipment and udders.	Farmers, cattle	B strain	40% log reduction of *Staphylococcus aureus* in fresh milk sample.	Reduction of bacteria in fresh milk.	Both qualitative data from farmers and quantitative data from milk.	No statistics on whether statistically significant.	Malaysia	Medium
Small Scale	Loayza-Villa et al., 2021	RWA for two generations.	Swine	R strain	No statistically significant reduction in antimicrobial-resistant coliforms in faecal samples compared to control group.	Two generations of RWA were not enough to see a reduction in antimicrobial resistant coliforms.	Followed more than one generation. Control group. Randomised. Looked at range of resistance genes.	Pooling of faecal samples. Could only follow two generations as pigs sent for slaughter.	Ecuador	High
Small Scale	Luyckx et al., 2015	Cleaning Protocols for *E.coli* (commercial solution containing sodium hydroxide).	Broilers	B strain	Number of *E. coli* positive swabs reduced by 86% (1 - 3% difference depending on soaking & water temperature).	Reduction in presence of *E.coli* in broiler house.	Multiple cleaning protocols and factors assessed.	No differentiating between ESBL and others.	Belgium	Medium
Small Scale	Mourand et al., 2017	*E. coli* pro-biotic strain ED1a	Swine	R strain	Four trials - most comparisons between control and intervention groups showed no statistical significance.	Limited effect on shedding of ESBL-*E.coli*.	Trialled different doses of ED1a, across multiple data points for each group.	Artificial contamination with pathogenic *E.coli* strain. Might not reflect real world situation.	France	High
Small Scale	Methner et al., 2019	Competitive exposure (CE) culture for ESBL and AmpC *E.coli* (EEC).	Broilers	R strain	Difference in EEC between CE culture and untreated controls: 4.0 vs. 5.0 log10 units on day 37 of age.	Reduced load of EEC in birds treated with EC.	Non-invasive, not ongoing. One off colonisation with bacteria.	Birds needs broad range of probiotic strains for protection.	Germany	Medium

(Continued)

10.3389/frabi.2023.1233698

TABLE 3 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Small scale	Nuotio et al., 2013	Competitive exposure (CE) (BROILACT). for ESBL and pAmpC *E.coli*	Broilers	R strain	Reduced prevalence of resistant isolates in ceca samples.	Reduced prevalence of resistance isolates in ceca of chicks.	Checked effect of already implemented existing intervention.	Study only continued 5 days after challenge with *E.coli.*	Finland	Medium
Small Scale	Pedroso et al., 2013	RWA, Pro- and Pre-biotics.	Broilers	R strain	No statistically significant reduction in tetracycline-resistant *E. coli* or class 1 integron resistance element.	No significant difference in resistance isolates between the groups.	Extended length of study. Many aspects were compared.	Two farms only considered; third farm testing not considered.	USA	High
Small scale	Rubio-García et al., 2015	Allostatic modulator in tap drinking water (48h before shipment) with 10h or 16h feed withdrawal for coliforms.	Broilers	B strain, Food	10h feed withdrawal produced 0.29 log10 CFU/ml carcass rinse coliforms. 16h feed withdrawal produced ~0.92 log10 CFU/ml coliforms at carcass rinse	Reduction in coliforms ($p = 0.014$) and total aerobic mesophilic bacteria ($p = 0.0001$).	Cost effective intervention. Swabs taken at production give indication of residue in meat.	Testing in experimental setting and not a production setting. Swabs only taken at production not in live birds.	Mexico	Medium
Small scale	Sapkota et al., 2014	Organic certification.	Broilers	R strain	Resistant *Salmonella Kentucky* isolates less prevalent in litter, water, and feed on organic farms: amoxicillin–clavulanate ($p= 0.049$), ampicillin ($p= 0.042$), cefoxitin ($p= 0.042$), ceftiofur ($p= 0.043$) and ceftriaxone ($p= 0.042$)	Antibiotic resistant *Salmonella Kentucky* less prevalent on organic farms.	Compared detection of *Salmonella Kentucky* isolates across the farms.	All farms under one feed mill and small area. All control group farms, no pre-post interventions.	USA	High
Small scale	C. Shen et al., 2020	Cessation of colistin as a feed additive to reduce mcr-1 resistance in *E.coli*	Swine	R strain, environment, food	Reduction of mcr-1 on farms (81% to 23% $p < 0.0001$), in pork (52% to 29%, $p < 0.0001$) as well as soil and water around slaughterhouses (49% to 27%, $p < 0.0001$).	Significant reductions in mcr-1-positive *E. coli* after cessation of colistin as a feed additive for swine.	Broad sampling pool both in terms of provinces and sample populations.	Sampled same 3-month period each time so may miss seasonal differences.	China	Medium
Small Scale	Thapaliya et al., 2017	RWA testing for MRSA.	Poultry, Cattle, Swine, Aqua culture	Food	0.4% ($n = 2/530$) of RWA meats, and 1.4% ($n = 39/2760$) of conventional meat were MRSA positive.	No statistically significant lower levels of MRSA in RWA meat.	Larger sample size than other RWA meat studies.	Cannot differentiate pre/post slaughter contamination. Limited RWA meat.	USA	High
Small scale	Verrette et al., 2019	Cessation of ceftiofur use from hatcheries to reduce resistance	Broilers	R strain	ESBL/AmpC blaCMY-2 and blaCTX-M genes reduced by 7% and 6%, respectively, in meconium after cessation of ceftiofur, 0% and 20% respectively in faeces of broilers, 0% and 6% respectively in faeces of breeders. However, increased to or above levels prior with introduction of lincomycin-spectinomycin.	Decrease in resistance genes after stopping ceftiofur in ovo but increase after replacement with lincomycin-spectinomycin.	Several testing methods performed.	Whole flocks pooled for sampling, trends.	Canada	Medium

(Continued)

10.3389/frabi.2023.1233698

TABLE 3 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Small scale	Vikram et al., 2017	Non antimicrobial treated cattle (RWA) for a range of AMs	Cattle	R strain	Erythromycin-resistant Enterococcus sp. concentrations in faeces. RWA 61% less than control ($p < 0.01$).	Reduced erythromycin resistance but not MLS or tetracycline.	Considerable number of resistance genes assessed not just bacterial strains.	Faeces not collected and tested at processing plant to assess risk of exposure/contamination.	USA	High
Small scale	Wanninger et al., 2016	Intervention: RWA testing tetracycline resistance of *Clamydia Suis* (*C. suis*) with 2 controls. Control 1) herd level prophylactic oral AMU (trimethoprim, sulfadimidine, and sulfathiazole (TSS) Control 2) herd treatment with chlortetracycline +/- tylosin and sulfadimidine (CTS).	Swine	R strain	At the start and end of intervention 0% of *C. Suis* isolates resistant in RWA. Control 1) 67% (start) and 0% (end) Control 2) 38% (start) and 83% (end).	Absence of tetracycline treatment led to the absence of resistant insolates.	Provides details on who performed treatments: vet vs. para-veterinarian vs. other.	No evaluation of statistical significance. Small population. Control group 2 was unable to treat *C. suis* on herd level.	Switzerland	High

AMU, Antimicrobial use;
AMR, Antimicrobial resistance;
AMS, Antimicrobial stewardship;
CE, Competitive Exposure;
CFU, Colony forming unit;
ESBL, Extended spectrum beta lactamase;
E. coli, Escherichia coli;
MRSA, Methicillin resistant staphylococcus aureus;
RWA, Raised without antimicrobials;
B strain, Reduced frequency of bacterial strain;
R strain, Reduced frequency of resistance genes within bacterial strain;
Area, Reduced frequency of resistance genes detected/isolated in livestock spp at regional, national, or continental level.
Environment, Reduced frequency of resistance genes detected/isolated within herd/environment around herd.
Food, Reduced frequency of resistance genes detected/isolated in food products (meat, milk, egg, etc).
DDDA/Y, defined daily dosages animal per year.

10.3389/frabi.2023.1233698

TABLE 4 Change in knowledge of appropriate AMU practices, AMR, and AMS.

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
International	Food and Agriculture Organization of the United Nations, n.d.-	FAO-PMP-AMR - Help countries to create national action plans (NAPs).		AMU	See *Table 6: Surveillance strategies.*					N/A
International	World Organisation for Animal Health, 2021	OIE Calculation Tool, helps countries calculate AMU.			25% of countries reporting AMU to WOAH use tool to collect AMU product information and calculate active ingredients.	Defined targets for national AMR surveillance in food and agriculture sectors.	Presence of tool.	Limited information about tool.		N/A
National	Bradley et al., 2017	National mastitis control scheme: AHDB Dairy Mastitis Control Plan (DMCP) (includes surveillance and actions).	AHP, Farmers & Cattle	AMR, AMS	See *Table 1: Change in antimicrobial use practices of animal health professionals.*				United Kingdom	Medium
Regional	Powell et al., 2017	Cornwall One Health Antimicrobial Resistance Group.	AHP (+MHP)	AMR, AMS	One Health AMR education at Cornwall Veterinary Association conference. AMU decreased among MHP by 12.8% (in primary care). No data on AHP.	Increased knowledge targeting AHP and MHP. Reduction in human AMU.	One Health approach creating collaboration and understanding.	Limited evaluation of interventions. Limited veterinary involvement.	Cornwall, United Kingdom	Low
Regional	Rees et al., 2021	The Arwain Vet Cymru Project - Veterinary Prescribing Champions (VPC) (incl. webinars, workshops, discussion).	AHP	AMU, AMS	43 veterinarians being VPCs with knowledge about AMS.	Increased AMS knowledge in VPCs with the aim to disseminate this to practices.	Using peers to disseminate knowledge.	Labour intensive for creators and participators. Impact rather than research led.	Wales, United Kingdom	Low
Small Scale	Feyes et al., 2021	AMS programme in veterinary teaching hospital based on the CDC 7 core elements of hospital AMS program.	AHP	AMU, AMR, AMS	Surveillance of AMU and AMR, aim for students to have knowledge of AMS, AMR, and correct AMU (guidelines).	Surveillance data on AMU and AMR. Impact on other aims/ outcomes not reported in article.	Targeting AHPs before they start practicing hopefully creating good habits from the start.	Did not measure impact or outcome for most of AMS programme.	Ohio, USA	Low
Small Scale	Lee et al., 2017	Sanitation and education intervention about cleaning milking equipment and udders.	Farmers, cattle	Other	See *Table 3: Change in development and/or spread/distribution of AMR.*				Malaysia	Medium
Small Scale	Morgans et al., 2021	Facilitated farmer action groups	Farmers, cattle	AMU, AMR, AMS	Median reduction in HPCIA use was 3.484 mg/kg ($p<0.001$) Median reduction in General AMU was 0.360 mg/kg ($p = 0.719$). Qualitive assessment showed increase knowledge.	Statistically significant reduction in HPCIA but not in general AMU. An increased knowledge on AMR, AMU, and AMS.	Generates conversation and understanding not just action.	No control group used. Intervention time consuming (meet every 6 - 8 weeks).	United Kingdom	Medium

(Continued)

10.3389/frabi.2023.1233698

TABLE 4 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Small Scale	Musoke et al., 2020	One Health training - knowledge on AMR and sanitation (case studies, group discussions).	AHP (MHP)	AMS, AMU	See *Table 2: Change in uptake and use of AMS by animal health professionals.*				Uganda	Medium
Small scale	Pempek et al., 2022	AMS training for farmers in two parts: didactic presentations, calf-side training, and veterinarian feedback.	Farmers, Cattle	Other	See *Table 1: Change in antimicrobial use practices of animal health professionals.*				Ohio, USA	High
Small Scale	Roulette et al., 2017	Knowledge and innovations for: 1) prudent AMU (tape measures & dosage charts (calculate weight for more accurate dosage) and 2) pasteurization of milk (thermometers). Aim was to reduce resistant *E. coli.*	Farmers	AMR	70% of women used their innovations correctly (thermometer), men performed only 18% of dosage steps correctly. Men retained AMR knowledge (0.30) vs. women (0.14).	Two months after innovation some knowledge on AMR was retained and some use of innovation was still present.	Assessed whether knowledge was retained and had impact on action.	Cultural values need to be incorporated in interventions (data from this can be used going forward).	Tanzania	Medium
Small Scale	Sharma et al., 2022	Raising AMR awareness. Two intervention steps performed 1) Focus group and information pack 1 of 4 about AMR, animal health, animal health and AMR or focus group only and (2) Follow up questionnaire.	Farmers, AHP	AMR	Knowledge scores higher amongst farmers participating in intervention meetings ($p<0.05$) and received intervention 20 ($p=0.03$) or 3) ($p=0.01$).	Knowledge score of farmers is higher when given information on animal health or animal health plus AMR compared to those that just participated in meeting or had information on AMR.	Trialling various material vs. no material.	Only post intervention data considered. Focus group findings not considered.	India	Medium
Small Scale	L. Shen et al., 2021	One year of health education-based interventions (training sessions, speakerphone messages, poster, and handbooks) to improve AMU in pigs and humans.	Farmers, swine	AMU	Increase in knowledge around pigs and AMU not statistically significant.	No significant increase in farmer knowledge on AMU in swine.	Repetition within intervention and process evaluation during year.	Only half of farmers at the start of the intervention raised swine through to the end of the intervention.	China	Medium
Small Scale	van Dijk et al., 2017	Participating in AMU reduction policy making.	Farmers, AHP	AMU	See *Table 5: Change in attitudes and perceptions to AMU, AMR, and AMS.*				United Kingdom	Low

AHP, Animal Health Professional;
AMU, Antimicrobial use;
AMR, Antimicrobial resistance;
AMS, Antimicrobial stewardship;
FAO, Food and Agriculture Organisation of the United Nations;
MHP, Medical Health Professional;
Within 'Secondary outcome':
AMU, Change in knowledge of appropriate antimicrobial use practices;
AMR, Change in knowledge of AMR (e.g., increased understanding of AMR (microbiological, public health), how AMR spreads and what it effects, the role of farmers/animal health professions in reduction of AMR.
AMS, Knowledge of antimicrobial stewardship.

10.3389/frabi.2023.1233698

10.3389/frabi.2023.1233698

TABLE 5 Change in attitudes and perceptions to AMU, AMR, and AMS.

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
Small Scale	van Dijk et al., 2017	Participating in AMU reduction policy making.	Farmers, AHP	Role	Farmers and AHP reported thinking more about their AMU (dry cow therapy reduction and cephalosporin reduction), got ideas for moving Herd Health Plans forward.	Reported greater knowledge about AMU and thoughts on their role.	Using those that will be impacted by the policy to create the policy. Feeling more included may give better results.	Lack of a universal way to assess AMU. Limited comparison and overview.	United Kingdom	Low

AMU, Antimicrobial use;
AHP, Animal Health Professional;
Role, Changes in attitudes and/or perception on farmers/animal health profession's role in reduction of AMR.

AMR, potentially biasing the outcomes. There was also no follow-up post-intervention measurement performed to evaluate if improvement was ongoing, and this was made difficult by veterinarians being reluctant to provide information on curative antimicrobials.

In the final example, Gerber et al. (2021) measured general AMU, diagnosis-specific usage, and HPCIAs usage amongst cattle farmers in Switzerland. Farmers picked the interventions to implement in their herds/farms from a pre-defined list of 17 udder, uterine, or calf health interventions. Udder or uterine health strategies resulted in a reduction in AMU ($p < 0.04$). Calf health interventions did not result in reduction in AMU. Allowing the farmers to choose herd-specific interventions from a pre-defined list allowed farmers to have partial autonomy. Observed weaknesses were the test and control groups were of different herd-sizes, breeds, and milk yields, which made comparison and interpretation of outcomes challenging. In addition, no information was collected on why the farmers chose their specific interventions (Gerber et al., 2021).

The financial benefit of AMU reduction was only explored in one intervention in two papers (Rojo-Gimeno et al., 2016; Postma et al., 2017). Increased net profit was recorded for a broad intervention that included herd management, biosecurity, and vaccination strategy customised to age groups of swine. At the same time, a decrease in treatment incidence of 52% and 32%, from birth to slaughter and for breeding animals, respectively, was reported.

3.1.2 Interventions reporting reduction in HPCIAs

Reduction in the use of HPCIAs (n = 9/22) was reported as part of broader interventions (Kuipers et al., 2016; Postma et al., 2017; Gerber et al., 2021; Morgans et al., 2021; Moura et al., 2022). Postma et al. (2017) noted a reduction of long-acting ceftiofur in sucklers by 83% and Gerber et al. (2021) reported a reduction in HPCIAs for treatment of udder related ailments ($p = 0.05$). However, reduction in use of HPCIAs was not always coupled with a general AMU reduction. An intervention targeting cattle-farmer-facilitated action groups assessed both general AMU and use of HPCIAs in cattle farmers in the United Kingdom and reported a reduction in use of HPCIAs of 3.484 mg/kg ($p<0.001$) but an overall median AMU reduction of 0.360 mg/kg ($p = 0.719$) (Morgans et al., 2021). In the same study, participant knowledge about AMR, AMS, and AMU at pre and post intervention was assessed qualitatively and an increase in the measured outcomes was reported. The noted study limitations were the lack of a control group and a societal push for AMR awareness at the time (Morgans et al., 2021).

3.1.3 Reduction of volume of AMU for a specific diagnosis

Five interventions (5/22) were implemented at the regional, national, or continental level and all five addressed a specific diagnosis (5/20) (Table 1). Four of the 5 interventions were at the regional, national, or continental level and measured the following: AMU in European farmers and AHPs (The European Parliament and The Council of the European Union, n.d.), reduction in

TABLE 6 Surveillance strategies.

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
International	Food and Agriculture Organization of the United Nations, n.d.	FAO-PMP-AMR - Help countries to create national action plans (NAPs).		AMU, AMR	Aim to increase awareness, surveillance & research, promote responsible AMU (strengthen governance and allocate resources on country level).	Individual country impact not evaluated.	Working toward unified goal/ framework of NAP.	Limited information about tool.		N/A
Continental	EFSA, 2022	Antimicrobial Resistance in zoonotic and indicator bacteria.	Livestock	AMR	Monitoring of zoonotic and indicator bacteria for AMR with data from 27 EU member states (incl. *Salmonella*, *E.coli* (ESBL/ AmpC).	Data available for AMR for both humans and animals of zoonotic and indicator bacteria.	Supranational programme, overview of EU - reflection and discussion of each strain.	Does not include clinical break points.	Europe	N/A
Continental	European Medicines Agency, 2022	European Surveillance of Veterinary Antimicrobial Consumption (ESVAC).	AHP, farmers	AMU	Collation of veterinary antibiotic sales in 31 EU countries.	Overview of veterinary antibiotic sales in EU by country and antimicrobial class.	Leverage point and accountability by other EU nations by having overview.	Sales to not equate usage directly.	Europe	N/A
Continental	Mader et al., 2021, 2022	European Antimicrobial Resistance Surveillance network in veterinary medicine (EARS-Vet).	Livestock	AMR	Aim to create surveillance system and collate veterinary clinical AMR isolates from EU countries.	Not yet fully in practice.	Not yet fully in practice.	Not yet fully in practice.	Europe	N/A
National	AURES, 2017	Austrian Report on Antimicrobial Resistance.	Farmer, AHP (MHP)	AMR, AMU	Campylobacter and AMU monitoring for veterinary/ food sector (also human sector).	Data on campylobacter and AMU in veterinary sector + data on AMU and other resistance in human.	Joint coordination between human and veterinary.	Limited ability to compare human and veterinary due to different tests and cut offs.	Europe	N/A
National	Bradley et al., 2017	National mastitis control scheme: AHDB Dairy Mastitis Control Plan (DMCP) (includes surveillance and actions).	AHP, Farmers & Cattle	AMU	See *Table 1*: *Change in antimicrobial use practices of animal health professionals.*				United Kingdom	Medium
National	Breen et al., 2017	National mastitis control scheme: AHDB Dairy Mastitis	AHP, Farmers & Cattle	AMU	See *Table 1*: *Change in antimicrobial use practices of animal health professionals.*				United Kingdom	Medium

(Continued)

10.3389/frabi.2023.1233698

TABLE 6 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
		Control Plan (DMCP) (includes surveillance and actions).								
National	Cazeau et al., 2022	Resapath - French surveillance network for antimicrobial resistance in bacteria from diseased animals.	Livestock	AMR	AMR from 14 bacteria (incl. *E.coli*, *S. aureus*, *Streptococcus*) monitored across species.	Data on resistance levels from a range of veterinary bacterial pathogens.	Training runs annually to try and align result interpretation at partner labs. Broad/extensive resistance monitoring.	Voluntary submission. Testing run by partner labs.	France	N/A
National	Centers for Disease Control and Prevention, 2015	National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS).	Livestock (retail meat, humans)	AMR	AMR monitoring from bacteria incl. *Salmonella, Shigella, Campylobacter, E. coli O157, Vibrio.*	Data on resistance levels in bacteria incl. *Salmonella, Shigella, Campylobacter, E. coli O157, Vibrio.*	Whole genome sequencing.	Data or reports from 2015 forward available through other sources.	USA	N/A
National	Danish Veterinary and Food Administration, n.d.	VetStat	Farmers, AHP	AMU	Reporting of AMU (and other medication) for food producing animals from farmers, AHPs and pharmacists.	Data available for AMU of food producing animals.	Available on herd level for AHP and farmers to assess own usage.	AMU data not publicly available (through some published through DANMAP).	Denmark	N/A
National	DANMAP, 2022	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme.	Farmers, AHP, livestock (humans)	AMU, AMR	AMR and AMU monitoring.	Data available for AMU and AMR for both humans and animals.	Available and accessible materials and methods section.	AMU reported incl. purchase data, may not reflect species prescription or amount used.	Denmark	N/A
National	Dupont et al., 2017	Yellow card scheme	AHP, farmers, swine	AMU	See *Table 1*: *Change in antimicrobial use practices of animal health professionals.*				Denmark	Medium
National	Federal Office of Consumer Protection and Food Safety, 2020	Federal Office of Consumer Protection and Food Safety, 2020.	Livestock	AMR	Monitoring of zoonoses (incl. *salmonella, L. monocytogenes, E. coli*) and AMR within these in annual reports.	Data available of AMR within zoonoses.	Various stages of production evaluated - allows tracking along food chain.	Not presented alongside human data.	Germany	N/A
National	Federal Office of Public Health FOPH, 2015	Usage of Antibiotics and Occurrence of Antibiotic Resistance in Bacteria from	Livestock, AHP, farmers (and human)	AMU, AMR	Monitoring of zoonoses (incl. *salmonella, L. monocytogenes, E. coli*) and AMR within these along with indicator bacteria and	Data available of AMR within zoonoses, indicator bacteria as well as antimicrobial sales.	Sampling from both healthy animals and diagnostic samples.	Sales do does not equate usage. Mg/kg also does not reflect doses (no defined DDD for animals).	Switzerland	N/A

(Continued)

10.3389/frabi.2023.1233698

TABLE 6 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
		Humans and Animals in Switzerland.			veterinary antimicrobial sales in annual reports.					
National	Finnish Food Authority, 2022	Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents.	Livestock, AHP, farmers	AMU, AMR	Monitoring AMR of zoonoses, indicator bacteria as well as antimicrobial sales in annual reports.	Data on AMR of zoonoses and indicator bacteria & AMU.	Population adjusted sales, mg active ingredient per PCU (mg/PCU).	Narrow resistance testing. Sales does not equate usage. Mg/kg also does not reflect doses (no defined DDD for animals).	Finland	N/A
National	INFAAR, 2020	Laboratory-based surveillance of AMR in fisheries and aquaculture: 1) AMR from healthy fish, and 2) improve AMR awareness in community.	Farmers, aquaculture	AMR	Aim to establish surveillance of AMR in fisheries.	Seemly not yet completed.	Lack of information on the programme.	Lack of information on the programme.	India	N/A
National	JVARM, n.d.	Japanese Veterinary Antimicrobial Resistance Monitoring System.	Farmers, AHP, livestock	AMU, AMR	Monitoring AMU consumption (sales), Resistance in zoonotic and indicator bacteria in healthy animals. Resistance in pathogens in diseased animals.	Data on AMU consumption and resistance in both indicator and zoonotic bacteria as well as isolates from diseased animals.		No data on food product isolate testing as part of surveillance.	Japan	N/A
National	Korean Veterinary Antimicrobial Usage and Resistance Monitoring, 2022	Korean Veterinary Antimicrobial Resistance Monitoring System.	Farmers, AHP, livestock	AMU, AMR	Monitoring AMR in animals and carcass and antimicrobial sales.	Data on AMR and antimicrobial sales available on interactive database.	Unable to find data in English.	Unable to find data in English.	Korea	N/A
National	MARAN, 2021	Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands.	Farmers, AHP, Livestock (companion animals)	AMU, AMR	Monitoring AMR of food-borne pathogens, indicator bacteria and Enterobacteriaceae plus antimicrobial sales in annual reports.	Data on AMR from food-borne pathogens, indicator bacteria and Enterobacteriaceae as well as antimicrobial sales.	Dual reporting (however no comparison of isolates).	Limited resistance testing. Sales does not equate usage. Mg/kg also does not reflect doses (no defined DDD for animals).	Netherlands	N/A

(Continued)

10.3389/frabi.2023.1233698

TABLE 6 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
National	Simonsen et al, 2022	Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway.	Farmers, AHP, livestock (humans)	AMU, AMR	Monitoring of AMR in zoonotic pathogens, indicator bacteria, clinical isolates, and antimicrobial sales.	Data on AMR from zoonotic pathogens, indicator bacteria and clinical isolates and antimicrobial sales.	Comprehensive dual reporting to same agency.	Limited resistance testing. Sales does not equate usage. Mg/kg also does not reflect doses (no defined DDD for animals).	Norway	N/A
National	Public Health Agency of Sweden and National Veterinary Institute, 2022	SVARM - Sales of antibiotics and occurrence of antibiotic resistance in Sweden.	Farmers, AHP, Livestock	AMU, AMR	Monitoring of AMR in zoonotic pathogens, indicator bacteria, clinical isolates, and antimicrobial sales.	Data on AMR from zoonotic pathogens, indicator bacteria and clinical isolates and antimicrobial sales.	Comparative analysis between human and animal antimicrobial sales & AMR.	Sales to not equate usage directly.	Sweden	N/A
National	Teng et al., 2022	VISAVET Health Surveillance Centre.	Swine	AMR	National AMR surveillance of AMR in food producing pigs through faecal samples at abattoir.	Data on AMR in *Salmonella* from Swine for a 16-year period.	Thorough analysis of specific bacteria, multiple resistance genes and testing methods.	Different antimicrobials tested so data set could not be analysed. MICs and antibiotic susceptibility testing changed over the years. Only high-capacity abattoirs included, does not reflect farms not collaborating with them.	Spain	N/A
National	U.S. Department of Agriculture Animal and Plant Health Inspection Service, 2022	National Animal Health Monitoring System (NAHMS).	Livestock	AMU, AMR	National studies on health and health management of livestock and poultry. Nine studies on AMU, AMS, and AMR (generally *Salmonella, Campylobacter, E. coli*, and *Enterococcus*).	Available national data on AMS, AMR, AMU.	One Health - Integrated report/ data presentation for comparison, broader overview of health with AMU.		USA	N/A
National	U.S. Food & Drug Administration, 2022a	The National Antimicrobial Resistance Monitoring System (NARMS).	Livestock (retail meat and human)	AMR	Monitoring system of AMR in enteric bacteria from ill people (CDC), retail meats (FDA) and food animals (USDA).	Data on resistance levels in enteric bacteria.	Integrated data presentation for comparison, partner with Vet-LIRN.	Emphasis on clinical illness isolates only from humans and companion animals (and less on food producing animals.	USA	N/A
National	U.S. Food & Drug Administration, 2022b	Veterinary Laboratory Investigation and Response Network (Vet-LIRN).	Livestock	AMR	Track AMR, create AMS material, and promote AMS within veterinary hospitals.	Available material/ AMR educational resources and tracking AMR.	Laboratory network that all test to same standard partner with NARMS.	More emphasis on clinical illness isolates only from humans and companion animals.	USA	N/A
National	Veterinary Medicines Directorate, n.d.	Veterinary Antimicrobial Resistance and Sales	Farmers, AHP, livestock,	AMU, AMR	Monitoring AMR of zoonoses, commensal bacteria of healthy	Data available on AMR in both healthy animals	Antimicrobial sales but also AMU reported	Some antimicrobials sold to feed mills and exported. AMR within diagnostic samples not	UK	N/A

(Continued)

10.3389/frabi.2023.1233698

TABLE 6 Continued

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Location	Quality Grade
		Surveillance - UK VARSS.	swine, poultry, companion animals		slaughter animals, and clinical AMR surveillance as well as antimicrobial sales and usage in annual reports.	and diagnostics as well as antimicrobial sales.	on electronic medicine books by AHP & farmers.	representative of entire population.		
Small Scale	Feyes et al., 2021	AMS programme in veterinary teaching hospital based on the CDC 7 core elements of hospital AMS program.	AHP	AMU, AMR	*See Table 2: Change in uptake and use of AMS by animal health professionals.*				Ohio, USA	N/A
Small Scale	Ha et al., 2021	Created App for drug stores to report veterinary drug sales in over 3-week period.	Farmer, AHP	AMU	Sales data of veterinary antimicrobials collected from veterinary drug stores using App (on provided tablets) from veterinary drug shops.	Data available for veterinary drug sales from veterinary drug stores.	Ability for government to collect sales data.	3 weeks extrapolated to 1 year - variations in year not accounted for. No indication of compliance levels. Sales does not equate usage. No feed additives counted this way.	Vietnam	Low
Small Scale	Yusuf et al., 2018	iSIKHNAS (Indonesia's integrated animal health information system).	Farmers, livestock	AMU	Surveillance system for farmers to report medical usage and disease.	Self-reported data available on AMU for livestock farms.	Offers farms a way to track when there is not a national system. Offers data for future research.	Only covers most developed island - not true picture of remote areas. Not true number of animals in which antimicrobial is used.	Indonesia	N/A

AM, Antimicrobial;
AMU, Antimicrobial use;
AMR, Antimicrobial resistance;
AMS, Antimicrobial stewardship;
AHP, Animal Health Professional;
FAO, Food and Agriculture Organization of the United Nations;
MHP, Medical health professional;
Within 'Secondary outcome':
AMU, Surveillance of AMU/AM sales;
AMR, Surveillance of AMR.

10.3389/frabi.2023.1233698

TABLE 7 Other.

Size	Source	Tool/Intervention	Target population	Secondary outcome	Outcome	Impact	Strengths	Limitations	Country	Quality Grade
International	Food and Agriculture Organization of the United Nations, 2020	FAO Assessment Tool for Laboratories and AMR Surveillance Systems (FAO-ATLASS).		Review of Lab and AMR surveillance systems	28 countries have had assessments performed.	Defined targets for national AMR surveillance in food and agriculture sectors.	Availability of a working tool.	Limited information about tool.		N/A
International	World Organisation for Animal Health, 2019	OIE-Performance of Veterinary Services (PVS) Pathway.		Assessment of animal health situation, incl. AMR	Self-reported review/score of animal health in country.	Tool/score for countries to work towards improving.	Standardised scoring system for all countries.	Self-reporting requires countries to choose to submit. Risk of participation bias as only countries with resources and interest might participate.		N/A
International	WHO, 2022	Tripartite AMR country self-assessment survey (TrACSS).		AMR Monitoring and Surveillance network	Aim for countries to review progress in implementing actions to address AMR at the national level, and to report annually at the global level.	Standardised progress reports for countries to address AMR.	Overview of nations' effort, evaluate own efforts, see other countries efforts, multisectoral.	Self-reporting requires countries to choose to submit. Risk of participation bias as only countries with resources and interest might participate.		N/A
International	World Organisation for Animal Health, n.d.	Veterinary Legislation Support Program (VLSP).		Assessment of veterinary legislation	Report for 137 countries on veterinary legislation that is aimed at creating legislation that reduces biological threat and AMR.	Knowledge about gaps and weaknesses of veterinary legislation, including those pertaining to AMR.	Knowledge sharing about legislation.	Report/review does not equal action/change.		N/A
International	Food and Agriculture Organization of the United Nations, n.d.	Tool for a Situation Analysis of AMR risks in the Food and Agriculture Sectors on a national level.		Report on AMR risk and improvements	Aim to provide picture of current situation and guide decisions.	Individual report impact not evaluated on.	Added support for countries with less resources so they do not have to make their own evaluation.	Only as useful as the country implementing. No assessment if action plans are suitable for country.		N/A
Small Scale	van Dijk et al., 2017	Participating in AMU reduction policy making	Farmers, AHP	Policy created	*See: Table 5: Change in attitudes and perceptions to AMU, AMR, and AMS.*				United Kingdom	Low

AMU, Antimicrobial use;
AMR, Antimicrobial resistance;
AMS, Antimicrobial stewardship;
AHP, Animal Health Professional;
FAO, Food and Agriculture Organization of the United Nations.

10.3389/frabi.2023.1233698

ceftiofur use by AHPs and poultry farmers in Canada (Agunos et al., 2017; Agunos et al., 2018), AMU and HPCIAs reduction in AHPs and swine farmers in Denmark (Jensen et al., 2014; Dupont et al., 2017), and general AMU, AMR, diagnostic specific AMU reduction in dairy cattle farmers in the United Kingdom (Bradley et al., 2017; Breen et al., 2017), and HPCIAs reduction in dairy cattle farmers and AHPs in the Netherlands (Moura et al., 2022).

A fifth intervention, 'Yellow Card System', was at the national level and was aimed at reducing AMU and HPCIAs use in swine farmers and AHPs in Denmark (Jensen et al., 2014; Dupont et al., 2017). The intervention required swine farms to reduce their AMU to pre-set levels and resulted in 38.4 – 56.2% reduction of mg active ingredients/pig/day with increased use of vaccines, and decreased herd medication was reported as the biggest perceived influencing factor (Jensen et al., 2014; Dupont et al., 2017). However, these factors were only assessed in herds with > 10% reduction in AMU and self-reported by farmers and AHPs. This study included a large national sample size and excluded herds with < 10% reduction in AMU. Other national interventions overlapped with diagnostic-specific interventions. A national mastitis control scheme assessed AMU by AHPs and dairy cattle farmers in the United Kingdom (Bradley et al., 2017; Breen et al., 2017). This intervention resulted in a 40% reduction in use of intramammary medication in lactating cows and a 20% reduction in clinic mastitis rates, achieved through AHP and farmer training. However, this intervention was only noted in a letter and conference proceedings and no information was provided on strengths and limitations (Bradley et al., 2017; Breen et al., 2017).

3.1.4 Summary of change in AMU practices of AHPs and farmers

This category of interventions primarily focused on farmers and used herd-specific interventions to reduce AMU with success reported for both overall AMU reduction and reduction in use of HPCIAs. One study required farmers to select interventions from a pre-set list (Gerber et al., 2021). There was overlap of the primary outcomes measured across interventions and many studies also featured other primary outcome measures. Diagnosis-specific interventions were aimed at changing AMU in cases of mastitis and calf diarrhoea (Bradley et al., 2017; Gomez et al., 2017; Bailey et al., 2019). Studies involved pre- and post-intervention measurement of outcomes. Only a few studies reported use of control groups to account for other external influences (Kuipers et al., 2016; Becker et al., 2020).

3.2 Change in uptake and use of AMS by AHPs and farmers

Within this primary outcome, change in prescribing habits (n=4/8) was the most frequently measured, followed by increased adherence to guidelines (n=2/8) and increased use of diagnostics (n=2/8) (Table 2). There were additional aspects from 'Other' category of interventions reported within this primary outcome (n=6/8). Farmers (6/8) and AHPs (5/8) were almost equally targeted. Four interventions were implemented in Europe (4/8), two in Africa (2/8), and two in North America (2/8). The interventions were distributed across the levels: continental (1/8), national (1/8), regional (2/8), and small-scale (4/8). Of these interventions, two (2/8) were legislative interventions. The quality of intervention design ranged from high (1/7), medium (4/7), to low (2/7). The interventions, impact, outcome, and limitations are described in Table 2.

3.2.1 Change in prescribing habits

The studies reporting change in prescribing habits focussed on herd health plans and educational interventions. As an example, a study in Uganda used a One Health approach and focused on change in prescribing, guideline use, and diagnostic use in medical, healthcare, and AHPs (Musoke et al., 2020). Medical health professionals self-reported improved handwashing (57.3%), guideline use (52.9%), treatment based on diagnostics (44.1%), and reduction in unnecessary AMU (51.3%). Participation of AHPs was low compared to medical health professionals (Musoke et al., 2020). A disadvantage of self-reporting is perception may not translate to actual action; just because the participant says they are doing something, it does not mean they are. The other interventions surrounding prescribing habits, including the intervention of prescribing champions and herd health plans, are discussed in other sections (Raasch et al., 2020; Rees et al., 2021; Pempek et al., 2022).

3.2.2 Change of AMS through legislation

Change in AMS through legislation was reported. Two examples are the 'California State Bill 27' aimed at farmers in California, USA (Abdelfattah et al., 2022), and Regulation (EU) 2019/6 on veterinary medicinal products and repealing Directive 2001/82/EC aimed at both farmers and AHPs in Europe (The European Parliament and The Council of the European Union, n.d.). The 'California State Bill 27' states that usage of antimicrobials of medical importance for humans for livestock requires a prescription. Assessment of this intervention indicated self-reported change in disease management including increased use of diagnostics (29.4%) and an increased use of alternatives to antimicrobials (26.8%) (Abdelfattah et al., 2022). This study was limited by a low response rate and possible response bias. As mentioned previously, self-reporting change may not translate to action. There was no report on AMU suggesting it was not evident whether self-reported change resulted in action (Abdelfattah et al., 2022). The latter, EU regulation, bans medication through feed or to groups for livestock use (The European Parliament and The Council of the European Union, n.d.). No data was presented within the legislation about the effect of this legislation (The European Parliament and The Council of the European Union, n.d.). In general, there were few studies evaluating legislation/bills.

3.2.3 Other reported aspects

Other aspects were reported under the primary outcome measure, change in uptake and use of AMS by AHPs and farmers. The first aspect was improving sanitation (i.e., improving

hand washing and biosecurity) in both AHPs in Uganda and California (Musoke et al., 2020; Abdelfattah et al., 2022). The second aspect was improving dosage accuracy in cattle farmers in Ohio, USA (Pempek et al., 2022). These interventions were described in earlier sections and under the primary outcome measure 'change in AMU of AHPs.'

3.2.4 Summary of change in uptake and use of AMS by AHPs and farmers

The interventions reported under this category illustrated how both voluntary programmes and legislation can create an impact on AMS. However, it is important to note the impact of many of these interventions were self-reported (Musoke et al., 2020; Abdelfattah et al., 2022). This carries the risk of response bias. Social and moral responsibility perceived by the reporting individuals may therefore influence the responses (Bradburn et al., 2004).

3.3 Change in development and/or spread of AMR

Change in development and/or spread of AMR was reported. The most frequent aspect measured was the reduced frequency of resistance genes within detected strains (21/30) followed by reduced frequency of bacterial strains (9/30), reduced frequency of resistance genes detected/isolated in food products (meat, milk, egg, etc) (5/30), and reduced frequency of resistance genes detected/isolated within the herd environment (3/30) (Table 3). The interventions were conducted in Europe (12/30), North America (10/30), Asia (4/30), South America (3/30), and Africa (1/30). The interventions were primarily small-scale (28/30) with two interventions conducted on a national level (2/30). The quality of study design was split between high (17/30) and medium (13/30) (Table 3).

3.3.1 Reduction in resistance strains

Reduced frequency of resistance genes within bacterial strains (19/26) was reported primarily at a small-scale level (17/19) and twice at a national level (2/19). Findings from the interventions at the national level are presented separately from the small-scale interventions.

3.3.1.1 National projects

Two interventions conducted at a national level focused primarily on ceftiofur resistance in broilers. The first intervention reported a voluntary reduction of ceftiofur and assessed the reduction of resistant strains in Japanese hatcheries (Hiki et al., 2015). Testing was performed on one faecal sample per farm with commercially available kits and to country standards. This longitudinal study did not have a control group or assess confounding but evaluated multiple resistance genes (Hiki et al., 2015). The second intervention, the Canadian Integrated Program for Antimicrobial Resistance Surveillance, tested for ceftiofur and other resistance genes in farms, abattoirs, and retail products (Agunos et al., 2017). All isolates were tested at national reference laboratories for continuity and to allow for comparison of results (Agunos et al., 2017). Neither intervention performed assessments of mortality or animal health. Both interventions used different reduction strategies and testing methods, but both reported reduced ceftiofur resistance in broiler production.

3.3.1.2 Small scale

Small-scale interventions were also reported and mainly focused on resistant strains in broilers (14/21), along with swine (7/21), cattle (5/21), goats (1/21), and a range of end meat products (1/21). The interventions assessed a range of parameters including biosecurity/sanitation (4/21) (Hao et al., 2013; Dorado-García et al., 2015a; Dorado-García et al., 2015b; Wanninger et al., 2016; Brusa et al., 2019), animals raised without antibiotics (6/21) (Pedroso et al., 2013; Wanninger et al., 2016; Thapaliya et al., 2017; Vikram et al., 2017; Haskell et al., 2018; Loayza-Villa et al., 2021), cessation of antimicrobials in feed (3/21) (Chinwe et al., 2014; Verrette et al., 2019; Shen et al., 2020) and competitive exposure (7/21) (Nuotio et al., 2013; Pedroso et al., 2013; Ceccarelli et al., 2017; Mourand et al., 2017; Methner et al., 2019; Dame-Korevaar et al., 2020a; Dame-Korevaar et al., 2020b). The distribution of the various interventions is summarised in Figure 4.

Competitive exposure was one of the interventions used in broilers and included use of commercial products, pre-and probiotics, as well as specifically created bacterial compositions with positive effects (Nuotio et al., 2013; Pedroso et al., 2013; Ceccarelli et al., 2017; Mourand et al., 2017; Methner et al., 2019; Dame-Korevaar et al., 2020a; Dame-Korevaar et al., 2020b). Two specific examples of interventions involving competitive exposure were the use of unselected fermented intestinal bacterial and/or a selection of pre- and pro/biotics in broilers in the Netherlands (Dame-Korevaar et al., 2020a), and use of a commercial natural live intestinal flora, Aviguard, to target ESBL-*E.coli* in broilers in The Netherlands (Dame-Korevaar et al., 2020b). The former intervention had no effect when unselected fermented intestinal bacterial and a selection of pre- and pro/biotics were given on the same day (Day 0) as the challenge ESBL *E.coli* (Dame-Korevaar et al., 2020a). A reduced excretion of CTX-M-1- *E.coli* was seen when the challenge was given on day 5 after unselected fermented intestinal bacterial and a selection of pre- and pro/biotics. The study was limited by the short time frame (5 days) and experimental conditions. There is a gap in information on whether reduction in

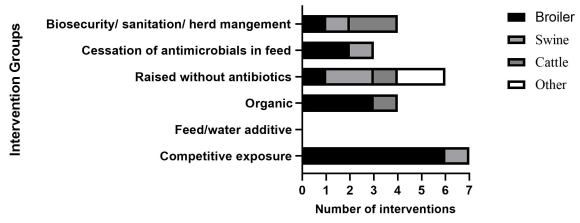


FIGURE 4
Distribution of interventions that assessed change in development of AMR with a focus on reducing AMR resistance genes. 'Other' in RWA accounts for intervention that includes broilers, swine, and cattle, and meat products.

resistance is linked to disease or end meat contamination (Dame-Korevaar et al., 2020a).

In the latter study, Aviguard was administered to chicks right after hatching and challenged with CTX-M-1-*E.coli* on day 5 (Dame-Korevaar et al., 2020b). Of the test group, 0/200 broilers were CTX-M-1-*E.coli* positive on day 21 vs. the control with 187/200 positive. Multiple scenarios were tested and CTX-M-1-*E.coli* swabbing occurred every day. A potential limitation of this study was performance in semi-field conditions under stringent biosecurity means results may not translate to field conditions (Dame-Korevaar et al., 2020b). Like the previous report, disease and end meat levels were not assessed. This study suggested competitive exposure was successful within certain criteria such as high biosecurity and short time frames, but more knowledge is needed on the effect of longer timeframes and mechanism of human transmission.

A reduction in resistance levels following herd management and sanitation interventions in livestock was reported. A study in the Netherlands reported a reduction of 31 MRSA-positive herds to 29, and a 44% reduction in AMU, defined daily dosages animal per year, in swine (Dorado-García et al., 2015a). This was achieved by improving personnel and farm hygiene as well as changing animal contact structure. Having separate water pipes from medication pipes, specific rooms for deliveries, and designated sow groups, were all positively correlated with reducing MRSA. A limited intervention period (18 months) and pooling of samples may however lead to inaccuracies in measurement of outcomes (Dorado-García et al., 2015a).

3.3.2 Reduction in bacterial strains

The range of interventions focused on reducing bacterial strains (Figure 5) was similar to those for resistant strains (Figure 4). Broilers were the most frequently targeted animal group (5/9). Others were cattle (1/9) and goats (1/9). Unlike interventions focused on resistance genes, there was a larger emphasis on feed/water additives (4/9) (Hao et al., 2013; Rubio-García et al., 2015; Wanninger et al., 2016; Kannan et al., 2019), on cessation of antimicrobials (1/9) (Chinwe et al., 2014), and biosecurity/sanitation (3/9) (Bahrndorff et al., 2013; Luyckx et al., 2015; Lee et al., 2017; Kannan et al., 2019) (Figure 5).

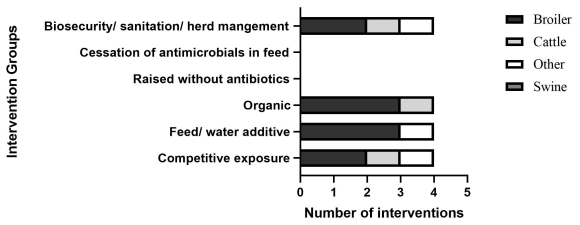


FIGURE 5
Distribution of interventions that assessed change in development of AMR with a focus on reducing bacterial strains. 'Other' in 'biosecurity/sanitation/herd management' and 'feed/water additive' is same intervention.

Two examples of feed and drink additives used in the interventions were dietary brown seaweed used to reduce rumen *E.coli* in goats (Kannan et al., 2019) and an allostatic modulator in drinking water and feed withdrawal from broilers to reduce coliforms (Rubio-García et al., 2015). The first intervention investigated microbiological contamination of goat carcasses in Georgia, USA (Kannan et al., 2019). To determine the effect of brown seaweed and chlorinated wash on microbiological contamination of carcasses, bucks were fed seaweed as a supplement and the feed was sprayed with 50 mg/L chlorinated water. Rumen but not skin *E.coli* count was reduced following feeding with seaweed ($p < 0.05$). Skin count was reduced after chlorinated wash ($p < 0.05$) (Kannan et al., 2019). No information was provided on the contamination of meat in a production (abattoir) setting or transmission to humans. The second intervention aimed to reduce coliforms in broilers and end meat in Mexico (Rubio-García et al., 2015). Broilers were given an allostatic modulator in tap drinking water and a ten- or sixteen-hour feed withdrawal before slaughter shipment. The allostatic modulator contained electrolytes, acetylsalicylic acid, and ascorbic acid. Allostatic modulators aim to reduce allostasis (chronic stress). Reduction in coliforms ($p = 0.014$) and total aerobic mesophilic bacteria ($p = 0.0001$) were reported and the intervention was considered financially reasonable and accessible. A limitation of this study was it was performed under experimental conditions only (Rubio-García et al., 2015).

Two interventions on biosecurity and sanitation were reported and these included implementation of education and cleaning protocols. In the first intervention conducted in Belgium, the reduction of bacterial strains detected in broilers was assessed through on-farm cleaning protocols used by farmers (Luyckx et al., 2015). Sanitation of the broiler houses with commercial products containing sodium hydroxide resulted in 86% reduction in the number of *E. coli*-positive swabs (1-3% difference depending on soaking and water temperature) (Luyckx et al., 2015). The second intervention investigated the reduction in bacterial strains in fresh milk samples of cattle in Malaysia (Lee et al., 2017). The intervention was education of farmers on udder and machine sanitation and resulted in a 40% log reduction of *Staphylococcus aureus* in fresh milk samples (Lee et al., 2017). A limitation of this study was that statistical significance was not reported and a clear description of how the training was performed was not provided.

3.3.3 AMR in the environment and food

Articles reported on interventions focussed on the environment ($n = 3$) and food products ($n = 4$). Three studies focused on the environment and area around herds. The first study investigated bacterial strains in broilers and the environment in China (Hao et al., 2013). This intervention focused on sanitation, specifically the use of acidic water (pH 5.0 – 6.0) wash containing chlorine to reduce *Salmonella* spp. and *E.coli* in broiler houses and resulted in a 16% reduction in *Salmonella* spp and *E.coli* in broiler houses (Hao et al., 2013). A limitation of this study was the intervention was not applicable to bird housing with cages. The second intervention focused on both sanitation and reducing AMU and investigated

resistant bacterial strains in veal cattle in the Netherlands (Dorado-García et al., 2015b). The intervention reported that cleaning and disinfecting negatively impacted the MRSA burden in the environment around veal cattle (Dorado-García et al., 2015b). This intervention was implemented for two production cycles with different techniques and under short time frames (12 weeks) making comparison of results difficult. A third study investigated bacterial strains on swine farms, in the surrounding farm environment, and in meat products in China (Shen et al., 2020). There were significant reductions in MCR-1-positive *E. coli* after the cessation of colistin as a feed additive. This was both at farm level ($p < 0.0001$), in food (pork) ($p < 0.0001$), and in the environment (soil and water around slaughterhouses) ($p < 0.0001$).

3.3.4 Summary of change in development and/or spread of AMR

The design of interventions under this category varied. Some interventions measured outcomes at pre and post intervention to assess change in outcomes, whereas other interventions used control herds. Experimental studies were used to evaluate outcomes within this category, more than for any other primary outcome measurement. Findings from experimental studies do not necessarily translate to or are feasible in field conditions. Replicating these findings in field conditions is an important next step to assess if the interventions work in the real-world situations. Some of the reported interventions run for a short time frame and no indication of disease level, transmission to humans, or end meat contamination was assessed (Dame-Korevaar et al., 2020a; Dame-Korevaar et al., 2020b; Shen et al., 2020).

3.4 Change in knowledge of appropriate AMU practices, AMR, and AMS

There were 14 reported interventions within the primary outcome, change in knowledge of appropriate AMU practices, AMR, and AMS. These included change in knowledge of appropriate AMU practices (n = 9), change in knowledge of AMR (n = 6), and change in knowledge of AMS (n = 6), with overlapping observed within the interventions (Figure 6). There were also interventions with aspects that did not fit within the predefined groupings (3/14). AHPs and farmers were targeted in 6/14 and 8/14 of the interventions. The interventions were conducted in high income countries in Europe (5/14) and North America (2/14) and less in LMICs within Africa (2/14), and Asia (3/14). Interventions featured across all levels; international (2/14), national (1/14), regional (2/14), and small scale (9/14) (Table 4). A description of interventions in high income countries and LMICs is provided below. The quality of design of these studies were scored as medium (7/12), low (4/12) and high (1/12).

3.4.1 Change in knowledge in high income countries

Interventions in this primary outcome measure were mostly in high income countries (Bradley et al., 2017; Powell et al., 2017; van Dijk et al., 2017; Feyes et al., 2021; Morgans et al., 2021; Rees et al., 2021) and overlapped with other primary outcomes. For example, the use of farmer-facilitated groups reduced AMU while increasing knowledge around AMU, AMR, and AMS for dairy farmers in the United Kingdom (Morgans et al., 2021). This intervention was considered time-consuming as required meetings every 6 – 8 weeks but allowed for conversation and discussion to create understanding (Morgans et al., 2021). In another study, the Arwain Vet Cymru Project created veterinary prescribing champions with the aim of changing the behaviour of AHPs in Wales and increasing knowledge of AMU and AMS through webinars, workshops, and discussions (Rees et al., 2021). The impact and limitations of the dissemination were not reported. This intervention was reported to be labour-intensive for the creators and participants (Rees et al., 2021). In another intervention, increased knowledge of farmers and reduction in AMU in calves in Ohio, USA was achieved through didactic presentations and calf-side training (Pempek et al., 2022). Limitations were not reported regarding knowledge acquisition, but other limitations were reported as noted earlier in the section on change in AMU practices of AHPs.

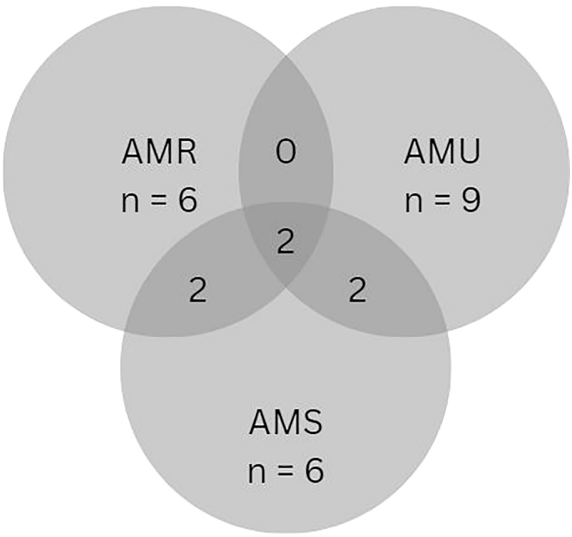


FIGURE 6
Distribution of interventions that assessed change in knowledge with a focus on AMU practices, AMR, and AMS within secondary outcome measurements.

3.4.2 Change in knowledge in LMICs

Interventions focussed on knowledge acquisition were also conducted in LMICs (Roulette et al., 2017; Musoke et al., 2020; Shen et al., 2021; Sharma et al., 2022). While knowledge acquisition was part of a broad intervention in high income countries, this was conducted as a single activity in LMICs. Two interventions that focused on assessing knowledge about AMR were reported in LMICs. The first intervention assess knowledge on AMR and animal health among farmers and AHPs in India (Sharma et al., 2022). The target participants attended meetings and were given 'knowledge packs' on AMR and/or animal health, to raise AMR

awareness. Higher knowledge scores were reported for farmers that participated in the meetings ($p<0.05$) and received information on animal health ($p=0.03$) or animal health and AMR ($p=0.01$). A key limitation of this study was it did not include translation of knowledge to actions or compare a pre-post intervention knowledge score (Sharma et al., 2022). In the second intervention, AMR was assessed after education on AMR was given to farmers in Tanzanian Masai communities (Roulette et al., 2017). Additionally, tape measures and antimicrobial dosage charts were given to men, and women received thermometers for milk pasteurization. At a 2-month follow-up, men retained more AMR knowledge (30%) compared to women (14%). However, 70% of women used their innovations correctly (thermometer) whereas men only performed 18% of dosage steps correctly (Roulette et al., 2017). A strength of this study was that cultural aspects and gender roles were taken into consideration. A limitation of this study was knowledge retention about AMR and innovation use were not evaluated as potential influences of each other. In general, there was limited information on interventions focussed on change in knowledge in LMICs and no demonstrated evidence of knowledge translating to action.

3.4.3 A summary of the change in knowledge of appropriate AMU practices, AMR, and AMS

The reported interventions illustrate that knowledge on AMR can be learned and retained (Roulette et al., 2017; Sharma et al., 2022) and assessed using pre- and post-intervention testing. However, there is need to gain more understanding of whether knowledge provided to farmers and AHPs translates to action and if there is sustainable change. The intervention conducted by Morgans et al. (2021) aimed to create sustained change through peer-to-peer learning and prescribing champions. However, there was no measurement of outcomes. Interventions evaluating outcome measurements are needed to understand the impact of these interventions.

3.5 Change in attitudes and perceptions to AMU, AMR, and AMS

Only one article investigated the change in attitudes and perceptions (n = 1) and did so as a small-scale qualitative assessment of farmers and AHPs in the United Kingdom (van Dijk et al., 2017) (Table 5). After participating in the creation of an AMU reduction policy, farmers and AHPs provided thoughts on their AMU practices and how these could be incorporated into their herd health plans. The responses were individual statements on an *ad hoc* basis (van Dijk et al., 2017). This study suggests that using stakeholders (such as AHPs and farmers) that will directly be impacted by policy to create policy could result in the stakeholders feeling more included and motivated potentially resulting in better policy outcomes.

3.6 Surveillance strategies

Interventions involving surveillance strategies (30/90) were primarily conducted on a continental (3/30) and national (23/30) level. Most of the surveillance strategies focused on both AMU and AMR (12/30), some focused solely on AMR (9/30), or AMU (4/30). Of those focussing on both AMU and AMR, some considered a One Health approach and provided human data (6/12). The largest number of surveillance strategies were reported in Europe (17/30) and less in Asia (5/30) and North America (5/30). No surveillance strategies were reported in Africa or South America. A detailed description of surveillance involving a One Health approach or focussed on AMU and AMR is provided below. Small-scale interventions (3/30) are presented separately.

3.6.1 One Health-focused strategies

Within national surveillance activities involving AMR and AMU, there was a focus on One Health. The most comprehensive surveillance strategies included AMR in zoonotic pathogens, indicator bacteria, and clinical isolates for both humans and animals along with antimicrobial sales in annual reports. These strategies were most reported in Europe and Asia. Half (6/12) of the reported strategies provided a comparison of human and veterinary isolates (JVARM, ; Federal Office of Public Health FOPH, 2015; MARAN, 2021; DANMAP, 2022; Korean Veterinary Antimicrobial Usage and Resistance Monitoring, 2022; Simonsen et al., 2022).

3.6.2 AMU-focused strategies

In other countries that did not report AMU and AMR together, surveillance strategies were split, or reported aspects on AMU or AMR or human and veterinary isolates, separately. Surveillance strategies focusing on AMU used medical sales data. As an example, on a continental level, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) collates antimicrobial sales data from 31 European Union (EU) countries, offering an overview and accountability for usage (European Medicines Agency, 2022). These exist at country level in Europe (Danish Veterinary and Food Administration, n.d.; Finnish Food Authority, 2022).

3.6.3 AMR-focused strategies

Surveillance of AMR on its own exists in multiple forms, on both a national and continental level primarily in Europe and North America. On a continental level, the EU collates AMR data through The European Food Safety Authority (EFSA). Monitoring is conducted for zoonotic and indicator bacteria for AMR (incl. *Salmonella* spp., *E.coli* (Extended Spectrum Beta-Lactamase (ESBL)/AmpC beta-lactamases (AmpC)) with data from both human and livestock isolates from 27 EU member states (EFSA, 2022). Under creation is the European Antimicrobial Resistance Surveillance Network in veterinary medicine (EARS-Vet) which will register veterinary clinical isolates (Mader et al., 2021). Clinical isolates from livestock are currently not collected by many surveillance systems. The French surveillance network for antimicrobial resistance in bacteria from diseased animals (RESAPATH) in France offers the voluntary submission of 14 clinical isolates (Cazeau et al., 2022). Another large surveillance strategy exists in the US. Data on resistant isolates are collected through the Veterinary Laboratory Investigation and Response Network (Vet-LIRN) in partnership with The National

Antimicrobial Resistance Monitoring System (NARMS) (U.S. Food and Drug Administration, 2022a; U.S. Food and Drug Administration, 2022b). NARMS monitors and publishes reports of AMR data from enteric isolates from retail meats, food animals, ill people, and companion animals.

3.6.4 Surveillance on a small scale

There were only three surveillance strategies that were considered small-scale. Two interventions were conducted in LMICs (Indonesia and Vietnam) (Yusuf et al., 2018; Ha et al., 2021) and one at a large veterinary teaching hospital in the USA (Ohio) (Feyes et al., 2021). Of the two studies in LMICs, the first study investigated AMU using sales data of veterinary antimicrobials from drug stores in Indonesia (Ha et al., 2021). An app was created for pharmacists to report sales data allowing them to monitor sales and making data available to monitor on a larger scale. The study acknowledges multiple limitations. Three weeks of sales data was extrapolated to 1 year and as such did not account for variations throughout the year. Furthermore, sales do not equate to usage, and no feed additives were accounted for (Ha et al., 2021). The second surveillance strategy measuring AMU was a self-reporting system for farmers in Vietnam (Yusuf et al., 2018). It involved reporting medical usage and disease via a tablet. This offered farmers a way to track their AMU, without a national system. The study followed farmers on the main island for 2 years, excluding rural settings (Yusuf et al., 2018). Two limitations were observed in this study; there was room for reporting inaccuracies and compliance levels were not reported.

3.6.5 Summary of surveillance strategies

Most surveillance strategies within the scope of this review were based in high income countries suggesting there is little data from LMICs – most likely due to the requirements for financial investment and infrastructure. Within the surveillance strategies that do exist, mandatory reporting at the national level appears widespread which helps ensure that isolates received reflect AMR distribution in each setting. However, reporting especially of clinical isolates is voluntary within systems (Cazeau et al., 2022) which risks a fractured picture of the clinical isolate presence and distribution. Another aspect of surveillance that operates with a margin of error is using sales data as a measure of AMU, as it does not account for off-label use and unused medication. Few surveillance interventions that are considered small-scale were reported, and with varying limitations.

3.7 Other interventions

The 'other' category of interventions (n = 6) included tools provided by the quadripartite to help countries with surveillance systems or self-assessment of their AMR situation (5/6) and one small-scale intervention that focused on policy related to reducing AMU by farmers and AHPs in the United Kingdom. Two examples of the tools from the quadripartite are the FAO Assessment Tool for Laboratories and AMR Surveillance Systems (FAO-ATLASS) (Food and Agriculture Organization of the United Nations, 2020) and WHO Tool for a Situation Analysis of AMR risks in the Food and Agriculture Sectors on a national level (Food and Agriculture Organisation of the United Nations, n.d.).

Using the FAO Assessment Tool, either in surveillance mode or laboratory mode, a baseline level of the country's setup can be assessed, steps for specific improvement identified, and progress made monitorable (Food and Agriculture Organization of the United Nations, 2020). There is limited information on the related impact or outcomes at national levels. The WHO Tool creates a national report on AMR risk and improvements aiming to provide a picture of the current situation and guide decisions based on One Health principles (Food and Agriculture Organisation of the United Nations, n.d.). There may be a gap between receiving a report and actioning change. These tools do not have a primary effect on the target population but generally offer guidance to a country's AMR plan.

4 Discussion

This scoping review summarises the existing evidence on interventions focussed on AMR, AMU and AMS in the animal health sector and provides insights into their impact, gaps, and limitations. Interventions targeting AHPs, farmers, and livestock were of interest. The review included 90 studies that reported interventions from around the world, with 19% of those in LMICs. Within the defined primary outcome measurements, there was a broad range of animal sector interventions. The reported interventions mainly focused on changing AMU levels and changing the development and spread of AMR. Within the primary outcome focused on reducing AMU, herd specific interventions with pre and post intervention measurements were common. Interventions aiming to reduce AMR were often experimental with few investigating the environment or end meat levels. There were few interventions focused on changing knowledge and/or attitudes and perceptions. Retention of knowledge and self-reported change was assessed in some of the reported interventions. Interventions involving surveillance were conducted at the national level and reported AMU determined from sales data and AMR based on detection of indicator bacteria.

Although interventions focusing on AMU were reported, it is important to note that a reduction in AMU does not automatically mean a reduction in AMR. Nonetheless, change in AMU is used as a measurement of impact. Evidence on the linkage between a reduction in AMU and AMR is mixed (Bennani et al., 2020). AMU is used, likely because it is more easily quantifiable and the data requires less resource to collect compared to that of resistant isolates (Bennani et al., 2020). The potential mismatch between AMU and AMR should be considered when assessing the impact of an intervention. Ideally, when assessing a reduction in AMU, the presence of AMR should also be measured.

None of the studies that investigated AMU practices assessed the duration of therapy in animals. Although there is a shortage of veterinary data, data in the human medicine sector indicates a change in therapy can have an impact on AMR without affecting

treatment efficacy (Llor and Bjerrum, 2014; de Waele and Martin-Loeches, 2018; Spellberg and Rice, 2019). Furthermore, the lack of data on duration of therapy may indicate that a reduction in AMU could be due to fewer animals being treated, or the same number of animals being treated for a shorter period, unless number of animals is accounted for. This aspect requires closer examination in interventions assessing AMU practices.

Interventions assessing changes in development and/or spread of AMR were carried out in both field and experimental conditions in different livestock (cattle, swine, broilers, goats). Although experimental conditions can negate the limitations of field conditions, interventions garnering results in these conditions may not do so in field conditions. For interventions involving experimental conditions, it is important to account for these differences or to follow up the experimental studies with field studies to reflect real-world conditions.

In general, the interventions focussed on change in behaviour, knowledge, and attitude were few or lacking. Aspects on attitude, behaviour, and knowledge were often implicit parts of interventions but not outcome measurements. Interventions that investigated these aspects featured one or two time points or a set knowledge 'bank' without determining if the change in knowledge translated into actions or long-term change. Beyond assessing change in knowledge, it is important to investigate if increased knowledge translates to actions that reduce AMR.

In the current review, the animal health interventions in LMICs were scarce; only 19% (16/83) were performed in LMICs. This is a higher percentage compared to the estimate in human medicine of 1 – 2% (Cox et al., 2017). All the animal health related interventions in LMICs were on a small scale (at herd or farm level). The design of the interventions were mostly deemed of low quality, only one study was of high quality. The reasons for the low number of interventions in LMICs are unclear, but it is possible lack of resources is a contributing factor. The quadripartite and other key players at the global level are making efforts to lessen the gap in skills and resources between LMICs and high-income countries. For example, there are several stewardship tools and road maps that were developed and made available to LMICs to facilitate the implementation of AMR policy and interventions at a national level (World Health Organisation, 2016; Seale et al., 2017). Two examples of these are the Wellcome Trust Road Map for LMICs to participate in the global antimicrobial surveillance system (Seale et al., 2017) and the WHO manual for LMICs to implement national action plans to reduce AMR in both human and animal sectors (World Health Organisation, 2016). These tools need to be coupled with research and to focus on barriers while tailoring the AMR interventions to specific country socioeconomic needs and ensuring the output trickles down to farmers and AHPs.

Differences in the target population (farmers, AHPs, etc) and access to antimicrobials were observed in interventions performed in LMICs vs. high-income countries. Within the reviewed studies, more heterogeneity of the target population (farmers, AHP, etc) was found in LMICs. The farmers in high-income countries run mainly large farms. However, in LMICs, there were small-holdings or small-scale farmers (Phu et al., 2021) and communities that keep livestock for their own consumption (Roulette et al., 2017). In general, in high-income countries, the AHPs encompassed licensed and registered veterinarians but in LMICs, this also included unregistered practitioners prescribing antimicrobials. The access to antimicrobials varied across the targeted populations. Access to over-the-counter antimicrobials (Ha et al., 2021), which is not legal in most high-income countries, and through feed mills (Chinwe et al., 2014) exists in LMICs. This contrasts with high-income countries such as in the EU where access to over the counter is restricted and there is a ban on growth promotors (The European Parliament and The Council of the European Union, n.d.). These nuances in the target population and the access to antimicrobials make for a complex environment to promote AMS and to implement related interventions. The above findings suggest there is a need to tailor interventions aimed at restricting access to antimicrobials and promoting AMS to the local LMIC settings and to the targeted population and regulatory environment.

The AMR surveillance strategies were primarily at a national level and were reported mainly in high-income countries. AMR surveillance strategies often involve collection and testing of a range of isolates. The capacity of laboratories, along with infrastructure, technology and human resources can be a major limitation of surveillance activities at a national level. Increasing the capacity and capability of these aspects in LMICs may provide the opportunity for models applied in high-income countries to be used more globally (Fall et al., 2019; Jayatilleke, 2020). Understanding of the current resistance patterns in given settings could help focus and tailor resources to where they are most needed to reduce AMR and to guide the type of interventions needed to change AMR levels.

Cost, or perceived cost of testing and monitoring can serve as a barrier to efforts to tackle AMR. This may be more pronounced for farmers and AHPs who depend largely on livestock for their livelihood (Golding et al., 2019). Only one herd health intervention on Flemish pig farms reported financial related data with respect to interventions, specifically increased profit, and production parameters (Rojo-Gimeno et al., 2016; Postma et al., 2017). Most studies included in this review did not include financial calculations. This information is relevant for farmers, AHPs, and other key players, to assess if given interventions are financially feasible. Demonstrating that an intervention is of value or of benefit in terms of financial gain or improvement of other parameters (e.g., herd health, feed conversion), can facilitate evidence-based decision making and may encourage the uptake and implementation of interventions considered of value by AHPs and farmers. Ensuring financial and practical feasibility in real-world situations and reducing barriers to uptake within AHPs and farming communities are therefore useful targets to consider when exploring strategies to reduce AMR in the animal health sector.

The assessment of AMU in surveillance strategies was mainly performed using sales data. A limitation with use of sales data is that it does not directly translate to usage of antimicrobials. Medicine can be used for a different animal group than it was licensed and sold for. Wastage or unused medicine is not accounted for either. This means the actual usage could be vastly different from the calculated usage. Furthermore, milligram per millilitre (mg/mL) differs between antibiotics resulting in a different number of doses per mL. Some interventions focused on reducing AMU addressed this issue by using dose instead. However, there is no universal way of denoting dose

amounts. The European Medicines Agency has defined daily dose based on active substance and administration route based on a mean. Other studies used other dose denominations. These do not account for discrepancies between different drugs and individual doses. Having a universal dose denomination for animal medicines would help make data comparable globally like it is in human medicine (World Health Organization, n.d.).

The findings in the current study should be viewed with limitations in mind. General overview searches across multiple sources (databases and websites of organisation) were performed using defined search terms. Even with such a broad search, articles could be missed. To get an increased sensitivity, all the searches could be performed on a country basis. However, this is not feasible within a reasonable time frame considering all the countries at a global level. The quality of the data has not been evaluated in depth. A light touch review of study design has been performed to ensure some level of quality assessment. The quality of some of the literature is limited as some interventions were based on self-reported information. Self-assessment comes with a social desirability bias (Bradburn et al., 2004). For example, participants reporting that they have changed behaviour as the result of an intervention does not necessary mean that this is the case. People tend to over-report "good behaviour" (Bradburn et al., 2004). Understanding the gap between what is reportedly happening and what is happening in relation to change in AMR is critical for generating reliable outcome measurement in AMR interventions.

In addition to self-assessment and social desirability bias, volunteer bias can be a factor in intervention studies. This is especially plausible in those studies assessing knowledge and/or behaviour change with voluntary participation. Many of the studies addressed this limitation but did not correct for it. It is possible that smaller-scale studies with presumed volunteer bias may have had different outcomes than broader mandatory national/regional interventions (Salkind, 2010).

This scoping review only included interventions that reported change in measured outcomes whether successful or not. It is possible additional unsuccessful or even successful interventions were not being published and therefore not accessed or reviewed. There was a range of study designs and types, with some studies performed in field conditions while others were performed in environments created solely for the intervention. This illustrates the importance of understanding and interpreting intervention outcomes within different settings and contexts.

In conclusion, changes in AMU practices of AHPs and farmers and changes in the development and/or spread of AMR were the most frequent primary outcomes measured in the reviewed studies. Change in uptake and use of AMS, along with change in attitude and knowledge changes were measured less. Small-scale and national-level interventions were more common compared to continental or international interventions. Most interventions were performed in field conditions while some AMR interventions were conducted in experimental conditions. Only 19% of interventions took place in LMICs and were conducted primarily on a small scale. Analysis of the financial aspect of interventions was limited along with an understanding of compliance levels. Self-assessment to measure impact was commonly performed which increases the risk of volunteer bias.

Going forward, a focus on implementing and evaluating interventions in LMICs is warranted to ensure that this underrepresented group is included in the international conversation on AMR. Robust interventions that include objective outcome measures (e.g., measurable outcomes vs. self-reporting) both in LMICs and around the world can increase the understanding of the true impact of AMR interventions. Studies that investigate the benefits and financial implications of interventions are necessary to inform feasibility and the impact of interventions and to encourage uptake of AMR interventions by animal health professionals and farmers.

Author contributions

Conceptualisation, methodology, analysis, and editing: AJ and AE. Data collection, data extraction, synthesis, result reporting, and writing: AJ and AE. Supervision and reviewing: AE and JO. Methodological analysis of intervention studies: AJ and JO. All authors have read and agreed to the submitted version of the manuscript.

Funding

This work was funded through a postgraduate research studentship provided by the University of Surrey.

Acknowledgments

We thank the staff at the University of Surrey library for helping retrieve hard to find articles through intra-library loans.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frabi.2023.1233698/full#supplementary-material

References

Abdelfattah, E. M., Ekong, P. S., Okello, E., Williams, D. R., Karle, B. M., Lehenbauer, T. W., et al. (2022). Factors associated with antimicrobial stewardship practices on california dairies: One year post senate bill 27. *Antibiotics (Basel)* 11 (2), p. 165. doi: 10.3390/ANTIBIOTICS11020165

Agunos, A., Arsenault, R. K., Avery, B. P., Deckert, A. E., Gow, S. P., Janecko, N., et al. (2018). Changes in antimicrobial resistance levels among Escherichia coli, Salmonella, and Campylobacter in Ontario broiler chickens between 2003 and 2015. *Can. J. Veterinary Res.* 82 (3), p.163-177.

Agunos, A., Léger, D. F., Carson, C. A., Gow, S. P., Bosman, A., Irwin, R. J., et al. (2017). Antimicrobial use surveillance in broiler chicken flocks in Canada 2013-2015. *PloS One* 12. doi: 10.1371/JOURNAL.PONE.0179384

AURES (2017). *Resistance Report Austria AURES 2016 Summary* (Vienna: Federal Ministry of Health and Women's Affairs). Available at: www.bmgf.gv.at.

Bahrndorff, S., Rangstrup-Christensen, L., Nordentoft, S., and Hald, B. (2013). Foodborne disease prevention and broiler chickens with reduced campylobacter infection - volume 19, number 3—March 2013 - emerging infectious diseases journal - CDC. *Emerg. Infect. Dis.* 19, 425–430. doi: 10.3201/EID1903.111593

Bailey, M. A., Taylor, R. M., Brar, J. S., Corkran, S. C., Velásquez, C., Novoa Rama, E., et al. (2019). Prevalence and antimicrobial resistance of Campylobacterfrom antibiotic-free broilers during organic and conventional processing. *Poult Sci.* 98, 1447–1454. doi: 10.3382/PS/PEY486

Becker, E., Projahn, M., Burow, E., and Käsbohrer, A. (2021). Are There Effective Intervention Measures in Broiler Production against the ESBL/AmpC Producer Escherichia coli? *Pathogens* 10 (5), 608. doi: 10.3390/PATHOGENS10050608

Becker, J., Schüpbach-Regula, G., Steiner, A., Perreten, V., Wüthrich, D., Hausherr, A., et al. (2020). Effects of the novel concept "outdoor veal calf" on antimicrobial use, mortality and weight gain in Switzerland. *Prev. Vet. Med.* 176, 104907. doi: 10.1016/J.PREVETMED.2020.104907

Bennani, H., Mateus, A., Mays, N., Eastmure, E., Stärk, K. D. C., and Häsler, B. (2020). Overview of evidence of antimicrobial use and antimicrobial resistance in the food chain. *Antibiotics* 9 (2), 49. doi: 10.3390/ANTIBIOTICS9020049

Bradburn, N., Sudman, S., and Wansink, B. (2004). *Asking questions: The definitive guide to questionnaire design–For market research, political polls, and social and health questionnaires* San Francisco: Jossey-Bass.

Bradley, A., Breen, J., Leach, K., Gibbons, J., Armstrong, D., and Green, M. (2017). AHDB dairy mastitis control plan. *Vet. Rec* 180, 154–155. doi: 10.1136/VR.J680

Breen, J. E., Jones, B., Jones, B., Jones, N., Jones, M., and Edwards, R. (2017). "Implementation of the AHDB Dairy Mastitis Control Plan to reduce clinical mastitis rate and antibiotic use in a large dairy herd," in *2017 British Mastitis Conference*, Worcester, UK, 15 November 2017. 31–42.

Brusa, V., Restovich, V., Signorini, M., Pugin, D., Galli, L., Díaz, V. R., et al. (2019). Evaluation of intervention measures at different stages of the production chain in Argentinian exporting abattoirs. *Food Sci. Technol. Int.* (ANSES, Lyon et Ploufragan-Plouzané-Niort, France) 25, 491–496. doi: 10.1177/1082013219836326

Cazeau, G., Collineau, L., Haenni, M., Jarrige, N., Jouy, E., Lupo, A., et al. (2022). Resapath - French surveillance network for antimicrobial resistance in bacteria from diseased animals.

Ceccarelli, D., van Essen-Zandbergen, A., Smid, B., Veldman, K. T., Boender, G. J., Fischer, E. A. J., et al. (2017). Competitive exclusion reduces transmission and excretion of extended-spectrum-β-lactamase-producing Escherichia coli in broilers. *Appl. Environ. Microbiol.* 83 (11). doi: 10.1128/AEM.03439-16

Centers for Disease Control and Prevention (2015) *National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) 2015 Integrated Report*. Available at: https://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialRe (Accessed January 30, 2023).

Chinwe, N., Thomas, A., Ifeanyichukwu, I., Chika, E., Emmanuel, N., Anthonia, O., et al. (2014). Cloacal feacal carriage and occurrence of antibiotic resistant Escherichia coli in chicken grown with and without antibiotic supplemented feed. *J. Veterinary Med. Anim. Health* 6, 91–94. doi: 10.5897/JVMAH2013.0267

Cicconi-Hogan, K. M., Belomestnykh, N., Gamroth, M., Ruegg, P. L., Tikofsky, L., and Schukken, Y. H. (2014). Short communication: Prevalence of methicillin resistance in coagulase-negative staphylococci and Staphylococcus aureus isolated from bulk milk on organic and conventional dairy farms in the United States. *J. Dairy Sci.* 97, 2959–2964. doi: 10.3168/JDS.2013-7523

Collineau, L., Rojo-Gimeno, C., Léger, A., Backhans, A., Loesken, S., Nielsen, E. O., et al. (2017). Herd-specific interventions to reduce antimicrobial usage in pig production without jeopardising technical and economic performance. *Prev. Vet. Med.* 144, 167–178. doi: 10.1016/J.PREVETMED.2017.05.023

Cox, J. A., Vlieghe, E., Mendelson, M., Wertheim, H., Ndegwa, L., Villegas, M. V., et al. (2017). Antibiotic stewardship in low- and middle-income countries: the same but different? *Clin. Microbiol. Infection* 23, 812–818. doi: 10.1016/J.CMI.2017.07.010

Dame-Korevaar, A., Fischer, E. A. J., van der Goot, J., Velkers, F., Ceccarelli, D., Mevius, D., et al. (2020a). Early life supply of competitive exclusion products reduces colonisation of extended spectrum beta-lactamase-producing Escherichia coli in broilers. *Poult Sci.* 99, 4052–4064. doi: 10.1016/J.PSJ.2020.04.025

Dame-Korevaar, A., Kers, J. G., van der Goot, J., Velkers, F. C., Ceccarelli, D., Mevius, D. J., et al. (2020b). Competitive exclusion prevents colonisation and compartmentalisation reduces transmission of ESBL-producing Escherichia coli in broilers. *Front. Microbiol.* 11. doi: 10.3389/FMICB.2020.566619

Danish Veterinary and Food Administration (n.d.) *VetStat*. Available at: https://vetstat.fvst.dk/vetstat/ (Accessed January 27, 2023).

DANMAP (2022) *DANMAP 2021 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark* (Copenhagen). Available at: https://www.danmap.org/reports/2021 (Accessed January 27, 2023).

de Waele, J. J., and Martin-Loeches, I. (2018). Optimal duration of antibiotic treatment in Gram-negative infections. *Curr. Opin. Infect. Dis.* 31, 606–611. doi: 10.1097/QCO.0000000000000491

Dorado-García, A., Dohmen, W., Bos, M. E. H., Verstappen, K. M., Houben, M., Wagenaar, J. A., et al. (2015a). Dose-response relationship between antimicrobial drugs and livestock-associated MRSA in pig farming. *Emerg. Infect. Dis.* 21, 950. doi: 10.3201/EID2106.140706

Dorado-García, A., Graveland, H., Bos, M. E. H., Verstappen, K. M., Van Cleef, B. A. G. L., Kluytmans, J. A. J. W., et al. (2015b). Effects of reducing antimicrobial use and applying a cleaning and disinfection program in veal calf farming: Experiences from an intervention study to control livestock-associated MRSA. *PloS One* 10 (9), e0139536. doi: 10.1371/JOURNAL.PONE.0135826

Dupont, N., Diness, L. H., Fertner, M., Kristensen, C. S., and Stege, H. (2017). Antimicrobial reduction measures applied in Danish pig herds following the introduction of the "Yellow Card" antimicrobial scheme. *Prev. Vet. Med.* 138, 9–16. doi: 10.1016/J.PREVETMED.2016.12.019

EFSA (2022). The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2019–2020. *EFSA J.* 20 (3), e07209. doi: 10.2903/J.EFSA.2022.7209

The European Parliament and The Council of the European Union (2022). *Sales of veterinary antimicrobial agents in 31 European countries in 2021 - Trends from 2010 to 2021 (Twelfth ESVAC report)*. doi: 10.2809/291108

European Parliament Council of the European Union Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC, (2019). *Off. J. Eur. Union.* L4/43. https://eur-lex.europa.eu/eli/reg/2019/6/oj (accessed August 09 2023)

Fall, I. S., Rajatonirina, S., Yahaya, A. A., Zabulon, Y., Nsubuga, P., Nanyunja, M., et al. (2019). Integrated Disease Surveillance and Response (IDSR) strategy: current status, challenges and perspectives for the future in Africa. *BMJ Glob Health* 4, e001427. doi: 10.1136/BMJGH-2019-001427

FAO UNEP WHO and WOAH (2022). *Global Plan of Action on One Health. Towards a more comprehensive One Health, approach to global health threats at the human-animal-environment interface* (Rome: WOAH (World Organisation for Animal Health)). doi: 10.4060/cc2289en

Federal Office of Consumer Protection and Food Safety (2020) *Zoonoses Monitoring 2020 - Summary of Findings and Conclusions* (Braunschweig). Available at: https://www.bvl.bund.de/SharedDocs/Downloads/01_Lebensmittel/04_Zoonosen_Monitoring/Zoonosen_Monitoring_Bericht_2020_en-summary.pdf;jsessionid=09C3B82CC0C06F572D757F22DCC3A995.1_cid372?:blob=publicationFile&v=3 (Accessed January 26, 2023).

Federal Office of Public Health FOPH (2015) *Usage of Antibiotics and Occurrence of Antibiotic Resistance in Bacteria from Humans and Animals in Switzerland* (Bern). Available at: www.bag.admin.ch/star (Accessed January 28, 2023).

Feyes, E. E., Diaz-Campos, D., Mollenkopf, D. F., Horne, R. L., Soltys, R. C., Ballash, G. A., et al. (2021). Implementation of an antimicrobial stewardship program in a veterinary medical teaching institution. *J. Am. Vet. Med. Assoc.* 258, 170–178. doi: 10.2460/JAVMA.258.2.170

Finnish Food Authority (2022). *FINRES-Vet 2021. Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents* (Helsinki: Finnish Food Authority).

Food and Agriculture Organisation of the United Nations *AMR situation analysis tool*. Available at: https://www.fao.org/antimicrobial-resistance/resources/tools/amr-situation-analysis-tool/en/ (Accessed November 22, 2022a).

Food and Agriculture Organisation of the United Nations *FAO Progressive Management Pathway for Antimicrobial Resistance (FAO-PMP-AMR)*. Available at: https://www.fao.org/antimicrobial-resistance/resources/tools/fao-pmp-amr/en/ (Accessed November 22, 2022b).

Food and Agriculture Organization of the United Nations (2020) *ATLASS*. Available at: https://www.fao.org/antimicrobial-resistance/resources/tools/fao-atlass/en/ (Accessed November 22, 2022).

Gerber, M., Dürr, S., and Bodmer, M. (2021). Reducing antimicrobial use by implementing evidence-based, management-related prevention strategies in dairy cows in Switzerland. *Front. Vet. Sci.* 7. doi: 10.3389/FVETS.2020.611682

Golding, S. E., Ogden, J., and Higgins, H. M. (2019). Shared goals, different barriers: A qualitative study of UK veterinarians' and farmers' beliefs about antimicrobial resistance and stewardship. *Front. Vet. Sci.* 6. doi: 10.3389/FVETS.2019.00132/BIBTEX

Gomez, D. E., Arroyo, L. G., Poljak, Z., Viel, L., and Weese, J. S. (2017). Implementation of an algorithm for selection of antimicrobial therapy for diarrhoeic calves: Impact on antimicrobial treatment rates, health and faecal microbiota. *Veterinary J.* 226, 15–25. doi: 10.1016/J.TVJL.2017.06.009

Gomez, D. E., Arroyo, L. G., Renaud, D. L., Viel, L., and Weese, J. S. (2021). A multidisciplinary approach to reduce and refine antimicrobial drugs use for diarrhoea in dairy calves. *Veterinary J.* 274, , 105713. doi: 10.1016/J.TVJL.2021.105713

Gozdzielewska, L., King, C., Flowers, P., Mellor, D., Dunlop, P., and Price, L. (2020). Scoping review of approaches for improving antimicrobial stewardship in livestock farmers and veterinarians. *Prev. Vet. Med.* 180, 105025. doi: 10.1016/j.prevetmed.2020.105025

Ha, L. T. T., RueanghIran, C., Giang, N. T. H., Thuy, D. P., Phu, D. H., Tuan Kiet, B., et al. (2021). Antimicrobial usage surveillance through sales at veterinary drug shops intended for livestock in Vietnam. *Front. Sustain Food Syst.* 5. doi: 10.3389/FSUFS.2021.784500

Hao, X. X., Li, B. M., Wang, C. Y., Zhang, Q., and Cao, W. (2013). Application of slightly acidic electrolyzed water for inactivating microbes in a layer breeding house. *Poult Sci.* 92, 2560–2566. doi: 10.3382/PS.2013-03117

Haskell, K. J., Schriever, S. R., Fonoimoana, K. D., Haws, B., Hair, B. B., Wienclaw, T. M., et al. (2018). Antibiotic resistance is lower in Staphylococcus aureus isolated from antibiotic-free raw meat as compared to conventional raw meat. *PloS One* 13(12), e0206712. doi: 10.1371/JOURNAL.PONE.0206712

Hiki, M., Kawanishi, M., Abo, H., Kojima, A., Koike, R., Hamamoto, S., et al. (2015). Decreased Resistance to Broad-Spectrum Cephalosporin in Escherichia coli from Healthy Broilers at Farms in Japan After Voluntary Withdrawal of Ceftiofur. *Foodborne Pathog. Dis.* 12, 639–643. doi: 10.1089/FPD.2015.1960

INFAAR (2020) *INFAAR-a research platform for accelerating laboratory-based surveillance of antimicrobial resistance in fisheries and aquaculture in India*. Available at: https://amr-review (Accessed January 26, 2023).

Jayatilleke, K. (2020). Challenges in implementing surveillance tools of high-income countries (HICs) in low middle income countries (LMICs). *Curr. Treat Options Infect. Dis.* 12, 191. doi: 10.1007/S40506-020-00229-2

Jensen, V. F., de Knegt, L. V., Andersen, V. D., and Wingstrand, A. (2014). Temporal relationship between decrease in antimicrobial prescription for Danish pigs and the "Yellow Card" legal intervention directed at reduction of antimicrobial use. *Prev. Vet. Med.* 117, 554–564. doi: 10.1016/J.PREVETMED.2014.08.006

JVARM *The Japanese Veterinary Antimicrobial Resistance Monitoring System : 動物医薬品検査所*. Available at: https://www.maff.go.jp/nval/tyosa_kenkyu/taiseiki/monitor/e_index.html (Accessed February 7, 2023).

Kannan, G., Lee, J. H., Kouakou, B., and Terrill, T. H. (2019). Reduction of microbial contamination of goat meat using dietary brown seaweed (Ascophyllum nodosum) supplementation and chlorinated wash. *Can. J. Anim. Sci.* 99, 570–577. doi: 10.1139/CJAS-2018-0156/ASSET/IMAGES/LARGE/CJAS-2018-0156F3.JPEG

Kassem, I. I., Kehinde, O., Kumar, A., and Rajashekara, G. (2017). Antimicrobial-resistant campylobacter in organically and conventionally raised layer chickens. *Foodborne Pathog. Dis.* 14, 29–34. doi: 10.1089/FPD.2016.2161

Kempf, I., Kerouanton, A., Bougeard, S., Nagard, B., Rose, V., Mourand, G., et al. (2017). Campylobacter coli in organic and conventional pig production in France and Sweden: Prevalence and antimicrobial resistance. *Front. Microbiol.* 8. doi: 10.3389/FMICB.2017.00955

Korean Veterinary Antimicrobial Usage and Resistance Monitoring (2022) *National Antimicrobial Usage and Resistance Monitoring - Livestock and fishery*. Available at: https://www.kdca.go.kr/nohas/en/common/main.do (Accessed January 28, 2023).

Kuipers, A., Koops, W. J., and Wemmenhove, H. (2016). Antibiotic use in dairy herds in the Netherlands from 2005 to 2012. *J. Dairy Sci.* 99, 1632–1648. doi: 10.3168/jds.2014-8428

Lam, T. J. G. M., Jansen, J., and Wessels, R. J. (2017). The RESET Mindset Model applied on decreasing antibiotic usage in dairy cattle in the Netherlands. *Ir Vet. J.* 70 (5). doi: 10.1186/s13620-017-0085-x

Lee, H. Y., Loong, S. K., Khoo, J. J., Lim, F. S., Chai, L. C., Suntharalingam, C., et al. (2017). Impact of hygiene intervention practices on microbial load in raw milk. *J. Pure Appl. Microbiol.* 11, 1281–1286. doi: 10.22207/JPAM.11.3.07

Llor, C., and Bjerrum, L. (2014). Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. *Ther. Adv. Drug Saf.* 5, 229. doi: 10.1177/2042098614554919

Loayza-Villa, F., Torres, A., Zhang, L., Trueba, G., De Robles, D., Pampite, Y., et al. (2021). Removal of antimicrobial prophylaxis and its effect on swine carriage of antimicrobial-resistant coliforms. *Sci. Prog.* 104, 1–17. doi: 10.1177/00368504211050279

Luyckx, K. Y., Van Weyenberg, S., Dewulf, J., Herman, L., Zoons, J., Vervaet, E., et al. (2015). On-farm comparisons of different cleaning protocols in broiler houses. *Poult Sci.* 94, 1986–1993. doi: 10.3382/PS/PEV143

Mader, R.behalf of EU-JAMRAI, on, , Bourély, C., Amat, J.-P., Broens, E. M., Busani, L., et al. (2022). Defining the scope of the European Antimicrobial Resistance Surveillance network in Veterinary medicine (EARS-Vet): a bottom-up and One Health approach. *J. Antimicrobial Chemotherapy* 77, 816–826. doi: 10.1093/jac/dkab462

Mader, R., Damborg, P., Amat, J. P., Bengtsson, B., Bourély, C., Broens, E. M., et al. (2021). Building the European Antimicrobial Resistance Surveillance network in veterinary medicine (EARS-Vet). *Eurosurveillance* 26 (4). doi: 10.2807/1560-7917.ES.2021.26.4.2001359

Mahase, E. (2021). Use of critically important antibiotics class has more than doubled on UK pig farms. *BMJ* 373, n1572. doi: 10.1136/BMJ.N1572

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic use in agriculture and its consequential resistance in environmental sources: Potential public health implications. *Molecules : A J. Synthetic Chem. Natural Product Chem.* 23 (4). doi: 10.3390/MOLECULES23040795

MARAN (2021) *Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands*. Available at: https://www.wur.nl/en/Research-Results/Research-Institutes/Bioveterinary-Research/In-the-spotlight/Antibiotic-resistance/MARAN-reports.htm (Accessed January 26, 2023).

Methner, U., Friese, A., and Rösler, U. (2019). Competitive exclusion: A tool to combat extended-spectrum β-lactamase-producing Escherichia coli strains in chickens. *Res. Vet. Sci.* 123, 124–128. doi: 10.1016/J.RVSC.2019.01.003

Millar, N., Aenishaenslin, C., Lardé, H., Roy, J. P., Fourichon, C., Francoz, D., et al. (2022). Evidence of a decrease in sales of antimicrobials of very high importance for humans in dairy herds after a new regulation restricting their use in Quebec, Canada. *Zoonoses Public Health* 69, 370–381. doi: 10.1111/ZPH.12929

Morgans, L. C., Bolt, S., Bruno-McClung, E., van Dijk, L., Escobar, M. P., Buller, H. J., et al. (2021). A participatory, farmer-led approach to changing practices around antimicrobial use on UK farms. *J. Dairy Sci.* 104, 2212–2230. doi: 10.3168/JDS.2020-18874

Moura, P., Sanders, P., Heederik, D., Van Geijlswijk, I. M., and Niza-Ribeiro, J. (2022). Use of a new antimicrobial consumption monitoring system (Vet-AMNet): Application to Dutch dairy sector over a 9-year period. *Front. Vet. Sci.* 9. doi: 10.3389/fvets.2022.984771

Mourand, G., Paboeuf, F., Fleury, M. A., Jouy, E., Bougeard, S., Denamur, E., et al. (2017). Escherichia coli Probiotic Strain ED1a in Pigs Has a Limited Impact on the Gut Carriage of Extended-Spectrum-β-Lactamase-Producing E. coli. *Antimicrob. Agents Chemother.* 61 (1). doi: 10.1128/AAC.01293-16

Mulchandaniid, R., Wangid, Y., Gilbert, M., and Van Boeckelid, T. P. (2023). Global trends in antimicrobial use in food-producing animals: 2020 to 2030. *PloS Global Public Health* 3, e0001305. doi: 10.1371/JOURNAL.PGPH.0001305

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 399, 629–655. doi: 10.1016/S0140-6736(21)02724-0

Musoke, D., Kitutu, F. E., Mugisha, L., Amir, S., Brandish, C., Ikhile, D., et al. (2020). A one health approach to strengthening antimicrobial stewardship in wakiso district, Uganda. *Antibiotics* 9, 1–16. doi: 10.3390/ANTIBIOTICS9110764

Nuotio, L., Schneitz, C., and Nilsson, O. (2013). Effect of competitive exclusion in reducing the occurrence of Escherichia coli producing extended-spectrum β-lactamases in the ceca of broiler chicks. *Poult Sci.* 92, 250–254. doi: 10.3382/PS.2012-02575

O'Neill, J. (2016) *Tackling Drug-Resistant Infections Globally: Final Report And Recommendations The Review On Antimicrobial Resistance Chaired By Jim O'Neill*. Available at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf (Accessed November 16, 2022).

Pedroso, A. A., Hurley-Bacon, A. L., Zedek, A. S., Kwan, T. W., Jordan, A. P. O., Avellaneda, G., et al. (2013). Can probiotics improve the environmental microbiome and resistome of commercial poultry production? *Int. J. Environ. Res. Public Health* 10, 4534–4559. doi: 10.3390/IJERPH10104534

Pempek, J., Masterson, M., Portillo-Gonzalez, R., Creutzinger, K., Cheng, T. Y., and Habing, G. (2022). The impact of antimicrobial stewardship training on calf producers' Knowledge, treatment behaviors and quantified antimicrobial use. *Microorganisms* 10 (8). doi: 10.3390/MICROORGANISMS10081525

Phu, D. H., Van, Cuong, N., Truong, D. B., Kiet, B. T., Hien, V. B., Thu, H. T. V., et al. (2021). Reducing antimicrobial usage in small-scale chicken farms in Vietnam: A 3-year intervention study. *Front. Vet. Sci.* 7. doi: 10.3389/FVETS.2020.612993

Postma, M., Vanderhaeghen, W., Sarrazin, S., Maes, D., and Dewulf, J. (2017). Reducing antimicrobial usage in pig production without jeopardizing production parameters. *Zoonoses Public Health* 64, 63–74. doi: 10.1111/ZPH.12283

Powell, N., Davidson, I., Yelling, P., Collinson, A., Pollard, A., Johnson, L., et al. (2017). Developing a local antimicrobial resistance action plan: the Cornwall One Health Antimicrobial Resistance Group. *J. Antimicrobial Chemotherapy* 72, 2661–2665. doi: 10.1093/JAC/DKX164

Public Health Agency of Sweden and National Veterinary Institute (2022) *Swedres-Svarm 2021. Sales of antibiotics and occurrence of resistance in Sweden* (Solna/Uppsala). Available at: www.folkhalsomyndigheten.sewww.sva.sewww.folkhalsomyndigheten.se/publicerat-material/oratwww.sva.se/swedres-svarm/ (Accessed January 27, 2023).

Raasch, S., Collineau, L., Postma, M., Backhans, A., Sjölund, M., Belloc, C., et al. (2020). Effectiveness of alternative measures to reduce antimicrobial usage in pig production in four European countries. *Porcine Health Manag* 6 (6). doi: 10.1186/S40813-020-0145-6

Rees, G. M., Bard, A., and Reyher, K. K. (2021). Designing a national veterinary prescribing champion programme for welsh veterinary practices: The arwain vet cymru project. *Antibiotics* 10, 1–18. doi: 10.3390/ANTIBIOTICS10030253

Rhouma, M., Soufi, L., Cenatus, S., Archambault, M., and Butaye, P. (2022). Current insights regarding the role of farm animals in the spread of antimicrobial resistance from a one health perspective. *Vet. Sci.* 9 (9), 480. doi: 10.3390/vetsci9090480

Rojo-Gimeno, C., Postma, M., Dewulf, J., Hogeveen, H., Lauwers, L., and Wauters, E. (2016). Farm-economic analysis of reducing antimicrobial use whilst adopting improved management strategies on farrow-to-finish pig farms. *Prev. Vet. Med.* 129, 74–87. doi: 10.1016/J.PREVETMED.2016.05.001

Roulette, C. J., Caudell, M. A., Roulette, J. W., Quinlan, R. J., Quinlan, M. B., Subbiah, M., et al. (2017). A two-month follow-up evaluation testing interventions to limit the emergence and spread of antimicrobial resistant bacteria among Maasai of northern Tanzania. *BMC Infect. Dis.* 17. doi: 10.1186/S12879-017-2857-Z

Rubio-García, M. E., Rubio-Lozano, M. S., Ponce-Alquicira, E., Rosario-Cortes, C., Nava, G. M., and Castañeda-Serrano, M. P. (2015). Improving appearance and microbiologic quality of broiler carcasses with an allostatic modulator. *Poult Sci.* 94, 1957–1963. doi: 10.3382/PS/PEV144

Ruckert, A., Fafard, P., Hindmarch, S., Morris, A., Packer, C., Patrick, D., et al. (2020). Governing antimicrobial resistance: a narrative review of global governance mechanisms. *J. Public Health Policy* 41, 515–528. doi: 10.1057/S41271-020-00248-9

Salkind, N. (2010). *Encyclopedia of Research Design.* (2455 Teller Road, Thousand Oaks California 91320 United States: SAGE Publications, Inc). doi: 10.4135/9781412961288

Sapkota, A. R., Kinney, E. L., George, A., Hulet, R. M., Cruz-Cano, R., Schwab, K. J., et al. (2014). Lower prevalence of antibiotic-resistant Salmonella on large-scale U.S. conventional poultry farms that transitioned to organic practices. *Sci. Total Environ.* 476–477, 387–392. doi: 10.1016/J.SCITOTENV.2013.12.005

Simonsen, G.S., Blix, H.S., Grave, K., Urdahl, A.M., Akselsen, P.E., Andersen, C.T., et al (2022). *NORM/NORM-VET 2021 Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway* (Veterinærinstituttet og Folkehelseinstituttet. 164. NORM/NORM-VET(1) Veterinærinstituttet.

Schreuder, J., Simitopoulou, M., Angastiniotis, K., Ferrari, P., Wolthuis-Fillerup, M., Kefalas, G., et al. (2022). Development and implementation of a risk assessment tool for broiler farm biosecurity and a health intervention plan in the Netherlands, Greece, and Cyprus. *Poult Sci.* 102 (2), 102394. doi: 10.1016/J.PSJ.2022.102394

Seale, A. C., Gordon, N. C., Islam, J., Peacock, S. J., and Scott, J. A. G. (2017). AMR surveillance in low and middle-income settings - A roadmap for participation in the Global Antimicrobial Surveillance System (GLASS). *Wellcome Open Res.* 2 (92). doi: 10.12688/WELLCOMEOPENRES.12527.1

Sharma, G., Mutua, F., Deka, R. P., Shome, R., Bandyopadhyay, S., Shome, B. R., et al. (2022). Comparing the effectiveness of different approaches to raise awareness about antimicrobial resistance in farmers and veterinarians of India. *Front. Public Health* 10. doi: 10.3389/FPUBH.2022.837594

Shen, L., Dyar, O. J., Sun, Q., Wei, X., Yang, D., Sun, C., et al. (2021). The effectiveness of an educational intervention on knowledge, attitudes and reported practices on antibiotic use in humans and pigs: A quasi-experimental study in twelve villages in Shandong Province, China. *Int. J. Environ. Res. Public Health* 18, 1–12. doi: 10.3390/IJERPH18041940

Shen, C., Zhong, L. L., Yang, Y., Doi, Y., Paterson, D. L., Stoesser, N., et al. (2020). Dynamics of mcr-1 prevalence and mcr-1-positive Escherichia coli after the cessation of colistin use as a feed additive for animals in China: a prospective cross-sectional and whole genome sequencing-based molecular epidemiological study. *Lancet Microbe* 1, e34–e43. doi: 10.1016/S2666-5247(20)30005-7

Speksnijder, D. C., Graveland, H., Eijck, I. A. J. M., Schepers, R. W. M., Heederik, D. J. J., Verheij, T. J. M., et al. (2017). Effect of structural animal health planning on antimicrobial use and animal health variables in conventional dairy farming in the Netherlands. *J. Dairy Sci.* 100, 4903–4913. doi: 10.3168/JDS.2016-11924

Spellberg, B., and Rice, L. B. (2019). Duration of antibiotic therapy: Shorter is better. *Ann. Intern. Med.* 171, 210. doi: 10.7326/M19-1509

Stanton, I. C., Bethel, A., Leonard, A. F. C., Gaze, W. H., and Garside, R. (2022). Existing evidence on antibiotic resistance exposure and transmission to humans from the environment: a systematic map. *Environ. Evid* 11, 1–24. doi: 10.1186/S13750-022-00262-2/FIGURES/21

Tang, K. L., Caffrey, N. P., Nóbrega, D. B., Cork, S. C., Ronksley, P. E., Barkema, H. W., et al. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. *Lancet Planet Health* 1, e316–e327. doi: 10.1016/S2542-5196(17)30141-9

Teng, K. T., Aerts, M., Jaspers, S., Ugarte-Ruiz, M., Moreno, M. A., Saez, J. L., et al. (2022). Patterns of antimicrobial resistance in Salmonella isolates from fattening pigs in Spain. *BMC Vet. Res.* 18, 1–17. doi: 10.1186/S12917-022-03377-3/FIGURES/12

Thapaliya, D., Forshey, B. M., Kadariya, J., Quick, M. K., Farina, S., O' Brien, A., et al. (2017). Prevalence and molecular characterisation of Staphylococcus aureus in commercially available meat over a one-year period in Iowa, USA. *Food Microbiol.* 65, 122–129. doi: 10.1016/J.FM.2017.01.015

Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P., and Van Boeckel, T. P. (2020). Global trends in antimicrobial use in food animals from 2017 to 2030. *Antibiotics (Basel)* 9, 1–14. doi: 10.3390/ANTIBIOTICS9120918

Toya, R., Sasaki, Y., Uemura, R., and Sueyoshi, M. (2022). Optimizing antimicrobial use by improving medication adherence among pig producers. *Anim. Sci. J.* 93, e13713. doi: 10.1111/asj.13713

Tricco, A. C., Lillie, E., Zarin, W., O'Brien, K. K., Colquhoun, H., Levac, D., et al. (2018). PRISMA extension for scoping reviews (PRISMA-scR): checklist and explanation. *Ann. Intern. Med.* 169, 467–473. doi: 10.7326/M18-0850

Turner, A., Tisdall, D., Barrett, D. C., Wood, S., Dowsey, A., and Reyher, K. K. (2018). Ceasing the use of the highest priority critically important antimicrobials does not adversely affect production, health or welfare parameters in dairy cows. *Vet. Rec* 183, 67. doi: 10.1136/VR.104702

U.S. Department of Agriculture Animal and Plant Health Inspection Service (2022) *National Animal Health Monitoring System.* Available at: https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/monitoring-and-surveillance/nahms (Accessed January 28, 2023).

U.S. Food and Drug Administration (2022a) *The National Antimicrobial Resistance Monitoring System.* Available at: https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/2019-narms-update-integrated-report-summary-interactive-version (Accessed January 28, 2023).

U.S. Food and Drug Administration (2022b) *Veterinary Laboratory Investigation and Response Network.* Available at: https://www.fda.gov/animal-veterinary/science-research/veterinary-laboratory-investigation-and-response-network (Accessed February 3, 2023).

US Food and Drug Administration (2021). *2020 Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals* US Food and Drug Administration, Silver Springs, MD.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2015). Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5649–5654. doi: 10.1073/PNAS.1503141112/SUPPL_FILE/PNAS.201503141SI.PDF

van Dijk, L., Hayton, A., Main, D. C. J., Booth, A., King, A., Barrett, D. C., et al. (2017). Participatory policy making by dairy producers to reduce anti-Microbial use on farms. *Zoonoses Public Health* 64, 476–484. doi: 10.1111/ZPH.12329

Verrette, L., Fairbrother, J. M., and Boulianne, M. (2019). Effect of cessation of ceftiofur and substitution with lincomycin-spectinomycin on extended-spectrum-β-lactamase/ampC genes and multidrug resistance in escherichia coli from a Canadian broiler production pyramid. *Appl. Environ. Microbiol.* 85 (13). doi: 10.1128/AEM.00037-19

Veterinary Medicines Directorate *Veterinary Antimicrobial Resistance and Sales Surveillance - UK VARSS.* Available at: https://www.gov.uk/government/publications/veterinary-antimicrobial-resistance-and-sales-surveillance-2021 (Accessed January 26, 2023).

Vikram, A., Rovira, P., Agga, G. E., Arthur, T. M., Bosilevac, J. M., Wheeler, T. L., et al. (2017). Impact of "Raised without antibiotics" Beef cattle production practices on occurrences of antimicrobial resistance. *Appl. Environ. Microbiol.* 83. doi: 10.1128/AEM.01682-17

Wanninger, S., Donati, M., Di Francesco, A., Hässig, M., Hoffmann, K., Seth-Smith, H. M. B., et al. (2016). Selective pressure promotes tetracycline resistance of Chlamydia suis in fattening pigs. *PloS One* 11 (11), e0166917. doi: 10.1371/JOURNAL.PONE.0166917

WHO (2022) *Global Database for Tracking Antimicrobial Resistance (AMR) Country Self- Assessment Survey (TrACSS)* (WHO). Available at: https://amrcountryprogress.org/#/visualization-view (Accessed February 3, 2023).

World Health Organization *Defined Daily Dose (DDD).* Available at: https://www.who.int/tools/atc-ddd-toolkit/about-ddd (Accessed March 8, 2023).

World Health Organisation (2016) *Antimicrobial Resistance A manual for Developing National Action Plans* (Geneva). Available at: https://www.who.int/publications/i/item/antimicrobial-resistance-a-manual-for-developing-national-action-plans (Accessed November 25, 2022).

World Health Organization (2018) *Critically Important Antimicrobials for Human Medicine* (Switzerland). Available at: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf (Accessed December 16, 2022).

World Health Organization (2019) *Antimicrobial resistance* (World Health Organisation). Available at: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance (Accessed April 5, 2023).

World Organisation for Animal Health *Veterinary Legislation Support.* Available at: https://www.woah.org/en/what-we-offer/improving-veterinary-services/pvs-pathway/targeted-support/veterinary-legislation-support/ (Accessed November 22, 2022).

World Organisation for Animal Health (2019) *OIE Tool for the Evaluation of the Performance of Veterinary Services* (Paris). Available at: https://www.woah.org/app/uploads/2021/03/2019-pvs-tool-final.pdf (Accessed November 22, 2022).

World Organisation for Animal Health (2021) *OIE Annual Report on Antimicrobial Agents Intended for Use in Animals* (Paris). Available at: https://www.woah.org/app/uploads/2021/05/a-fifth-annual-report-amr.pdf (Accessed November 21, 2022).

Yusuf, H., Idris, S., and Paul, M. (2018). Antimicrobial usage surveillance of cattle in Indonesia to address antimicrobial resistance. *Proceedings of the 1st International Conference Postgraduate School Universitas Airlangga : "Implementation of Climate Change Agreement to Meet Sustainable Development Goals" (ICPSUAS 2017).* doi: 10.2991/icpsuas-17.2018.77

frontiers | Frontiers in Antibiotics

TYPE Original Research
PUBLISHED 10 April 2024
DOI 10.3389/frabi.2024.1367936

OPEN ACCESS

EDITED BY
Kebede Amenu,
Addis Ababa University, Ethiopia

REVIEWED BY
Ashenafi Feyisa Beyi,
Iowa State University, United States
Abid Ali,
Texas A and M University, United States
Ronni Mol Joji,
Arabian Gulf University, Bahrain

*CORRESPONDENCE
Séverine Ferdinand
sferdinand@pasteur-guadeloupe.fr

RECEIVED 09 January 2024
ACCEPTED 25 March 2024
PUBLISHED 10 April 2024

CITATION
Caderhoussin A, Couvin D, Gruel G, Quétel I, Pot M, Arquet R, Dereeper A, Bambou J-C, Talarmin A and Ferdinand S (2024) The fly route of extended-spectrum-β-lactamase-producing *Enterobacteriaceae* dissemination in a cattle farm: from the ecosystem to the molecular scale. *Front. Antibiot.* 3:1367936. doi: 10.3389/frabi.2024.1367936

The fly route of extended-spectrum-β-lactamase-producing *Enterobacteriaceae* dissemination in a cattle farm: from the ecosystem to the molecular scale

Alann Caderhoussin[1], David Couvin[1], Gaëlle Gruel[1], Isaure Quétel[1], Matthieu Pot[1], Rémy Arquet[2], Alexis Dereeper[1], Jean-Christophe Bambou[3], Antoine Talarmin[1] and Séverine Ferdinand[1]*

[1]Transmission, Reservoir and Diversity of Pathogens Unit, Institut Pasteur, Les Abymes, France, [2]Tropical Platform for Animal Experiments, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), Le Moule, France, [3]Tropical Agroecology, Genetics and Livestock Systems Research Unit, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), Petit-Bourg, France

Introduction: This study aimed to understand the origin and to explain the maintenance of extended-spectrum β-lactamase (ESBL) *Enterobacteriaceae* isolated from food-producing animals in a third-generation cephalosporin (3GC)-free farm.

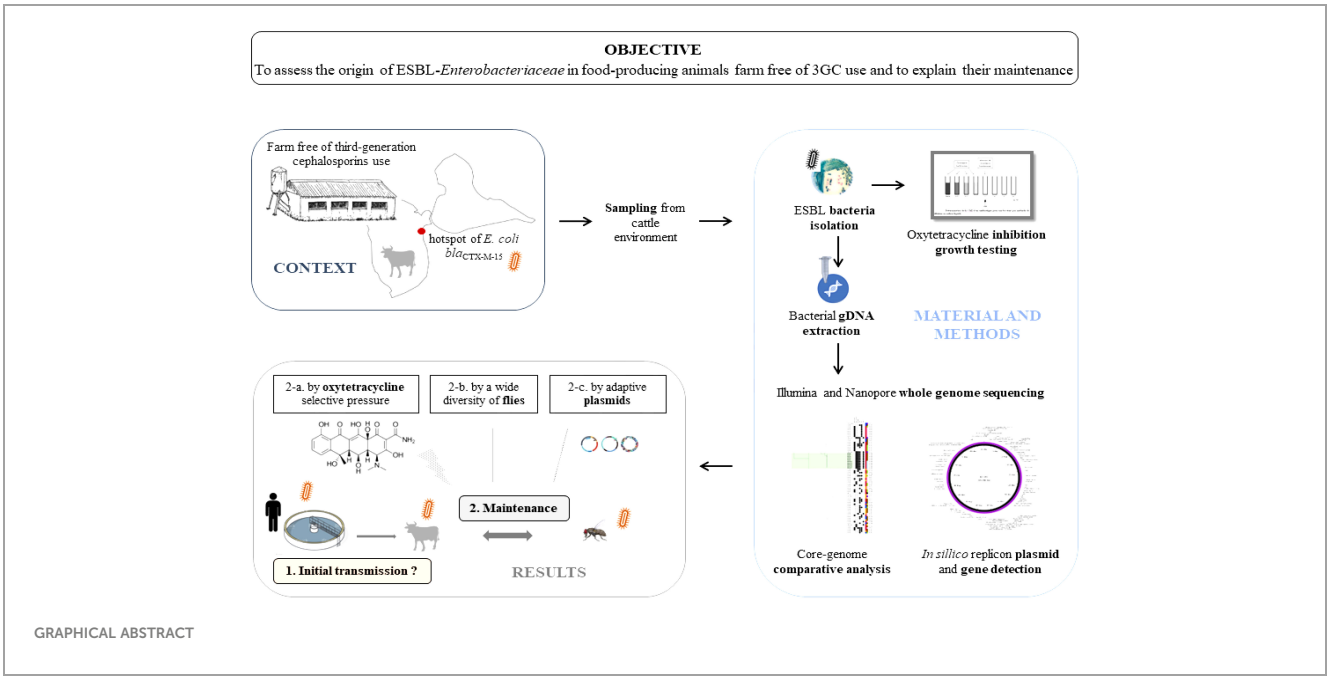
Methods: Culture and molecular approaches were used to test molecules other than 3GC such as antibiotics (tetracycline and oxytetracycline), antiparasitics (ivermectin, flumethrin, fenbendazol, and amitraz), heavy metal [arsenic, HNO_3, aluminum, HNO_3, cadmium ($CdSO_4$), zinc ($ZnCl_2$), copper ($CuSO_4$), iron ($FeCl_3$), and aluminum (Al_2SO_4)], and antioxidant (butylated hydroxytoluene) as sources of selective pressure. Whole-genome sequencing using short read (Illumina™) and long read (Nanopore™) technologies was performed on 34 genomes. *In silico* gene screening and comparative analyses were used to characterize the genetic determinants of resistance, their mobility, and the genomic relatedness among isolates.

Results: Our analysis unveiled a low diversity among the animal ESBL-producing strains. Notably, *E. coli* ST3268 was recurrently isolated from both flies (n = 9) and cattle (n = 5). These *E. coli* ST3268/$bla_{CTX\text{-}M\text{-}15}$/$bla_{TEM\text{-}1B}$ have accumulated multiple plasmids and genes, thereby representing a reservoir of resistance and virulence factors. Our findings suggest that flies could act as effective mechanical vectors for antimicrobial gene transfer and are capable of transporting resistant bacteria across different environments and to multiple hosts, facilitating the spread of pathogenic traits. A significantly higher mean minimum inhibitory concentration of oxytetracycline (841.4 ± 323.5 mg/L vs. 36.0 ± 52.6 mg/L, p = 0.0022) in ESBL *E. coli* than in non-ESBL *E. coli* and $bla_{CTX\text{-}M\text{-}15}$ gene overexpression in oxytetracycline-treated vs. untreated ESBL *E. coli* (RQ_{Oxy} = 3.593, p = 0.024) confirmed oxytetracycline as a source of selective pressure in ESBL *E. coli*.

Discussion: The occurrence of ESBL *E. coli* in a farm without 3GC use is probably due to an as yet undefined human origin of *Enterobacteriaceae* $bla_{CTX\text{-}M\text{-}15}$ gene transmission to animals in close contact with cattle farm workers and the maintenance of the local ESBL *E. coli* reservoir by a high fly diversity and oxytetracycline selective pressure. These findings highlight the critical need for stringent vector control to mitigate antimicrobial resistance spread for preserving public health. Addressing this issue necessitates a multifaceted approach combining microbial genetics, vector ecology, and farm management practices.

KEYWORDS

ESBL, *Enterobacteriaceae*, ST3268, oxytetracycline, selective pressure



GRAPHICAL ABSTRACT

1 Introduction

Antimicrobial resistance (AMR) is currently one of the most important public health problems in the world (O'Neill, 2014). It has dramatically increased morbidity and mortality in both humans and animals (Eurosurveillance editorial team, 2015). The emergence of AMR is mainly due to the selective pressure of antibiotics used in both human and veterinary medicine (Nóbrega and Brocchi, 2014).

Food-producing animals are not only potential reservoirs of AMR but also central conduits through which resistance can be transmitted to humans. This transmission occurs *via* several vectors: the food chain (Antunes et al., 2020), direct contact, environmental contamination through waste (Heuer et al., 2011), and even indirectly through water sources (Juhna et al., 2007; Dierikx et al., 2013). Flies have been suggested to be involved in the dissemination of clones of antimicrobial-resistant bacteria and in the widespread dissemination of plasmids containing antimicrobial resistance genes between farms (Usui et al., 2015). Furthermore, it has been suggested that flies act as reservoirs of antimicrobial-resistant bacteria throughout their life cycle and may therefore be involved in their maintenance and circulation in the farm environment (Fukuda et al., 2019). However, the role of insects such as flies as vectors in the transmission of resistant bacteria within the complex ecosystem of a cattle farm has not been extensively studied. This gap is of particular importance given the ability of flies to bridge diverse ecological niches and move between animal waste, livestock, and human habitations, potentially serving as a critical conduit for pathogen spread. Studies have documented the transfer of extended-spectrum β-lactamase (ESBL) *E. coli* and

genes from animals to farm workers, highlighting the complexity of these pathways (Dahms et al., 2015).

The threat posed by *Enterobacteriaceae* carrying ESBLs is alarming and global, with *E. coli* identified as the predominant species harboring ESBLs across in both humans (Ewers et al., 2012; Dahms et al., 2015) and animals (Dahms et al., 2015; Alonso et al., 2017). The presence of plasmids from distinct Inc groups (Michael et al., 2015) and phylogenetic lineages underscores the ability of these bacteria to spread efficiently and acquire resistance traits (Ewers et al., 2012; Lupo et al., 2018). In addition to antibiotics, other agents used in agriculture such as heavy metals and biocides (Wales and Davies, 2015) may also exert selective pressures that contribute to resistance.

Our study focuses on a cattle farm with a hotspot of ESBL *E. coli* $bla_{\text{CTX-M-15}}$ carriers despite rational antimicrobial use and the absence of 3GC treatments (Gruel et al., 2021). Indeed the proportion of ESBL *E. coli* was significantly higher in this farm than in other farms (47.1% vs. 7,1%, $p = 0.003$). This result was difficult to explain. Furthermore, we demonstrated the role of animal food production systems as a reservoir of mobile genetic elements carrying multiple resistance determinants. However, the origin, spread, and maintenance of resistance were not established, and further studies are warranted to better define the genetic background of ESBL *E. coli* isolates and the context of antibiotic resistance in Guadeloupe, especially in food-producing animals not exposed to third-generation cephalosporins. Mechanisms other than the selective pressure of these antimicrobials in the emergence of antibiotic resistance remain to be elucidated. We investigate the hypothesis that other selective pressures, such as oxytetracycline and environmental factors, may play a role in the persistence of ESBL *Enterobacteriaceae*. Moreover, we explore the potential for human–animal transfer as a source of AMR. This work aims to elucidate the origins and maintenance mechanisms of AMR in cattle, potentially offering insights into mitigation strategies that address these resistance pathways at the ecosystem level.

2 Materials and methods

2.1 Sampling and collection

A total of 16 farms were visited and sampled between February 2018 and November 2019 (Supplementary Data Set S1). We focused our investigations on one farm, number 13, which had the highest rate of ESBL *E. coli* (Gruel et al., 2021). Between February 2018 and May 2019, 74 samples were collected only once at that farm. Fresh fecal samples were randomly collected from cattle living in the stall ($n = 32$) or in the field ($n = 13$) and from stalled goats ($n = 10$) immediately after defecation. We did not actually sample manure or goat feed. Flies that landed around cattle feces ($n = 1$), manure ($n = 1$), or goat breastfeeding food (pool $n = 4$) and adult mosquitoes in unused goat feeders (pool $n = 1$) were trapped using a 6-V mechanical aspirator. The mechanical aspiration technique used allowed the collection of pools of several flies: around cattle feces ($n = 1$) yielded 42 flies, manure ($n = 1$) yielded 81 flies, and goat breastfeeding food ($n = 4$) yielded 34 flies. A total of 157 flies were collected from six samples. Drinking water ($n = 3$) and untreated agricultural water ($n = 2$) were sampled. Wastewater samples ($n = 3$) were collected downstream of the administration building. Cattle feed ($n = 1$), solubilized goat milk ($n = 1$), milk powder ($n = 1$), and pellets ($n = 1$) were collected aseptically. All samples were stored and transported in sterile cups or bags on ice to the laboratory of the Institut Pasteur within 4 h.

2.2 Isolation and identification of bacteria

A 10-µL loop of each fecal sample was mixed in 10 mL of Luria–Bertani (LB) broth (BD Difco™, Humeau, La Chapelle-sur-Erdre, France). Suspensions of pellet, powdered milk, and food were prepared by mixing 30 g in 200 mL of LB. The flies and mosquitoes were crushed manually with a micropestle in 1 mL LB. A volume of 1 mL of wastewater sample was suspended in 10 mL of LB. The water (500 mL) was filtered through a 0.45-µm membrane (Millipore, Guyancourt, France), and the filter was incubated in 10 mL LB with 4 mg/L ceftriaxone for enrichment. The suspensions were supplemented with or without 4 mg/L ceftriaxone and incubated at 37°C for 24 h. Selective enrichments with 4 mg/L ceftriaxone were streaked onto chromogenic coliform agar plates (CHROMagar™, Paris, France) supplemented with 4 mg/L ceftriaxone. Non-selective enrichments were streaked onto chromogenic coliform agar plates without 4 mg/L ceftriaxone. All plates were incubated at 37°C for 24 h. Metallic blue colonies were randomly picked from the non-selective ($n = 1$) and selective ($n = 4$) chromogenic coliform agar, respectively. These isolates were then identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on an Axima high-performance spectrometer (Shimadzu Corp, Osaka, Japan). The susceptibility of all isolates to 17 antimicrobials in six different classes was assessed by the standard disk diffusion method on Mueller–Hinton agar, as previously described (Gruel et al., 2021).

2.3 Measurement of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) values were used to compare the relative resistance levels of ESBL isolates with those of non-ESBL isolates. The MIC was determined using the EUCAST reference broth microdilution method (https://www.eucast.org/publications_and_documents/consultations/). Antibiotics (cefotaxime, ceftriaxone, tetracycline, and oxytetracycline), antiparasitics (ivermectin, flumethrin, fenbendazol, and amitraz), heavy metal [arsenic, HNO_3, aluminum, HNO_3, cadmium ($CdSO_4$), zinc ($ZnCl_2$), copper ($CuSO_4$), iron ($FeCl_3$), aluminum (Al_2SO_4)], and antioxidant (butylated hydroxytoluene) molecules were tested. Serial dilutions were inoculated with a pure bacterial suspension at 0.5 McFarland turbidity within 2 h of preparation. After overnight incubation at 37°C, the optical density at 620 nm (OD_{620}) was measured using a microplate reader (Multiscan™ FC, Thermo Fisher Scientific). The MICs were read as the lowest concentrations that produced no visible growth. *E. coli* ATCC

25922 was used as the control strain. The listed MIC values presented are the mean of three independent experiments.

2.4 Molecular identification of flies

Flies (n = 157) from the sample pool were divided into eight groups based on their morphological characteristics. The taxonomic assignment of the fly species was performed on one fly from each of the eight morphotype groups. DNA was extracted individually from seven morphologically different flies using NucleoSpin® Tissue DNA Extraction Kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. A fragment of the genes encoding cytochrome oxidase I (COI) (710 bp) was amplified in all flies as previously described (Folmer et al., 1994). Amplified PCR products were sequenced (Eurofins, Cologne, Germany) and compared to known COI gene sequences in the GenBank database by multiple sequence alignment using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All matching sequences were submitted to the phylogenetic tree reconstruction pipelines available on the Phylogeny.fr platform (Dereeper et al., 2008). The tree was constructed using the "Advanced" option, which allows the statistical evaluation of branch support values using 100 bootstraps, and plotted using iTOL (Letunic and Bork, 2021) v6.7.4.

2.5 *bla*$_{\text{CTX-M-15}}$ gene expression

To assess the selective advantage of ESBL *E. coli* under oxytetracycline, ivermectin, and copper selective pressure, *bla*$_{\text{CTX-M-15}}$ gene expression was quantified and compared between treated and untreated isolates. The *bla*$_{\text{CTX-M-15}}$ gene expression was determined in 14 ESBL *E. coli* isolates using a two-step RT-qPCR strategy described in detail in Supplementary Material M1. Briefly, bacterial samples were obtained from overnight-cultured ESBL and non-ESBL *E. coli* in Luria–Bertani broth media supplemented or not with oxytetracycline at a subinhibitory concentration. The bacterial density was measured by using a photometer and pelleted to adjust the concentration to 10^8 cells/mL. Total RNA was extracted immediately using the NucleoSpin® RNA isolation kit following the manufacturer's recommendations (Macherey-Nagel). A maximum of 2 µg of RNA was then reverse-transcribed to the corresponding cDNA using the SuperScript™ VILO™ Master Mix (Thermo Fisher Scientific), in a total volume of 20 µl, according to the manufacturer's instructions. cDNA was then used in qPCR using the TaqMan™ Gene Expression Master Mix and thanks to a 7500 Real-Time PCR system (Thermo Fisher Scientific). 16S was the reference gene. For each run, a standard curve was generated in duplicate using a 10-fold serial dilution of a quantification calibrator of untreated *E. coli* cDNA. The $2^{-\Delta\Delta CT}$ algorithm was used to estimate the relative expression level of *bla*$_{\text{CTX-M-15}}$ transcripts for the two populations studied using the RQ application module on the Thermo Fisher Cloud. Each real-time PCR run included the gene expression measurements of the endogenous 16S rRNA gene and the target *bla*$_{\text{CTX-M-15}}$ gene in the corresponding samples.

2.6 Whole-genome and multiplex long read sequencing

A total of 34 genomes of *E. coli* isolates (n = 23) and *Enterobacter cloacae* complex Taxon 4 (n = 11) were obtained from farm number 13. To assess the genomic relatedness and dynamics of ESBL transmission, high-throughput whole-genome sequencing (WGS) of 79 isolates [34 ESBL *Escherichia coli* (n = 23) and *E. cloacae* complex Taxon 4 (n = 11) isolates from farm number 13 and 45 from other farms in Guadeloupe (Gruel et al., 2021)] was performed at the Biomics Platform, C2RT (Institut Pasteur, Paris, France). The preparation of the WGS libraries, the sequencing process, and the detailed analysis are described in Supplementary Material M2. Briefly, libraries were prepared using the Nextera XT kit (Illumina), and sequencing was performed on the NextSeq 500 system (Illumina), generating 35–151-bp paired-end reads for an average depth of coverage of 85-fold (minimum 78-fold, maximum 92-fold). The reads were trimmed and filtered. The genomes were assembled, and final quality was assessed. Annotation of the assembled genomes was performed, and then a core genome was extracted. Maximum likelihood phylogenetic reconstruction was performed and plotted on a tree. *In silico* screening and annotation of replicon plasmid types, antimicrobial resistance, virulence genes, and multilocus sequence typing (MLST) were performed. The same software tools were used to characterize plasmids (Chen et al., 2005; Wirth et al., 2006; Zankari et al., 2012; Carattoli and Hasman, 2020). The phylogenetic tree was constructed as described above. Genomic identification of *Enterobacter* strains was performed using the different approaches described in our previous manuscript (Pot et al., 2022). To fully reconstruct and characterize the major plasmids, 14 *bla*$_{\text{CTX-M-15}}$ ESBL *E. coli* isolates were sequenced using Oxford Nanopore sequencing long-reads technology on a MinION device. The preparation of the MinION libraries, the sequencing procedure, and the detailed analysis are described in Supplementary Material M3. Briefly, libraries were constructed from 1 µg of unfragmented bacterial gDNA following the protocol instructions for native barcoded genomic DNA (using EXP-NBD104, EXP-NBD114, and SQK-LSK109). The final library was loaded onto a R9.4.1 flow cell (FLO-MIN106D) according to the manufacturer's instructions and run on a laptop (MinKNOW Core v3.6.5). Single-flow cell sequencing data from multiplexed barcoded isolates were run on the MinION for 48 h. Base calling of MinION raw signals was performed. Fastq files were extracted and split by barcode. *De novo* genome assembly was performed using a hybrid strategy on combined nanopore long reads and previously available Illumina short reads. The fully resolved assemblies were generated and visualized. Quality control of nanopore data was performed. The plasmids were aligned graphically and annotated. Mobilization module characterization was performed.

3 Results

3.1 ESBL Enterobacteria carriage in wastewater, cattle, and fly species

A total of 12 out of 74 samples (16.2%) were ESBL-positive. Of these, 25 ESBL Enterobacteria were isolated: 14 *E. coli* (Table 1, Figure 1) were isolated mainly from cattle in stalls and from five different fly species (Supplementary Figure S1) collected around goat breastfeeding food and manure. No ESBL isolates were detected elsewhere in the environmental samples from farm number 13. A total of 11 ESBL-producing Enterobacter isolated from wastewater downstream of the administration building were identified as belonging to *E. cloacae* complex Taxon 4 species according to the latest nomenclature (Feng et al., 2021). Their sequence type (ST) was ST598, and they differed from one to 23 single-nucleotide polymorphism (SNPs) (Supplementary Figure S2).

3.2 A reservoir of $bla_{\text{CTX-M-15}}$ ESBL isolates

A total of 25 ESBL Enterobacteriaceae genomes were sequenced from the 12 ESBL-positive samples, and nine additional genomes were provided from ESBL-negative samples. The 25 ESBL genomes from the 12 ESBL-positive samples were distributed as follows: five *E. coli* genomes from two pooled fly samples, nine *E. coli* genomes from nine cattle, and 11 *E. cloacae* genomes from one human wastewater sample. A total of 34 genomes of *E. coli* isolates (n = 23) and *E. cloacae* (n = 11) from farm number 13 were sequenced. Among the ESBL producers (n = 25), most of them carried the $bla_{\text{CTX-M-15}}$ gene (21/25, 84.0%), followed by the $bla_{\text{CTX-M-1}}$ gene (5/25, 20.0%; Table 2). Replicon genes from incompatible FIB group plasmids were found in all ESBL isolates from the three biotopes (25/25, 100.0%). However, there were differences between bacterial species (Figure 1). The IncFIB [F-:A-:B42] and IncFIB [F-:A-:B70] replicon sequence types were found in ESBL *E. coli* and ESBL *E.*

TABLE 1 Description of samples and ESBL enterobacteria collected from the farm environment.

Origin	Sample						Isolate		
n (%)	Total (n = 74)		ESBL + (n = 12)		Total[a] (n = 34)		ESBL + (n = 25)		Taxonomy
Cattle feces									*Escherichia coli*
In stall	32	(43.2)	9	(12.2)	18	(52.9)	9	(26.5)	
In field	13	(17.6)	0	(0.0)	0	(0.0)	0	(0.0)	
Goat feces									*Escherichia coli*
In stabulation	10	(13.5)	0	(0.0)	0	(0.0)	0	(0.0)	
Flies									*Escherichia coli*
Around cattle feces	1	(1.4)	0	(0.0)	0	(0.0)	0	(0.0)	
Around manure	1	(1.4)	1	(1.4)	4	(11.8)	4	(11.8)	
Around goat breastfeeding food	4	(5.4)	1	(1.4)	1	(2.9)	1	(2.9)	
Mosquitoes									*Escherichia coli*
In goat feeder	1	(1.4)	0	(0.0)	0	(0.0)	0	(0.0)	
Water									*Escherichia coli*
Agricultural	2	(2.7)	0	(0.0)	0	(0.0)	0	(0.0)	
Drinking	3	(4.1)	0	(0.0)	0	(0.0)	0	(0.0)	
Milk									*Escherichia coli*
Solubilized	1	(1.4)	0	(0.0)	0	(0.0)	0	(0.0)	
Powder	1	(1.4)	0	(0.0)	0	(0.0)	0	(0.0)	
Food									*Escherichia coli*
Pellets	1	(1.4)	0	(0.0)	0	(0.0)	0	(0.0)	
Grass	1	(1.4)	0	(0.0)	0	(0.0)	0	(0.0)	
Wastewater									*Enterobacter cloacae*
Administration building	3	(4.1)	1	(1.4)	11	(32.4)	11	(32.4)	

ESBL, extended-spectrum β-lactamase producer; +, positive sample or isolate from corresponding sample.
[a]Isolates resistant to at least one of the following antibiotics: ampicillin, streptomycin, nalidixic acid, tetracycline, and trimethoprime-sulfamethoxazol.

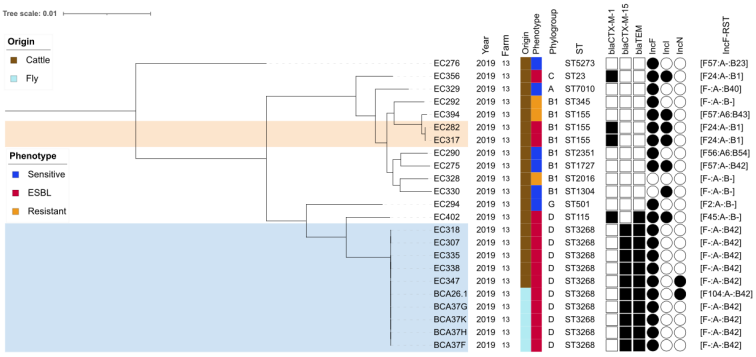


FIGURE 1
Core genome maximum likelihood phylogenetic tree of 23 *E. coli* isolates from farm number 13. Clusters ST3268 and ST155 represent groups of similar core genomes (0 to 25 single-nucleotide polymorphism difference). Hosts and antimicrobial susceptibility phenotypes are indicated by vertical colored stripes. The resistant phenotype is assigned to isolates that are resistant to at least one of the 17 antimicrobials tested. ST, sequenced type based on the Achtman MLST scheme (Wirth et al., 2006). Corresponding β-lactamase-associated resistance-coding genes are indicated by black squares and plasmids by black circles. IncF, plasmid incompatibility group F; RST, replicon typing system.

cloacae complex Taxon 4, respectively. The IncN-pST3 replicon type was found only in cattle and fly ESBL *E. coli* ST3268.

3.3 An ecosystem with a high potential for resistance spread and persistence

Sequence assembly using long reads revealed that the $bla_{\text{CTX-M-15}}$ gene of fly and bovine ESBL *E. coli* and wastewater ESBL *E. cloacae* was carried on the IncFIB [F-:A-:B42] and IncFIB [F-:A-:B70] replicon types, which differed in size and gene composition (Supplementary Figure S3). At the molecular level, plasmid reconstruction allowed the clustered ST3268 isolates to be divided into two new subclusters. Cluster ST3268.1 included the ST3268 isolates EC347 from cattle and BCA26.1 from flies, which simultaneously harbored three major plasmid backbones, and the ST3268.2 isolates (EC307, EC318, EC338 cattle and BCA37F, -G, -H, -K flies; Figure 2), which shared two plasmids with ST3268.1.

The $bla_{\text{CTX-M-15}}$ gene was located in a transposon carried by a non-mobilizing multi-replicative plasmid IncFIB(K)_1_Kpn3_JN233704 (560 bp)/IncFIB(AP001918)_1_AP001918, cointegrated with a truncated IncN_1_AY046276 (85,190 bp), containing many mobile genetic elements (transposons, integrons, and insertion sequences) and several associated resistance genes. The conjugative replicon plasmid (IncN_1_AY046276-pST3, 50,979 bp), absent in ST3268.2, carrying the $bla_{\text{TEM-1B}}$ gene with a cassette of resistance genes and virulence genes involved in the type IV secretion system (T4SS) was carried by ESBL *E. coli* strains common to cattle and flies. The third, a phage plasmid (47,973 bp), contained prophage regions from *Vibrio* and *Bacillus* without resistance genes and a toxin HigB/antitoxin HigA system involved in pathogenicity regulation. The EC335 isolates shared only the IncF 85,190-bp plasmid (not shown in Figure 2) with the other isolates from ST3268. ESBL *E. coli* ST3268-$bla_{\text{CTX-M-15/TEM-1B}}$ was found here in cattle and flies (Figures 1, 2). These results revealed an ESBL *E. coli* ST3268 cluster containing multiple plasmid backbones (Supplementary Figure S3), some of which are mobilizable with multiple associated resistance and virulence genes.

3.4 A first described IncF replicon [F-:A-:B42] in ST3268 ESBL *E. coli*

The collection of ST3268 isolates from other geographical origins found on Enterobase presents only ESBL producers (n = 22) (Supplementary Figure S4). Of these strains, 68.2% were isolated from humans (15/22). This sequence type was identified in many countries and was also found in wild and domestic animals with a $bla_{\text{CTX-M-15}}$ gene. However, it has never been identified in insects, and the IncF replicon [F-:A-:B42] was only identified in farm number 13. No clonal relationship was found between the Guadeloupean isolates and those identified internationally.

TABLE 2 Distribution of $bla_{\text{CTX-M}}$ gene and replicon type in extended-spectrum β-lactamase isolates.

	Total		$bla_{\text{CTX-M-15}}$		$bla_{\text{CTX-M-1}}$		IncFIB		IncN	
n (%)	(n = 25)		(n = 21)		(n = 4)		(n = 25)		(n = 2)	
Cattle	9	(36.0)	5	(20.0)	4	(16.0)	9	(36.0)	1	(4.0)
Fly	5	(20.0)	5	(20.0)	0	(0.0)	5	(20.0)	1	(4.0)
Wastewater	11	(44.0)	11	(44.0)	0	(0.0)	11	(44.0)	0	(0.0)

bla, β-lactamase.

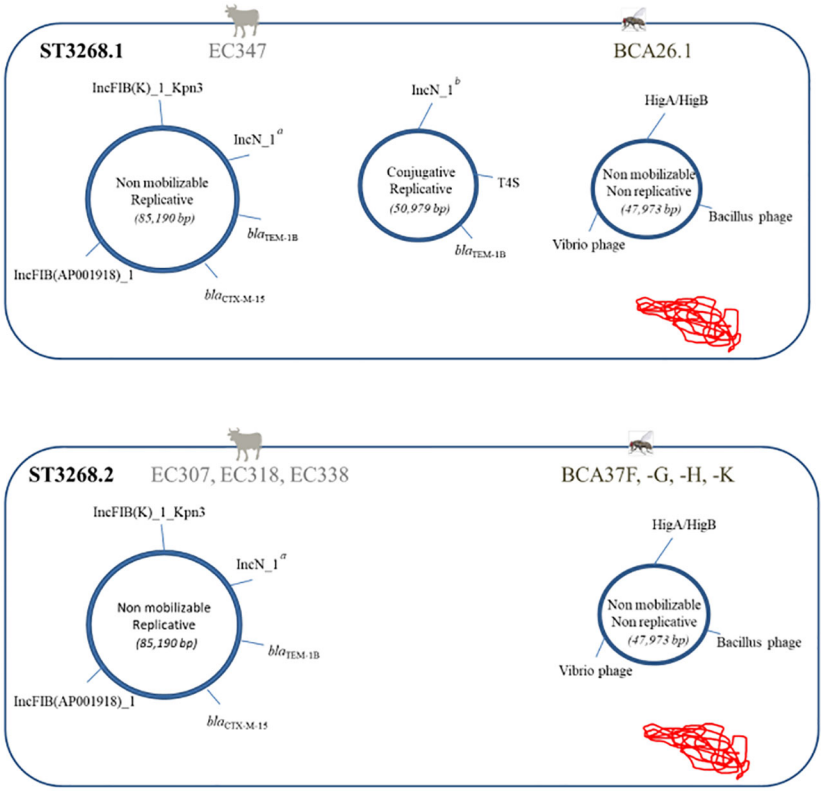


FIGURE 2
Schematic representation showing two combinations of the ultrastructural genetic background of the ST3268.1 ESBL *E. coli* subclusters (n = 7 isolates: BCA37F, -G, -H, -K, EC307, EC318, and EC338) and ST3268.2 (n = 2 isolates: BCA26.1 and EC347). Plasmids are shown as circles annotated for replicon, β-lactamase resistance genes, secretion system (T4S), toxin/antitoxin system (HigA/HigB), and phage-encoded protein genes. Supercoiled chromosomal DNA is schematically shown in red. a, truncated IncN_1_AY046276 replicon (247 bp). b, complete IncN_1_AY046276-pST3 replicon (512 bp).

3.5 Oxytetracycline selective pressure in favor of the emergence of ESBL *E. coli* IncN carriers

We compared the MIC of 17 antiparasitic, antioxidant, antibiotic, and heavy metal compounds in ESBL (n = 14) *vs.* non-ESBL *E. coli* (n = 5) from farm number 13 (Table 3) and in ESBL *E. coli* full IncN carriers (ST3268.1, n = 3) *vs.* non-carriers (ST3268.2, n = 6). Our results showed a significantly higher mean MIC of oxytetracycline (841.4 ± 323.5 mg/L vs. 36.0 ± 52.6 mg/L, p = 0.0022) and arsenic (125.0 ± 0.0 mg/L vs. 78.1 ± 31.3 mg/L, p = 0.0019) in ESBL *E. coli* than in non-ESBL *E. coli*. Cefotaxime and ceftriaxone were used as 3GC-positive controls and confirmed a selective advantage of ESBL *E. coli*. Our results showed a higher tetracycline MIC (256 ± 0.0 mg/L vs. 170.7 ± 73.9 mg/L, p = 0.0325) in ESBL *E. coli* carrying the complete IncN conjugative T4SS replicon plasmid than in non-carriers. For arsenic, copper, and ivermectin, no difference in mean MIC was observed between ESBL *E. coli* complete IncN carriers (ST3268.1) and non-carriers (ST3268.2). In addition to MIC, our results showed a significant $bla_{\text{CTX-M-15}}$ gene overexpression in oxytetracycline-treated vs. untreated ESBL *E. coli* (RQ_{Oxy}=3.593, p = 0.024) (Figure 3). No difference in $bla_{\text{CTX-M-15}}$ gene expression was observed with the ivermectin and copper treatments.

3.6 Acquisition of distinct *E. coli* lineages

To investigate the dynamics of *E. coli* circulation at a regional scale, we considered the 23 *E. coli* isolates from farm number 13 and 45 additional isolates from other farms surveyed in Guadeloupe during the same period. A total of 68 *E. coli* from 16 farms, including six cattle, six pig, and five poultry farms (one farm was a cattle and poultry producer), were included (Figure 4). A total of 29 isolates (42.6%) were grouped into seven clusters with similar core genomes (0 to 25 SNP difference). Four clusters representing (16/68, 23.5%) ESBL *E. coli* isolates were farm specific (10 ST3268 fly and cattle isolates from farm number 13, two ST115 poultry isolates from farm number 18, two ST1630 poultry isolates from farm number 18, and two ST155 cattle isolates from farm number 13), while three clusters (n = 13 ESBL isolates: ST2705, ST2015, and ST115) were from 11 different farms in the three food animal systems (Figure 4). The two largest clusters (ST3268 and ST2015) contained eight to 10 ESBL *E. coli* harboring a $bla_{\text{CTX-M-1}}$ (ST2015) or a $bla_{\text{CTX-M-15}}$ (ST3268) gene. Globally, the population structure of *E. coli* tends to show a higher proportion of unclustered isolates. When clustered, the isolates tend not to be farm specific. These results reflect sporadically acquired isolates from different lineages rather than the active spread of major clones. Cluster ST3268

TABLE 3 Association of antibiotics, antiparasitics, heavy metals, and antioxidants with ESBL phenotype in *E. coli* isolates.

MIC (mg/L)	ESBL *E. coli* (*n* = 14)		Non-ESBL *E. coli* (*n* = 5)		*p*-value
	Mean	(± SD)	Mean	(± SD)	
Antimicrobials					
Cefotaxime	310.9	(± 139.4)	51.6	(± 114.3)	**0.0041**
Ceftriaxone	676.6	(± 276.9)	205.4	(± 457.6)	**0.0214**
Tetracycline	226.5	(± 56.1)	20.4	(± 27.6)	**0.0004**
Oxytetracycline	841.4	(± 323.5)	36.0	(± 52.6)	**0.0022**
Antiparasitics					
Ivermectin	512.0	(± 0.0)	460.8	(± 114.5)	0.0943
Flumethrin	438.9	(± 211.3)	307.2	(± 114.5)	0.1574
Fenbendazol	563.2	(± 264.4)	512.0	(± 443.4)	0.4673
Amitraz	384.0	(± 132.8)	307.2	(± 114.5)	0.2563
Heavy metals					
Arsenic (HNO_3)	125.0	(± 0.0)	78.1	(± 31.3)	**0.0019**
Aluminum (HNO_3)	200.0	(± 64.5)	218.8	(± 62.5)	0.6101
Cadmium ($CdSO_4$)	151.8	(± 53.2)	150.0	(± 55.9)	0.9478
Zinc ($ZnCl_2$)	571.4	(± 181.6)	600.0	(± 223.6)	0.7697
Copper ($CuSO_4$)	2000.0	(± 0.0)	2000.0	(± 0.0)	–
Iron ($FeCl_3$)	4285.7	(± 1069.0)	4800.0	(± 1,788.9)	0.4338
Aluminum (Al_2SO_4)	4000.0	(± 0.0)	4000.0	(± 0.0)	–
Antioxidant					
Butylated hydroxytoluene	329.1	(± 120.0)	256.0	(± 0.0)	0.1904

MIC, minimum inhibitory concentration; ESBL, extended-spectrum β-lactamase.
Mean ± SD or median IQR.
Statistically significant p-values are highlighted in bold

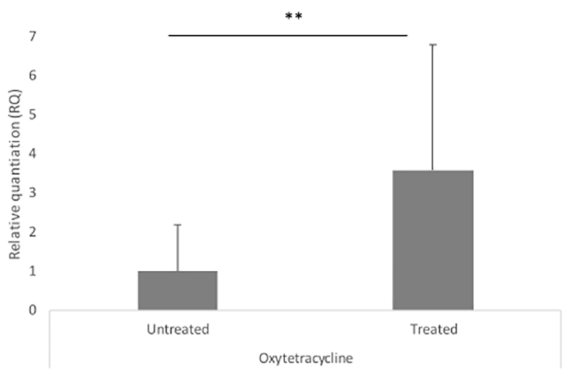


FIGURE 3
Modulation of $bla_{CTX\text{-}M\text{-}15}$ gene expression under *in vitro* selective pressure. Relative quantification of $bla_{CTX\text{-}M\text{-}15}$ gene expression under oxytetracycline treatment in ESBL *E. coli* isolates. Error bars represent the standard deviation of at least two independent experiments. RQ, relative quantification. Error bars indicate the range between RQ min and RQ max. ** Statistically significant p-value < 0.05.

showed a close genomic relationship between 10 CTX-M-15 producing *E. coli* from both fly and cattle sources. These *E. coli* ST3268/$bla_{CTX\text{-}M\text{-}15}$/$bla_{TEM\text{-}1B}$ have accumulated and maintained multiple plasmids and genes, thereby representing an extensive reservoir of resistance and virulence factors. Our results suggest that flies could act as vectors and highlight a clear link between cattle and flies in the spread of CTX-M-15 producing *E. coli*. This underscores the role of flies in increasing the risk of transmission of such resistance factors from livestock to the wider environment. This refined statement underscores the importance of understanding these dynamics in addressing the spread of antibiotic resistance.

4 Discussion

This study investigates the origin of ESBL *E. coli* in a farm of food-producing animals not exposed to third-generation cephalosporins, allowing the identification of a local cattle and fly reservoir of *E. coli* ST3268/$bla_{CTX\text{-}M\text{-}15}$/$bla_{TEM\text{-}1B}$. This ST was not

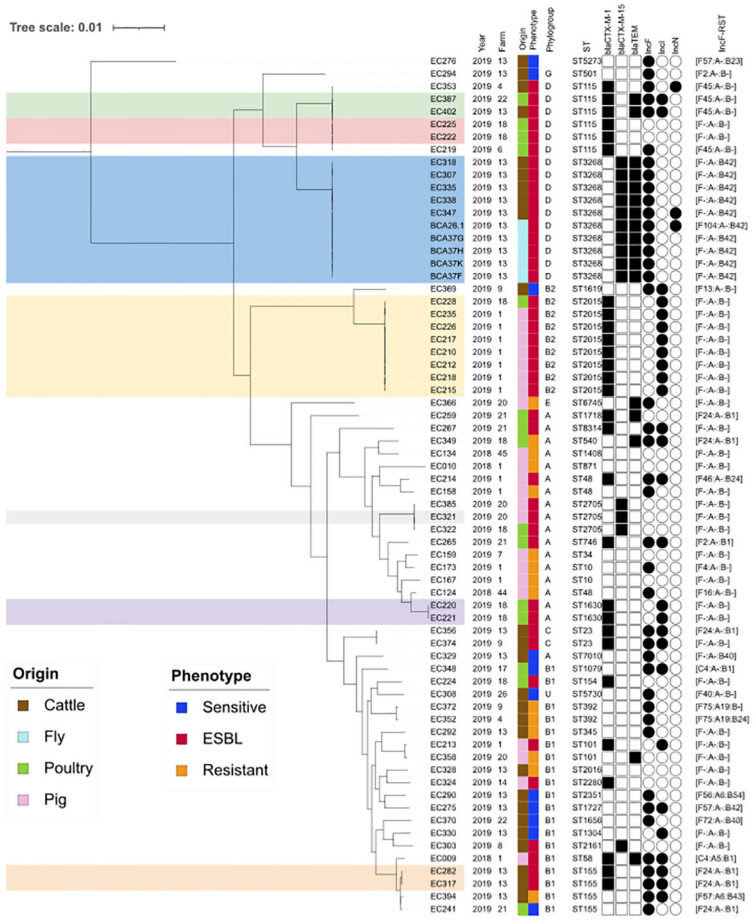


FIGURE 4
Core genome comparative analysis of 68 *E. coli* isolates from food-producing animals and flies. Maximum likelihood phylogenetic tree of 68 *E. coli* isolated from farms in Guadeloupe between 2018 and 2019. The farm number refers to our previous reference number (Gruel et al., 2021). Associated hosts and antimicrobial susceptibility phenotypes are indicated by vertical colored stripes. The resistant phenotype is assigned to isolates that are resistant to at least one of the 17 antimicrobials tested. Of these, 43 were ESBL producers. The colored clusters (ST115, ST3268, ST2015, ST2705, ST1630, and ST155) represent groups of similar core genomes (≤25 SNP). Only β-lactamase-encoding genes are indicated by black squares. Plasmid replicons are indicated by a black circle, and only the IncF RST was detailed. IncF, plasmid incompatibility group F; RST, replicon sequence typing.

found elsewhere in Guadeloupe (Sadikalay et al., 2018; Guyomard-Rabenirina et al., 2020; Gruel et al., 2021, 2022). This ST was also rarely found in genomic databases. However, it has been recovered from different compartments worldwide (Zhou et al., 2020). Its association with the $bla_{\text{CTX-M-15}}$ gene was first identified in humans in France in 2010 (Zamudio et al., 2022). ST3268 ESBL *E. coli* was subsequently described in cattle (Hordijk et al., 2019) and in raccoons (Zhou et al., 2020). Although the reservoirs (flies and cattle) of *E. coli* ST3268/$bla_{\text{CTX-M-15}}$/$bla_{\text{TEM-1B}}$ are limited to one farm and the human compartment still seems to be sporadically affected by this ST, special caution is required as we are facing a new reservoir of an emerging zoonotic *E. coli* ST3268 lineage (Hammerum et al., 2020). The emergence of a novel *E. coli* lineage, ST3268, harboring resistance genes common to both cattle and flies is significant. It suggests that vectors such as flies may play a role in the maintenance and spread of novel and important resistance genes, with potential implications for both animal and human health. This reinforces the need for integrated veterinary and public health surveillance and control strategies. To reduce the risk of flies as vectors, we advocate improved farm hygiene and waste management practices, the use of biosecurity measures such as insect screens and zappers, and further research into environmentally friendly insect control methods.

In our study, *E. coli* ST3268/$bla_{\text{CTX-M-15}}$/$bla_{\text{TEM-1B}}$ isolated from flies and cattle have accumulated multiple plasmids and genes and represent a reservoir of resistance and virulence factors. In all ESBL *E. coli* isolates, the $bla_{\text{CTX-M-15}}$ gene was carried by a non-mobile multireplicon plasmid (2 IncFIB) cointegrating with a truncated IncN_1_AY046276 replicon. ESBL *E. coli* $bla_{\text{CTX-M-15}}$/$bla_{\text{TEM-1B}}$ of the ST38 clonal group has already been found in Japan on unsequenced but transferable IncFIB plasmids (Usui et al., 2013) shared between cattle and flies. However, to the best of our knowledge, our multidrug resistance structure of IncFIB/$bla_{\text{CTX-M-15}}$/bla_{TEM1B} multi-FIB replicon cointegrating IncN has never been described in animal ESBL *E. coli*. The conjugative plasmid IncN-pST3 found in flies and cattle is enriched in resistance and virulence

genes that can spread to humans and cause severe infections that are difficult to treat with current antibiotics. Since the IncF/$bla_{CTX\text{-}M\text{-}15}$ non-mobilized plasmid backbone differed between animal [IncFIB (F-:A-:B42), 85,190 bp] and wastewater [IncFIB (F-:A-:B70), 106,354 bp], the origin of the human $bla_{CTX\text{-}M\text{-}15}$ genes observed in flies and cattle is a consequence of multiple and cumulative origins of ESBL bacteria rather than the active horizontal spread of a single successful clone or plasmid. We investigated here the main sources of ESBL *E. coli* originated from animals, insects, water, feeds, and human wastewater. The ESBL-producing *E. cloacae* Taxon 4 ST598 found in administrative building wastewater was previously found in hospital wastewater in Guadeloupe and also isolated from patients (Pot et al., 2022). These findings highlight the importance of investigating non-animal or non-human reservoirs of antibiotic-resistant bacteria, as they may play a key role in the spread of resistance and may reach humans through various transmission routes. Other possible sources of ESBL *E. coli* include incoming animals, soil (Gelalcha et al., 2022), or wild fauna (Guyomard-Rabenirina et al., 2020) not investigated here. As no ESBL *E. coli* were detected in cattle in the field or in the grass and no manure was applied on soil, our hypotheses did not support a soil source of ESBL *E. coli* (Collis et al., 2022). Incoming animals are not involved in our agroecosystem. Thus, the most alternative source of ESBL *E. coli* on farm number 13 may be from wild fauna, particularly rats, which are very present in farm housing and have already been described as carriers of ESBL *E. coli* (Guyomard-Rabenirina et al., 2020). Due to the hygienic measures taken after our visit, no rat feces were found at farm number 13.

In our study, several ESBL *E. coli* isolates combined broad and narrow host range plasmids (Rozwandowicz et al., 2018). In addition, the plasmids in each group contain different combinations of resistance and virulence genes. Taken together, these results may explain the successful persistence and spread of ESBL isolates and plasmids on the farm and suggest a complex transmission dynamics of resistance and virulence genes, plasmids, and *Enterobacteriaceae* strains. This reflects the spread of multiple persistent ESBL isolate lineages rather than a single epidemic circulating clone. *E. coli* ST3268 was found to host multiple plasmids carried by different fly species. Due to their strong flight capabilities (Nazni et al., 2005), flies appear to be the primary vector for the spread of ESBL isolates in our ecosystem and could act to spread resistance genes (Usui et al., 2015). The current dogma dictates that antimicrobial resistance is associated with a fitness cost. The fitness cost of plasmids in our Enterobacteriaceae has not been evaluated, but we are likely facing contemporary ESBL *E. coli* strains that may be more "fit" and able to persist in the gut with a significant colonization burden despite a lack of antibiotic exposure (Kremer et al., 2022). Indeed some plasmids have evolved to have little effect on host strains (Cottell et al., 2012). Therefore, the persistence of antibiotic resistance genes and their vectors can be expected in the absence of antibiotic selection pressure, regardless of antibiotic stewardship. Other means of reducing plasmid stability are needed to prevent the persistence of these vectors and the antibiotic resistance genes that they carry. Differences in plasmid characteristics between samples highlight the complexity of transmission dynamics. Our study contributes to the understanding of how resistance genes spread, with implications for approaches to monitoring and controlling AMR on farms, and the importance of considering a variety of genetic vehicles in these processes. The coexistence of multiple resistant and mobilizable plasmids has serious implications for both the agricultural ecosystem and public health. It demonstrates the ability of pathogens to evolve rapidly in response to environmental pressures and the need for comprehensive genomic surveillance strategies to monitor and understand this genetic exchange.

The isolation of *E. coli* resistant to the 3GC cefotaxime from cattle with no previous exposure to cefotaxime has recently been reported (Udikovic-Kolic et al., 2014; Mir et al., 2016; Liu et al., 2020). In our study, the occurrence of ESBL isolates in cattle was probably due to the co-selection of multiple resistance genes in the same plasmid by other antibiotics such as oxytetracycline. A metagenomic study of bacterial communities showed that tetracycline resistance is frequently found and transmitted with ESBL-containing plasmids (Tacão et al., 2014). The widespread use of tetracyclines in Guadeloupe (Gruel et al., 2021) may explain some of the discrepancy between the high prevalence of resistance and the moderate use of 3GC. Tetracyclines that are not listed as critical for human treatment may promote resistance to more important molecules. Our results show a selective advantage of ESBL *E. coli* carrying IncN_1_AY046276 over non-carriers under oxytetracycline selective pressure, but this needs to be strengthened by more consistent sampling to increase the robustness of the assays and confirm the trends. These results confirm previous conclusions that the maintenance of plasmids in bacteria, and thus the $bla_{CTX\text{-}M}$ genes, is a contribution of genetic determinants mediating non-β-lactam resistance mechanisms acquired through co-selection (Woodford et al., 2009). In addition, the presence of ESBL *E. coli* in a 3GC-free environment suggests that alternative selective pressures may be at play. It highlights the possibility of other contributing factors, such as the use of different antimicrobials such as oxytetracycline, heavy metal exposure, and non-antibiotic selective agents, which could co-select for resistance. Our work calls for a re-evaluation of the current understanding of AMR transmission and highlights the need to consider a wider range of selective pressures. The identification of oxytetracycline as a potential selective agent for ESBL-producing bacteria highlights the need for comprehensive stewardship that includes all antibiotics, not just those thought to directly select for resistance. It contributes to a more nuanced approach to antibiotic use in agriculture.

While the design of this study primarily focused on investigating the role of the human compartment through the analysis of wastewater, we acknowledge the opportunity to extend our research by exploring ESBL *Enterobacteriaceae* presence among farm workers. Such an extension would not only complement our current findings but also offer a more comprehensive understanding of contamination origins, thereby enhancing the robustness of proposed risk mitigation strategies. Despite this, the integrity and relevance of the results presented remain intact. Future investigations, including longitudinal monitoring of strains among farm workers, are indeed recommended to fill this gap, further strengthening the study's impact on preventing the emergence and spread of ESBL clones in such environments.

5 Conclusion

We demonstrated that the high level of ESBL *E. coli* in a farm without 3GC use is likely due to the maintenance of the local ESBL *E. coli* animal reservoir by a high fly diversity and oxytetracycline pressure. This is the first observation of multiple *E. coli* IncFIB/IncN::$bla_{CTX\text{-}M\text{-}15}$/$bla_{TEM\text{-}1B}$ replicon plasmids clustering in animals. While the likely human origin of this plasmid observed in flies and cattle remains to be clarified, our study highlights the importance of considering environmental factors and antibiotic stewardship in managing antimicrobial resistance. It shows that multiple factors, including the use of specific antibiotics, contribute to the selection of resistance genes, requiring a comprehensive strategy that includes monitoring drug use, regulating potential environmental contributors to AMR, and implementing biosecurity to reduce vector spread. These findings call for a One Health approach that integrates human, animal, and environmental health to inform policy and improve agricultural practices.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject, PRJNA1001660.

Ethics statement

As fecal samples were taken from animals in the field after excretion, "use of live animals for scientific purposes" (within the meaning of the Rural Code, Art R214-87 and following) was not relevant; no invasive procedure was conducted on live animals. Furthermore, the project was considered to be outside the scope of the regulations on animal experimentation by the chairman of the Ethics Committee on animal experimentation of the Antilles and Guyane (registered with the French Ministry of Higher Education, Research and Innovation No. 69). Thus, according to French national law for the protection of animals (No. 2013–118), which reproduces European directive 2010/63/UE on the protection of animals used for experimental and other scientific purposes, no ethics committee approval was deemed necessary according to Article 7.1 on recommendations for animal welfare and Article 7.8 on use of animals in research and education of the World Organization for Animal Health Terrestrial Animal Health Code, used in France. The entity responsible for the animals was the owners. Sampling of animals feces was authorized verbally by the owners, who are responsible for the animals. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AC: Data curation, Formal Analysis, Investigation, Writing – review & editing, Validation. DC: Writing – review & editing, Data curation, Formal Analysis, Software, Validation, Resources. GG: Data curation, Writing – review & editing, Investigation. IQ: Data curation, Writing – review & editing, Formal Analysis, Software, Visualization. MP: Formal Analysis, Visualization, Writing – review & editing, Validation, Investigation. RA: Writing – review & editing, Investigation, Resources. AD: Resources, Writing – review & editing, Software, Validation, Data curation, Formal Analysis. J-CB: Writing – review & editing, Investigation, Methodology. AT: Writing – review & editing, Funding acquisition. SF: Project administration, Validation, Writing – review & editing, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Supervision, Writing – original draft, Visualization.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the European Union on the Guadeloupe Region under the European Research and Development Fund (ERDF) 2014–2020 program (2018-FED-1084). GG is funded by a PhD fellowship from the Guadeloupe Region. The funders had no role in the study design, data collection, analysis, and interpretation, or writing of the manuscript.

Acknowledgments

We would like to thank all the technicians and students who participated in this project at the Institut Pasteur de la Guadeloupe. The authors would like to thank Ludovic Arthein and Alain Farant (PTEA, Guadeloupe) for their help with sampling during the field work on the farm. We thank Raphaël Pasquier (C3i, Université des Antilles, Guadeloupe) for his useful contribution to the development of the scripts. We thank the team of the Biomics Platform, C2RT, C. Baum, L. Lemée (Institut Pasteur, Paris, France), supported by France Génomique (ANR-10-INBS-09) and IBISA.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frabi.2024.1367936/full#supplementary-material

References

Alonso, C. A., Zarazaga, M., Ben Sallem, R., Jouini, A., Ben Slama, K., and Torres, C. (2017). Antibiotic resistance in *Escherichia coli* in husbandry animals: the African perspective. *Lett. Appl. Microbiol.* 64, 318–334. doi: 10.1111/lam.12724

Antunes, P., Novais, C., and Peixe, L. (2020). Food-to-humans bacterial transmission. *Microbiol. Spectr* 8, 2–26. doi: 10.1128/microbiolspec.MTBP-0019-2016

Carattoli, A., and Hasman, H. (2020). PlasmidFinder and in silico pMLST: identification and typing of plasmid replicons in whole-genome sequencing (WGS). *Methods Mol. Biol.* 2075, 285–294. doi: 10.1007/978-1-4939-9877-7_20

Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y., et al. (2005). VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res.* 33, D325–D328. doi: 10.1093/nar/gki008

Collis, R. M., Biggs, P. J., Burgess, S. A., Midwinter, A. C., Brightwell, G., and Cookson, A. L. (2022). Prevalence and distribution of extended-spectrum β-lactamase and AmpC-producing *Escherichia coli* in two New Zealand dairy farm environments. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.960748

Cottell, J. L., Webber, M. A., and Piddock, L. J. V. (2012). Persistence of transferable extended-spectrum-β-lactamase resistance in the absence of antibiotic pressure. *Antimicrob. Agents Chemother.* 56, 4703–4706. doi: 10.1128/AAC.00848-12

Dahms, C., Hübner, N.-O., Kossow, A., Mellmann, A., Dittmann, K., and Kramer, A. (2015). Occurrence of ESBL-producing *escherichia coli* in livestock and farm workers in mecklenburg-western pomerania, Germany. *PloS One* 10, 1–13. doi: 10.1371/journal.pone.0143326

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469. doi: 10.1093/nar/gkn180

Dierikx, C. M., van der Goot, J. A., Smith, H. E., Kant, A., and Mevius, D. J. (2013). Presence of ESBL/ampC -producing *escherichia coli* in the broiler production pyramid: A descriptive study. *PloS One* 8, e79005. doi: 10.1371/journal.pone.0079005

Eurosurveillance editorial team (2015). WHO member states adopt global action plan on antimicrobial resistance. *Euro Surveill* 20, 1. doi: 10.2807/ese.20.21.21137-en

Ewers, C., Bethe, A., Semmler, T., Guenther, S., and Wieler, L. H. (2012). Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin. Microbiol. Infect.* 18, 646–655. doi: 10.1111/j.1469-0691.2012.03850.x

Feng, Y., Hu, Y., and Zong, Z. (2021). Reexamining the association of ampC variants with enterobacter species in the context of updated taxonomy. *Antimicrob. Agents Chemother.* 65, e0159621. doi: 10.1128/AAC.01596-21

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3, 294–299.

Fukuda, A., Usui, M., Okamura, M., Dong-Liang, H., and Tamura, Y. (2019). Role of flies in the maintenance of antimicrobial resistance in farm environments. *Microb. Drug Resist.* 25, 127–132. doi: 10.1089/mdr.2017.0371

Gelalcha, B. D., Ensermu, D. B., Agga, G. E., Vancuren, M., Gillespie, B. E., D'Souza, D. H., et al. (2022). Prevalence of antimicrobial resistant and extended-spectrum beta-lactamase-producing *escherichia coli* in dairy cattle farms in east tennessee. *Foodborne Pathog. Dis.* 19, 408–416. doi: 10.1089/fpd.2021.0101

Gruel, G., Couvin, D., Guyomard-Rabenirina, S., Arlet, G., Bambou, J.-C., Pot, M., et al. (2022). High Prevalence of *bla* (CTXM-1)/IncI1-Iγ/ST3 Plasmids in Extended-Spectrum β-Lactamase-Producing *Escherichia coli* Isolates Collected From Domestic Animals in Guadeloupe (French West Indies). *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.882422

Gruel, G., Sellin, A., Riveiro, H., Pot, M., Breurec, S., Guyomard-Rabenirina, S., et al. (2021). Antimicrobial use and resistance in *Escherichia coli* from healthy food-producing animals in Guadeloupe. *BMC Vet. Res.* 17, 116. doi: 10.1186/s12917-021-02810-3

Guyomard-Rabenirina, S., Reynaud, Y., Pot, M., Albina, E., Couvin, D., Ducat, C., et al. (2020). Antimicrobial resistance in wildlife in Guadeloupe (French west indies): distribution of a single blaCTX–M–1/incI1/ST3 plasmid among humans and wild animals. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.01524

Hammerum, A. M., Porsbo, L. J., Hansen, F., Roer, L., Kaya, H., Henius, A., et al. (2020). Surveillance of OXA-244-producing *Escherichia coli* and epidemiologic investigation of cases, Denmark, January 2016 to August 2019. *Eurosurveillance* 25, 1–9. doi: 10.2807/1560-7917.ES.2020.25.18.1900742

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14, 236–243. doi: 10.1016/j.mib.2011.04.009

Hordijk, J., Fischer, E. A. J., van Werven, T., Sietsma, S., Van Gompel, L., Timmerman, A. J., et al. (2019). Dynamics of faecal shedding of ESBL- or AmpC-producing *Escherichia coli* on dairy farms. *J. Antimicrob. Chemother.* 74, 1531–1538. doi: 10.1093/jac/dkz035

Juhna, T., Birzniece, D., Larsson, S., Zulenkovs, D., Sharipo, A., Azevedo, N. F., et al. (2007). Detection of *Escherichia coli* in biofilms from pipe samples and coupons in drinking water distribution networks. *Appl. Environ. Microbiol.* 73, 7456–7464. doi: 10.1128/AEM.00845-07

Kremer, A., Whitmer, G., Diaz, A., Sajwani, A., Navarro, A., and Arshad, M. (2022). ESBL *escherichia coli* isolates have enhanced gut colonization capacity compared to non-ESBL strains in neonatal mice. *Microbiol. Spectr* 10, e0058222. doi: 10.1128/spectrum.00582-22

Letunic, I., and Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. doi: 10.1093/nar/gkab301

Liu, Y., Dyall-Smith, M., Marenda, M., Hu, H.-W., Browning, G., and Billman-Jacobe, H. (2020). Antibiotic resistance genes in antibiotic-free chicken farms. *Antibiotics* 9, 1–12. doi: 10.3390/antibiotics9030120

Lupo, A., Saras, E., Madec, J. Y., and Haenni, M. (2018). Emergence of *bla*CTX-M-55 associated with *fos*A, *rmt*B and *mcr* gene variants in *Escherichia coli* from various animal species in France. *J. Antimicrob. Chemother.* 73, 867–872. doi: 10.1093/jac/dkx489

Michael, G. B., Freitag, C., Wendlandt, S., Eidam, C., Feßler, A. T., Lopes, G. V., et al. (2015). Emerging issues in antimicrobial resistance of bacteria from food-producing animals. *Future Microbiol.* 10, 427–443. doi: 10.2217/fmb.14.93

Mir, R. A., Weppelmann, T. A., Johnson, J. A., Archer, D., Morris, J. G. J., and Jeong, K. C. (2016). Identification and characterization of cefotaxime resistant bacteria in beef cattle. *PloS One* 11, e0163279. doi: 10.1371/journal.pone.0163279

Nazni, W. A., Luke, H., Wan Rozita, W. M., Abdullah, A. G., Sa'diyah, I., Azahari, A. H., et al. (2005). Determination of the flight range and dispersal of the house fly, *Musca domestica* (L.) using mark release recapture technique. *Trop. Biomed.* 22, 53–61.

Nóbrega, D. B., and Brocchi, M. (2014). An overview of extended-spectrum beta-lactamases in veterinary medicine and their public health consequences. *J. Infect. Dev. Ctries* 8, 954–960. doi: 10.3855/jidc.4704

O'Neill, J. (2014). Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist.* 1–20. Available at: https://wellcomecollection.org/works/rdpck35v.

Pot, M., Reynaud, Y., Couvin, D., Dereeper, A., Ferdinand, S., Bastian, S., et al. (2022). Emergence of a Novel Lineage and Wide Spread of a *bla*(CTX-M-15)/IncHI2/ST1 Plasmid among Nosocomial Enterobacter in Guadeloupe. *Antibiot (Basel Switzerland)* 11, 1–20. doi: 10.3390/antibiotics11101443

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., et al. (2018). Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 73, 1121–1137. doi: 10.1093/jac/dkx488

Sadikalay, S., Reynaud, Y., Guyomard-Rabenirina, S., Falord, M., Ducat, C., Fabre, L., et al. (2018). High genetic diversity of extended-spectrum β-lactamases producing *Escherichia coli* in feces of horses. *Vet. Microbiol.* 219, 117–122. doi: 10.1016/j.vetmic.2018.04.016

Tacão, M., Moura, A., Correia, A., and Henriques, I. (2014). Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. *Water Res.* 48, 100–107. doi: 10.1016/j.watres.2013.09.021

Udikovic-Kolic, N., Wichmann, F., Broderick, N. A., and Handelsman, J. (2014). Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc. Natl. Acad. Sci. U S A* 111, 15202–15207. doi: 10.1073/pnas.1409836111

Usui, M., Iwasa, T., Fukuda, A., Sato, T., Okubo, T., and Tamura, Y. (2013). The role of flies in spreading the extended-spectrum β-lactamase gene from cattle. *Microb. Drug Resist.* 19, 415–420. doi: 10.1089/mdr.2012.0251

Usui, M., Shirakawa, T., Fukuda, A., and Tamura, Y. (2015). The role of flies in disseminating plasmids with antimicrobial-resistance genes between farms. *Microb. Drug Resist.* 21, 562–569. doi: 10.1089/mdr.2015.0033

Wales, A. D., and Davies, R. H. (2015). Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. *Antibiotics* 4, 567–604. doi: 10.3390/antibiotics4040567

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L. H., et al. (2006). Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol. Microbiol.* 60, 1136–1151. doi: 10.1111/j.1365-2958.2006.05172.x

Woodford, N., Carattoli, A., Karisik, E., Underwood, A., Ellington, M. J., and Livermore, D. M. (2009). Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. *Antimicrob. Agents Chemother.* 53, 4472–4482. doi: 10.1128/AAC.00688-09

Zamudio, R., Boerlin, P., Beyrouthy, R., Madec, J.-Y., Schwarz, S., Mulvey, M. R., et al. (2022). Dynamics of extended-spectrum cephalosporin resistance genes in *Escherichia coli* from Europe and North America. *Nat. Commun.* 13, 7490. doi: 10.1038/s41467-022-34970-7

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhou, Z., Alikhan, N.-F., Mohamed, K., Fan, Y., and Achtman, M. (2020). The EnteroBase user's guide, with case studies on Salmonella transmissions, *Yersinia pestis* phylogeny, and Escherichia core genomic diversity. *Genome Res.* 30, 138–152. doi: 10.1101/gr.251678.119

Frontiers in Antibiotics

Addresses the evolving challenges of antibiotic development and resistance

Provides a forum to explore solutions to antibiotic resistance, development and delivery to improve the health of the global population.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact