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Insights in veterinary epidemiology and economics 2023

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Insights in veterinary epidemiology and economics: 2023

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Editorial: Insights in veterinary epidemiology and economics: 2023

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Editorial on the Research Topic
Insights in veterinary epidemiology and economics: 2023

The field of veterinary epidemiology and economics continues to evolve, driven by a growing need to protect animal health, support sustainable food systems, and address emerging public health challenges in the face of climate change and global travel and trade (1, 2). This Research Topic brings together a collection of studies that highlight both recent advancements and ongoing challenges within the discipline. The studies demonstrate the importance of data-driven approaches, transdisciplinary collaboration particularly in a One Health context, and both refinement of existing tools and the development of innovative methodologies to address global animal health concerns.

Water quality remains an essential factor influencing livestock health and productivity. By conducting an integrated approach using aerobic mesophilic counts, pathogen isolation, and antimicrobial resistance testing to investigate the microbial quality of poultry drinking water on farms in Austria, Mustedanagic et al. revealed persistent contamination despite various water line treatments. Their findings underscore the complexity of eliminating opportunistic pathogens, such as *Pseudomonas* spp., and highlight the risks posed to both poultry and farm personnel. This research serves as a reminder of the critical need for effective water management strategies to mitigate antimicrobial resistance (AMR) and ensure poultry welfare and demonstrates how existing methods can be combined to provide additional insights to ongoing challenges.

The global challenge of AMR in both human and veterinary medicine was also a focus of a study by Marco-Fuertes et al. of non-traditional small companion mammals in Spain, which highlighted the role these animals may play as reservoirs for AMR *Staphylococci*. The findings, which show alarming levels of multidrug resistance, underscore the importance of a One Health approach to address the interconnected health of humans, animals, and the environment. Vigilant monitoring and interdisciplinary collaboration are essential to curtail the spread of resistant pathogens.

Planned investments in animal health are foundational to sustainable agricultural practices. Schrobback et al. explored on-farm investments into dairy cow health across 15 countries, offering insights into the allocation of resources for veterinary care and medicine. The study demonstrated that while direct health expenditures represent a modest portion of total production costs, their impact on overall productivity and animal welfare is significant. This research demonstrates how economic analyses are critical in directing benefits in animal health and provides valuable benchmarks for policymakers and farm managers aiming to optimize health investments in dairy production systems worldwide. In addition to ongoing health management, planning responses to infectious disease incursions and the fight against transboundary diseases such as foot-and-mouth disease (FMD) remain a priority in veterinary epidemiology. The theme of evidence-based decision making is continued in the Research Topic in a study by Cardenas et al., in which FMD spread was simulated in Brazil to evaluate control measures. This study demonstrated the use of a multi-host stochastic multilevel model and the value of real-world data to determine the effectiveness of rapid response strategies, including vaccination and depopulation. The findings provide critical insights for policymakers on managing FMD outbreaks and highlight the importance of preparedness and scalable interventions.

Expanding health planning to wildlife populations is another innovation highlighted in this Research Topic. In a perspective about utilizing livestock health planning models for wildlife management, Patterson identifies how structured health planning approaches that are commonly used in livestock management could be adapted to structured health plans for conservation and ecosystem health. While challenges remain in adapting such frameworks to wild populations, the perspective presents a compelling case for applying evidence-based planning to wildlife health initiatives to inform decision making around resource allocation and intervention implementation, to enhance wildlife health management, conservation and public health benefits through reduced opportunity for pathogen transmission.

Causal inference is a cornerstone of epidemiology, and its importance is highlighted through contributions that address its principles and the critical process of variable selection in veterinary epidemiology (Ruple et al.; Sargeant et al.). Sargeant et al. underscore the necessity of embracing causal inference by advocating for the articulation of clear hypotheses and the meticulous selection of confounding variables to ensure validity and reproducibility. Ruple et al. call for rigorous methodologies in exposure variable selection and validation to minimize measurement errors; when proxy variables are necessary, they should be thoughtfully chosen and transparently reported so evidence informing health policies and interventions is reliable and contributes to improved population health outcomes. Together, these studies highlight the need for adoption of more rigorous frameworks for observational research that would align veterinary epidemiological practices with the methodological rigor observed in other branches epidemiology.

Lastly, effective communication between researchers and stakeholders is crucial for translating scientific findings into actionable policies. Renter et al. advocate for a stakeholder-driven approach to promote the alignment of study outcomes with the practical needs of end-users and thus enhance the utility and impact of scientific findings. By using a case example in which interventions for bovine respiratory disease were evaluated, they highlight the need to considering multiple outcomes—such as antimicrobial use, animal welfare, and economic factors—to meet stakeholder decision-making requirements. Whether the beneficiaries are livestock owners, pet guardians, or public health officials, research outcomes must be relevant and reliable to inform decision-making processes effectively.

Collectively, these contributions underscore the dynamic and multifaceted nature of veterinary epidemiology and economics. They address critical contemporary challenges including antimicrobial resistance, the spread of transboundary diseases, methodological rigor in research, and effective stakeholder engagement. By offering new insights and practical solutions, this Research Topic advances the field and contributes to the broader objective of safeguarding animal and public health in an increasingly interconnected global landscape. Looking ahead, it is evident that collaboration, innovation, and a commitment to scientific rigor will be indispensable in tackling the complex challenges facing veterinary epidemiology and economics.

Author contributions

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On-farm investments into dairy cow health: evidence from 15 case study countries

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Managing investments in dairy cow health at a national and global scale, requires an improved understanding of current on-farm expenses for cow health (e.g., expenditure for medicine and veterinary consultations). The aim of this study was to assess on-farm health investments for typical dairy farms in 15 case study countries, including Argentina, Australia, Bangladesh, Brazil, Canada, India, China, Colombia, Indonesia, Kenya, New Zealand, Uganda, UK, Uruguay, and USA. The study was conducted using a descriptive analysis of a secondary data set that was obtained from the International Farm Comparison Network (IFCN). The results suggest that health expenditures take up a relatively small proportion (<10%) of the annual total production costs per cow across all countries in the sample. The means of production costs (e.g., feed, machinery) can take up to 90% of the total production costs for highly intensive systems, while these costs can be as low as 9% for extensive systems. This study highlights the importance of understanding on-farm animal health investments as a contribution to improved national and global decision making about animal health in the dairy sector.

KEYWORDS

dairy, costs, cow, disease, health, investment, livestock, production

1. Introduction

The global dairy sector is an important source of protein and other nutrients that contribute to ensuring food security and nutrition, and also provides income generation opportunities for rural communities worldwide (1). Yet, the global dairy sector is experiencing a range of pressures (e.g., climate change impacting feed availability, habitat shifts and heat stress; changing consumer expectations and shifts); with the prevention, treatment, and management of diseases being one of the key challenges (2, 3).

The health of dairy cattle can impact their productivity, production profitability, zoonotic risks, international trade (e.g., biosecurity risk associated with transboundary infectious diseases), and animal welfare (4). To improve the sustainability of dairy cattle production, national and on-farm investments in dairy cattle health (e.g., biosecurity regulations and enforcement, vaccine and medicine application, herd health monitoring) are vital to prevent diseases and to manage them effectively if they occur (5).

The literature offers a range of studies which focus on the on-farm costs or expenditures for managing specific diseases in dairy cattle (e.g., lameness, mastitis, metritis, retained placenta, left-displaced abomasum, ketosis, and hypocalcemia) in selected countries [e.g., (6–10)]. There

are also studies that assess the economic impact of specific dairy cattle diseases, for example, Johne's disease (11, 12), mastitis (13), or food and mouth disease (14–16). However, there is not - to the best of our knowledge - a proper understanding about farm-scale health expenditures for dairy cattle. This need has also been identified by Perry et al. (17).

The aim of this study was to address this gap by assessing on-farm health costs for dairy cows in 15 case study countries representing a diversity of dairy production systems, including a comparison to other production costs (e.g., feed, labor), milk yields and animal losses, and its variations among different countries.

Information generated in this study is to be considered as a proof of concept, emphasizing the value of systematically collected production and animal health data at farm scale. The findings may be useful for intergovernmental organizations, national governments, dairy industry associations, and veterinarians to collaboratively address the data gaps around global farmed animal health. Insights into global on-farm animal health investments are also of interest for the Global Burden of Animal Disease (GBADs) program,[1] as a component of the animal health loss envelope (18) which provides a baseline for assessments of the costs and benefits of investments in improved animal health to global society.

2. Materials and methods

2.1. Data material

To gain an improved understanding of current global investments into on-farm dairy cow health, a secondary data set from the International Farm Comparison Network (IFCN) was acquired. A case study approach, including 15 countries (using 2021 as the reference year), was selected to demonstrate the value of information about on-farm animal health and production data to understand global differences in animal health and disease management.

The 15 countries were selected on the basis of: (a) availability within the IFCN database for 2021, (b) income level according to the The World Bank (19) classification (i.e., high-income, upper middle-income, lower middle-income, low-income), (b) share of global milk production, and (c) geographic region, with the aim of including a diverse range of production systems within our analysis. This resulted in the selection of the following countries: Argentina, Australia, Bangladesh, Brazil, Canada, China, Colombia, India, Indonesia, Kenya, New Zealand, Uganda, United Kingdom, United States of America (United States), and Uruguay (see Figure 1). It should be noted that Uganda was the only low-income country available in the IFCN database for 2021, which is due to the difficulties in establishing research partnerships, including collaborative data collection, in these countries (IFCN, personal communication in March 2023).

The 15 case studies countries together produced nearly half (47.2%) of the total global milk production output in 2021 as illustrated in Figure 2 (20).

For each of the 15 countries, data for two different farm types were provided in the IFCN dataset (21). For larger countries such as Brazil, China, India, and the United States, data for four farm types were available. The total number of farm types included in the analysis was 38. Importantly, these farm types were characterized by IFCN [e.g., (21)] in the context of each individual country, e.g., typical dairy farming systems observed in each country. This implies that the farm types may not be directly comparable between countries.

For each farm type, the data set included standardized annual information about dairy production systems for 2021. A wide range of production system variables that describe the farm types were available, including number of milking cows, predominant breed, average milk yield per milking cow (e.g., kilogram per cow/year), cow losses (e.g., proportion of cows died), size of farms, stocking rates, production system type, and production costs. Data about the average size of dairy land and the total land size of dairy farms was also provided, yet detailed information about the composition of these land types was not included. No information was available about the whole dairy herd size and the age structure of the herd kept on the farms, including heifers, claves, and breeding bulls.

A range of cost types were provided in the annual production data set for 2021 which we categorized into:

a. means of production costs: cost of feed (feed, forage, fertilizer, seed, pesticides), machinery (maintenance, depreciation, contractor), energy and water (fuel, energy, lubricants, water), buildings (maintenance, depreciation), animal purchases, insurance taxes, other dairy enterprise inputs (e.g., milk supplies, herd testing, fees for pedigree records, bedding, fees for disease prevention board, hauling, promotion, milk quota-not used), other whole farm enterprise inputs (e.g., accounting and book keeping fees, phone and utilities costs), insemination, and value added tax balance,
b. health costs (one aggregate for all types of veterinary and medicine expenses),
c. land costs (one aggregate for all types of land costs, e.g., land tax),
d. labor costs (one aggregate for all types of labor expenses, e.g., hired, family), and.
e. capital costs (one aggregate for all types of equity and liabilities).

The cost data was provided as unit of USD/100 kg milk (solid corrected milk (SCM)). This unit cost value per farm type was multiplied with the average milk yield per milking cow which was provided as unit of kg SCM/milking cow. Information about national programs that provide free or subsidized animal health care services, e.g., medicine, vaccines, health consultations, was not given in the dataset. Annual farm gate milk price (i.e., the price farmers got paid) data was also available for each farm type represented in USD / 100 kg SCM. More detailed information about the data collection method of the original data set is described by Hemme et al. (22) and Hemme (22).

The research team considered a further disaggregation of the health cost aggregate provided in the secondary data set [see category (b) above] using expert interviews (e.g., veterinarians, dairy industry representatives, government extension officers) in each of the 15 countries. This included disaggregation of health costs into different medicine expenses, health professional consultation cost, other health costs such as surgeries, disease prevention costs and treatment costs

1 https://animalhealthmetrics.org/

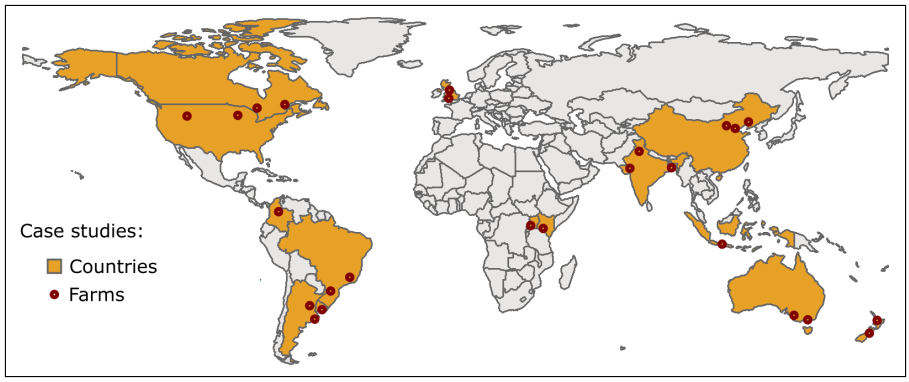

FIGURE 1
Map of case study countries and approximate data collection areas. Notes: 'Farms' indicate the locations within a country where the secondary data was collected.

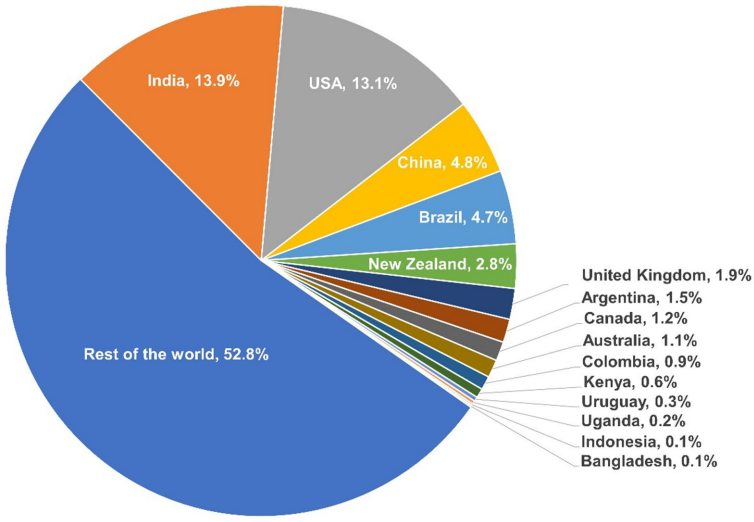

FIGURE 2
Case study country's contribution to the total global milk production output in 2021. Source: FAO (20).

per cow. However, when testing this method of data collection with participants in several countries (23 in total), a range of issues were identified, including difficulty in identifying knowledgeable experts who were willing to participate in interviews, and a large variance in responses for individual countries. These issues resulted in the decision to discontinue the expert interviews and subsequently the attempt to further disaggregate the health cost aggregate that was available in the IFCN data set. The Supplementary material provides information about the interview questionnaire and key learnings from the interviews, which may be of interest for the reader.

2.2. Methods

All costs in the data set were reported in USD, which reflect USD 2021 average exchange rate adjusted cost values that were originally collected in local currency units (LCU) by IFCN. However, for a meaningful comparison of on-farm health costs across countries, these values needed to be adjusted by the purchasing power parity (PPP) (23). The purchasing power varies greatly in different countries (e.g., the amount of feed that farmers can purchase with 100 USD is different in Uganda compared to the United States) which can lead to misinterpretation of cost and price differentials, especially when comparing absolute production costs and milk prices. To adjust the production cost and milk price data for PPP, they were first converted back into their LCU (e.g., USD back to Ugandan Schilling) using average annual market exchange rates of LCUs to the USD, and then normalized by the PPP conversion rates provided by The World Bank (24) (see in the Supplementary material Table S1).

The data set only offered observations for one production year, i.e., 2021, and 2–4 observations for farm types per country which limited the data assessment to a descriptive analysis. This included an assessment of the absolute and proportional on-farm dairy production costs based on the cost components: means of production (e.g., feed, machinery, fuel, labor, veterinary and medicine, insemination, buildings, other costs), labor, land, capital, and health. For example,

the proportion of health costs, C_H, compared to the total production costs, C_T, can be expressed as:

$$\frac{C_H}{C_T} \quad (1)$$

Furthermore, total production costs and health costs per cow *per annum* were analyzed based on the average milk yield per cow, Y, represented, respectively, by:

$$\frac{C_T}{Y} \text{ and } \frac{C_H}{Y}. \quad (2)$$

The analysis considered the identification of trends in on-farm expenditures by the income category of countries using the The World Bank (19). The country categories were: Low-income country included Uganda, lower middle-income countries included Bangladesh, India, Indonesia, Kenya, upper middle-income countries included Argentina, Brazil, China, Colombia, high income countries included Australia, Canada, New Zealand, United Kingdom, United States, and Uruguay.

An analysis by agri-ecological zone and production classification system was also considered, but ultimately not carried out. The small number of countries included in this analysis and the lack of precise location information where the dairy farm data was collected would have limited the relevance of such analysis.

3. Results

3.1. Description of dairy farms

An overview of the key variables that describe the data for the production systems in the 15 case study countries is presented in Table 1. Different farm types for each country are identified using the codes A-D, and we reiterate that these farm types may not be comparable between countries. The number of milking cows across farm types varies significantly within the data set, e.g., 2–2,600. Holstein Friesian (HF) was the most common cow breed observed, yet other dairy breeds (e.g., Jersey), dual purpose breeds (e.g., Ankole) and crossbreds were also present.

Most farm types in the data set have a very high proportion (over 90%) of land used for dairy production proportional to the total size of farms. Lower proportions for the ratio of dairy land and total land may indicate that these are mixed system farms (e.g., dairy and cropping) which can still be grazing/pasture-based systems. There are also farm types in the sample with 'no' dairy land (e.g., China-A-D) which suggests a zero grazing/pasture farming system such as a feedlot but not necessarily landless dairy systems.

3.2. Decomposition of total production costs

Figure 3 presents the breakdown of the total production costs per cow *per annum* by country and farm type adjusted by PPP for cross-country comparability. Cost components include means of production (i.e., feed and forage, energy and water, machinery, building, insurance taxes, other inputs to dairy enterprise, other inputs, and VAT balance), labor, capital, land, and health (i.e., medicine and veterinary consultations) expenses.

The results indicate that the means of production costs take up the major share of the total production costs across all case study countries (median: 66.2%, ranging from 8.9–86.6%) followed by labor costs (median: 17.6%, ranging from 6.5–47.5%), capital costs (median: 4.0%, ranging from 1.9–29.7%) and land costs (median: 6.5%, ranging from 0.1–22.6%; Figure 3). The share of health costs is relatively small across all countries (median: 2.5%, ranging from 0.2–12.5%) compared to other cost components but are equal or higher than capital and land costs in selected cases (e.g., Canada-A, Brazil-A).

Three of the four Chinese dairy farm types (i.e., B, C, D) were the most cost intensive dairy systems within the data set. These farm types had a high number of cows and no dairy land (Table 1), implying that this is an intensive, zero grazing system. All feed and forage for these farm types is purchased outside the farm, which explains the high costs of means of production (80–85% of the total annual production costs).

The results suggest that the average total on-farm costs per cow increase with the wealth of a country up to the upper middle-income category and then decreases slightly for high income countries (Figure 4). Notable is the sharp increase of average total production costs per cow from low-income to lower middle-income country category. However, as there is only one country in the data set categorized as low-income, Uganda, these results may not be representative for other low-income countries. Hence, this outcome needs to be interpreted cautiously.

The increase in average total production costs per cow with increasing wealth of a country appears to be driven by dynamics in the means of production costs across all country income categories (Figure 4). For example, upper middle-income countries appear to spend the highest average costs on means of production inputs, which then decreases for high-income countries. Average on-farm costs per cow for labor, capital, land, and health costs appear to vary only slightly across lower middle-, upper middle- and high-income countries (Figure 4A). The results also suggest that there is some variation in the range of total production costs for all income groups, except the low-income category, as shown in Figure 4B.

3.3. Health costs

The average on-farm health costs per cow range between a median of 3–250 USD *per annum* across all country income groups (Figure 5A) important data in the estimation of the animal health loss envelope in the GBADs program (18). However, the dispersion around the median values is relatively large, specifically for upper middle-income countries as indicated by the boxplots. Low-income countries spend about 10% (median) of their total production costs for animal health related expenses (Figure 5B). This proportion appears to decrease to 1.7–2.9% (median) for other country income groups.

3.4. Total production costs, health costs vs. milk yield

Milk yield tends to increase with rising total costs of production per cow (Figure 6A). This result may be due to higher yielding

TABLE 1 Descriptive statistics of case study countries' dairy production systems.

Country	Country category	Farm type	Average number of milking cows per farm	Predominant breed	Proportion of farm area used for dairy production (%)	Stocking rate (cows/ha)	Average milk yield (kg SCM/ cow/year)	Production system	Selected references describing county's dairy sector
Argentina	UMI	A	180	HF	100	1.2	5,020	GF	Lazzarini et al. (25)
		B	400	HF	100	1.5	6,308	GF	
Australia	HI	A	307	HF	75	1.9	6,465	GF	Sheng et al. (26), Dairy Australia (27)
		B	420	HF	75	2.5	7.338	GF	
Bangladesh	LMI	A	2	Local	16	23.0	927	SSF	Datta et al. (28), Uddin et al. (29), Hossain et al. (30)
		B	14	Local x Shahiwal, HF	20	25.1	1,262	SSF	
Brazil	UMI	A	34	HF	100	1.5	6,995	FF	Balco et al. (31), Daros et al. (32)
		B	64	HF	72	2.9	8,001	FF	
		C	180	HF	100	1.1	4,563	GF	
		D	320	HF	100	1.2	4,969	GF	
Canada	HI	A	66	HF	93	1.6	9,117	SBF	Mc Geough et al. (33), van Kooten (34), Charlebois et al. (35)
		B	140	HF	35	1.1	9,705	FSBF	
China	UMI	A	320	HF	0	0	7,252	FSBF	Li et al. (36), Huang et al. (37)
		B	1,828	HF	0	0	11,662	FF	
		C	289	HF	0	0.0	9,023	FF	
		D	2,250	HF, Jersey	0	0.0	10,381	FF	
Colombia	UMI	A	6	HF	100	2.8	4,607	GF	Carulla and Ortega (38)
		B	108	HF	93	2.7	6,129	GF	
India	LMI	A	2	Murrah buffalo crossbred	50	2.2	4,140	SSF	Kumar et al. (39), Landes et al. (40)
		B	8	HF crossbred, Murrah buffalo	53	4.9	2,595	SSF	
		C	70	HF	70	21.0	4,385	FSBF	
		D	300	HF crossbred, Jersey	92	4.9	5,051	FSBF	
Indonesia	LMI	A	3	HF	81	1.8	3,093	SSF	Susanty et al. (41), Umberger (42), Apdini et al. (43)
		B	10	HF	99	5.4	4,060	SSF	

(Continued)

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TABLE 1 (Continued)

Country	Country category	Farm type	Average number of milking cows per farm	Predominant breed	Proportion of farm area used for dairy production (%)	Stocking rate (cows/ha)	Average milk yield (kg SCM/ cow/year)	Production system	Selected references describing county's dairy sector
Kenya	LMI	A	2	HF, HF crossbreed	70	2.6	2,591	SSF	Onono et al. (44), Kibiego et al. (45), Odero-Waitituh (46)
		B	10	HF, HF crossbreed	65	2.4	2,868	SSF	
New Zealand	HI	A	380	HF x Jersey	84	2.7	5,466	GF	Dairy NZ (47), Foote et al. (48)
		B	1,171	HF x Jersey	79	3.4	6,323	GF	
Uganda	LI	A	3	HF x Ankole cattle	63	2.8	3,099	SSF	Kirunda et al. (49), Waiswa et al. (50), Waiswa and Günlü (51)
		B	13	Ankole cattle	69	3.5	679	SSF	
UK	HI	A	160	HF	94	1.7	8,403	FSBF	Arnott et al. (52), Wilkinson et al. (53)
		B	259	HF	79	1.6	7,949	FSBF	
USA	HI	A	80	HF	96	1.2	10,445	SBF	Khanal et al. (54), von Keyserlingk et al. (55)
		B	500	HF	98	1.8	11,081	FSBF	
		C	1,200	Swedish Red/HF	94	3.2	11,020	FSBF	
		D	2,600	HF	100	17.8	12,119	FSBF	
Uruguay	HI	A	129	HF	71	1.1	5,369	GF	Fariña and Chilibroste (56), Méndez et al. (57), Stirling et al. (58)
		B	367	HF	66	1.1	5,699	GF	

LI for low income, LMI of lower middle income, UMI for upper middle income, HI for high income, SCM for solid corrected milk, HF for Holstein Friesian, GF for grazing farm, SSF for small scale farm, FF for feedlot farm, SBF for stanchion barn farm, FSBF for free stall barn farm. Stocking rate refers to livestock heads per hectare of dairy land. The proportion of farm area used for dairy production was derived by the ratio of reported 'dairy land' size and 'total land' size. Details on the composition of different land types were unavailable. Source: IFCN (59).

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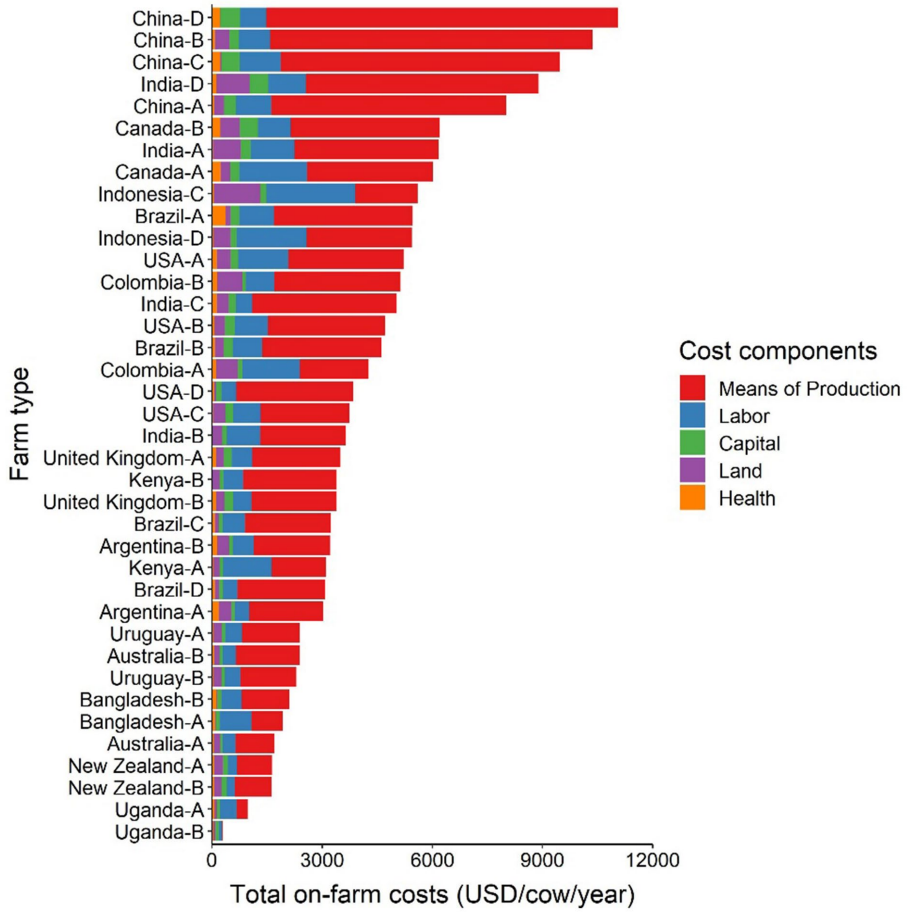

FIGURE 3
Total production costs per cow *per annum* by country, farm type and cost components. A-D for farm types available in the data set, see Table 1. Values are presented in International Dollars (i.e., USD value adjusted by PPP for each country). Source: IFCN (59).

animals that are larger, require more feed and forage and are fed higher quality diets which may be more expensive. Yet, these results also show that it is more expensive for lower income countries to achieve a higher yield compared to higher income countries. This could be due to an absence of competition in input provision and subsequent higher input prices. This association is also reflected by the cost of means of production in relation to milk yield (Figure 6B), which is not surprising considering that the means of production costs contribute a major share of total production cost per cow in all farm types. Furthermore, a similar but less clear relationship can also be observed for health costs per cow and milk yield (Figure 6C).

Low-income countries appear to spend a higher proportion of total production costs on animal health (Figure 7). This result is influenced by the relatively low means of production costs as a proportion of the total production costs for low-income countries (see Figure 3). This result could also be explained by the breeds used in these extensive dairy systems (e.g., local dual purpose and crossbred animals). Interestingly, there appears to be a negative relationship between the proportion of health costs investment spent of the total production costs in the milk yield for all country income categories. This result suggests that the yield of cows may be mostly associated with other input factor costs rather than health investments.

3.5. Total production costs, health costs vs. milk price

The total on-farm production costs and health costs appear to rise with increasing milk price (Figures 8A,B). This relationship is evident for all country income categories, except for low-income countries, which could be due to a small sample bias, i.e., one country in this category. Yet, the causation of the positive cost–price relationships is unclear, i.e., higher production costs leading to higher milk prices, or higher milk prices leading to higher production costs. Furthermore, the results for the production cost-milk price relationship should also be interpreted with caution as the milk price in different countries can be distorted by market interventions such as subsidies. There can also be a lack of farmer's price bargaining power which affects the milk price across and within different countries, and other financial sources may be available to farmers to cross-finance dairy production input costs (e.g., in crop-dairy production systems). Yet, such information was not available in the data.

3.6. Health costs vs. number of cows

On-farm health costs per cow do not appear to change significantly, e.g., staying below 100 USD, based on the herd size, except for herds of

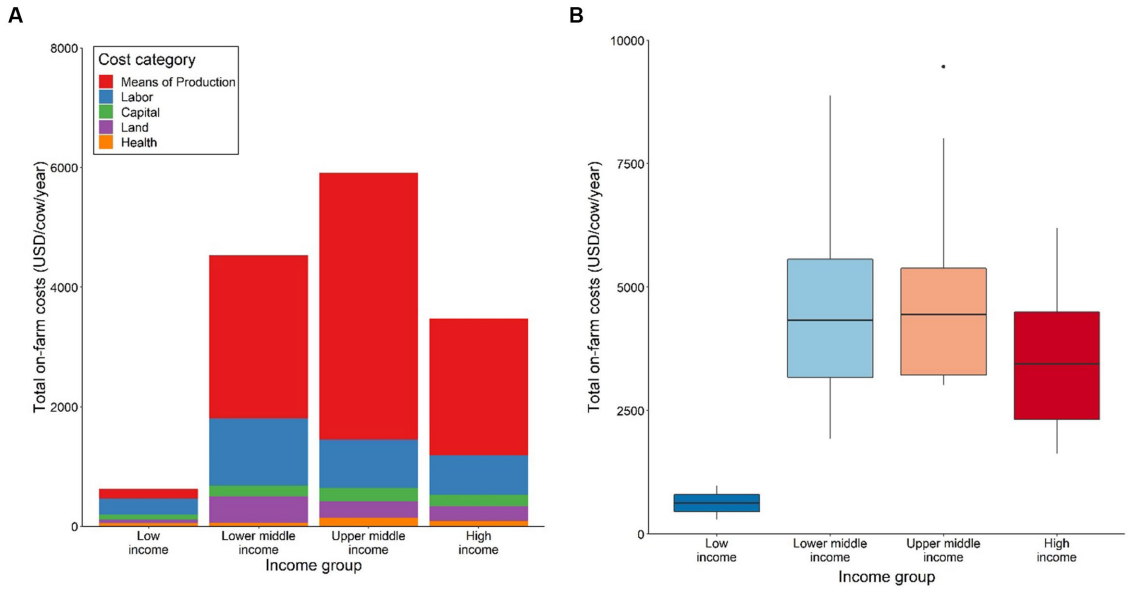

FIGURE 4
Structure of total production costs by country income group; **(A)** Average contribution of means of production, labor, capital, land, and health to total production cost, **(B)** Range of total production costs. Low-income group includes $n = 1$, lower middle-income group includes $n = 4$, upper middle-income group includes $n = 4$, high-income group includes $n = 6$. Values are presented in International Dollars (i.e., USD value adjusted by PPP for each country). Source: IFCN (59).

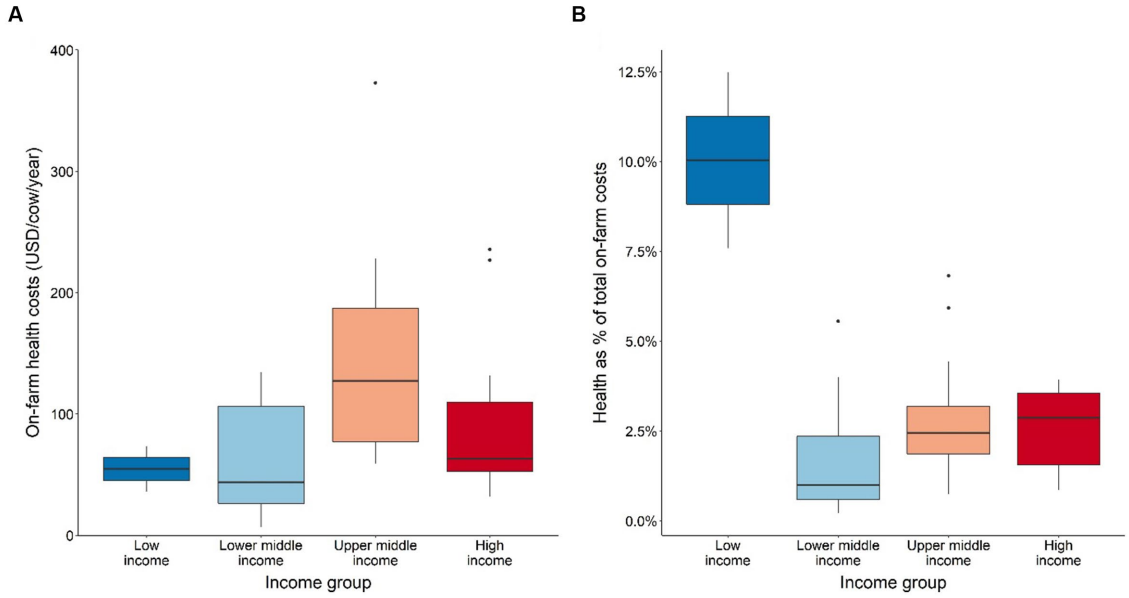

FIGURE 5
(A) Absolute value of on-farm health costs per cow and country income-group, and **(B)** On-farm health costs per cow as a proportion of total costs by country income group. Values are presented in International Dollars (i.e., USD value adjusted by PPP for each country). Source: IFCN (59).

between 100 and 500 animals for which health costs per cow seem to increase in some countries (Figure 9A). This relationship becomes more evident in Figure 9B, in which health costs as a proportion of total production costs are compared to the number of cows on farms. This figure also shows that low-income countries, i.e., Uganda, with small herds (13 or less cow) appear to spend a relatively high proportion of their total cost per cow on health compared to other countries.

3.7. Heath costs vs. losses of cows

A comparison of on-farm health costs with the proportion of cows that die (Figure 10) suggests that an increase in on-farm health costs may lead to a decrease in mortality (i.e., proportion of cows that die) for high-income countries. This trend is also observed for lower middle-income countries, but it is less clear. The opposite relationship

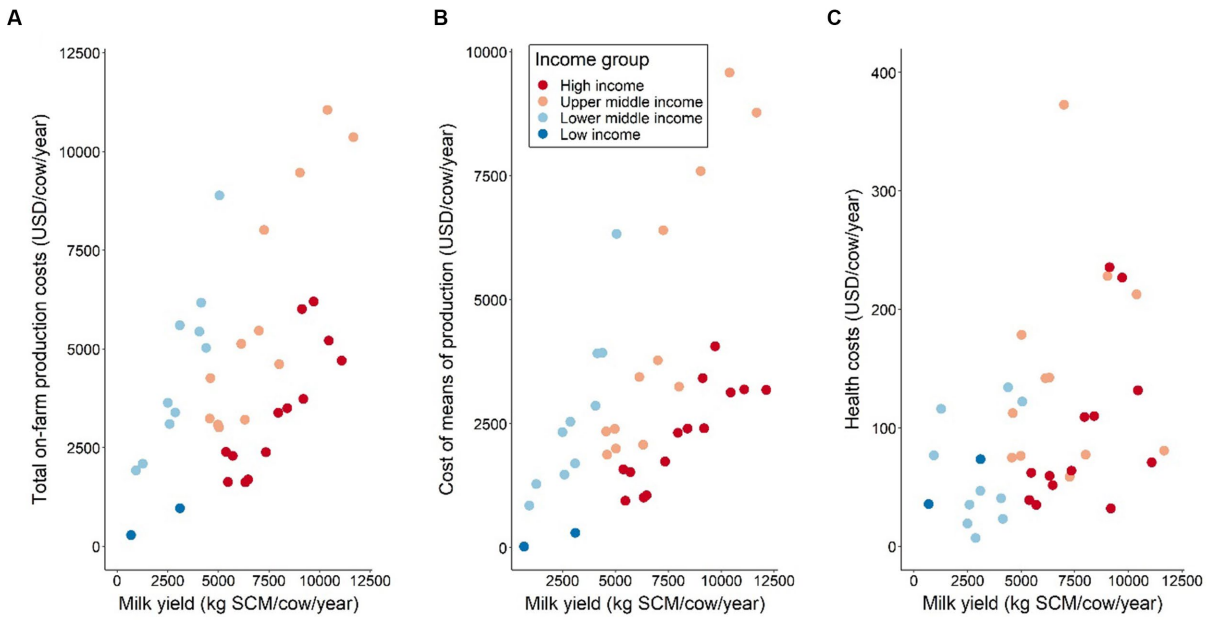

FIGURE 6
(A) Total on-farm production costs by milk yield and country income group, **(B)** Costs of means of production by milk yield and country income group, and **(C)** Health costs by milk yield and country income group. Values are presented in International Dollars (i.e., USD value adjusted by PPP for each country). Source: IFCN (59).

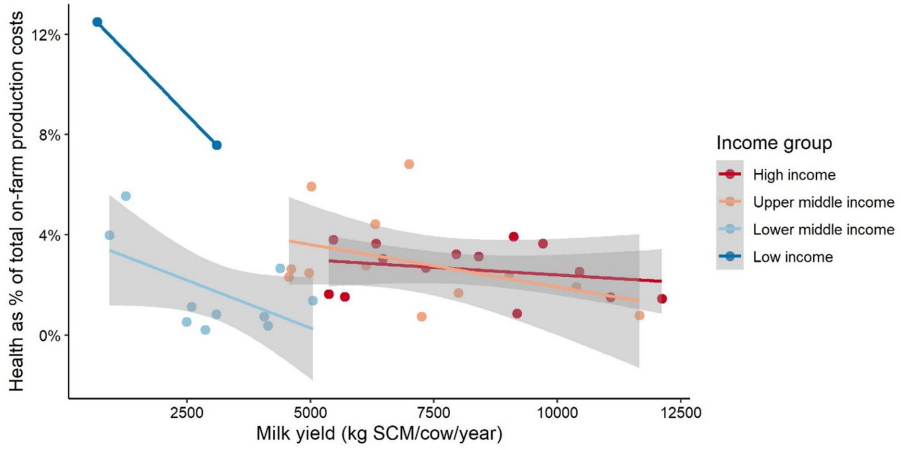

FIGURE 7
Trends in health cost–milk yield relationship. The gray area represents the confidence interval around the smoothed geometric mean. The smaller the gray area around the mean trend line, the more similar the observations within a country income group and vice versa. Cost values are presented in International Dollars (PPP adjusted values). Source: IFCN (59).

was identified for upper middle-income countries and lower income countries, i.e., higher health expenses leading to higher mortality. An explanation for this result could be that the quality of, and access to, animal health care and monitoring only significantly increases once a country reaches a high-income level. Furthermore, external health support or directive such as national animal health programs (e.g., mandatory vs. reactive vaccination programs) may indirectly impact this relationship, which cannot be verified in the absence of data. Farm system specific aspects, e.g., health cost per cow and management of herds in fully confined systems may be different compared to pasture-based or semi-confined systems which may also indirectly be reflected in the results. Yet, caution should be used in generalizing these results since Figure 10 shows outliers to this trend. Additional results (e.g., on losses, assessment by fixed and variable cost types) are presented in the Supplementary material.

4. Discussion

The results presented in this study offer new insights into on-farm health expenditure patterns for dairy cattle across different countries and a comparison of animal health expenditures to other production

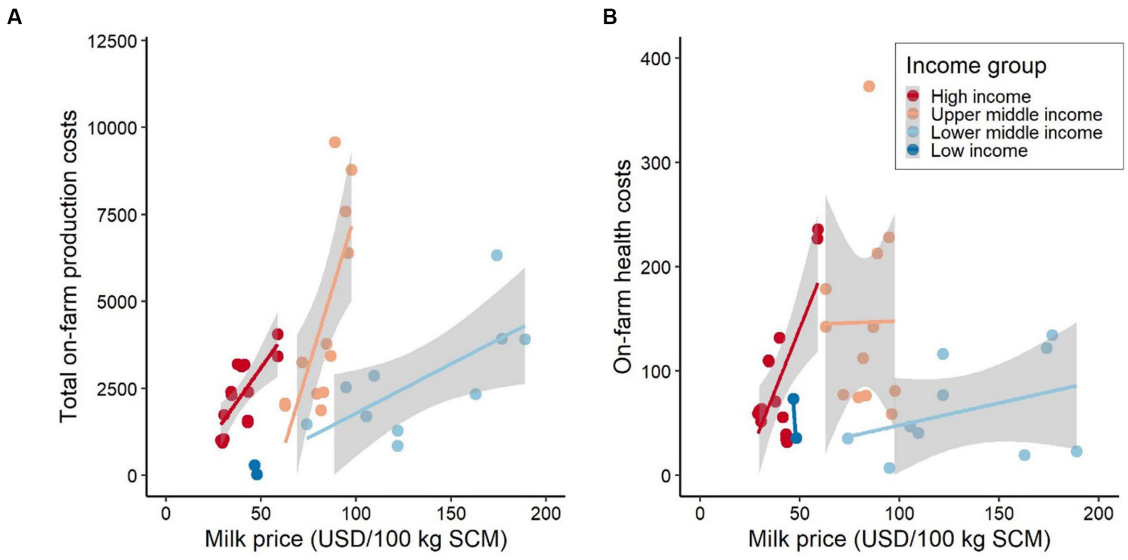

FIGURE 8
(A) Total on-farm production costs vs. milk price, and **(B)** health costs vs. milk price. Costs values are presented in International Dollars (PPP adjusted values). Source: IFCN (59).

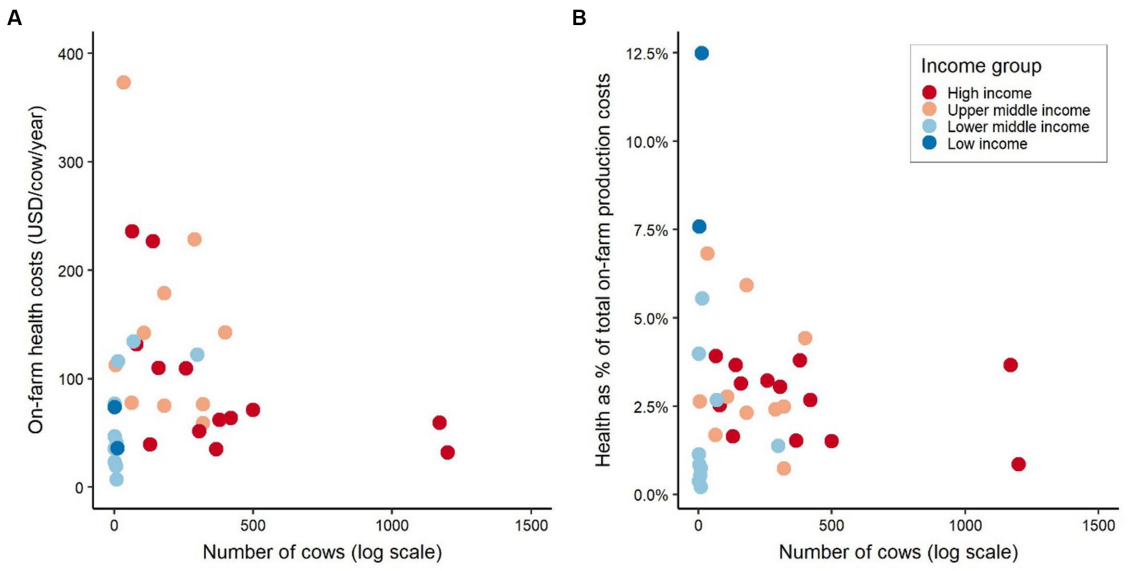

FIGURE 9
(A) On-farm health costs by number of cows and country income group, and **(B)** Health costs as proportion of total on-farm production costs by number of cows (log scale) and country income group. Costs values are presented in International Dollars (PPP adjusted values). Source: IFCN (59).

cost types (e.g., feed, labor, capital), milk yields and cow losses. These are critical data for the GBADs program and ones that are not easily accessible.

A key finding suggests that on-farm health expenditures across all countries are relatively low, ranging between 0.2–12.5% of the total production costs per cow *per annum* (Figure 5), which is similar to previous reports [e.g., (60, 61)]. Farms in low-income countries (Uganda) tend to have lower overall expenditure and spend a higher proportion of their total costs per cow per year on animal health (e.g., 10%) than farms in higher income countries (Figure 5). These findings are supported by Waiswa and Günlü (51) who found that veterinarian, drugs, acaricides, and vaccination costs combined can take up an even higher share, i.e., up to 24.9%, of total production costs for Ugandan dairy farms.

The results also indicate that the means of production costs can take up to 90% of the total costs for intensive feedlot dairy systems (Figure 3), where most of the feed is purchased. Means of production costs are also likely to be high in systems where feed is produced on-farm with high levels of inputs such as seed, fertilizer, and pest/weed control. This aligns with findings by Ruviaro et al. (62), who showed that feed costs alone (which were here treated as a part of means of production costs) can take up to 87% of the total production

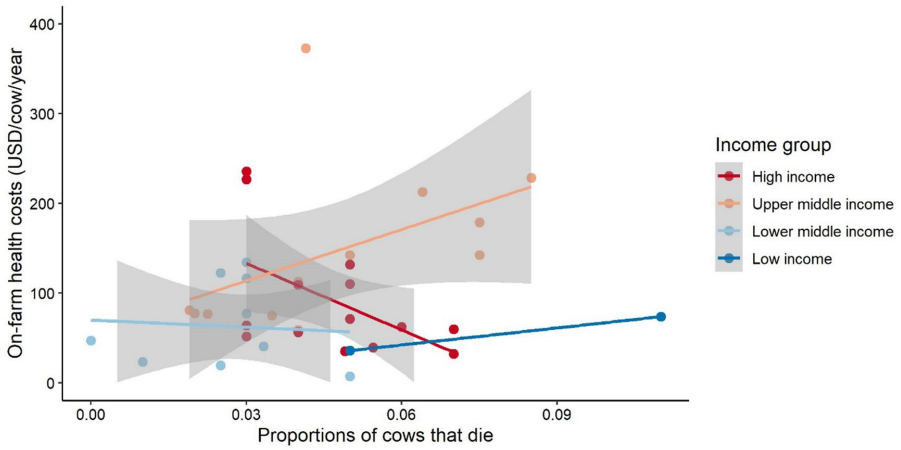

FIGURE 10
Health costs by proportion of cows that died. The gray area represents the confidence interval around the smoothed geometric mean. The smaller the grey area around the mean trend line the more similar the observations within a country income group and vice versa. Cost values are presented in International Dollars (PPP adjusted values). Source: IFCN (59).

costs in semi-confined feedlot systems in Brazil. However, the analysis revealed that means of production costs can also be as low as 9–29% in low-input systems, e.g., for the Uganda farm types A and B. Again, this finding aligns with Waiswa and Günlü (51) who report that feed costs for dairy farms in Uganda take a share of 11.4% of the total production costs. This is likely because dairy cattle in Uganda are predominantly managed in extensive grazing systems with much lower inputs compared to dairy farms in higher income countries (63, 64).

A further finding from the analysis was that the total production costs per cow appear to increase with the country's wealth. This may not be surprising since with increasing wealth of a country, higher quality production inputs may become available, but these may also be more expensive than in less wealthy countries (e.g., opportunity cost of land, cost of higher quality feed, advanced equipment and machinery) [e.g., (65, 66)].

The results show interesting patterns with respect to countries' income status. For example, total on-farm costs (Figure 4A) and on-farm health costs (Figures 5, 10) were higher for farms in upper middle-income countries than both lower middle-income countries and high-income countries. This finding may be attributed to the farm types, e.g., very high number of cows, intense feedlot systems, in the upper middle-income country cluster (i.e., Brazil, China) and overrepresentation of these by including four farm types for Brazil and China in the data set in comparison to Argentina and Colombia for which only two farm types were available. Hence, the findings for the upper middle-income country category may be due to a country selection bias (e.g., high milk volume producing countries) and should be interpreted cautiously. A larger sample could offer more robust and clearer results about these aspects.

While this study offers insights into the value of systematically collected on-farm cost data for a global assessment of investments into dairy animal health, there are data gaps that should be addressed in future. For example, the lack of data for low-income countries, where the burden of animal disease has likely higher social and economic implications, is a concern. Furthermore, data that disaggregates on-farm health expenditures into different components should be collected, together with health management practices (e.g., frequency of animal vaccinations, veterinary health consultations) and prevailing diseases (see Supplementary material). Such information would assist the modeling of disease spread (e.g., nationally, regionally, and globally) and their impact on dairy herds. It would also provide the opportunity to measure the socio-economic impact of cattle diseases and their management as a potential basis for national and international investments in improved disease prevention. These data needs align and complement the list of data needs proposed by other authors such as Perry et al. (17) and Waiswa et al. (50).

Animal health is a public good since it can affect global human food security and human health (67–69). Hence, collected data on animal health aspects should be accessible for researchers and policy makers at a national and global scale as a basis for decision making. Currently, data sets that describe dairy production systems, including animal health aspects (e.g., health expenses, disease prevalence) exist, but mostly in silos and are not collated and harmonized. These data sets are typically inaccessible or only accessible at a cost for public good research purposes. This is a barrier to gaining an improved understanding about global on-farm animal health investments. Therefore, effective collaborations about the collection, analysis, and use of on-farm animal health data (including the transfer of technologies, methods, skills in developing data collection processes) need to be developed between the key stakeholders (e.g., research institution, industry associations, NGOs, private companies, national governments, and intergovernmental organizations). While establishing these collaborations may appear to be challenging, the Covid-19 pandemic has demonstrated that such partnerships between key stakeholders (e.g., pharmaceutical companies, national governments, World Health Organization and research institutions) can be effective if public human health is at risk [e.g., (70, 71)]. Learnings from this experience should be adapted to the global animal health/One Health context to avoid risks due to animal diseases for global food security and human health (e.g., zoonosis) (67, 69, 71).

A limitation of this study is the small sample size of countries and the lack of time series data for each country which affects the robustness and generalization of results (e.g., missing trends). A larger data set would offer the opportunity to include more advanced analytical approaches to establish potential causes for the observed production cost structures. This would also allow an assessment of

on-farm costs by agri-ecological zone and production classification system. Data about on-farm dairy herd age structure, e.g., number of animals by age and use, as well as age and use specific production costs of the animals in the dairy herd would have been beneficial for more detailed health investment analysis.

A further limitation is the use of production cost data for 2021, which was a year in which Covid-19 was prevailing. Implications of Covid-19 restrictions, e.g., social distancing, labor shortages, limited logistics options, may have affected production costs and the availability of input factors. This may imply that the structure of on-farm costs may be different for this production year compared to previous production years. This highlights the need to compare expenses for on-farm animal health investments over time and assess changes and drivers for changes (e.g., policies, subsidized medicine, human pandemics).

Moreover, national animal health programs that provide financial incentives for on-farm animal health management (e.g., subsided medicine and vaccines) likely vary across countries and may affect on-farm health expenditures differently. This has not been included in the analysis but offers scope for future research. For example, a comparison of the 15 national animal health management strategies and policies, including investments in prevention and management of diseases, could provide insights to how national health programs may influence on-farm health expenditures and farmers' decision making. This aligns with the research needs identified by Capper and Williams (5), e.g., the need to better understand producers' and veterinarians' perceptions and behaviors toward the disease management. An extension to the present work could identify potential gaps in countries' governance of animal diseases and options how to address these as a global community with an interest in animal health and food security.

5. Conclusion

This study provides a proof of concept, using a subset of a global farm comparison dataset to demonstrate the value of systematically collected data about on-farm health expenditure and comparisons across countries. Such information offers insights into farm production cost structures, which can be useful for national governments and intergovernmental organizations to identify investment gaps in animal health as a public good that needs to be addressed through targeted policies. For example, our analysis highlights an imbalance in on-farm expenditure, with farms in low-income countries investing proportionally more in animal health compared to farms in higher-income countries. We also highlight data gaps, both in the geographical spread and diversity of farms surveyed, and the types of data collected, e.g., on-farm dairy herd age structure, prevailing diseases their management, disaggregation of heath expenses at national or even sub-national scales, which limit our ability to adequately corelate on-farm investments in animal health with animal health and productivity outcomes.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Data sharing agreement with data owner prevents the sharing of data with third parties. Requests to access these datasets should be directed to Name of data owner: IFCN; Website: https://ifcndairy.org/; Email: info@ifcndairy.org.

Ethics statement

The studies involving humans were approved by CSIRO ethics and privacy committee (approval number 054/22). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft. CGF: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. DM: Funding acquisition, Resources, Supervision, Writing – review & editing. MH: Funding acquisition, Supervision, Writing – review & editing.

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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/fvets.2023.1288199/full#supplementary-material

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Assessment of microbial quality in poultry drinking water on farms in Austria

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The quality of poultry drinking water has a significant effect on broiler health and performance. This study conducted an analysis of aerobic mesophilic counts (AMC), *Enterobacteriaceae* (EB), *Pseudomonadaceae* (PS), and screened for the presence of *Campylobacter* spp. in water samples collected from a total of 14 farms in Austria, with either a public or private water source. The efficacy of two water line treatment methods was evaluated: a chemical treatment of the water lines with 4.0 ppm ClO_2 (T1) and a combined chemical (4.0 ppm active ClO_2 and 3.0% peracetic acid) and mechanical treatment (purging of the water lines with a high-pressure air pump; T2). However, both the T1 and T2 treatments failed to reduce the AMC counts below the maximum acceptable microbial limit of 4.0 log_{10} CFU/ml in water samples. In addition, no significant reduction in EB and PS counts was observed in water samples after either T1 or T2 water line treatment. The water samples showed a high level of microbial diversity with 18 to 26 different genera. The genus *Pseudomonas* was most frequently isolated across all poultry farms, while *Campylobacter jejuni* was identified in a single sample collected before water line treatment. Isolate analysis revealed the presence of opportunistic pathogens in water samples both before (T1 43.1%, T2 30.9%) and after (T1 36.3%, T2 33.3%) water line treatment. Opportunistic pathogens belonging to genera including *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp., were most frequently isolated from poultry drinking water. These isolates exhibited multidrug resistance and resistance phenotypes to antimicrobials commonly used in Austrian poultry farms. The findings of this study emphasize the potential risk of exposure to opportunistic pathogens for poultry and personnel, underscoring the importance of efficient water line management.

KEYWORDS

water line treatment, opportunistic pathogens, poultry health, *Pseudomonas*, antimicrobial susceptibility

1 Introduction

Poultry is one of the main sources of meat production worldwide (1). In 2020, more than 97 million chickens were processed in Austria, representing 124.000 tons of processed poultry meat (2). Drinking water is a vital nutrient for commercial poultry and has a significant impact on poultry health, liveweight, feed conversion ratios, and overall performance (3, 4). The water consumption of poultry is approximately twice the amount of feed intake (5). Poultry health and water intake are directly influenced by microbial water quality (4, 6, 7).

In Europe, the water quality standards for poultry drinking water have been adapted from water quality regulations intended for human drinking water consumption (8), EC Directive 98/83/EC (Drinking Water Directive [DWD] 9). According to the Austrian Poultry Hygiene Regulation (10) drinking water used for poultry production must not exceed a total aerobic mesophilic count (AMC) of 2.0 $\log_{10}$ and 1.3 $\log_{10}$ colony forming units (CFU/ml) at 22° and 37°C, respectively. Currently, there is no legal requirement to examine microbial contamination inside the drinking water lines (11). Hence, maintenance of water line hygiene is primarily the responsibility of the poultry producer, and it is typically conducted between the production cycles (12). The standard water line practices involve mechanical cleaning by flushing the water lines, followed by oxidative disinfection, primarily using chlorination or acidifiers (7, 12–14).

While water line treatment is a crucial component of an effective biosecurity program, its effectiveness does not ensure the complete elimination of the microorganisms within the water lines (15–17). *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. have been detected in poultry drinking water (7, 18). Elevated temperatures and low water flow rates in enclosed water line systems have been found to adversely affect water quality, as indicated by previous studies (4, 12). These conditions are favorable for the accumulation of dissolved organic substances, minerals, and solid particles, which facilitate growth and promote the formation of biofilms. Among biofilm-forming bacteria, primarily *Pseudomonas* and *Stenotrophomonas* are responsible for biofilm formation on surfaces of poultry drinking lines (12). Biofilms may provide a favorable surface for attachment of opportunistic pathogens (OP), such as such as *Acinetobacter*, *Aeromonas, Citrobacter, Enterobacter,* and *Klebsiella* whose members are natural inhabitants of plumbing systems and adapted to survival in drinking water (19). Although these bacteria are generally not pathogenic, some have the potential to cause infections in susceptible poultry and farm workers (20). Hence, the detachment of pathogen and OP rich biofilms and their contamination of the water system present a significant risk for waterborne transmission of these bacteria, posing a potential threat to both poultry and human health. Moreover, the administration of medication to poultry through drinking water, which is a preferred route, has been linked to presence of multidrug-resistant (MDR) bacteria (21, 22).

Microbial water quality is frequently evaluated at its source, but assessments at the end of the drinking lines are infrequent, despite the potential for substantial variations in microbial quality between the source and endpoint (12). Thus, the objective of this study was to evaluate the microbial quality of water samples collected at the end of a production cycle of five to six weeks and shortly before restocking for the subsequent production cycle, following the water line treatment. Previous studies have demonstrated the presence of pathogens such as *Campylobacter* spp. in poultry water on farms with private water supplies compared to those with a public supply (23, 24). This highlights the critical role of poultry drinking water as a potential source of *Campylobacter* spp. infection on the farm (25, 26). The presence of *Campylobacter* spp. in drinking water on poultry farms may indicate lapses in biosecurity, contaminated water source, ineffective and/or incorrectly applied water line cleaning procedures (11, 18). Therefore, one of our objectives was to assess the microbial quality of poultry drinking water in farms with either public or private water supply. We applied ISObased reference methods to assess bacterial load and presence of *Campylobacter* spp. in poultry drinking water, followed by partial 16S rRNA sequencing of bacterial isolates. Antibiotic susceptibility patterns of commonly isolated OP were then determined.

2 Materials and methods

2.1 Water line treatment and sample collection

Twenty-eight poultry farms producing broilers for local slaughterhouses in Austria voluntarily participated in the study between May 2019 and August 2020, some of which had private ($n = 11$) and others public ($n = 17$) water supplies. The fattening period at the participating poultry farms in Austria was five to six weeks. The poultry farms were divided into two distinct groups based on whether the farms employed solely chemical (T1) or a combination of chemical and mechanical (T2) water line treatment methods. An overview of the poultry farms included in the study is presented in Figure 1. Cleaning and water line treatment at the poultry farms was performed by the farmer. Since the participation of poultry farms in the study was voluntary, poultry farms 6, 8, 9, 12, and 13 withdrew their participation after T1 and were substituted by the poultry farms 15–19 during T2. The study was conducted in collaboration with a private laboratory (HYGIENICUM GmbH, Graz, Austria), which provided training on the water line cleaning procedures to be implemented at the poultry farms to the participating farmers.

During T1 water line treatment, water lines were drained and filled with a commercially-available solution of which the main disinfecting component contained 4.0 ppm active chlorine dioxide (ClO_2) solution (Calgonit CD-K1/K2, Calvatis GmbH, Ladenburg, Germany). The commercial solution was retained in the water lines for 24 h. Measurements of free ClO_2 inside the waterlines were not obtained. Subsequently, the water lines were washed with the supply water by continuous flushing for 10 min. Under normal operating conditions. The T2 water line was performed by continuous pumping of acidic cleaner containing 3.0% peroxyacetic acid (PAA) and hydrogen peroxide (Calgonit DS 625, Calvatis GmbH, Ladenburg, Germany) continuously for 30 min using highpressure air pump. The water lines were then washed with the supply water and purged using a high-pressure air pump until no inorganic and organic debris were visible in the water. Subsequently, the water line disinfection was performed using a commercial disinfection solution containing 4.0 ppm active ClO_2 solution (Calgonit CD-K1/K2) which was retained in the water lines for 24 h. Subsequently, the water lines were washed with supply water by flushing for 10 min. Under normal operating conditions.

Water samples were collected by employees from the private laboratory, samples were taken from the end nipple of the drinking

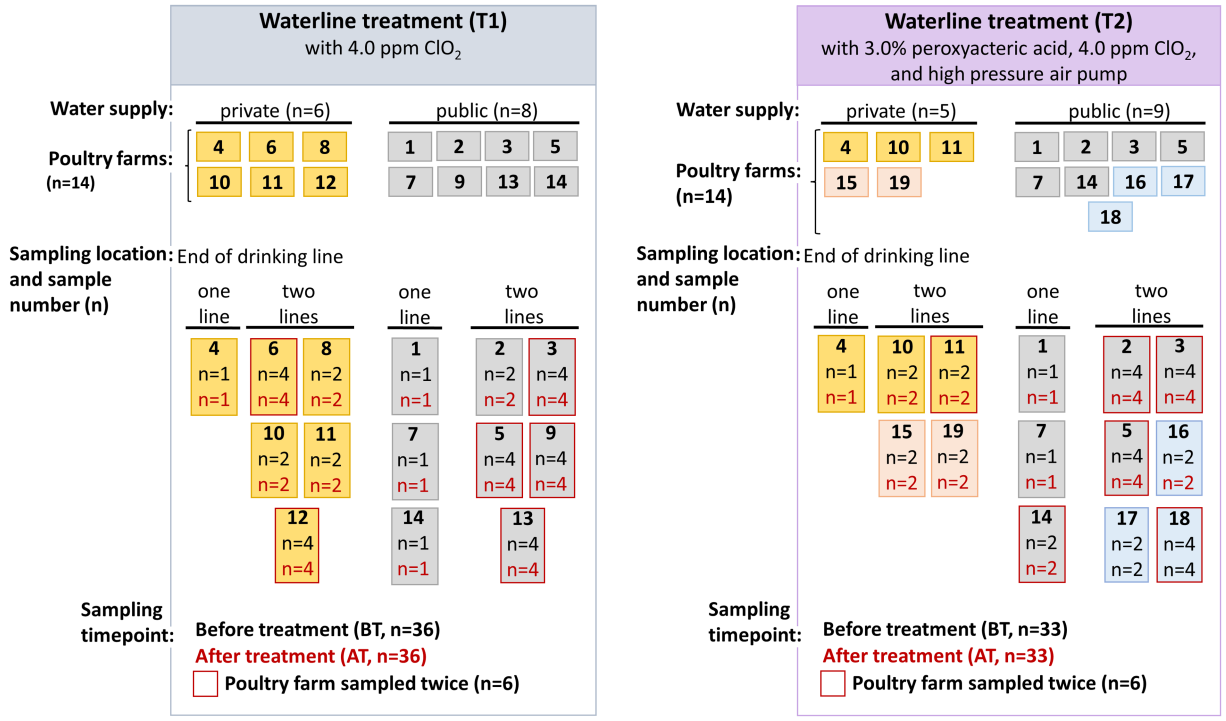

FIGURE 1
An overview of the sampling conducted in fourteen poultry farms during chemical (T1) and combined chemical with mechanical (T2) water line treatment. Poultry farms 6, 8, 9, 12, and 13 withdrew their participation after T1 and were substituted by the poultry farms 15 and 19 (indicated by pink color) with private water supply, and 16–18 (indicated blue color) with public water supply during T2 water line sampling.

water line inside the vacant poultry house (HYGIENICUM GmbH, Graz, Austria). One water line was sampled at four and five poultry farms, while two water lines (line 1 and 2) were sampled at ten and nine poultry farms during T1 and T2 water line treatments (Figure 1). Two sampling timepoints were chosen, namely before treatment (BT) at the end of fattening period of 5–6 weeks, and after the water line treatment (AT) before restocking of the subsequent production cycle. As shown in Figure 1 and Supplementary Table S1, in six poultry farms during the T1 and T2 water line treatment, water samples were collected at two different sampling intervals, while other poultry farms were sampled only once. Additionally, at some poultry farms from some water lines the duplicate samples were collected, while from other poultry farms only a single sample was collected. Therefore, in total 36 (T1) and 33 (T2) BT and corresponding AT samples were collected for the microbial analysis in the present study. The water samples were collected in sterile 500 mL bottles by the private laboratory and immediately transported to the laboratory at 4°C for microbial analysis.

2.2 Sample processing and microbial analysis

Prior to analysis, 500 mL of water samples were centrifuged at 8000 rpm for 30 min at 4°C (Thermo Scientific, Sorvall Lynx 4000 centrifuge). All but 10 mL of the supernatant was discarded, the remainder was then resuspended using a serological 10 mL pipette (Greiner Bio One, Frickenhausen, Germany) and vortexed for 30 s.

Campylobacter selective enrichment and isolation were performed according to the ISO 10272-1:2006 standard for the detection of *Campylobacter* spp. in foodstuff (27). Five milliliters of the supernatant were transferred to 45 mL of Bolton broth (Thermo Fisher Scientific Ltd., Hampshire, United Kingdom) supplemented with 5% hemolyzed horse blood (Oxoid Ltd., Hampshire, United Kingdom). The Bolton broth enrichment was incubated for up to 48 h at 42°C under microaerobic conditions (10% CO_2, 3% O_2, 87% N_2). After incubation modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid Ltd) was inoculated by fractionated loop inoculation (10 µL) and incubated at 42°C for 48 h under microaerobic conditions. Quantification of aerobic mesophilic count (AMC), *Enterobacteriaceae* (EB), and *Pseudomonadaceae* (PS) counts was carried out according to ISO reference methods (28, 29). For enumeration of AMC, EB, and PS, 5 mL of the re-suspended supernatant was transferred to 45 mL buffered peptone water (BPW) (Biokar Solabia diagnostics, Pantin Cedex, France). Subsequently, serial ten-fold dilutions were prepared up to dilution 10^{-5} in BPW (Biokar Solabia diagnostics, Pantin Cedex, France). The AMC were enumerated on trypto-caseine soy agar with 0.6% yeast extract (TSAYE) (Biokar Solabia diagnostics), while EB and PS were enumerated on red bile glucose agar (VRBG) (Merck KGaA, Darmstadt, Germany). Each dilution step (100 µL) was plated on selective agar media for the enumeration of AMC, EB, and PS counts. For dilution 10^{-1} the volume of 1 mL was divided (333 µL) on three agar plates per selective medium. Agar plates were incubated at 30°C (AMC) and 37°C (EB, PS) aerobically for up to 48 h. The EB and PS counts on VRGB agar were differentiated by their ability to ferment

glucose, leading to pink colonies with or without precipitation and pale colonies for PS. Presumptive EB and PS isolates were confirmed using oxidase reaction (BioMerieux, Marcy l'Etoile, France). The minimum and maximum limits for the determination of the AMC, EB, and PS in the samples ranged between 10 and 300 CFU.

Microbial quality of water samples before (BT) and after (AT) sanitation were categorized according to AMC, EB, and PS load in two contamination levels, <4.0 $\log_{10}$ CFU/ml and ≥4.0 $\log_{10}$ CFU/ml based on existing studies (4, 7, 12).

2.3 Isolation and identification of bacterial and *Campylobacter* spp. isolates

The predominant bacterial colony morphologies were collected from each water sample for further confirmation. Specifically, 1–5 colonies were selected from TSAYE ($n = 224$), VRBG ($n = 206$) and mCCDA agar ($n = 41$) and then subcultured on the respective medium. The isolate list is provided in the Supplementary Table S1. The purified colonies, comprising isolates from T1 BT samples ($n = 123$), T1 AT samples ($n = 113$), T2 BT samples ($n = 139$), T2 AT samples ($n = 96$) were stored at – 80°C in brain heart infusion broth (Biokar Solabia diagnostics) supplemented with 25% (v/v) glycerol (Merck KgaA).

For DNA extraction of *Campylobacter* spp. isolates 10 µL loop of bacterial material was resuspended in 100 µL of 0.1 M Tris–HCl buffer pH 7 (Sigma Aldrich, St. Louis, MO, United States) and mixed with 400 µL Chelex® 100-Resin (BioRad, Hercules, CA, United States) (30). The bacterial Chelex® 100Resin suspension was heated at 100°C for 10 min on a block heater (Thermo Fischer Scientific Inc.), followed by short centrifugation step at 15,000 ×*g* (Eppendorf Centrifuge 5,425) for 5 s. The supernatant (100 µL) was transferred to a maximum recovery tube (Corning Incorporated Life Sciences, Reynosa, Mexico) and stored at −20°C until analysis. *Campylobacter* spp. were identified using multiplex PCR targeting genes including the conserved genus-specific 23S rRNA gene, the *Campylobacter jejuni* hippuricase gene (*hipO*) and the *Campylobacter coli* serine hydroxymethyltransferase (*glyA*) gene, as previously described (31). Briefly, a single reaction mixture (20 µL) contained diethylpyrocarbonate (DEPC) treated water (Sigma Aldrich), 1× PCR buffer, 2 mM $MgCl_2$, 500 nm *hipO* forward and reverse primer, 1,000 *glyA* forward and reverse primer, 200 nm 23S forward and reverse primer, 200 µM dNTP mix, 1.5 U of Platinum Taq DNA polymerase (Platinum™ Taq DNA Polymerase, DNAfree, Thermo Fisher Scientific Inc., Waltham, MA, United States), and 5 µL template genomic DNA. The amplification was performed in T100™ Thermal Cycler (Bio-Rad, Hercules, CA, United States). The PCR cycling conditions included initial denaturation at 94°C for 2 min, 30 cycles of denaturation (94°C for 30 s), primer annealing (59°C for 30 s), elongation (72°C for 30 s) and final elongation (72°C for 7 min). The gel electrophoresis of PCRamplicons was performed in a 1.5% agarose gel containing 0.5× TrisBorateEDTA (TBE) buffer (Sigma Aldrich, St. Louis, MO, United States) and 3.5 µL peqGREEN DNA gel stain (VWR International, Radnor, United States), at 120 V for 30 min. The DNA standard Thermo Scientific™ GeneRuler™ 100 bp (Thermo Fisher Scientific Inc., Waltham, United States) was applied for fragment length comparison. We utilized the following control isolates for the DNA extraction and multiplex PCR: *C. jejuni* strain DSM 4688 and *C. coli* strain DSM 4689, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

For DNA extraction of isolates from TSAYE and VRBG, bacterial cells were lysed by boiling the suspension. A 10 µL loop of bacterial material was re-suspended in 100 µL 0.1 M Tris–HCl pH 7 buffer (Sigma Aldrich, St. Louis, MO, United States), briefly vortexed and heated at 100°C for 15 min (Thermo Scientific™ block heater, Thermo Fischer Scientific Inc.). The suspension was then centrifuged for 5 s at 15,000 ×*g* (Eppendorf Centrifuge 5,425, Hamburg, Germany) and the supernatant (70 µL) was transferred into maximum recovery tubes (Corning Incorporated Life Sciences, Reynosa, Mexico) and stored at −20°C until analysis. For identification of bacteria isolates ($n = 471$) the partial amplification of 16S rRNA gene was performed following the methods of (32, 33), using universal primer pairs 616F (5'AGAGTTTGATYMTGGCTC3') and 1492R (5'GGYTACCT TGTTACGACTT3') (both Microsynth AG, Blagach, Switzerland). A single PCR reaction (45 µL) contained 1× PCR buffer, 2 mM $MgCl_2$, 200 nM forward and reverse primer, 250 µM dNTP mix, 2 U of Platinum Taq DNA polymerase (Platinum™ Taq DNA Polymerase, DNAfree, Thermo Fisher Scientific Inc.) and 5 µL template genomic DNA. The DNA amplification was performed in T100™ Thermal Cycler (BioRad, Hercules, CA, United States). The PCR cycling conditions included initial denaturation at 95°C for 5 min, 35 cycles of denaturation (94°C for 30 s), primer annealing (52°C for 30 s), elongation (72°C for 60 s) and final elongation (72°C for 7 min). Subsequently, the PCR amplicons were sent for purification and sanger sequencing to LGC Genomics (LGC Genomics GmbH, Berlin, Germany). The gel electrophoresis of PCR-amplicons was performed in a 1.5% agarose gel containing 0.5× TrisBorateEDTA (TBE) buffer (Sigma Aldrich, St. Louis, MO, United States) and 3.5 µL peqGREEN DNA gel stain (VWR International, Radnor, United States), at 120 V for 30 min. The DNA standard Thermo Scientific™ GeneRuler™ 100 bp (Thermo Fisher Scientific Inc., Waltham, United States) was applied for fragment length comparison. The PCR amplicons were sequenced using a 1492R (5'GGYTACCTTGTTACGACTT3') primer. The nucleotide sequences were qualityevaluated by using Finch TV 1.4.0 (34) and MEGA X (35). The bacterial nucleotide Basic Local Alignment Search Tool (BLAST) algorithm from the National Centre for Biotechnology Information (NCBI)[1] was used for taxonomy assignment. Sequences were assigned to genus or species level according to best matches and highest similarities (1,040 to 1,120 bp fragment length, similarity cutoff ≥97.0%). The partial rRNA gene sequence data from the isolates were deposited in the GenBank database under accession numbers MZ642358 to MZ643011.[2] Subsequent identification of opportunistic pathogens among identified isolates was performed using the bacterial metadata base BacDive (36) and List of Prokaryotic names with Standing in Nomenclature (LPSN) (37).

2.4 Antimicrobial susceptibility testing

Opportunistic pathogens with clinical relevance isolated from water samples during T1 and T2 water line treatment were subjected

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

2 https://www.ncbi.nlm.nih.gov/genbank

to antimicrobial susceptibility testing (AST). The set of isolates included most frequently isolated OP, such as *Pseudomonas* spp. ($n = 17$), *Ochrobactrum* spp. ($n = 4$), *Stenotrophomonas* spp. ($n = 3$), and human relevant opportunistic pathogens including *Citrobacter* spp. ($n = 2$), *Enterobacter* spp. ($n = 2$), *Klebsiella* spp. ($n = 1$), and *Aeromonas* spp. ($n = 1$).

AST was performed for a total of 30 bacterial isolates using Sensititre™ Avian AVIAN1F Vet AST Plate (ThermoFischer Scientific Inc., Waltham, MA, United States), according to the manufacturer's instructions. Briefly, single colonies were picked from fresh cultures grown on TSAYE for 24 h at 30°C, suspended in in sterile water to an optical density of a 0.5 McFarland standard (~ 10^8 CFU/mL). 50 µl volumes of the bacterial suspension were transferred to wells containing different concentrations of lyophilized antimicrobials. Plates were sealed and incubated at 30°C for 24 to 48 h, after which minimum inhibitory concentrations (MIC) were read visually and defined as the lowest concentration of a given antibiotic at which no growth of the test organism was observed. *E. coli* strain ATCC 25922 was used as the internal quality control isolate. The minimum inhibitory concentration (MIC) breakpoints and definitions for multi-drug resistance (MDR; resistance to two or more antibiotic classes) (38) were determined following the standards provided by the Clinical and Laboratory Standards Institute (CLSI) manuals (39–41).

2.5 Data analysis

A descriptive analysis was carried out (mean, median, and standard deviation) for AMC, EB, and PS counts. The normal distribution of each data set (T1 and T2) was investigated using the Shapiro–Wilks test. Due to nonnormal distribution of data, the median values of AMC, EB, and PS counts were calculated. The Wilcoxon–Mann–Whitney rank sum test performed as a twosided test was applied to identify whether there was a significant difference between median AMC, EB and PS counts of BT and AT samples. Median AMC, EB, and PS counts in AT samples were compared for different water supplies (public vs. private), water line treatments (T1 vs. T2), following $\log_{10}$ transformation, using Wilcoxon–Mann–Whitney rank sum test. Values of $p < 0.05$ were considered as statistically significant. Statistical analyses were carried out using the R software package for statistical computing.[3]

3 Results

3.1 Aerobic mesophilic count, *Enterobacteriaceae*, and *Pseudomonadaceae* count in poultry drinking water

Ninety-nine BT samples and their corresponding AT water samples were microbiologically assessed, with a maximum acceptable microbial limit of 4.0 $\log_{10}$ CFU/ml for AMC, EB, and PS counts (Table 1). Due to non-normal distribution of the data, we used the Wilcoxon–Mann–Whitney twosided rank sum test to assess the median values for AMC, EB, and PS counts. No significant differences ($p \geq 0.05$) were observed between the median AMC, EB, and PS counts of the BT and AT samples after T1 water line treatment (Table 1). Furthermore, we did not observe any significant difference between median AMC, EB, and PS counts in poultry farms with private and public water supply. Among the water samples, the highest median AMC counts were observed in BT (5.9 ± 1.02 $\log_{10}$ CFU/ml, median ± MAD; MAD: median absolute deviation) and AT (6.0 ± 1.17 $\log_{10}$ CFU/ml) samples. Higher median AMC counts in BT and AT samples were observed in poultry farms with a private well than those with a public water supply (Table 1). The lowest median counts were observed for EB in both BT (3.6 ± 2.13 $\log_{10}$ CFU/ml) and AT (2.3 ± 1.52 $\log_{10}$ CFU/ml) samples. In AT samples higher median EB counts were observed in poultry farms with public water supply. The PS resulted in the second highest median counts, which remained unchanged in BT (4.7 ± 1.44 $\log_{10}$ CFU/ml) and AT (4.7 ± 2.48 $\log_{10}$ CFU/ml) samples. Higher median PS counts were detected in both BT and AT samples in poultry farms with public water supply.

After T1 water line treatment, high (>4.0 $\log_{10}$ CFU/ml) AMC, EB, and PS counts from BT samples decreased below the maximum acceptable microbial limit in 8/36, 7/36, and 9/36 AT samples, respectively (Supplementary Table S2). The AMC, EB, and PS below the microbial limit were observed in 1/36, 18/36, and 7/36 BT and AT samples, respectively. The AMC, EB, and PS counts above the maximum acceptable microbial limit were observed in 27/36, 11/36, and 20/36 AT samples, respectively, after T1 treatment.

During T2 water line sampling, no significant differences ($p \geq 0.05$) were observed in the median AMC, EB, and PS counts between the BT and AT samples (Table 1). No significant difference was observed between median AMC, EB, and PS count in poultry farms with private and public water supply. The highest median counts were for AMC counts in both BT (4.6 ± 1.55 $\log_{10}$ CFU/ml) and AT (4.7 ± 1.85 $\log_{10}$ CFU/ml) samples, followed by the PS counts in BT (3.5 ± 1.62 $\log_{10}$ CFU/ml) and AT (3.1 ± 2.05 $\log_{10}$ CFU/ml) samples The lowest counts were observed in the median EB counts of BT (2.4 ± 1.63 $\log_{10}$ CFU/ml) and AT (1.6 ± 0.42 $\log_{10}$ CFU/ml) samples. Higher median AMC, EB, and PS counts were detected in AT samples in poultry farms with public water supply.

After T2 water line treatment, high (>4.0 $\log_{10}$ CFU/ml) AMC, EB, and PS counts from BT samples decreased below the maximum acceptable microbial limit in 8/33, 5/33, and 14/33 AT samples, respectively (Supplementary Table S3). The AMC, EB, and PS counts below the microbial limit were detected in 4/33, 25/33, and 10/33 samples in both BT and AT, respectively. The AMC, EB, and PS counts remained above the maximum acceptable microbial limit in 21/33, 3/33, and 9/33 AT samples, respectively, after T2 water line treatment.

The impact of T1 and T2 water line treatment on private and public water supply was evaluated by calculating the $\log_{10}$ ratio from CFU $\log_{10}$ counts detected in BT and AT water samples (Table 1). No significant differences ($p \geq 0.05$) in $\log_{10}$ ratios were observed for AMC, EB, and PS counts after T1 and T2 water line treatment. The $\log_{10}$ ratio was not significantly different ($p \geq 0.05$) between private and public supplied poultry farms after T1 and T2 water line treatment. The median AMC, EB, and PS ratios after T1 waterline treatment were -0.2 ± 2.13, -0.6 ± 1.79, and 0.0 ± 2.26, respectively. The analysis of $\log_{10}$ ratios after T2 waterline treatment resulted in median values of -1.1 ± 2.13 for AMC, 0.0 ± 2.94 for EB, and 0.0 ± 3.12 for PS counts. Although $\log_{10}$

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TABLE 1 The median aerobic mesophilic count (AMC), *Enterobacteriaceae* (EB), and *Pseudomonadaceae* (PS) in poultry drinking water samples were determined before (BT) and after waterline treatment (AT) during T1 and T2 waterline treatment using culture-dependent methods.

Treatment (T)	Water supply	Median AMC log_{10} CFU/ml		Median AMC log_{10} ratio	Median EB log_{10} CFU/ml		Median EB log_{10} ratio	Median PS log_{10} CFU/ml		Median PS log_{10} ratio	*Campylobacter* spp.	
		BT	AT		BT	AT		BT	AT		BT	AT
1	Private	5.8 ± 1.30	5.4 ± 1.99	−0.5 ± 2.37	3.6 ± 2.13	1.6 ± 0.53	−1.1 ± 1.85	4.9 ± 1.30	3.7 ± 2.01	−0.7 ± 2.71	0/15	0/15
1	Public	5.9 ± 0.81	6.4 ± 0.88	−0.2 ± 1.68	3.5 ± 2.13	3.2 ± 2.77	−0.6 ± 1.01	4.6 ± 1.63	5.3 ± 1.95	0.3 ± 2.14	1/21	0/21
2	Private	5.0 ± 1.47	4.1 ± 0.82	−1.1 ± 1.68	1.3 ± 0.00	1.3 ± 0.00	0.0 ± 0.00	3.1 ± 2.11	2.5 ± 1.84	−1.8 ± 2.00	0/9	0/24
2	Public	4.5 ± 1.54	4.8 ± 2.15	−1.2 ± 2.88	2.6 ± 1.36	1.9 ± 0.83	−0.3 ± 3.74	3.7 ± 1.47	3.4 ± 1.54	0.8 ± 3.83	0/9	0/24
Total after T1		5.9 ± 1.02	6.0 ± 1.17	−0.2 ± 2.13	3.6 ± 2.13	2.3 ± 1.52	−0.6 ± 1.79	4.7 ± 1.44	4.7 ± 1.44	0.0 ± 2.26	1/36	0/36
Total after T2		4.6 ± 1.55	4.7 ± 1.85	−1.1 ± 2.13	2.4 ± 1.63	1.6 ± 0.42	0.0 ± 2.94	3.5 ± 1.62	3.1 ± 2.05	0.0 ± 3.12	0/33	0/33

The AMC, EB, and PS values are provided as median values (log_{10} CFU/g) and standard deviations. The presence (+) or absence (−) of *Campylobacter* spp. in water samples identified by the multiplex PCR assay. MAD, median absolute deviation.

ratios between poultry farms with private and public water supplies were not significantly different, we observed higher median log_{10} reduction of AMC, EB, and PS counts at poultry farms with private water supply. During T2 water line treatment higher median log_{10} reduction was observed for AMC and EB counts at poultry farms with public water supply, while higher median log_{10} reduction for PS counts was observed in poultry farms with private water supply.

Out of the 14 poultry farms assessed, five farms exhibited microbial counts below the acceptable microbial limit (<4.0 log_{10} CFU/ml) subsequent to the T1 water line treatment (Figures 2A–C). Among these farms, three had a private water supply, while the remaining two had public water supplies. Notably, poultry farm 7, which had a public water supply, exhibited an AMC count below the maximum acceptable microbial limit in both BT and corresponding AT water sample. Furthermore, 11 poultry farms exhibited EB counts below the maximum acceptable microbial limit. Of these, nine poultry farms demonstrated EB counts below the microbial limit in both BT and corresponding AT samples. Additionally, among 14 poultry farms examined, a total of eight poultry farms exhibited PS counts below the microbial limit. Out of these, four poultry farms demonstrated PS counts below the microbial limit in both BT and corresponding AT samples. Among the poultry farms that underwent two samplings, poultry farms 12 and 13 exhibited AMC and PS counts exceeding the microbial limit in one of the sampling events. Furthermore, poultry farm 12 demonstrated EB counts above the microbial limit on one of two sampling occasions.

During T2 waterline treatment AMC counts below the microbial limit were observ ed in six out of 14 poultry farms (Figures 3A–C). Of these, two poultry farms demonstrated AMC count below the microbial limit in both BT and corresponding AT samples (Figure 3B). EB counts below the microbial limit were observed in 12 out of 14 poultry farms, and among them, nine poultry farms had EB counts below the microbial limit in both the BT and corresponding AT samples. Similarly, PS counts below the microbial limit were observed in ten from 14 poultry farms, and among them, three poultry farms demonstrated PS counts below the microbial limit in BT and corresponding AT samples. Among the poultry farms subjected to two samplings, poultry farm 18 demonstrated AMC, PS and EB counts below the microbial limit during one of the sampling occasions. However, after second sampling, the AMC load in water samples exceeded the microbial limit. Notably, the PS and EB counts remained below the microbial limit during both sampling occasions.

3.2 Bacterial isolate identification in poultry drinking water

Isolate taxonomic assignment was performed using partial sequencing of 16S rRNA gene. In the present study, isolate sequences showed ≥97.0% similarity to the reference sequence in the NCBI database. In BT samples, 123 isolates corresponded to 24 genera and 55 species, while in AT samples, the 113 isolates corresponded to 22 genera and 40 species. Further analysis of bacterial isolates revealed that in BT and AT samples, 43.1% ($n = 41$ isolates) and 36.3% ($n = 53$ isolates) of sequenced isolates were assigned to OP, found in 29/36 BT and 17/36 AT samples (Table 2). The isolates from BT samples contained OP represented by 16 genera and 19 species, while isolates from AT samples contained OP represented by 12 genera and 12 species OP. Furthermore, *C. jejuni* was detected using multiplex PCR in a single BT water sample from a poultry farm with a public water supply.

During the T2 water line treatment, 139 isolates in the BT corresponded to 26 genera and 46 species, whereas 96 isolates in AT samples corresponded to 21 genera and 33 species (Table 2). Among the sequenced isolates, 30.9% ($n = 43$ isolates) and 33.3% ($n = 33$ isolates) corresponded to OP, isolated from 20/33 BT and 14/33 AT samples, respectively. The OP in the BT samples comprised 10 genera, and 14 species, while the OP in the AT samples comprised 11 genera and 14 species. No *Campylobacter* spp. were detected in poultry drinking water samples during the T2 water line treatment.

Figures 4A,B represents the taxonomic classification of assigned isolate sequences at phylum, and genus level. The predominant phyla in BT and AT samples were *Pseudomonadota,* followed by *Bacillota, Actinomycetota*, and *Bacteroidota* (Figure 4A). The frequently isolated genera during both T1 and T2 water line treatment in BT and AT samples were *Aeromonas, Bacillus, Citrobacter, Enterobacter, Pseudomonas* and *Stenotrophomonas* (Figure 4B). Among these, *Pseudomonas* (BT 38.2%; AT 32.7%) and *Bacillus* (BT, 13.0%, AT, 11.5%) were most commonly observed genera during T1 water line treatment. Similarly, during T2 water line treatment, *Pseudomonas* (BT, 31.7%; AT, 33.3%) and *Bacillus* (BT, 10.1%; AT, 11.5%) were predominant genera in BT and AT samples. The Figure 4B depicts the percentage identification of other observed genera during T1 and T2 water line treatments. The majority of sequenced isolates classified as OP in BT and AT samples during T1 and T2 water belonged to the *Pseudomonas* spp., followed by *Stenotrophomonas* spp., *Citrobacter* spp., *Ochrobactrum* spp., and *Acinetobacter* spp. (Figure 4C).

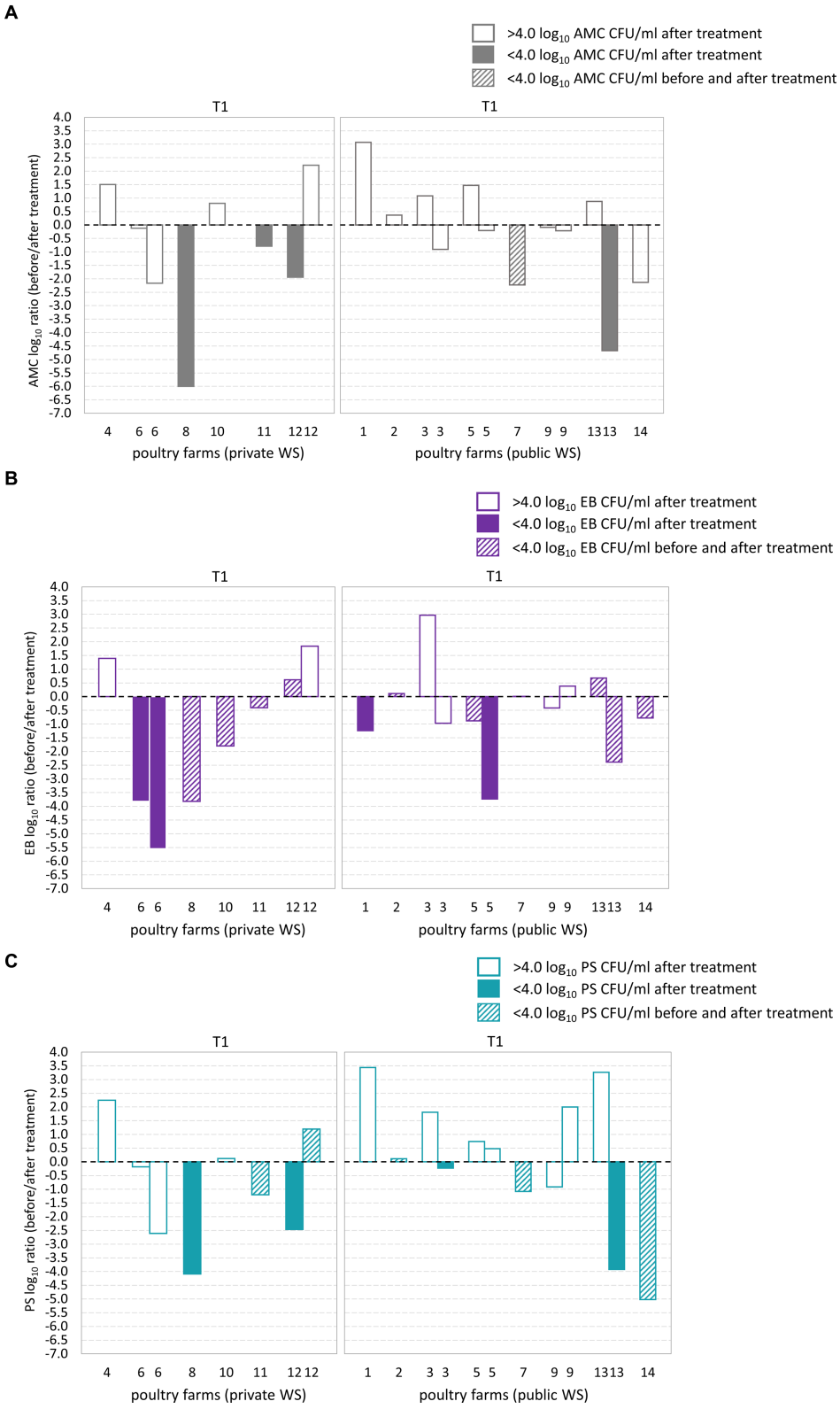

FIGURE 2
Log_{10} transformed average fold changes (before/after waterline treatment) obtained from aerobic mesophilic counts (AMC) **(A)**, *Enterobacteriaceae* (EB) **(B)**, and *Pseudomonadaceae* (PS) **(C)** in poultry drinking water. The x-axis indicates the comparison between poultry farms with private and public water supply (WS) after T1waterline treatment. The y-axis shows the log_{10} AMC, EB, and PS count ratio. The log_{10} AMC, EB, and PS ratio was not significantly different between poultry farms with private and public water supply. No significant differences were observed in the AMC, EB, and PS log_{10} ratio after T1 waterline treatment between poultry farms with private and public WS.

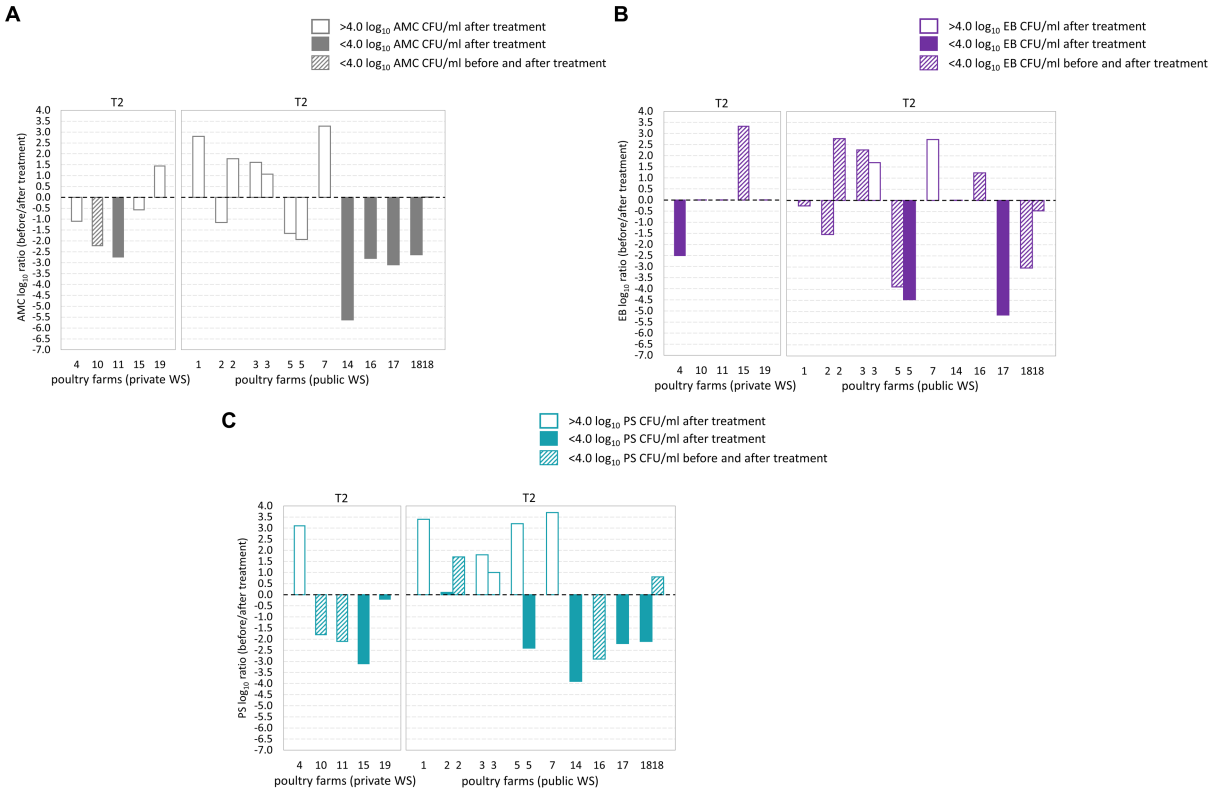

FIGURE 3
Log_{10} transformed average fold changes (before/after waterline treatment) obtained from aerobic mesophilic counts (AMC) **(A)**, *Enterobacteriaceae* (EB) **(B)**, and *Pseudomonadaceae* (PS) **(C)** in poultry drinking water. The x-axis indicates the comparison between poultry farms with private and public water supply (WS) after T2 waterline treatment. The y-axis shows the log_{10} AMC, EB, and PS count ratio. The log_{10} AMC, EB, and PS ratio was not significantly different between poultry farms with private and public water supply. No significant differences were observed in the AMC, EB, and PS log_{10} ratio after T2 waterline treatment between poultry farms with private and public WS.

TABLE 2 An overview of the number of isolate sequences assigned to the different phyla and genera using similarity cut-off of ≥97.0% after partial sequencing of 16S rRNA gene.

	T1		T2	
	Isolate diversity		Isolate diversity	
Sampling timepoint and isolate number	BT (*n* = 123)	AT (*n* = 113)	BT (*n* = 139)	AT (*n* = 93)
	n	*n*	*n*	*n*
Phylum	4	3	4	3
Genus	24	18	26	21
Opportunistic pathogens (≥97.0% sequence similarity)	*n*=53	53	43	33

The assigned bacterial isolate sequences encompass the classification of opportunistic pathogens present in water samples collected before (BT) and after (AT) the T1 and T2 water line treatment.

Furthermore, isolates of *Enterobacter* spp. and *Klebsiella* spp. genera were isolated during T1 and T2 sampling. The *Pseudomonas* spp. isolates identified as OP were most frequently observed bacteria sequences during both T1 (BT, 22/123 isolates; AT, 10/113 isolates) and T2 (BT, 13/139 isolates; AT, 10/96 isolates) sampling.

Before and after the T1 water line treatment, *Pseudomonas* spp. was isolated from BT and AT samples in 12/14 and 9/14 poultry farms, respectively (Table 3). Isolate sequences of OPs were detected in BT samples of 11 out of 14 poultry farms and in AT samples of 9 out of 14 poultry farms. Among the frequently observed genera before and after T2 treatment, the genus *Pseudomonas* was isolated from the BT and AT samples in 12 out of 14 poultry farms and 9 out of 14 poultry farms, respectively (Table 4). The OP were observed in 10 out of 14 poultry farms in BT samples and in 9 out of 14 poultry farms in AT samples after T2 water line treatment.

3.3 Antibiotic susceptibility patterns of bacterial isolates obtained from poultry drinking water

The susceptibility of bacterial isolates recovered from BT ($n = 14$) and AT ($n = 16$) water samples during T1 and T2 water line treatments to 18 antibiotic agents commonly used in poultry production was evaluated using Avian AVIAN1F Vet AST susceptibility plates (Table 5). The goal was to investigate AMR in the most frequently isolated OP isolates, including isolates belonging to *Pseudomonas* spp., *Stenotrophomonas* spp., *Ochrobactrum* spp., as well as AMR in specific waterborne OP important to human health, such as *Aeromonas* spp., *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp.

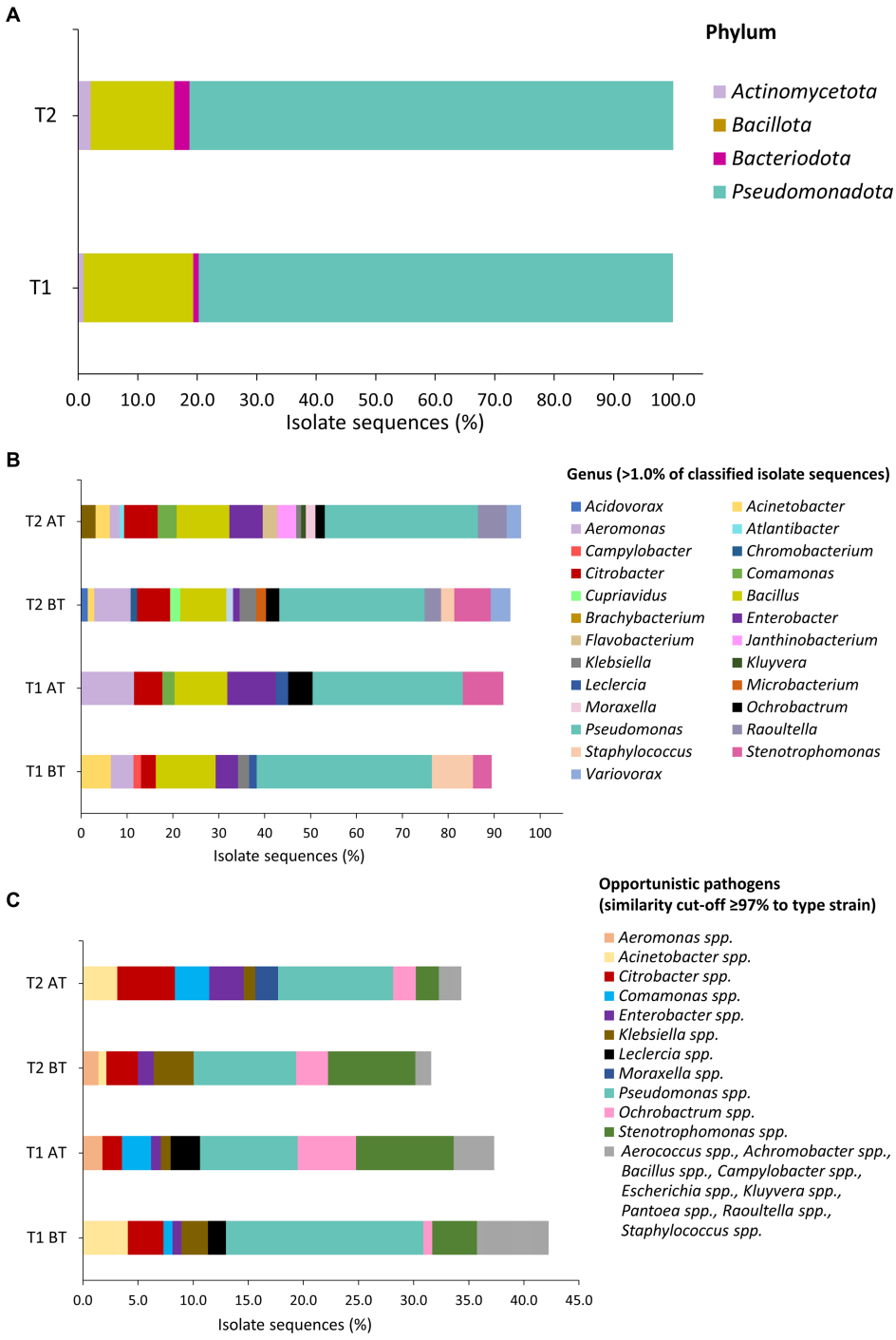

FIGURE 4
Taxonomic classification of isolates based on partial sequencing of 16S rRNA gene on phylum **(A)**, genus **(B)**, and opportunistic pathogens **(C)** level in water samples during T1 and T2 waterline treatments. Sequence similarity cut-off of ≥97.0% was applied for assignment of isolate sequences (1,040 to 1,120 bp fragment) to type strain was applied. **(C)** The bacterial sequences that were isolated from water samples one to two times are indicated by the grey color.

The highest level of AMR was observed against spectinomycin and sulfadimethoxin (90.0%; 27/30 isolates each), followed by ceftiofur (83.3%; 25/30 isolates), florfenicol (66.6%; 20/30 isolates), and neomycin (53.5%, 16/30 isolates). Further, some isolates were resistant to enrofloxacin (23.3%; 13/30 isolates), trimethoprim-sulfamethoxazole (23.1%; 3/13 isolates), sulfathiazole (20.0%; 6/30 isolates), streptomycin (16.7%, 5/30 isolates), gentamicin (13.3%; 4/30 isolates), and trimethoprim-sulfamethoxazole (10.0%, 3/30 isolates).

The MDR was exhibited among the isolates of *Pseudomonas* spp., (17/17 isolates), and *Stenotrophomonas* spp. (1/3 isolates), *Ochrobactrum* spp. (4/4 isolates), *Citrobacter* spp. (2/2 isolates), and *Enterobacter* spp. (1/2 isolates). All *Pseudomonas* spp. isolates showed

TABLE 3 The isolate diversity in poultry drinking water samples was assessed using partial sequencing of 16S rRNA gene of cultured isolates collected during chemical waterline treatment with 4.0 ppm active ClO_2 waterline treatment (T1) at poultry farms.

		Waterline treatment (T1)														Per sample isolation	
	Water supply	Private	Private	Private	Private	Private	Private	Public	Public	Public	Public	Public	Public	Public	Public	BT (n = 36)	AT (n = 36)
	Poultry farm	4	6	8	10	11	12	1	2	3	5	7	9	13	14		
	Water sample	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT		
Phylum (n = 4)	Genus (n = 29)																
Pseudomonadota (n = 20)	*Achromobacter*												1/1[1]			1	1
	Acinetobacter	1/0	1/0	2/0					4/0							5	0
	Aeromonas	0/1			0/1			1/0	2/9	1/0	0/2			2/0		5	5
	Atlantibacter													0/1		0	1
	Campylobacter													2/0		1	0
	Citrobacter									3/5	1/0		2/0			2	3
	Comamonas		0/2				1/0						0/1			1	2
	Enterobacter		2/0				1/1			0/1			2/10		1/0	4	6
	Escherichia		0/1													0	1
	Klebsiella									2/1	1/0					2	1
	Kluyvera				1/0											1	0
	Leclercia			1/0			1/3									2	1
	Ochrobactrum				0/1				0/5						1/0	1	3
	Pantonea				0/1											0	1
	Phytobacter												0/1		1/0	1	1
	Pigmentiphaga							1/0								1	0
	Pseudomonas	3/3	10/7		2/2	3/0	3/7	2/2	3/0	2/6	7/6	2/0	7/1	3/3		26	18
	Raoultella		1/0													1	0
	Rhizobium													0/1		0	1
	Stenotrophomonas			1/0			3/1		0/1		0/5		3/2	1/1	1/0	5	10
Actinomycetota (n = 2)	*Brachybacterium*													1/0		1	0
	Microbacterium			1/0												1	0
Bacillota (n = 5)	*Aerococcus*	1/0														1	0
	Bacillus			1/0	1/0	2/2			4/6	4/1	4/3				0/1	10	7
	Lysinibacillus						1/0									1	0
	Planococcus								1/0							1	0
	Staphylococcus		1/0		1/1		3/0		1/0					3/0	2/0	10	1
Bacteroidota (n = 2)	*Chryseobacterium*				0/1											0	1
	Sphingobacterium		1/0													1	0
Bacterial diversity on poultry farm		3/2	6/3	5/0	4/6	2/1	7/4	3/1	6/4	5/5	4/4	1/0	5/6	6/4	5/1		
Identified opportunistic pathogens		1/0	4/1	0/0	2/1	0/0	4/3	1/1	2/2	3/2	4/3	0/0	4/3	1/1	0/0	29/36	17/36

The occurrence of each genus in sample collected before treatment (BT, n = 36) and after treatment (AT, n = 36) was determined. The percentage of isolate occurrence was calculated based on cultured isolates from BT (n = 123) and AT (n = 113) samples. The isolate diversity at each poultry farm was evaluated in both BT and AT samples, and the presence of opportunistic pathogens was also determined. [1]Number of bacterial isolates isolated from BT and AT samples.

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TABLE 4 The isolate diversity in poultry drinking water samples was assessed using partial sequencing of 16S rRNA gene of cultured isolates collected during combined chemical (3.0% peroxyacetic acid [PAA] and 4.0 ppm active ClO_2) with mechanical (purging of waterlines with a high-pressure air pump) waterline treatment (T2) in poultry farms.

		Waterline treatment (T2)														Per sample isolation	
	Water supply	Private	Private	Private	Private	Private	Public	Public	Public	Public	Public	Public	Public	Public	Public	BS (n = 33)	AS (n = 33)
	Poultry farms	4	10	11	15	19	1	2	3	5	7	14	16	17	18		
	Water sample	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT		
Pylum (n = 4)	Genus (n = 33)																
Pseudomonadota (n = 24)	*Acidovorax*					0/2[1]						2/0			0/1	5	4
	Acinetobacter			1/0			0/1	1/0		0/2						2	2
	Aeromonas	0/1						4/1				1/0			6/0	7	2
	Atlantibacter						0/1									0	1
	Brevundomonas				0/1											0	1
	Buttiauxella							1/0								1	0
	Chromobacterium												2/0			2	0
	Citrobacter	0/2			0/3		1/0	0/1	3/0	4/0	1/1				1/0	8	4
	Comamonas							0/4								0	3
	Cupriavidus			1/0	1/0								1/0			3	0
	Enterobacter					1/0			1/3						0/4	2	3
	Janthinobacterium							0/1	0/3							0	3
	Klebsiella				0/1		3/0			2/0						2	1
	Kluyvera						0/1									0	1
	Moraxella								0/2							0	1
	Ochrobactrum							2/2							2/0	3	1
	Pantonea							0/2								0	1
	Phytobacter						1/0									1	0
	Pigmentiphaga														1/0	1	0
	Pseudaeromonas												1/0			1	0
	Pseudomonas	1/1	5/0	1/0	6/0	4/1	0/2	2/1	0/8	5/6	3/3	5/0	1/0	4/3	7/7	19	16
	Raoultella							1/0					4/0			3	0
	Stenotrophomonas			2/0	1/0	2/1			6/0	0/5						7	3
	Variovorax	2/0		2/0	1/0	0/1									1/2	5	2
Actinomycetota (n = 2)	*Brachybacterium*													2/0		1	0
	Microbacterium	1/0		1/0											1/0	3	0
Bacillota (n = 4)	*Bacillus*	1/1		3/0		1/0		6/5	2/0	1/5						10	8
	Jeotgalicoccus														1/1	1	1
	Staphylococcus							1/0						2/0	1/1	3	1
	Trichococcus									1/0						1	0

(Continued)

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TABLE 4 (Continued)

		Waterline treatment (T2)														Per sample isolation	
	Water supply	Private	Private	Private	Private	Public	Public	Public	Public	Public	Public	Public	Public	Public	Public	BS ($n = 33$)	AS ($n = 33$)
	Poultry farms	4	10	11	15	19	1	2	3	5	7	14	16	17	18		
	Water sample	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT		
Pylum ($n = 4$)	Genus ($n = 33$)																
Bacteroidota ($n = 3$)	*Chryseobacterium*			1/0												1	0
	Flavobacterium						0/1		0/2			1/0				1	3
	Pedobacter									1/0						1	0
Bacterial diversity on poultry farm		4/4	1/0	9/0	4/3	4/4	3/5	8/8	4/5	6/4	2/2	4/0	5/0	3/1	9/6		
Identified opportunistic pathogens		0/1	0/0	2/0	1/1	2/0	1/1	2/2	3/2	3/2	1/1	1/0	0/0	1/1	2/3	19/33	14/33

The occurrence of each genus in sample collected before treatment (BT, $n = 33$) and after treatment (AT, $n = 33$) was determined. The percentage of isolate occurrence was calculated based on cultured isolates from BT ($n = 139$) and AT ($n = 96$) samples. The isolate diversity at each poultry farm was evaluated in both BT and AT samples, and the presence of opportunistic pathogens was also determined. [1]Number of bacterial isolates isolated from BT and AT samples.

resistance patterns exhibiting resistance to a minimum of four and a maximum of eight antibiotics. Tested *Stenotrophomonas* spp. isolates also demonstrated resistance patterns to a minimum of four and a maximum of six antibiotics. All tested *Ochrobactrum* spp. were resistant to four antibiotics. The isolates of *Citrobacter* spp. were resistant to six antimicrobial classes and nine different antibiotics. The isolates of *Enterobacter* spp. showed resistance patterns to a minimum of two and a maximum of four antibiotics. The isolates of *Klebsiella* spp. were resistant to five antibiotics, while *Aeromonas* spp. isolate was susceptible to all tested antibiotic agents.

4 Discussion

Providing poultry with water that meets the highest quality standards is essential to ensure the safety and quality of the products derived from these animals. The presence of high microbial loads and biofilms in the drinking water lines can have a negative effect on poultry health and performance (14). Moreover, when health issues arise within a poultry flock, antibiotics are often administered through drinking water. This practice increases the risk of antibiotic resistance within poultry farms, presenting a potential threat to both animal and human health (12).

We assessed microbial quality of poultry drinking water at the end of the drinking line based on established limits from previous studies, where AMC, EB, and PS counts below 4.0 $\log_{10}$ CFU/ml were deemed acceptable (4, 7, 12). At the end of the fattening period, AMC exceeded acceptable limits in most poultry farms tested, with similar trends observed for PS counts. However, EB remained within acceptable levels in the majority of farms. Environmental factors, such as ambient temperatures (±25°C), low water flow rates, pipeline installation type, and feed additives (often mixed with glucose) provided ample nutrients for bacteria, contributing to a high microbial load at the end of the fattening period (42). Poultry farms opt to chlorinate and/or acidify their drinking water systems due to the easy application, cost-effectiveness, and broad antimicrobial properties of these treatment systems (12). Additionally, mechanical cleaning helps remove biofilm from surfaces inside the drinking water system. Surprisingly, plate count analysis did not show a significant reduction of microbial load (AMC, EB, and PS counts) in AT samples after chemical water line treatment (T1) or combined chemical with mechanical treatment (T2). Unlike previous reports associating poultry farms with a private water supply with elevated microbial loads, we did not observe significant differences in microbial load between poultry farms with private or public water supplies (43). The microbial counts observed in our study were similar to those found on surfaces inside poultry house drinking water systems, which were typically above 6.0 $\log_{10}$ CFU (12). This suggests a limited disinfection effectiveness likely due to low concentration of applied disinfectant. Despite mechanical cleaning and subsequent disinfection, high microorganism levels persisted in the water lines, indicating that the disinfectant concentration post-mechanical treatment was insufficient to eliminate the majority of microorganisms. However, our study focused solely on microbiological parameters, overlooking vital factors such as water hardness, pH, temperature, and free ClO_2 residues within the water lines. This limited our ability to comprehensively evaluate the

TABLE 5 Antimicrobial resistance among bacterial isolates before (BT) and after (AT) waterline treatment to a panel of veterinary antimicrobials commonly used in the poultry production.

				Antimicrobial class[1] (in μg/ml):										
				Aminoglycosides				Fluoroquinolones	Cephalosporins	Tetracyclines	Phenicols	Sulfonamides		Diaminopyrimidine/ sulfonamides
Opportunistic pathogens[2]	Treatment[3]	Time-poin[4]	Isolates (*n*)	GEN	SPE	NEO	STR	ENR	XNL	TET and OXY	FFN	SDM	STZ	SXT
				≥8	≥64	≥32	≥1,024	≥2/1	≥4	≥8	≥8	≥256		≥2/38
Citrobacter spp.	1	BT	1	0/1	1/1	1/1	0/1	1/1	0/1	1/1	1/1	1/1	1/1	1/1
	2	AT	1	0/1	1/1	1/1	0/1	1/1	0/1	1/1	1/1	1/1	1/1	1/1
Enterobacter spp.	1	AT	1	0/1	0/1	0/1	0/1	0/1	1/1	1/1	1/1	1/1	0/1	0/1
	2	AT	1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	1/1	0/1	0/1
Klebsiella spp.	2	BT	1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1	1/1	1/1	0/1
Ochrobactrum spp.	1	AT	1	0/1	1/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1
	2	BT	2	0/2	2/2	2/2	0/2	0/2	2/2	0/2	0/2	2/2	0/2	0/2
	2	AT	1	0/1	1/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1
Pseudomonas spp.	1	BT	8	0/8	8/8	0/8	1/8	3/7	8/8	NA[5]	8/8	8/8	1/8	NA
	1	AT	6	0/6	6/6	6/6	2/6	2/6	2/6		6/6	6/6	1/6	
	2	BT	1	1/1	1/1	0/1	0/1	0/1	1/1		1/1	1/1	0/1	
	2	AT	2	0/2	2/2	0/2	1/2	0/2	2/2		2/2	2/2	0/2	
Aeromonas spp.	2	AT	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Stenotrophomonas spp.	1	AT	1	1/1	1/1	1/1	1/1	0/1	1/1	NA	0/1	0/1	0/1	0/1
	2	BT	1	0/1	1/1	1/1	0/1	0/1	1/1		0/1	1/1	1/1	1/1
	2	AT	1	1/1	1/1	1/1	0/1	0/1	1/1		0/1	0/1	0/1	0/1
BT (*n*/*N*)[6]			14	2/14	14/14	5/14	1/14	4/14	12/14	1/14	10/14	14/14	4/14	2/14
AT (*n*/*N*)			16	2/16	13/16	11/16	4/16	4/16	13/16	3/16	10/16	13/16	2/16	1/16
Total (*n*/*N*)			30	4/30	27/30	16/30	5/30	7/30	25/30	4/10	20/30	27/30	6/30	3/30

[1]The resistance breakpoints for selected antimicrobial classes represented by the antimicrobial agents in μg/ml for ≥8 gentamicin (GEN); ≥64 spectinomycin (SPE), ≥32 neomycin (NEO); ≥1,024 streptomycin (STR); ≥2/1 enrofloxacin (ENR); ≥4 ceftiofur (XNL); ≥8 tetracycline (TET) and oxytetracycline (OXY); ≥8 florfenicol (FFN); ≥256 sulfadimethoxine (SDM) and sulfathiazole (STZ); ≥2/38 trimethoprim-sulfamethoxazole (STX). [2]Bacteria species identified by partial sequencing of 16S rRNA gene. [3]Waterline treatment type. [4]Isolate identification in water sample before treatment (BT) and after treatment. [5]NA: not applicable, bacteria have intrinsic resistance against the antimicrobial agent. [6]*n*/*N*: number of isolates resistant to particular antimicrobial agent/total isolates tested.

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efficiency of the 4 ppm active ClO_2 and 3% PAA during water line treatments. Previous studies have highlighted the limited effectiveness of water line disinfection practices using oxidizing agents such as chlorine or hydrogen peroxide (12). This limitation primarily arises from applied concentrations being lower than recommended by suppliers, which is in alignment with our observations of high microbial load in AT samples. In addition, inconsistencies were noted in AT water samples among poultry farms that were sampled twice, emphasizing the need for frequent water quality checks in a closed system. Even with the addition of typical concentrations of hydrogen peroxide (25–50 ppm) and free chlorine (2–5 ppm) to poultry drinking water during fattening, biofilm formation was observed in minimally contaminated water (7). Therefore, regular monitoring of microbial water quality, combined with consistent water line treatment during the fattening period, is a crucial aspect of robust biosecurity programs at poultry farms. Moreover, specialized contractors have been noted to achieve more effective water line treatment compared to farmers (42, 44). Finally, Zou et al. (45) demonstrated a significant reduction of *E. coli*, *Salmonella*, *Staphylococcus aureus*, and mold in poultry drinking water after treatment with sodium dichloroisocyanurate, correlating positively with poultry health.

The presence of high microbial load in water samples led to a wide taxonomic variety among isolates in both BT and AT samples, ranging between 18 and 26 genera. While definitive taxonomic conclusions require further extensive studies, the frequent presence of genera such as *Aeromonas, Bacillus, Citrobacter, Enterobacter, Pseudomonas* and *Stenotrophomonas,* commonly associated with waste and surface waters, underscores an increased risk to both poultry and human health in this study (19, 46). Identification of genera, including *Pseudomonas, Stenotrophomonas*, and *Ochrobactrum,* were in line with the isolates found on surfaces in poultry drinking water system (12). The majority of the identified bacteria found at poultry farms independent of their water supply were OP, specifically those belonging to *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp. The OP belonging to *Pseudomonas* spp. are linked to secondary infections in both poultry and humans. In poultry, these infections can manifest as septicemia, skin lesion infections, and hemorrhagic pneumonia (47). In immunocompromised humans, they can lead to septicemia, pneumonia, and urinary tract infections (48). Previous studies have also emphasized an increased mortality rate in poultry following *P. aeruginosa* OP infection (49, 50). A previous study demonstrated enhanced adhesion to abiotic surfaces, tissue invasion through cytotoxic effects, resistance to 0.2 mg/mL chlorine, and increased AMR among *P. aeruginosa* isolates from water (51). Moreover, *Stenotrophomonas maltophilia* and *Ochrobactrum intermedium* are emerging human environmental pathogens causing infections, primarily in immunocompromised patients (52). *S. matophilia* and *P. aeruginosa* are often co-isolated from the lungs of cystic fibrosis patients, and previous research findings suggest that *S. maltophilia* modulates the virulence of *P. aeruginosa* in a multispecies biofilm (53). While *S. maltophilia* and *O. intermedium* have been recognized to cause infections in immunocompromised humans, no established link between water quality and disease development in poultry production involving these bacterial species has been reported yet. Nevertheless, notable characteristics of these bacteria, such as resistance to disinfection and heat, slow growth, and biofilm formation, emphasize the potential risk of poultry and farmer infection through direct contact with drinking water, along with the risk of cross-contamination of chicken meat products during post-slaughter processing.

During T1 water line treatment, *C. jejuni* was detected in one water sample collected before water line treatment at a poultry farm with a public water supply, while other analyzed samples tested negative. The detection of *Campylobacter* spp. in water depends on factors such as sample volume, sample number, and bacterial concentration (54, 55). Furthermore, *Campylobacter* spp. can enter a viable but nonculturable state (VBNC) under environmental stress, potentially hindering growth on conventional culture media due to limited metabolic activity (56). Consequently, *Campylobacter* spp. might have been overlooked in other analyzed water samples due to limitations in the processing method. These limitations include a small sample volume, the absence of water sample filtration, and the potential presence of *Campylobacter* spp. in the VBNC state, which cannot be detected using the ISObased methods used in the current study. While this approach may have led to missing *Campylobacter* spp., our assessment of bacterial load and diversity in the water samples examined provided a comprehensive insight into both quantitative and qualitative microbial content in poultry drinking water. Notably, previous research emphasizes that a significant presence of *Pseudomonas* spp. in poultry drinking water heightens the risk of *Campylobacter* spp. infection, as *Campylobacter* sp. isolates from poultry can persist for extended periods within *P. aeruginosa* biofilms in drinking water (57–59).

Previous studies have established poultry farms as significant reservoirs of antimicrobial resistance genes, contributing to the emergence of AMR and transmission dynamics of MDR bacteria at the humananimalenvironment interface (60–62). Our findings align with these observations, revealing MDR patterns in all tested isolates of both *Pseudomonas* spp. and *Ochrobactrum* spp. isolates from BT and AT water samples. Furthermore, a single *Stenotrophomonas* spp. from BT water sample exhibited MDR pattern. The consistent AMR patterns observed in both BT and AT water samples align with our observations of ineffective water line treatment characterized by limited disinfectant concentrations that allow for the survival and persistence of AMR bacteria within the water lines. The antimicrobials permitted for poultry treatment in Austria at the time of this study include enrofloxacin, doxycycline, trimethoprimsulfamethoxazole, amoxicillinclavulanic acid, colistin sulfate, tetracycline, and gentamicin (63–67). For the isolates we utilized in the AST, information or protocols regarding the current or past treatment of poultry on these farms were not available to the authors; therefore a detailed analysis of the potential causes of AMR in these isolates was not possible. The isolates from both BT and AT water samples exhibited increased resistance patterns to spectinomycin, sulfadimethoxin, ceftiofur, florfenicol, and neomycin, likely attributed to their widespread use in poultry health management on farms. This raises concerns, as elevated streptomycin resistance in *E. coli* isolates from broilers in several countries in Europe, including Poland, Germany, Great Britain, France and Spain was previously reported (68). Additionally, resistance to streptomycin and sulfadimethoxin was previously reported in *Salmonella* spp. isolates from poultry farms in Canada and the United States (69–72). Furthermore, these isolates exhibited resistance to ceftiofur and enrofloxacin, both of which are

recognized as top priority critically important antimicrobials by the World Health Organization (73). This antimicrobial resistance raises concerns, as it can be indirectly transmitted through horizontal gene transfer to *E. coli, Salmonella* spp., *Campylobacter* spp. and other potential poultry and human pathogens. Heinemann et al. (42) reported isolation of extendedspectrum betalactamaseproducing bacteria (ESBL) such as *P. aeruginosa*, *Enterobacter* spp., *Klebsiella* spp., and *Acinetobacter baumanni* from poultry drinking water lines and sprinkler systems. ESBL bacteria can hydrolyze extendedspectrum cephalosporins, monobactams, and penicillins and thus lead to elevated morbidity and mortality, further complicating therapeutic choices, particularly among elderly and immunocompromised individuals (74–76). The observed AMR resistance patterns in poultry drinking water isolates highlight the potential for acquiring antimicrobial resistance through wateradministered medication, posing a risk and limiting treatment options in both veterinary and human medicine (1, 42, 77–79).

The study emphasizes the persistent challenge of maintaining microbial quality in poultry drinking water. The high microbial load observed is attributed to established microbiota in the water system, resistant to suboptimal disinfectant concentrations used during cleaning. Furthermore, our findings suggest that current poultry treatment and antibiotic usage may elevate the presence of AMR bacteria in drinking water due to inefficient management. Addressing this issue necessitates regular water monitoring, consistent water line treatment, and improved farmer education. Enhancing understanding of biological processes in drinking water systems and microorganism viability can lead to better guidance on herd health and farm productivity. Identifying and mitigating onfarm water quality risks, including assessing waterline technologies affecting microbiota in drinking water and water lines, is essential for controlling pathogen and antibiotic transmission in poultry production.

5 Conclusion

In conclusion, the majority of poultry farms in Austria exhibited high microbial loads in drinking water, largely attributed to inadequate water line management practices, including the use of suboptimal disinfectant concentrations and inconsistent treatment. Notably, there were no significant differences observed between chemical and combined chemical and mechanical water line treatments. The prevalent microbiota in poultry included *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp. Moreover, these isolates from both before and after water line treatment samples displayed increased resistance patterns to commonly used antimicrobials to treat bacterial infections in poultry. Our results underscore the need for future studies to consider appropriate water supply management on poultry farms in terms of the One Health approach, to protect public health, and to raise awareness among farmers and veterinarians.

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.

Author contributions

AM: Data curation, Writing – original draft, Investigation, Methodology, Software, Visualization. MM: Data curation, Methodology, Writing – review & editing. KW: Data curation, Methodology, Writing – review & editing. AS: Methodology, Writing – review & editing, Investigation. IK: Investigation, Methodology, Writing – review & editing. CF: Writing – review & editing. IL: Formal analysis, Methodology, Writing – review & editing. MW: Conceptualization, Writing – review & editing. BS: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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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/fvets.2023.1254442/full#supplementary-material

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What question are we trying to answer? Embracing causal inference

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This study summarizes a presentation at the symposium for the Calvin Schwabe Award for Lifetime Achievement in Veterinary Epidemiology and Preventive Medicine, which was awarded to the first author. As epidemiologists, we are taught that "correlation does not imply causation." While true, identifying causes is a key objective for much of the research that we conduct. There is empirical evidence that veterinary epidemiologists are conducting observational research with the intent to identify causes; many studies include control for confounding variables, and causal language is often used when interpreting study results. Frameworks for studying causes include the articulation of specific hypotheses to be tested, approaches for the selection of variables, methods for statistical estimation of the relationship between the exposure and the outcome, and interpretation of that relationship as causal. When comparing observational studies in veterinary populations to those conducted in human populations, the application of each of these steps differs substantially. The *a priori* identification of exposure–outcome pairs of interest are less common in observational studies in the veterinary literature compared to the human literature, and prior knowledge is used to select confounding variables in most observational studies in human populations, whereas data-driven approaches are the norm in veterinary populations. The consequences of not having a defined exposure–outcome hypotheses of interest and using data-driven analytical approaches include an increased probability of biased results and poor replicability of results. A discussion by the community of researchers on current approaches to studying causes in observational studies in veterinary populations is warranted.

KEYWORDS

causation, observational studies, veterinary, variable selection, confounding

Introduction

Early in every epidemiology student's training, they are indoctrinated with the mantra that "correlation/association does not imply causation." Numerous examples of non-causal associations exist; one such example is the finding that the number of human births over time is correlated ($p = 0.008$) with the number of stork breeding pairs in European countries (1), and yet it would be ludicrous to conclude that storks cause babies. The association is either random or related to the presence of a confounding variable.

There are two main reasons why associations do not imply causation: temporal ambiguity and spurious (non-causal) associations (2). Temporal ambiguity occurs because the temporal sequence of the two correlated variables may not be clear or identifiable. For instance, although stork density and human birth rates are correlated over time in Eastern Europe, the correlation does not address whether stork densities are antecedent or a consequence of human birth rates. Non-causal relationships may also explain apparent associations. These may include confounding factors. For instance, it is plausible that the apparent association between stork density and human birth rates is related to the confounding effects of socioeconomic status; when times are good, people may be more likely to add a child to their family, and there also may be more food waste during good economic times, increasing the number of storks in an area.

From a research perspective, issues of temporal ambiguity and confounding can both be addressed by random allocation to the intervention group (2). For this reason, experiment approaches such as randomized controlled trials are considered the strongest research design for establishing causation. Nonetheless, observational studies are common in veterinary medicine. It may not be ethical or feasible to randomly allocate modifiable risk factors to study subjects, and the necessary sample sizes may be prohibitively expensive, especially for interventions allocated at higher organizational levels, such as pen or herd. In addition, the study populations in observational studies may be more representative of source populations than in experimental trials, and observational settings may better reflect multifactorial disease causation (3). Finally, large observational datasets may exist for animal populations (e.g., medical record systems for companion animals and production databases for food animals), and these may be available for researchers (2).

Identifying causal associations is a common purpose in observational research

Observational studies may be conducted for several reasons: to estimate a single parameter such as incidence or prevalence, to predict an outcome (e.g., to identify at-risk individuals or populations or for prognostic purposes), to identify possible exposures for further study (exploratory or "hypothesis-generating" studies), or to identify causal relationships ("hypothesis-testing" studies). In observational studies in animal populations, identifying causal relationships is a common purpose; in an evaluation of 200 observational studies in the veterinary literature published between 2020 and 2022, causal wording was used in 86% of the articles (4). Additionally, a further evaluation of 100 randomly selected studies from the Sargeant et al. (4) study found that 70% of the studies did not state that the purpose was prediction and they either discussed the potential for confounding (a causal construct) or conducted multivariable statistics. Therefore, it might reasonably be assumed that the purpose of these 70 studies was to identify causal relationships.

Comparison of observational approaches to studying causes in the human versus veterinary literature

Ahern proposed a four-step framework for studying causal relationships in human health research (5). The steps include the following: (1) articulating the causal question (identifying exposure: outcome pairing(s) of interest and describing the causal parameter of interest); (2) linking causal and statistical parameters by considering the assumptions under which the exposure groups are equal (identification of confounding variables); (3) estimating the statistical parameter (controlling for confounding); and (4) interpreting the findings as causal effects (theoretical considerations). How are these steps applied in studies in veterinary populations where the intent is to identify causal relationships? Is the approach to identifying causal relationships in the veterinary literature the same as the approach in the human literature? To address these questions, we evaluated the 70 studies (above) where the purpose was assumed to be the identification of causal relationships and compared the results to those from a study by Staerk et al. on observational studies conducted in human populations (6).

Staerk et al. evaluated methodological approaches in 272 observational studies of human populations published in 2019 in four epidemiological journals (Epidemiology, American Journal of Epidemiology, European Journal of Epidemiology, and International Journal of Epidemiology) (6). Staerk et al. distinguished between "hypothesis generating" (exploratory) studies and "hypothesis testing" (causal) studies (6). The definition of causal studies was that the authors defined one or more exposure–outcome pairings of interest, which is the first component of articulating a causal question. Of the 272 observational studies of human populations, 94% included one or more defined exposure–outcome pairings of interest, as compared to 15 of 70 (21%) in observational studies of veterinary populations. This is not a direct comparison because the study of human populations selected articles from four epidemiology journals, whereas the study in veterinary populations did not include any discipline-specific journal restrictions. Nonetheless, it appears that causal studies—or at least the identification of exposure–outcome pairings of interest—are more common in observational studies of human populations. Additionally, only 3 of the 70 studies in the veterinary literature included a statement that the purpose of the study was causal, and none of these explicitly defined the causal parameter of interest (i.e., direct or total causal effect).

The second and third steps in the framework for causal studies involve the identification of confounding variables and the approaches to their control. It is recognized that a preferred approach for the selection of confounding variables is the use of prior knowledge of the underlying causal structure, ideally using Directed Acyclic Graphs (DAGs), with data-driven methods less appropriate to adequately control for confounding (7). Data-driven (algorithm-based) methods for controlling confounding include the use of *p*-values for variable selection (e.g., stepwise selection), methods based on changes in beta-coefficients, and selection of variables to identify individual predictors for inclusion in multivariable model building (univariable screening). Evaluations of the approaches to selecting confounding variables have been conducted on observational studies in human populations published in 2008 (8), 2015 (9), and 2019 (6). In all three of these studies, the observational studies were published in the American

Journal of Epidemiology, the European Journal of Epidemiology, Epidemiology, and the International Journal of Epidemiology, all considered to be high-impact journals. The results of these studies and the 70 observational studies in veterinary populations are shown in Table 1. In observational studies of human populations, the use of prior knowledge to select confounding variables has increased over time, from 28 to 73%. Although trends over time were not assessed for observational studies in veterinary populations, prior knowledge was used to select variables in 14% of the studies published in the veterinary literature during approximately the same time period as the study by Staerk et al. (6), reporting that 73% of observational studies in human populations used prior knowledge for variable selection. In the human population studies, the use of data-driven methods to select variables decreased from 37% for studies published in 2008 to 16% for studies published in 2019. However, data-driven methods to select confounding variables are the norm in observational studies of veterinary populations, with these techniques employed in 93% of studies published between 2020 and 2022. The main reason for the differences between observational studies in human versus veterinary populations appears to be the high proportion of studies in veterinary populations where univariable screening and/or *p*-value-based selection approaches are used.

The final step in the framework for causal inference pertains to interpreting the results as causal. One way to consider whether a causal interpretation is appropriate is to consider the guidelines proposed by Sir Bradford Hill (10). These include strength of association, specificity of association, consistency, temporality of exposure and outcome, biological gradient, biological plausibility, coherence with current knowledge, experimental confirmation, and analogy. The application of Hill's criteria was not accessed in the study by Staerk et al. (6). Based on the information provided in the discussion section of the 70 publications that were deemed to be causal studies of veterinary populations, the concepts provided in Hill's guidelines were seldom discussed. Although biological plausibility and coherence were routinely addressed, these discussions tended to be framed as general comparisons to the results of other studies without discussing whether these comparisons strengthened or weakened a causal argument for any of the associations identified in the study. A discussion of the temporal sequence of the exposure relative to the outcome was included in seven publications, and the need for experimental confirmation was discussed in four publications. For three studies, the authors explicitly stated that the study design used was not appropriate for causal inference. It should be noted that Sir Bradford Hill did not intend the guidelines to be used as "causal criteria," and not all of the concepts are necessary or achievable. Ioannidis argues that consistency, temporality, and experimental confirmation are the most relevant concepts for causal inference, although even these are not always possible or straightforward to determine (11). Nonetheless, it appears that the discussion sections in literature from observational studies in veterinary populations are neither strengthening nor disputing causal claims.

Implications of differences in approaches between causal observational studies in the human versus veterinary literature

The application of the four steps for causal studies in the veterinary literature suggests that there are substantive differences in approaches in the human literature and that inappropriate (or less than ideal) approaches are common in studies of veterinary populations. This then begs the question, "does it matter?." We argue that it does matter; if the purpose of an observational study is to identify causal associations, then not having one or more defined (and *a priori*) exposure–outcome pairs of interest and using data-driven methods to identify confounding variables may lead to biased results due to inappropriate control of confounding, inappropriate uses of *p*-values, and the use of questionable research practices such as HARKing (hypothesizing after the results are known), p-hacking, and data dredging.

Data-driven methods to identify confounders may be problematic, as a computer algorithm cannot distinguish between confounding

TABLE 1 Variable selection methods for control of confounding in observational studies in human epidemiology journals over time and veterinary populations between 2020 and 2022.

Variable selection method[a]	Human epidemiology journals, 2008 (*N* = 300, results as %)[b]	Human epidemiology journals, 2015 (*N* = 292, results as %)[c]	Human epidemiology journals, 2019 (*N* = 272, results as %)[d]	Causal studies in veterinary populations, 2020–2022 (*N* = 70, results as %)
Prior knowledge	28%	50%	73%	14%
Prior knowledge using DAGs	NA	NA	13%	7%
Data-driven methods	37%	24%	16%	93%
Change in estimate	15%	12%	7%	7%
Use of p-values (e.g., stepwise selection)	29%	5%	4%	69%
Univariable screening	NA	9%	4%	76%
Other methods	3%	2%	3%	9%
Not described	35%	37%	16%	6%

[a]Variable selection categories are not mutually exclusive. Therefore, percentages within columns may sum to more than 100%. [b]Walter and Tiemeier, 2009 (8). [c]Talbot and Massamba, 2019 (9). [d]Staerk et al., 2024 (6).

variables, colliders, or intervening variables. This can lead to bias in estimating the exposure effect size (6, 12, 13). Another example of inappropriate control of confounding is illustrated by the "Table 2 fallacy." The Table 2 fallacy refers to the presentation of results from a multivariable model as though each variable can be considered an exposure of interest with the remaining variables corresponding to confounders (14). This interpretation assumes that the causal structure (and therefore the confounding variables that need to be included) is the same for each of the variables in the model, an assumption that may not be true. Table 2 shows that fallacies appear to be common in veterinary medicine. In the 70 observational studies in veterinary populations explored herein, there were four publications where only univariable results were presented (but were categorized as causal because confounding was discussed), four publications where it was not possible to distinguish whether the results represented univariable or multivariable models, and four publications where there were one or no significant results. Of the remaining 58 publications where multivariable results were presented, 54 (93%) included results that could be considered a Table 2 fallacy.

There is a plethora of information on the uses and abuses of *p*-values related to inference about the effect size (i.e., null hypothesis significance testing), and the interested reader is referred to available resources on this topic [for example (15, 16)]. In the context of variable selection for causal studies, there are issues related to p-values and the importance of the effect size. *p*-values do not provide information on the clinical or biological importance of an association (e.g., the effect size that would represent an appreciable benefit or harm of applying an intervention). Additionally, some studies likely are not sufficiently powered to find meaningful differences as statistically significant for multiple variables that were identified as possible exposures *post hoc*. The confidence intervals on an effect size may, therefore, include an association representing a meaningful difference and yet not meet an arbitrary significance cut point for inclusion in a multivariable model.

Not having one or more exposure–outcomes of interest defined *a priori* may lead to the use of techniques involving cherry-picking results or question trolling, such as HARKing and p-hacking. These approaches can lead to biased results (17, 18). These and similar practices may be associated with an increased probability of type I errors. Statistically significant results are more likely to be reported within a manuscript, and studies with statistically significant results are more likely to be published (19). Cherry-picking results or question trolling can lead to type I errors, biased estimates becoming theory, and results for observational studies not being replicable.

In defense of HARKing

It should, however, be noted that although data-driven approaches to variable selection may lead to biased results, subject-matter knowledge may not always be sufficient to provide clear input to the identification of potentially confounding variables that need to be considered (20). Therefore, *post hoc* data-driven analyses may be of value for moving knowledge in a subject forward (21). However, the analyses should be reported as *post hoc,* and the results should be reported as exploratory. Hollenbeck and Wright refer to this practice as THARKing (Transparently HARKing) (21). However, from the dataset of 70 causal observational studies in veterinary populations, only 3 of the 55 that did not define one or more exposure–outcome pairings of interest reported that their analyses and results were exploratory. Thus, there is considerable room for improvement in the transparency of reporting.

Discussion

The comparison between observational studies of causal associations conducted in human populations versus veterinary populations highlights some substantive differences in approaches. In particular, approaches to research question formulation and confounding variable selection in studies in the veterinary literature may be prone to providing biased results. If observational studies of causal associations in the veterinary literature are to remain relevant in the broader epidemiological literature, these issues need to be addressed. Short-term solutions, which could be implemented immediately, include clearly describing the purpose of an observational study as causal, exploratory, or predictive. Methods and material sections could be expanded to include a stronger rationale for the identification and control of confounding variables, and ideally a DAG of the hypothesized causal pathways. Discussion sections could be modified to include an explicit discussion of the strength of causal arguments (causal studies), needed research (exploratory studies), or predictive strength of the model (predictive studies). In the longer term, there is a need for epidemiologists conducting observational studies in veterinary populations to discuss the implications of differences in our approach from studies in the human literature and to determine a path forward. Change will require concerted efforts by not only researchers but also mentors of the next generation of researchers, peer-reviewers, and journal editors. In this era of "One Health," it is time to embrace "One Epidemiology."

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: anonymized data available for research purposes from the first author upon request. Requests to access these datasets should be directed to JS, sargeanj@uoguelph.ca.

Author contributions

JS: Conceptualization, Formal analysis, Writing – original draft. AO'C: Conceptualization, Writing – review & editing. DR: Conceptualization, Writing – review & editing. AR: Conceptualization, Writing – review & editing.

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Exposure variables in veterinary epidemiology: are they telling us what we think they are?

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This manuscript summarizes a presentation delivered by the first author at the 2024 symposium for the Calvin Schwabe Award for Lifetime Achievement in Veterinary Epidemiology and Preventive Medicine, which was awarded to Dr. Jan Sargeant. Epidemiologic research plays a crucial role in understanding the complex relationships between exposures and health outcomes. However, the accuracy of the conclusions drawn from these investigations relies upon the meticulous selection and measurement of exposure variables. Appropriate exposure variable selection is crucial for understanding disease etiologies, but it is often the case that we are not able to directly measure the exposure variable of interest and use proxy measures to assess exposures instead. Inappropriate use of proxy measures can lead to erroneous conclusions being made about the true exposure of interest. These errors may lead to biased estimates of associations between exposures and outcomes. The consequences of such biases extend beyond research concerns as health decisions can be made based on flawed evidence. Recognizing and mitigating these biases are essential for producing reliable evidence that informs health policies and interventions, ultimately contributing to improved population health outcomes. To address these challenges, researchers must adopt rigorous methodologies for exposure variable selection and validation studies to minimize measurement errors.

KEYWORDS

exposure variables, variable selection, observational studies, veterinary epidemiology, causation

1 Introduction

John Snow, considered the father of modern epidemiology, published his conclusions regarding the Broad Street pump being the source of the Cholera epidemic in the Soho district of London in 1855. In terms of scientific advances this is still a relatively modern development and epidemiology is thus a relatively young science. To put this in perspective, we are equidistant from John Snow's publication "On the mode of communication of cholera" now as he was from Sir Isaac Newton's publication about the laws of motion (1687) at the time he presented that publication.

Given the foundations of this branch of science and the most pressing health-related issues facing human populations at the time, it is no surprise that the early developments in the field

of epidemiology were rooted in determining the cause(s) of infectious diseases. In this model, causal factors are those that are responsible for health impacts or modifications of health and each factor that contributes to disease occurrence is considered a component cause of disease. Any combination of factors that produce disease are considered a sufficient cause of disease, and causal factors that are required for the disease to develop are termed necessary causes. However, as we have moved from studying infectious causes of disease to non-infectious disease outcomes, such as cancer and aging in both humans and other animal species, we have increased the complexity of exposure measurement within the field. This is because with non-infectious outcomes there may be no necessary cause for a particular health outcome. In fact, any single component cause may only make a small contribution to the disease etiology. This perspective aims to elucidate the importance of appropriate selection of exposure variables within the field of veterinary epidemiology, though many of the concepts apply to human populations as well.

2 Challenges with exposure variables

Rothman and Greenland (1) described the concept of causation due to multiple component causes as being an incomplete causal mechanism unless or until all of the component conditions or events that are necessary for the outcome to occur have reached a set of minimal conditions or thresholds. Thus, each of those components must be accurately measured to determine causality. An additional complexity is that most diseases can be caused by more than one causal mechanism, a concept called multicausality, and each of these mechanisms involves the collective action of a multitude of component causes (1). Knowledge of which components are part of the multiple component causes and how they should be measured is necessary prior to occurrence of the outcome of interest in order to determine causality.

When measuring exposures, it is also important to consider the timing of the exposure on the individual or population in terms of when the exposure occurs in relation to the individual's development or life stage. This is important because the timing of the exposure can cause tremendous variability in the outcomes that may occur. An excellent example is the exposure to the steroidal alkaloid, cyclopamine, in sheep during pregnancy. Ewes can become exposed to this potent teratogen through ingestion of the plant *Veratrum californicum* resulting in synophthalmia (cyclopia) formation in the embryonic lamb. However, cyclopamine is rapidly eliminated from the ewe and ingestion of the plant only on gestational days 13 or 14 results in craniofacial malformations being exhibited (2).

In addition to the timing of exposure in relation to the individual's development, duration of exposure may also be associated with outcomes. In a prospective human birth cohort study conducted in Cincinnati, Ohio, early life exposure to traffic-related air pollution was associated with wheezing regardless of the age at which exposure occurs (3). However, increased risk for asthma was only identified in children exposed to traffic-related air pollution from birth to the age of seven (3). This illustrates that, even within the same population cohort, the duration of time exposed to the same exposure risk did influence disease occurrence.

Another complication is that many of the observational studies used in veterinary epidemiology are retrospective. However, it is not always possible to measure exposure variables retrospectively, as it is often the case that there are no measurable indicators of past exposures. For instance, dietary intake during childhood has been shown to affect adult risk of breast cancer in human females (4), but there are few adult individuals who have detailed descriptions of the types and amounts of foods they consumed as toddlers.

The total number of exposures of interest have increased considerably, too. In a recently published manuscript by Sargeant et al. (5), the authors evaluated 200 observational studies published in the veterinary literature between 2020 and 2022. The number of variables assessed during the screening step in these studies averaged over 20, with a maximum of more than 175. The average number of independent variables evaluated in the final models used in the studies was approximately 14.

The exposure variables being examined themselves have also become much more complex. For instance, food selections for companion animals have become more diverse (6), and different diet types have been associated with different health outcomes (7, 8). Environmental risk factors being examined in relation to health outcomes in animals include those related to the natural environment (9), built environment (10), and the chemical environment (11). Researchers are examining the role that psychosocial (12) and cognitive states (13) play in health outcomes in animals as well. Of course, we also are learning more about the role that genetic predispositions play in the outcome of disease, especially cancers (14), in animal species.

This increasing complexity and numeracy of exposures of interest has likely contributed to an increase in errors related to measurement of exposures (15, 16). It is thought that inaccurate exposure measurements are one of the main sources of bias in epidemiologic research. The magnitude of this bias is likely underappreciated (16). For instance, if we have a well measured variable that correlates with the true exposure of interest with a correlation coefficient of 0.7, we might consider that to be an acceptably strong relationship between the two variables. However, in this instance if we observe a risk ratio of 1.7 in our exposure variable with a correlation coefficient of 0.7, it would indicate that the true risk ratio associated with the exposure of interest is 3.0, nearly two-fold higher than what was measured. Of course, exposure estimates can be either under- or overestimated when measurement errors occur (17).

With the era of veterinary medical "Big Data" having begun (18), one might assume that measurement errors can be overcome by the use of enormous datasets with large numbers of observations. This assumption likely originates from the probability theory known as the law of large numbers wherein by taking the average of an increasing number of random observations sampled from a population it allows for convergence on the true value of the mean. However, measurement errors impact epidemiologic data analyses in several ways, including creating bias in, and affecting the precision of, the exposure effect estimate (17). Thus, a larger sample size will not necessarily move exposure effect estimates closer to their real values and may affect the precision of the estimate, but not the bias resulting in a very precise, but biased estimate. So a larger sample size might be able to compensate for the loss in precision that is caused by measurement error, but the bias created when the reliability of the measurement is low may need a 50-fold or more increase in sample size in order to compensate for the error (19, 20).

It is not uncommon for veterinary researchers to use proxy variables in lieu of directly measuring the true variable of interest. One type of proxy measure that is used with some frequency in epidemiologic research is distance. That is to say that we use the *distance* from an exposure of interest as a proxy measure for the *amount* of exposure. In many cases, investigators are able to measure distance from the exposure with a high degree of accuracy, but the true amount of exposure may not always be equal at equal distances from the source of exposure. For instance, a virus or fine particulate matter that is dispersed through the air and travels from a source of exposure like a silver mine (21) or a poultry house (22) does not travel uniformly in all directions away from the source of exposure. Factors such as wind direction and speed, the deposition process, and pathogen decay rate must be considered in order for true exposure to be estimated. Similarly, all animals in a closed barn may not receive the same exposure from an airborne pathogen due to differences in air flow within the building based on location of fans and doors and variables such as temperature and humidity. However, distance is regularly used as a proxy measure for exposure without accounting for variables that might differentially impact the way in which distance from a source of exposure should be interpreted in both human (23) and animal (24) health research.

It is also not uncommon for veterinary researchers to create variables to define exposures of interest. For example, there have been several studies that have examined the effect of brachycephaly, or a shortened skull shape, on health outcomes in dogs (25–27). However, there is not a standardized definition of the term brachycephaly being used across these studies. One study (25) used morphometric measurements to define dogs as brachycephalic, another (26) used a list of 13 dog breeds to define their brachycephalic cohort, and a third (27) used a list of more than 30 dog breeds to define their brachycephalic cohort, and that list did not incorporate all of the 13 breeds included in the previous study. Thus, the same exposure variable was ostensibly being examined, but on close inspection it becomes apparent that though the same label is being affixed, the term does not mean the same thing in each of these instances. This means that at least some of the animals or even entire breeds being studied must be misclassified when we compare results across studies.

3 Proposed solutions

Given that inaccurate exposure measurements are one of the main sources of bias in epidemiologic research, it seems prudent that we, as a discipline, make every effort to reduce the impact on our understanding of health. One of the most straightforward ways we can do this is by directly measuring exposure variables of interest. Foregoing the use of proxy measurements whenever feasible and realistic to do so will decrease bias and increase the accuracy of our exposure measurements. This will in turn allow us to observe risk ratios that are closer to the true effect and will enhance our understanding of disease etiologies.

When it is not possible to directly measure the exposure variable of interest, it is imperative that rational proxy measurements are used. Thoughtfully considering how the proxy measure may vary from the true exposure variable and taking those variables into account is crucial. Furthermore, it is imperative that the process through which the proxy variable was decided upon by the investigators be described in the methods section of the report associated with the work. Transparency around the decision-making process is critical so that readers can evaluate and determine how close a proxy measurement is to the true variable of interest.

Directed acyclic graphs (DAGs) or causal diagrams can also be used for selecting appropriate exposure variables as they provide a clear representation of the assumed causal relationships between variables. By mapping out these relationships, DAGs help to identify and distinguish between confounders, mediators, and colliders, thus preventing biased estimates of the exposure-outcome association (28). When used to guide the selection of variables to control for, they help to ensure that the chosen variables isolate the causal effect of the exposure on the outcome, rather than introducing bias or masking the true relationship.

Further, we must be consistent in our use of defined exposures. Using similar terminology with different inclusion criteria across studies makes research replication difficult, if not impossible. Our profession has a strong history of successfully using consensus statements to provide our community with information about topics as varied as the diagnosis and treatment of diseases to reporting guidelines for use when conducting research (29–34). Consensus statements also can be used to define exposure variables that can be uniformly applied across research endeavors.

Lastly, failure to recognize the impact of poorly measured exposure variables should not be tolerated. They should, in fact, be considered a serious flaw in research proposals and manuscripts submitted for publication. Erroneous measurements can lead to biased results that may not be sufficiently understood, even when they are recognized by the researchers. Several methods of quantitative bias analysis and "good practices" for their application have been developed (35). Acknowledging the presence of errors in the measurement of exposure variables in the discussion section of a manuscript should not be considered an adequate or acceptable practice.

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

AR: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. JS: Conceptualization, Writing – review & editing. AO'C: Conceptualization, Writing – review & editing. DR: Conceptualization, Writing – review & editing.

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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.

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Non-traditional small companion mammals in Spain as reservoirs of antimicrobial-resistant *Staphylococci*

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Introduction: The increasing prevalence of antimicrobial resistance (AMR) and multidrug resistance (MDR) in microorganisms poses a significant concern in both human and veterinary medicine. Non-traditional companion animals (NTCAs), particularly popular amongst households with children, play a crucial role in AMR epidemiology due to their rising population. Indeed, it is known that some of these animals may act as reservoirs of zoonotic pathogens and thus be able to spread and transmit them to family members, along with their AMR, through their shared environment. It is therefore imperative to address this concern with the involvement of human, animal and environmental health professionals. This pilot study aimed to assess the prevalence and AMR patterns of *Staphylococcus* spp. strains obtained from commensal mucosal and skin infection samples in NTC small mammals, with a focus on strains like methicillin-resistant *Staphylococcus* spp. (MRS) that are critical in public health.

Methods: For this purpose, 81 animals of different small mammal species were sampled, assessing antimicrobial susceptibility to 27 relevant antimicrobial agents (AMAs) in human health using minimum inhibitory concentration assays, and interpreting them according to EUCAST and CLSI guidelines. The isolated *Staphylococci* strains were identified by MALDI-TOF, with the predominant species being *Mammalicoccus sciuri* and *Staphylococcus aureus*.

Results and discussion: Including all strains isolated, AMR was observed against all 27 AMAs, including six last-resort AMAs in human medicine. Additionally, over 85% of the strains exhibited MDR. These findings underscore the need to monitor AMR and MDR trends in companion animals and emphasise the potential role of NTCAs in spreading resistance to humans, other animals, and their shared environment, calling for a comprehensive "One Health" approach.

KEYWORDS

antimicrobial resistance, methicillin resistance, non-traditional companion animals, small mammals, *Staphylococcus* spp.

1 Introduction

Non-traditional companion animals (NTCAs), including small mammals (such as rabbits or ferrets), snakes, lizards or exotic birds, currently account for almost 30% of all companion animals in Europe. In particular, there has been a remarkable increase in the number of small mammals, to 29 million in European households today (1).

Small mammals, such as rabbits, guinea pigs or rodents, are considered ideal companion animals for children because of their manageable size, relatively easy maintenance and low risk of injury. They are socially interactive and can bond with children, providing opportunities to learn about responsibility and animal behaviour. These animals adapt easily to small spaces, making them easy to care for in domestic settings, and their presence offers children the chance to learn about nature and the basic needs of living things (2). For this reason, this growing trend in keeping NTCAs favours their close contact with their owners, becoming particularly important in households shared with at-risk populations (3). In addition, it has been observed that these animals can harbour different microorganisms, such as commensal and pathogenic bacteria, and transmit them together with their antimicrobial resistance (AMR) (4).

AMR is characterised by the ability of microorganisms to evolve over time and become resistant to the drugs used to fight the infections they induce (5, 6). This is especially critical due to the emergence of multidrug resistant (MDR) strains, which are strains of bacteria that have developed resistance to several classes of antimicrobial agents (AMA) (5), and therefore limited resources are currently available for effective intervention (7). In fact, due to the challenges posed by AMR and MDR in both human and veterinary medicine, the World Health Organisation (WHO) has declared them as one of the major threats to public health today (6), as these AMRs are not exclusive to a single species and can spread through the shared environment between humans and animals, underlining the need to address this issue through a "One Health" strategy (4, 8).

Traditionally, the importance of AMR in livestock has been studied, together with its association with farmers (9), but few studies have been conducted in domestic animals despite the importance of its impact on owners, who are often children. In fact, different AMR monitoring and surveillance programmes have been implemented in the European Union (EU) for zoonotic and commensal bacteria in food-producing animals by the European Food and Safety Authority (EFSA) (10), and in human medicine by the European Centre for Disease Prevention and Control (ECDC) (11). However, particularly in the EU, each Member State has additionally implemented its own programmes, for example the National Antimicrobial Resistances Plan (PRAN, from its Spanish acronym Plan Nacional Resistencia Antibióticos) in Spain (12). Currently, there is a need to homogenise all these programmes to compare the available data and establish the current AMR epidemiological situation, including in food-producing and companion animals. For this reason, the EU intends to set up the European Antimicrobial Resistance Surveillance Network in Veterinary medicine (EARS-Vet) (13, 14), but only traditional companion animals, such as dogs and cats, are included in this project, leaving aside NTCAs.

In AMR epidemiological studies, the species within the *Staphylococceae* family are of special relevance as they are part of the commensal microbiota of the skin and mucosa of animals and humans, yet they are also considered opportunistic pathogens that can cause both human and animal infections (15). This family is divided into coagulase-positive *Staphylococci* (CoPS) and coagulase-negative *Staphylococci* (CoNS). Both groups have been identified as pathogenic bacteria with significant potential to cause severe infections in both human and veterinary medicine (16). In particular, one of the main bacteria monitored worldwide is *Staphylococcus aureus* (CoPS), which is one of the most widely distributed *Staphylococcus* species, as it is widely present in both humans and animals and has also been designated by the WHO as one of the high priority bacteria for research and development of new AMAs due to its high resistance (17). This is of special importance due to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains, one of the high priority pathogens listed by the WHO (17). However, the need to monitor all methicillin-resistant *Staphylococci* (MRS) strains due to its public health importance must be highlighted (18). In addition, it is important not to forget CoNS strains, as many of them have also been reported to be methicillin-resistant, potentially leading to therapeutic failures in the treatment of infections caused by these challenging strains (19, 20).

Nevertheless, despite all this information, there are no available programmes focused on NTCAs, although they are considered carriers of *Staphylococcus* spp. and can transmit them to their owners. Furthermore, few studies on NTCAs have been carried out in Europe, and the few that have been done have focused mainly on rabbits or rodents, the most popular NTC small mammals (21–24). Therefore, more studies are needed to achieve a global vision of AMR and MDR in the different animal species included in this bacterial group. Thus, to obtain a comprehensive initial overview, the aim of this pilot study was to assess the prevalence and AMR patterns of *Staphylococcus* spp. strains isolated from commensal mucosal samples and skin infection samples taken from NTC small mammals. Additionally, the study also aimed to investigate the presence of MDR and MRS in these strains.

2 Materials and methods

2.1 Experimental design

The Animal Ethics Committee of the UCH-CEU University (research number CEEA 22/04) reviewed and approved the present animal study carried out in Valencia Region.

For this purpose, an important veterinary centre (VC), which exclusively deals with exotics and NTCAs, was invited to participate on a voluntary basis. This centre deals with almost 70% of the exotic animal population of the Valencian Community, as it receives animals derived from several clinics and hospitals in Valencia, which makes it an exhaustive and representative sampling site for the study.

2.2 Epidemiological data collection

With the aim of taking the samples and collecting all epidemiological information on these sampled animals, informed consent was first requested from all animal owners. First, an epidemiological questionnaire was filled out by the veterinarians in the practise, which contained details on the origin of the animals. The second part provided

general data on the animals including their sex, age, whether they shared the household with other animals and whether they had outdoor access. Lastly, the third and final section of the questionnaire focused on clinical data related to the animals. It included information on whether the animal had any chronic diseases, whether it was currently taking any daily medication, details about its most recent AMAs treatment, and a record of specific AMAs administered throughout its lifetime. In addition, to study the impact that AMAs have in the development of AMR and MDR, four groups were made to classify animals depending on when they were last treated: (I) Never; (II) In the last 6 months: (III) In the last month; (IV) Under treatment at the time of sampling. The questionnaire is available in the Supplementary material.

2.3 Sample collection

To study the prevalence of *Staphylococcus* spp., its AMR patterns and multidrug resistance from NTC small mammals, samples were collected between January and June 2023, from any animals attending the VC. Two types of samples were taken: for the first, a swab (Cary-Blair sterile transport swabs, DELTALAB, Barcelona, Spain) was introduced in the nasal and then in the auricular cavity, from healthy asymptomatic small mammals, based on previous studies (25–27). To verify the health status of the animals, the veterinarians carried out a clinical examination, assessing vital signs, such as corporal temperature (T^a), and cardiac, respiratory and corporal condition (28), to ensure that they were within normal ranges, so that they could be classified as asymptomatic healthy animals. The second sample was taken to isolate infection-causing *Staphylococcus* spp. To this end, a swab (Cary-Blair sterile transport swabs, DELTALAB, Barcelona, Spain) was taken from animals with active skin infections, which was introduced in apparently skin infected wounds.

For further analyses, all samples were transported to the microbiology laboratory at the Faculty of Veterinary Sciences of the University CEU Cardenal Herrera, preserved in Cary-Blair transport medium and refrigerated at ≤4°C within 24 h of collection.

2.4 *Staphylococcus* spp. isolation and identification

The sample swabs were subjected to pre-enrichment in buffered peptone water (BPW; Scharlau, Barcelona, Spain) at a ratio of 1:10 vol/vol and then incubated at 37 ± 1°C for 24 h. Then, the suspension was seeded on non-specific agar, Columbia CNA agar with 5% Sheep Blood, Improved II (BD, Becton Dickinson, Madrid, Spain), and incubated at 37 ± 1°C for 24 to 48 h. Observation of the plates occurred at both the 24 and 48 h marks. Suspected colonies showing typical *Staphylococcus* spp. morphology on blood agar, along with a positive catalase test result, were identified by MALDI-TOF MS Biotyper System (Bruker Daltonics, Madrid, Spain) at the Microbiology Service of the *Consorcio Hospital General Universitario de Valencia*. The Standard Bruker criteria, ranging from 0.00 to 3.00, were used to interpret the results obtained (29). These scores are classified into three groups: the range of 2.00–3.00 means a high confidence identification by species; ranges between 1.70 and < 2.00 provided a low confidence identification by species (only reliable to genus level); and finally, ranges <1.70 do not provide a reliable identification. Only scores above 2.00 were included in this study.

2.5 Antimicrobial susceptibility testing

The antimicrobial susceptibility testing, which included important AMAs for public health, was performed following the protocol described in previous studies (30). In addition, MDR was defined as acquired resistance to at least one agent in three or more antimicrobial classes (5).

However, since little is known on the epidemiological status of NTC small mammals regarding their AMR for *Staphylococcus* spp., and there is no specific monitoring and surveillance programme for their AMR, two panels of AMAs were performed. The first panel, carried out with the GPALL1F Gram-Positive Sensititre Plate (Thermo Scientific™ Sensititre™, Madrid, Spain) (Table 1), included 20 AMAs of public health relevance and clinically important AMAs for human medicine and included in the EARS-Vet programme (32). Additionally, the plate had two D-test wells, combining clindamycin (CLI) and erythromycin (ERY). These wells indicated whether the strain tested had inducible resistance to CLI in the presence of ERY, which could lead to therapeutic failure. Interpretation of the results was performed following the guidelines of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC) (33). The second panel, which was performed with the EU Surveillance *Staphylococcus* EUST2 Sensititre Plate (Thermo Scientific™ Sensititre™, Madrid, Spain) (Table 1), included the AMAs with relevance in public health set out in Decision (EU) 2023/1017 as regards the monitoring of MRSA in fattening pigs (34), the only available legislation currently regarding this bacterium in the EU.

To this end, analyses were performed according to the manufacturer's instructions (ThermoFisher Scientific™, Madrid, Spain) (35). Manual reading of the plates was performed using a Sensititre Vizion (Thermo Scientific™ Sensititre™ Vizion™ Digital MIC Viewing System, ThermoFisher Scientific, Madrid, Spain).

All the results were interpreted based on the guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in its latest report (14th ed., 2024) (36). MRS strains were examined by assessing AMR against cefoxitin (the antibiotic used for screening MRSA and methicillin-resistant coagulase-negative *Staphylococci* (MR-CoNS) strains), and agains oxacillin + 2% NaCl, the antibiotic used for screening methicillin-resistant *Staphylococcus pseudintermedius* (MRSP). However, as some MIC values of these antibiotics for screening MR-CoNS and MRSP are not currently available in EUCAST, the Clinical and Laboratory Standards Institute (CLSI) recommendations, specified in M100 (37) and VET01 (38), were followed in those cases.

2.6 Statistical analysis

Once the analyses were complete and all study data had been obtained, they were analysed using a generalised linear model (GLM) with a probit link function, assuming a binomial distribution. This was done to examine the influence of intrinsic and external epidemiological factors of each animal on the occurrence of AMR and MDR patterns in small mammalian *Staphylococcus* spp. The objective of this analysis was to determine associations with categorical variables, including animal origin, sex, cohabitation with other animals, relationship with animals outside the household, and clinical information regarding chronic diseases, daily medication, and previous antibiotic treatments.

TABLE 1 Antimicrobial agents, latest WHO antimicrobial classification and their studied concentrations included in GPALL1F Gram-Positive Sensititre Plate and EU Surveillance *Staphylococcus* EUST2 Sensititre Plate (both Thermo Scientific™ Sensititre™, Madrid, Spain).

Antimicrobial agent group	Antimicrobial agent	Abbreviation	WHO	Concentration
Aminoglycosides	Gentamycin[1]	GEN	CIA	2-16 µg/mL
	Kanamycin[1,2]	KAN	CIA	4-32 µg/mL
	Streptomycin[1,2]	STR	CIA	4-32 µg/mL
Amphenicols	Chloramphenicol[1,2]	CHL	HIA	2-16 µg/mL
Ansamycins	Rifampicin[1,2]	RIF	CIA	0.015-4 µg/mL
Cephalosporins	Cefoxitin[1,2]	CXI	HIA	0.5-16 µg/mL
Folate Inhibitor Pathway	Trimethoprim / Sulfamethoxazole[1]	TRS	HIA	1/19-8/152 µg/mL
	Trimethoprim[2]	TMP	HIA	1-16 µg/mL
	Sulfamethoxazole[2]	SXM	HIA	64-512 µg/mL
Fusidates	Fusidic acid[2]	FUS	HIA	0.25-4 µg/mL
Glycopeptides	Vancomycin[1,2]	VAN	NA	0.25-32 µg/mL
Glycylcyclines	Tigecycline[1]	TIG	NA	0.03-0.5 µg/mL
Lincosamides	Clindamycin[1,2]	CLI	HIA	0.12-4 µg/mL
Lipopeptides	Daptomycin[1]	DAP	NA	0.5-4 µg/mL
Macrolides	Erythromycin[1,2]	ERY	CIA	0.25-8 µg/mL
Nitrofurans	Nitrofurantoin[1]	NIT	NA	32-64 µg/mL
Oxazolidinones	Linezolid[1,2]	LIN	NA	1-8 µg/mL
Penicillins	Ampicillin[1]	AMP	HIA	0.25-8 µg/mL
	Oxacillin + 2 % NaCl[1]	OXA+	HIA	0.25-4 µg/mL
	Penicillin[1,2]	PEN	HIA	0.06-8 µg/mL
Pleuromutilins	Tiamulin[2]	TIA	IA	0.5-4 µg/mL
Pseudomonic acid	Mupirocin[2]	MUP	NA	0.5-256 µg/mL
Quinolones	Levofloxacin (FQ) [1]	LEV	HPCIA	0,25-4 µg/mL
	Ciprofloxacin (FQ) [1,2]	CIP	HPCIA	1-2 µg/mL
	Moxifloxacin (FQ) [1]	MOX	HPCIA	0.25-4 µg/mL
Tetracyclines	Tetracycline[1,2]	TET	HIA	0.05-16 µg/mL
Streptogramins	Quinupristin / Dalfopristin[1]	QUD	HIA	0.5-4 µg/mL
D-test	Erythromycin (E) + Clindamycin (C)	DT[1]		4 µg/mL (E) + 0.5 µg/mL (C)

FQ: Fluoroquinolone. WHO: World Health Organisation [This column indicates the last updated of the classification of medically important antimicrobials authorised by WHO for human and animal use in order to protect public health, updated in 2023 (31)]. HIA: highly important antimicrobial. CIA: critically important antimicrobial. HPCIA: highest priority critical important antimicrobial. NA: not authorised for animal use (31). [1]antimicrobial agents included in GPALL1F Gram-Positive Sensititre Plate. [2]antimicrobial agents included in EU Surveillance *Staphylococcus* EUST2 Sensititre Plate. Additionally, both plates had two positive control wells.

A significance level of p-value ≤ 0.05 was considered indicative of a statistically significant difference. Statistical analyses were performed using the R software (version 4.3.1) packages EMMs (39), car (40) and multcompView (41).

3 Results

3.1 Epidemiological results

In the present study, 81 small mammals of nine different species were sampled. All of them and the number of samples taken by each animal species are in Table 2.

First, epidemiological information, including gender and age, was gathered on all the animals in this study. Nevertheless, due to the diverse nature of the study population, which includes several animal species from different families, the data are not directly comparable. Regarding their style-life, 61.7% (50/81) of the animals cohabited in the same household with other animals, but none of them went out of their house. Secondly, according to the clinical information gathered, 68% (55/81) of the animals presented a chronic disease, and 14.8% (12/81) were taking daily medication. Finally, of all the animals, 70.4% (57/81) had been previously treated with AMAs at some point in their lives. The data presented in Figure 1 show the AMAs treatment history of the study population, detailing the specific AMAs group and the date of the last treatment.

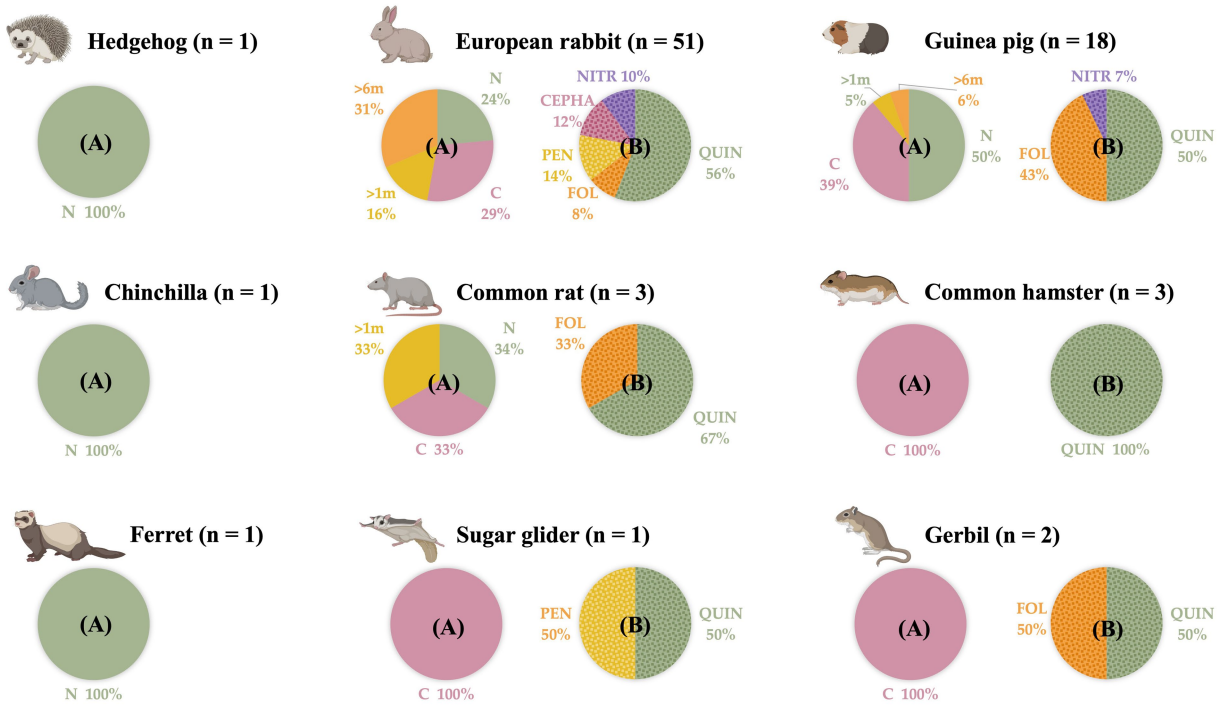

FIGURE 1
Distribution by animal species of the small mammal population studied, according to when they were last treated with antimicrobial agents and with which antimicrobial agents group. n: number of animals sampled. **(A)** Moment of the last antimicrobial agents administration. N: never. C: currently. >1 m: in the last month. >6 m: in the last 6 months. **(B)** Antimicrobial agents groups administered in the study population at some point of their lives. QUIN: quinolones. FOL: folate inhibitor pathway. CEPHA: cephalosporins. PEN: penicillins. NITR: Nitroimidazoles. (Created by Biorender).

TABLE 2 Number and percentage of the different animal species sampled.

Animal species (common name)	*n* (%) of animals sampled
Oryctolagus cuniculus (European rabbit)	51 (63.1)
Cavia porcellus (Guinea pig)	18 (22.2)
Rattus norvegicus (common rat)	3 (3.7)
Cricetinae (common hamster)	3 (3.7)
Gerbillinae (gerbil)	2 (2.5)
Chinchilla laniguera (chinchilla)	1 (1.2)
Erinaceinae (hedgehog)	1 (1.2)
Mustela putorius furo (ferret)	1 (1.2)
Petaurus breviceps (sugar glider)	1 (1.2)
Total	81

n: number, %: percentage.

3.2 *Staphylococcus* spp. prevalence

Of the 81 specimens sampled, 72 were asymptomatic animals and 9 presented a skin infection. Of all of them, the total prevalence of *Staphylococcus* spp. was 48.2% (39/81), of which 42% (34/81) and 6.2% (5/81) were commensal and infection-causing *Staphylococcus* spp., respectively. All *Staphylococcus* spp. isolated from each of the small mammals, together with the type of sample from which they were derived, are listed in Table 3.

3.3 Antimicrobial susceptibility in *Staphylococcus* spp. strains

3.3.1 Methicillin-resistant strains

In the present study, all MRS strains came from the commensal bacteria isolates, and none of the strains isolated from active skin infections showed methicillin resistance. MRS strains represented a 14.7% (5/34), belonging each one to a different species: *Mammaliicoccus sciuri, S. aureus, S. xylosus, S. haemolyticus* and *S. epidermidis*.

3.3.2 Antimicrobial resistance profile

Regarding the *Staphylococcus* spp. and *Mammaliicoccus* spp. strains isolated from commensal samples in the present study, all the strains (34/34) showed AMR to at least one of the 27 AMAs studied, and 85.3% (29/34) were MDR. Of all the commensal strains, 17.6% (6/34) were positive to the D-test performed. The AMR values for the AMAs groups, where more than one AMAs was studied, were 34.3% for quinolones, 29.4% for penicillins, 17.6% for folate inhibitor pathway. For the remaining AMAs groups, only one AMA from each group was studied, so Figure 2 shows the AMR for each AMA individually, with the exception of oxacillin, which was tested against a single strain of *S. pseudintermedius* and found to be susceptible. The AMR observed for each of the isolates, these are detailed in the Supplementary Table 1.

For all infection-causing *Staphylococcus* spp. isolated from animals with active skin infections, all of them (5/5) were resistant to at least one of the studied AMAs, and 40% (2/5) were MDR. Moreover, the

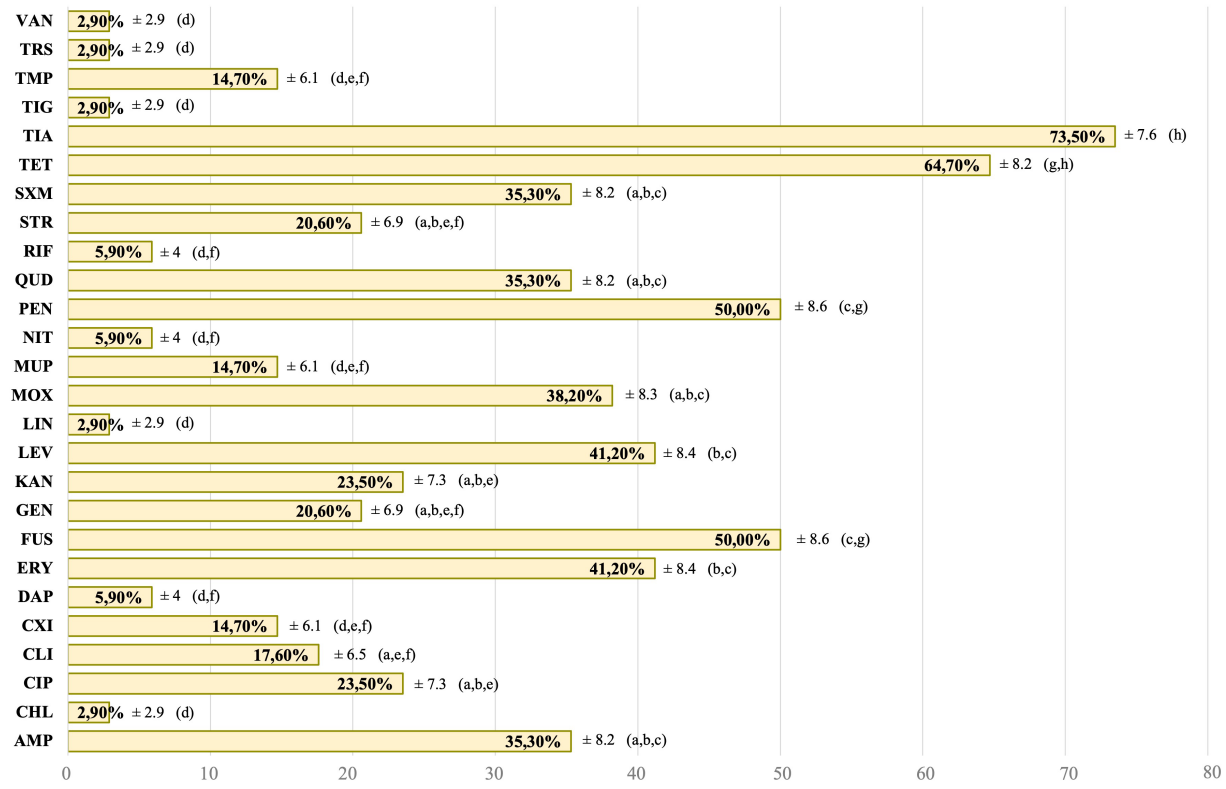

FIGURE 2
Antimicrobial resistance of the total commensal *Staphylococcus* spp. strains. AMP: ampicillin. CHL: chloramphenicol. CIP: ciprofloxacin. CLI: clindamycin. CXI: cefoxitin. DAP: daptomycin. ERY: erythromycin. FUS: fusidic acid. GEN: gentamycin. KAN: kanamycin. LEV: levofloxacin. LIN: linezolid. MOX: moxifloxacin. MUP: mupirocin. NIT: nitrofurantoin. PEN: penicillin. QUD: quinupristin/dalfopristin. RIF: rifampicin. STR: streptomycin. SXM: sulfamethoxazole. TET: tetracycline. TIA: tiamulin. TIG: tigecycline. TMP: trimethoprim. TRS: trimethoprim/sulfamethoxazole. VAN: vancomycin. a–h: different letters indicate significant statistically differences between the antimicrobial agents studied.

D-test performed in these strains was positive in 80% (4/5) of them. For AMAs groups with more than one AMA studied, AMR rates were 60% for penicillins and 20% for quinolones. For folate inhibitor pathway, no AMRs were shown. Figure 3 shows the AMR for each individual AMA studied. Regarding the AMR observed in each of the isolates, these are detailed in the Supplementary Table 1.

Furthermore, no relationship was observed between the epidemiological and clinical data collected in the questionnaire, and the occurrence of AMR and MDR, neither in commensal nor in those infection-causing *Staphylococcus* spp. strains (p-value >0.05).

Lastly, no discernible pattern in overall AMR trends was observed in this study. Amongst the 39 *Staphylococcus* spp. and *Mammaliicoccus* spp. isolates, 37 distinct AMR patterns were identified, indicating a diverse range of AMR profiles. Only two patterns were duplicated, one to folate inhibitor pathways together with pleuromutilins and quinolones, and the second to fusidates, pleuromutilins and tetracyclines, both in two commensal *Staphylococcus* spp. isolates (2/39). The list of AMR patterns can be found in the Supplementary Table 1.

4 Discussion

The *Staphylococcaceae* family is one of the most common bacteria overall, and particularly amongst gram-positive bacteria, as this microorganism is part of the normal microbiota on the skin and mucous membranes of humans and most animals. Recently, new phylogenomic studies of this family have been carried out, relocating some *Staphylococcus* spp. into other genera, such as the former *S. sciuri*, now called *Mammaliicoccus sciuri*. However, the importance of this strain remains the same, as it is considered the evolutionary reservoir of the *mecA* gene, which encodes methicillin resistance. Encompassing all the bacterial species found in this study, the observed prevalence is consistent with that found in other studies of both NTC and free-living small mammals (21, 42, 43), with most of the isolates being CoNS. In the present study, *M. sciuri* was the most prevalent bacterium from commensal samples, followed by *S. aureus*. In skin infection isolates, *S. aureus* was the most prevalent species. Although a high variability was found, as 10 additional bacterial species have been observed, as reported in other studies carried out in small mammals (44). One of the hypotheses for this high diversity of *Staphylococci* in NTC small mammals could be their household environment. They share it with humans of all ages, and more than 60% of the animals shared it with other companion animals, of the same or other species such as dogs, that go outside daily, making the environmental microbiome in homes richer and thus favouring different bacterial species colonising the mucosa of NTCAs. Another possible reason could be the high rate (more than 70%) of previous antimicrobial treatment in the study population, which puts pressure on bacterial communities and may favour the growth of selected bacterial species.

TABLE 3 Prevalence of *Staphylococcus* species isolated from commensal mucosa and skin infection samples from small mammals.

Type of sample	Prevalence of *S.* by class	*S.* species	*N* and (%) prevalence of each *S.* species	(*N*) of *S.* strains per animals' species
Commensal mucosa	CoPS – 17.6%	*S. aureus*	5 (14.6)	*Oryctolagus cuniculus* (3) *Gerbillae* (1) *Rattus norvegicus* (1)
		S. pseudintermedius	1 (3)	*Oryctolagus cuniculus* (1)
	CoNS – 82.4%	*S. borealis*	2 (5.9)	*Cavia porcellus* (2)
		S. cohnii	3 (8.8)	*Oryctolagus cuniculus* (3)
		S. epidermidis	1 (3)	*Oryctolagus cuniculus* (1)
		S. haemolyticus	3 (8.8)	*Oryctolagus cuniculus* (2) *Cavia porcellus* (1)
		S. hominis	1 (3)	*Mesocricetus auratus* (1)
		S. microti	3 (8.8)	*Cavia porcellus* (2) *Ernaceinae* (1)
		S. saprophyticus	2 (5.9)	*Oryctolagus cuniculus* (1) *Mesocricetus auratus* (1)
		S. sciuri[1]	6 (17.7)	*Cavia porcellus* (4) *Mesocricetus auratus* (1) *Oryctolagus cuniculus* (1)
		S. warneri	2 (5.9)	*Oryctolagus cuniculus* (2)
		S. xylosus	5 (14.6)	*Cavia porcellus* (1) *Oryctolagus cuniculus* (4)
Skin infection	CoPS – 60%	*S. aureus*	3 (60)	*Oryctolagus cuniculus* (1) *Cavia porcellus* (1) *Gerbillae* (1)
	CoNS – 40%	*S. epidermidis*	1 (20)	*Oryctolagus cuniculus* (1)
		S. xylosus	1 (20)	*Oryctolagus cuniculus* (1)

N: number of strains isolated. CoPS: coagulase-positive *Staphylococcus*. CoNS: coagulase-negative *Staphylococcus*. *S.*: *Staphylococcus*. [1]Due to a new phylogenomic study on the family *Staphylococcaceae, S. sciuri* now belongs to a new genus and has been renamed as *Mammaliicoccus sciuri*. However, in biochemical and MALDI-TOF identification, it is still identified as *S. sciuri*.

Moreover, the rise of AMR and MDR in *Staphylococcus* spp. strains in veterinary medicine poses a global public health challenge. Research indicates that these resistant strains can persist in the environment and be transferred between animals and humans (45, 46). This underlines the need to assess the prevalence of such resistances in both commensal and pathogenic bacteria, requiring a comprehensive "One Health" approach. Addressing this problem is vital not only to avoid therapeutic failures in veterinary medicine, but also to safeguard human health, especially with the results observed in this study, where all the strains were resistant to at least one of the AMAs studied, and more than 85% of them were MDR, with a diverse range of AMR profiles, not following any discernible pattern. Similar results have been observed in other NTCAs (21, 47) and traditional companion animals (48), highlighting this global problem. In addition to MDR, the surveillance of MRS strains is crucial, mainly due to their resistance to common AMAs, which complicates the selection of effective treatments. Moreover, MRS strains are known for their ability to spread rapidly in health care facilities. In this study, MRS strains (14.7%) have been observed in both CoPS and CoNS, as reported in studies carried out in other countries, such as Austria (47) or Turkey (49), in dog, cats and NTCAs, which underlines the global need to monitor these strains.

Regarding each AMA, AMR observed against tiamulin (TIA) stands out above the others with 73.5%. TIA is an AMA exclusively used in veterinary medicine, particularly for food-producing animals, especially pigs and poultry, for which similar AMR rates have been observed (50). However, it is also approved for use, although to a lesser extent, in meat-producing rabbits (51), which may contribute to their use in rabbits kept as companion animals and not for production purposes. The following AMAs with higher AMR were tetracycline (TET; 64.7%) and fusidic acid (FUS; 50%), both AMAs belonging to the highly important antimicrobials (HIA) category in the latest WHO categorisation (31). It is therefore to be expected that higher percentages of AMR will be observed against these AMAs (52) and not against those belonging to higher categories. Other AMAs in this category, which are one of the first line treatments, are folate inhibitor pathways, such as trimethoprim (TMP), sulfamethoxazole (SMX) or the combination of both (trimethoprim-sulfamethoxazole, TRS). Although these AMAs can be administered separately, higher AMR resistance rates were seen individually (TMP, 14.7%; SMX, 35.5%) than those observed to TRS (2.9%) in combination, which highlights

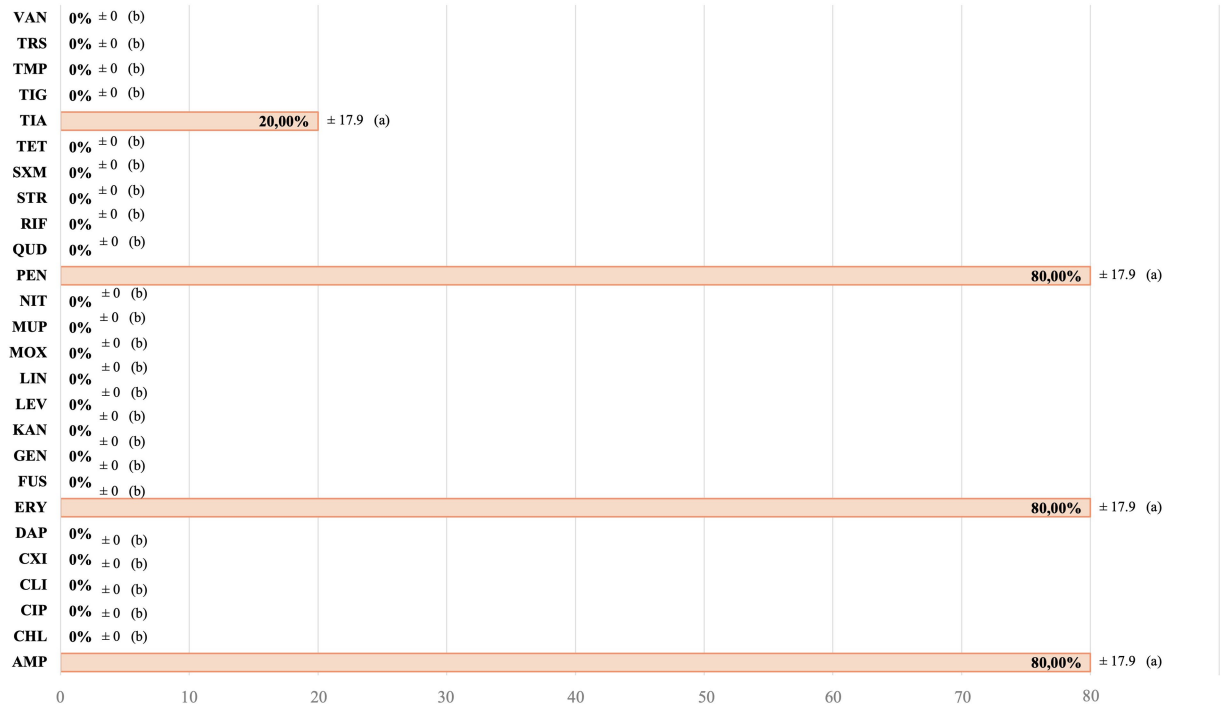

FIGURE 3
Antimicrobial resistance of the total infection-causing *Staphylococcus* spp. strains. AMP: ampicillin. CHL: chloramphenicol. CIP: ciprofloxacin. CLI: clindamycin. CXI: cefoxitin. DAP: daptomycin. ERY: erythromycin. FUS: fusidic acid. GEN: gentamycin. KAN: kanamycin. LEV: levofloxacin. LIN: linezolid. MOX: moxifloxacin. MUP: mupirocin. NIT: nitrofurantoin. PEN: penicillin. QUD: quinupristin/dalfopristin. RIF: rifampicin. STR: streptomycin. SXM: sulfamethoxazole. TET: tetracycline. TIA: tiamulin. TIG: tigecycline. TMP: trimethoprim. TRS: trimethoprim/sulfamethoxazole. VAN: vancomycin. a,b: different letters indicate significant statistically differences between the antimicrobial agents studied.

the importance of using this combination in veterinary medicine, until this therapeutic option is exhausted (53). However, higher AMR has been seen in this combination in traditional companion animals, reaching almost 50% (30, 54).

Of the AMAs studied, erythromycin (ERY) represents one of the first treatments of choice for *Staphylococcal* infections, especially in patients with penicillin allergies (55). The high AMR rates found in this study for ERY aligns with those found in other studies in small mammals (42), dogs and cats in Spain (48) and Canada (56), or in dogs and their owners in Italy (27), which indicates that first therapeutic options to treat these infections may begin to fail. For this reason, it is important to explore the AMR to other therapeutic options, such as clindamycin (CLI), a HIA category AMA, but used to treat community-acquired skin infections probably due to MRS (55). However, to evaluate whether this AMA can be used in the practise or not, the D-test should be performed, to confirm whether an inducible CLI resistance phenotype is present or not (57). In the present study, 17.6 and 80% of commensal and infection-causing strains, respectively, were positive to the D-test. This result indicates that, although CLI alone may appear to be effective, the bacteria can develop resistance during treatment, which can have serious implications for infection management, as AMR can compromise treatment (58). Moreover, inducible resistance to CLI confirms the macrolides, lincosamides, streptogramin ß and pleuromutilin (MLS_B-P) group resistance phenotype, as resistance genes which induce resistance to CLI can also induce resistance to MLS_B-P, which are antibiotics commonly used for the treatment of MRSA (59). This may be one of the reasons why high rates of AMR to these AMAs were observed in this study.

When all other therapeutic options fail, the last AMAs that can be used in veterinary medicine are the highest priority critical important antimicrobials (HPCIAs), including the quinolones (31), although in the study population, quinolones were the most administered AMAs group. In this study, the three quinolones evaluated: levofloxacin (LEV), ciprofloxacin (CIP) and moxifloxacin (MOX), are AMAs only approved for use in human but not in veterinary medicine in the EU (31). Therefore, the high AMR observed (34.3%) to this group, similar to that observed in another study in rabbits (21), is of concern due to the therapeutic failures it could pose, and the possibility of transmission of these AMRs to other pathogenic bacteria (60).

Finally, the last category available when all the others have failed is reserved for human medicine, and is not authorised for veterinary medicine, being more commonly known as last-resort AMAs (31). The AMAs of this category studied were vancomycin (VAN), tigecycline (TIG), linezolid (LIN), daptomycin (DAP), nitrofurantoin (NIT) and mupirocin (MUP). These AMAs are usually reserved for severe or life-threatening infections that do not respond to standard AMA therapies using the above categories. Regarding the AMR observed, a low prevalence of almost all AMAs was found, aligning with other studies conducted in small mammals in the Czech Republic (43) and in dogs, cats, and rabbits in Lithuania (61), but not for MUP. This AMA is for topical use only, utilised for complicated skin infections, including those caused by MRS, and for decolonising nasal carriers of *S. aureus*. Although given the importance of this AMA, a lower percentage of AMR should be observed, the prevalence reported in this study (14.7%) is within normal ranges, considering that in Spain the AMR

for this AMA in CoNS isolates is around 40% and for *S. aureus* between 8 and 10%, in human medicine (62).

The present study is focused on assessing the prevalence and AMR patterns of *Staphylococcaceae* strains isolated from mucosal samples and skin infections in small mammals. The results highlight the high prevalence of AMR and MDR in small mammals, underlining the need for a comprehensive "One Health" approach to address this issue, as these animals share the domestic environment with humans and other animals. Moreover, the diversity of bacterial species and the high rate of previous antimicrobial treatments suggest significant selective pressure, which may favour the emergence of AMR. This research is an initial step for future initiatives to control and prevent the proliferation of AMR and MDR in NTCAs. However, further research is essential to validate our results in a larger and more representative study population.

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 authors.

Ethics statement

The animal studies were approved by Animal Ethics Committees of the Universidad Cardenal Herrera-Ceu. 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

AM-F: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. CM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. JV-G: Methodology, Resources, Supervision, Validation, Visualization, Writing – review & editing. CG-C: Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing. VA-M: Investigation, Methodology, Supervision, Validation, Writing – review & editing. SV: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. LM-D: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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.

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Supplementary material

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Aligning valid research outcomes with stakeholder values—what do they need for decision-making?

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This paper is derived from a presentation given by the first author at the 2024 Symposium for the Calvin Schwabe Award, presented to Dr. Jan Sargeant for Lifetime Achievement in Veterinary Epidemiology and Preventive Medicine. Researchers must work toward ensuring validity throughout the research process, but we also should ensure that our resulting outcomes are specified to appropriately inform and enable decision-making by the end-users. Given the scope and diversity of topics addressed by veterinary researchers, the potential beneficiaries or stakeholders of our research also varies. Stakeholders or end-users may include veterinary practitioners, other researchers, livestock owners, "pet parents," government officials, corporate entities, or the general public in the case of public health or food security and safety issues. Current research in animal agriculture provides an opportunity to consider research outcomes in a sustainability framework which concurrently values social, economic, and environment impacts of animal health and management decisions. In companion animals, contemporary issues of affordability and access to care, quality of life, or compliance effects on efficacy, also extend the spectrum of relevant research outcomes. In these cases, traditional measures of animal health, such as morbidity, mortality, or weight gain, may not be the most relevant for the end-users. Furthermore, if studies are not designed and analyzed with well-defined primary outcomes that are informed by stakeholders' values, but rather post-hoc considerations of these values are made based on indirect or surrogate measures, there is the potential to incorporate error and bias into our conclusions and the end-users' decision-making processes.

KEYWORDS

outcomes, veterinary, validity, research impact, outcomes research

1 Introduction

When decision-makers strive to be informed by evidence or science, results from research are an essential and foundational component of this process. Research is often performed to determine whether a factor(s)—which may be described as an exposure, treatment, risk factor, intervention, or independent variable—is associated with an outcome or dependent variable. The selection of an appropriate outcome(s) to compare among groups is critical to maximizing research value and relevance to the end-user or stakeholders (1).

In the context of evidence-based medicine, the end-user of veterinary research is typically considered a clinician; yet, in a broader context, veterinary researchers may consider the end-user or stakeholders of their research to also include livestock owners or care-takers, "pet parents," other researchers, government agencies, private industry, or even society in general. Outcomes can be defined at both the conceptual and operational level (1). For example, "health" may be a conceptual outcome of interest, but operational outcomes, that can be measured to evaluate "health," could be various measures of morbidity or mortality. In animal agriculture, the framework of sustainability provides three conceptual domains: environmental, economic, and societal; within each of these domains, measurable operational outcomes for research purposes can be defined. Similarly, conceptual outcomes such as access to care, quality of life, animal welfare/well-being, or food safety and security can be further refined into operational outcomes that can be measured and incorporated into research plans. Regardless of the domain, research outcomes should be valid and relevant to the end-users for the research to have value and to enable appropriate decision-making. Here and elsewhere, discussions of research methodologies, including study design, implementation, analysis, and reporting, typically focus on operational outcomes—the things that are measured and analyzed.

A common research purpose is to determine differences in outcomes attributed to an intervention, where an intervention can be defined as many things, such as dietary changes or supplementations, changes to husbandry or management practices, implementation of vaccinations, or use of pharmaceuticals (1). While observational study designs are common in veterinary research, randomized controlled trials are considered the strongest empirical research design for establishing that an observed difference in the outcome was due to the intervention (1, 2). Thus, the concepts discussed in the remainder of this paper, while also relevant to other research approaches, will be presented primarily in the context of clinical trials.

There is increasing awareness of a need to address problems with research wastage and reproducibility in veterinary research; those issues are described in detail elsewhere (1, 3, 4). However, appropriate study designs and methods that address relevant research questions with well-defined outcomes valued by decision-makers are critical areas to be addressed in order to minimize research wastage and maximize value (1, 3). Therefore, the objective of this paper is to discuss the need and opportunities to define research outcomes that are relevant to stakeholders and to design studies that are driven directly by those defined outcomes.

2 Aligning outcomes with stakeholder values—what do they need for decision-making?

While stakeholder's needs and input can have a tremendous impact on research relevance and value, the mechanism to engage stakeholders or understand their values can be as diverse as the spectrum of veterinary research. Broadly speaking, applied veterinary research can span from the "micro level," where the end-user is a clinician or their clients, to the "macro level" in which decision-making can impact policy or a much larger population of animals or society (5). However, the critical question a researcher might ask is—what do "they" need for decision-making?

In some cases, the stakeholder(s) may indicate that decision-making simply requires a single key operational outcome that differs enough among intervention groups to be statistically significant and of a magnitude that is relevant clinically or economically for example. However, if decision-making is more complex, the stakeholder(s) may need research that demonstrates the impacts of an intervention on multiple conceptual outcome areas, each with key operational outcome measures. As an example, in Horton et al. (6), a stakeholder (cattle producer) was directly involved in a clinical trial to evaluate interventions for bovine respiratory disease with the primary goal of exploring options to reduce antimicrobial use. While antimicrobial use was the primary conceptual outcome, decision-making required other conceptual outcomes that also could be impacted by changes in antimicrobial use. Therefore, the study was designed with a primary operational outcome related to the number of antimicrobial doses, but also included other operational outcomes within the conceptual outcome areas of animal well-being, protein (beef) production, economics, and environmental impacts to address the stakeholder's decision-making needs (5). Although multiple outcomes were used, these were defined *a priori* which is important in the context of appropriate study design, analysis, and reporting (1, 3, 7).

Given the scope and diversity of topics addressed by veterinary researchers, the type of research that stakeholders need also may vary tremendously. Thus, there can be no one standard process for gathering input from stakeholders before research is initiated. Depending on the research topic and scope, forming advisory groups, surveying content experts, connecting with professional networks, initiating stakeholder surveys, or other approaches may be beneficial and necessary for collecting *a priori* information on the needs of stakeholders. Regardless of the mechanism, it is critical to engage stakeholders early in the research process and understand their values in order to maximize the relevance and value of research. A challenging, but critically important component is that the stakeholder(s) and researcher(s) define the primary outcome measures needed for decision-making as these primary outcomes directly impact the research design (1).

3 Primary outcomes—designing, analyzing and reporting research accordingly

For all studies, the primary outcome(s) should be the outcome that is most relevant for decision-making by the target audience, and should be defined, reported, and used for the study design, including for calculating the necessary sample size to detect a meaningful difference in the magnitude of that primary outcome (1). By defining primary versus secondary outcomes, the end-user of the research should be able to determine which outcomes the study was powered to detect meaningful, statistically significant differences (primary) versus outcomes (secondary) not specifically used for the study design (1). The way in which a difference in the primary outcome will be considered meaningfully relevant should be defined with stakeholder input, and may be considered in terms that are related to any measure that stakeholders consider relevant to the issue of study, for instance an economic endpoint or one related to quality of life. This process of defining what difference (or lack thereof) in outcome that is meaningful to stakeholders should be followed regardless of the

study design, or study purpose, even though the context differs for superiority, noninferiority, or equivalence studies (1).

Despite the importance of defining and reporting primary outcomes, implementation for applied veterinary research studies is generally poor. A scoping review of feedlot trials that included an economic outcome domain found that the primary outcome was stated in only 36% (41/113) of the trials (7). Similarly, of 91 dairy cattle trials published in 2017 with more than one outcome, the primary outcome was only identified in only 4 trials (8). In other reviews of veterinary literature, the reporting of primary outcomes was also low, and much lower compared to reports from human medical journals (1).

Failure to define and report primary outcomes, and appropriately design and analyze studies accordingly, can lead to several problems with research validity, reproducibility, and wastage that have been discussed in more detail elsewhere (1, 3, 7). Three of four major reasons for research wastage– addressing research questions that are not relevant, inadequate study design and methods, and biased or unusable results (1)—may be directly affected by poorly selected or defined outcomes. The lack of consistency in selection and reporting of outcomes also limits the ability to extend the value of individual studies through research synthesis methods (1).

The lack of appropriate selection, definition, and measurement of outcomes in designing, analyzing, and interpreting research studies can lead to bias and error (1, 3, 7). While errors are bound to happen even with well executed research and statistical methods (3), the traditional types of statistical errors—namely, type I and type II errors—can be inflated in studies that fail to avoid or address multiplicity in outcomes or fail to ensure adequate replication in relation to relevant differences in the primary outcome(s). Setting a type I error rate (α), of 5% for example, relates to a hypothesis test for a single outcome, and when multiple outcomes are analyzed (independently), the probability of type I errors can be quite large (1, 3). This problem can be further exacerbated—leading to bias—when reporting only the statistically significant results (1) or when only the statistically significant results are used for calculating some composite outcome(s), which involves combining multiple related outcomes into a single measure. As an example of the latter, consider that data are collected for multiple outcomes from cattle health and production data, but only those with statistically significant differences are used to calculate (*post hoc*) a composite economic outcome (7). This approach assumes that the variables omitted from the composite outcome are completely unaffected by the intervention which is a very strong, and often unrealistic assumption. In reality, the intervention may affect the omitted variables, but the study was underpowered to detect those differences so the composite variable is incorrect. Further, if there are no operational outcomes directly addressing the primary outcome domain (economics for example), but multiple surrogate operational outcomes are used based on results of hypothesis testing, type II errors also may occur (3, 7). Thus, in the context of the outcome domain of most relevance to the stakeholder, the results may be biased, and affected by some unknown combination of both type I and type II errors.

4 Discussion—outcomes research and potential solutions

The diversity and complexity of stakeholders and topics seem to make the provision of a standardized solution unrealistic for veterinary research as a whole. However, veterinary researchers can find guidance and potential solutions by looking to existing reporting guidelines for veterinary research or to other discipline areas such as those used for outcomes research, human health, and social sciences. For examples, recommendations for reporting outcomes for trials in pets (PETSORT) and livestock (REFLECT) are directly relevant and excellent resources (9–12). Reporting guidelines from other discipline areas, such as CHEERS for reporting economic assessments in human health studies, also are useful resources that have been used by veterinary researchers (7). However, none of the reporting guidelines provides guidance on how to appropriately identify and prioritize outcomes relative to stakeholder values.

A review on maximizing value and minimizing wastage in veterinary clinical trial research provides an excellent discussion on selection and reporting of outcomes (1). Among other topics, the authors discuss that one potential solution to improve consistency of outcome measures and reporting is the creation of core outcome sets, which represent an agreed upon minimum set of outcomes that should be reported in a specific topic area (1). The rationale and development of core outcome sets have been discussed in more detail elsewhere, but this approach has been applied in human healthcare much more frequently than in veterinary medicine (1). The ISPOR, a professional society for health economics and outcomes research developed nearly 30 years ago for human healthcare decision-making, has recently included animal- and one-health topics (4), and also provides resources including standards for health economics and outcomes research (13).

Two recent peer-reviewed reports on the relevance, value, and potential impacts of outcomes research in animal health and veterinary medicine provide excellent discussion of this discipline area (4, 14). While outcomes research principles are well-established in human medicine, their formal application in animal health and veterinary medicine are relatively new. The relevance here is that outcomes research explicitly focuses on defining both the potential effectiveness of an intervention (or policy) and the values of the stakeholders or research end-users (5). Thus, regardless of the domain, researchers prioritize outcomes that are valid and relevant to the end-users to maximize research value and to enable appropriate decision-making. That, in fact, should be the goal of researchers—to ensure validity throughout the research process, while also ensuring resulting outcomes appropriately inform and enable evidence- or science-based decision-making by the end-users.

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

DR: Conceptualization, Writing – original draft. JS: Conceptualization, Writing – review & editing. AO'C: Conceptualization, Writing – review & editing. AR: Conceptualization, Writing – review & editing.

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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.

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Modeling foot-and-mouth disease dissemination in Rio Grande do Sul, Brazil and evaluating the effectiveness of control measures

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Introduction: Foot-and-mouth disease (FMD) affects multiple food-animal species and spreads rapidly among ungulate populations, posing significant challenges for disease control. Understanding the dynamics of FMD transmission and evaluating the effectiveness of control measures are critical for mitigating its impact. This study introduces a multiscale compartmental stochastic model to simulate FMD spread and assess countermeasures.

Methods: We developed a model that integrates population dynamics, including births, deaths, and species-specific transmission dynamics, at both the between-farm and within-farm levels. Four scenarios were created to evaluate different control strategies: the base scenario included vaccinating 20 farms and depopulating four infected farms, while alternative scenarios increased vaccination and depopulation capacities or omitted vaccination altogether.

Results: Our simulations showed that bovines were the most frequently infected species, followed by swine and small ruminants. After 10 days of initial spread, the number of infected farms ranged from 1 to 123, with 90.12% of simulations resulting in fewer than 50 infected farms. Most secondary spread occurred within a 25 km radius. An early response to control actions significantly reduced the time spent managing outbreaks, and increasing daily depopulation and vaccination capacities further enhanced control efforts.

Discussion: Emergency vaccination effectively reduced the magnitude and duration of outbreaks, while increasing depopulation without vaccination also eliminated outbreaks. These findings highlight the importance of rapid response and capacity scaling in controlling FMD outbreaks, providing valuable insights for future decision-making processes in disease management.

KEYWORDS

dynamical models, infectious disease control, epidemiology, transmission, targeted control, FMD (foot-and-mouth disease), simulation

1 Introduction

Foot-and-mouth disease (FMD) is an infectious disease in cloven-hoofed animals that affects multiple species, including bovine, swine, small ruminants, and wildlife (1). This disease can also impact the economies of affected countries. During the 2001 FMD epidemic in the U.K. and the Netherlands, more than 6.7 million animals were slaughtered, including healthy ones (preemptive culling) (2). In both outbreaks, multiple species were infected, including goats on mixed dairy-goat/veal-calf farms (2, 3), and there were additional costs to other sectors, such as tourism, with a total expenditure of approximately 2.7 to 3.2 billion euros (4). The official World Organisation for Animal Health (WOAH, 2022) database recorded more than 2,484,001 outbreaks in 80 countries from 2015 to 2023, showing that 73.43% of FMD cases were associated with cattle, 3.02% with swine, 14.38% with small ruminants, and 4.8% with buffaloes (5). In South America, no large outbreaks have been reported since 2001, when 2,027 farms in Uruguay were affected, with cattle and small ruminants being the predominant infected species (6), up to date, the most recent outbreaks reported happened in Colombia between 2017 and 2018 (7); since then no epidemics have been officially reported in America, despite Venezuela's absence of official international status for FMD (8).

Despite substantial evidence that all susceptible species can contribute to significant FMD epidemics, response plans frequently focus on controlling the spread among cattle populations. This approach often overlooks the role of other domestic species (9). This makes it important to consider that the pathogenesis and transmission dynamics vary among species, given differences in viral loads needed to cause infection, variability in latency, and infection duration (10–13). For instance, infected swine shed more viral particles than cattle and sheep, historically resulting in widespread epidemics expected when infected (12). Thus, it is pivotal to consider such heterogeneity in transmission dynamics when modeling within and/or between-farm FMD dissemination (9). In the same vein, field observations and experimental trials have demonstrated the spread of FMD occurs between-farm transmission primarily occurs through direct contact among susceptible and infected animals (10, 14), and via indirect contact with fomites and long-distance transport of aerosols, a process known as spatial transmission (14).

Mathematical models have been widely used to investigate FMD epidemic propagation (15, 16). Although significant technical and computational advancements have been achieved, simplifications of complex dynamics are required because of computational costs or the lack of population data, such as details about herd structure (e.g., number of individuals, number born alive) or animal movements, for example (15). The most common model simplification involves limiting the dynamics to a single species (4, 17, 18). Despite the different applications and efforts in modeling FMD, outstanding questions remain regarding measurements of the epidemic trajectory and epidemic control strategies given heterogeneous transmission dynamics among the different susceptible species coexisting on the same premises (9, 16).

Here, we developed a multi-host, single-pathogen, multiscale model designed to capture the dynamics of various transmission patterns across different host species to (i) simulate the spread of FMD disease within the Rio Grande do Sul state in Brazil; (ii) describe the geodesic distances from the initial outbreak to secondary cases; and (iii) compare control action strategies, including emergency vaccination, depopulation, various restrictions of between-farm movements, and surveillance activities within control zones, taking into account the initial number of infected farms at the onset of control measures.

2 Materials and methods

2.1 Data sources

2.1.1 Population data

A comprehensive dataset was compiled from official records of 355,676 farms registered in Rio Grande do Sul, Brazil (19) hosted in the Agricultural Defense System (SDA) (20). The dataset encompassed the number of animals per farm individually for cattle, buffalo, swine, sheep, and goats. Following stringent criteria, 70,853 premises were excluded due to missing geographical coordinates, instances without animal stock, and the absence of incoming and or outgoing movements during the study period spanning from August 24, 2022, to August 24, 2023. Consequently, the final dataset comprised 284,823 farms with accurate and reliable information. To simplify the analysis, population, and movement data from cattle and buffalo farms were merged into a single category denoted as "bovines." Similarly, sheep and goats were classified as "small ruminants." The total number of farms by category was as follows: 243,047 bovine farms, 80,664 swine farms, and 41,831 small ruminant farms. Of the total farms, 97,828 raised more than one host species. Supplementary Figure S1 presents farm-level population distribution, and Supplementary Figure S2 presents the geographical farm density distribution.

2.1.2 Birth and death

Producers are required to disclose to SDA their total number of animals born alive and the number of deaths, including those due to natural causes, at least once a year. Here, we collected data on birth and death from SDA, comprising 273,787 individual records associated with births and 268,790 deaths. The daily births and deaths, categorized by species, are depicted in Supplementary Figure S3.

2.1.3 Movement data

From August 24, 2022, to August 24, 2023, 763,448 unique between-farm and from farm-to-slaughterhouse movements were recorded and collected from the SDA centralized traceability database. Upon evaluation of the movement data, 106,481 records (13.9%) were removed due to various reasons: (a) lacking origin or destination identifications; (b) zero animals moved; (c) exact origin and destination premises; and (d) movements from or to premises not registered in the population data or to premises outside the state of Rio Grande do Sul. Ultimately, 413,939 unique between-farm and 243,028 slaughterhouse movements were analyzed. The daily farm-to-farm and farm-to-slaughterhouses movements, categorized by species, are depicted in Supplementary Figure S3.

2.2 Outbreak simulation

Rio Grande do Sul has over two hundred thousand farms, of which a sample was drawn and used as initial infected premises. Our sample was multistage and stratified, using the number of farms and species by municipality (21). The sample size was determined considering a prevalence of 50%, with a 95% level of significance and a margin of error

of 1.1%, resulting in a total of 10,294 farms (Supplementary Figure S4). Our model simulation was carried out in two steps: First, FMD was seeded randomly via one infected animal into sample farms between August 24, 2022, and August 24, 2023. For farms with multiple species, for instance, farms with bovine, swine, and small ruminants, FMD was seeded into bovine; for farms with swine and small ruminants, FMD was seeded into swine, and for farms with cattle and small ruminants, FMD was seeded via one infected bovine. We assumed that all animals were susceptible to FMD, as the annual vaccination campaign in Rio Grande do Sul had been suspended since May 2021 (22).

2.3 Model formulation

We implemented a multi-host, single-pathogen, coupled multiscale model to simulate FMD epidemic trajectories (23) and subsequently applied countermeasures actions. The model led to the development of an R and Python package, entitled "MHASpread: A multi-host animal spread stochastic multilevel model" (version 0.3.0) more details can be consulted in https://github.com/machado-lab/MHASPREAD-model. MHASpread allows the explicit specification of species-specific transmission probabilities and latent and infectious periods of a disease that infects multiple species. The within-farms level includes births and deaths for each species. The between-farm level consists of the entry and exit of animals due to between-farm movements and movements to slaughterhouses (Figure 1).

2.3.1 Within-farm dynamics

For the within-farm dynamics, we assume populations were homogeneously distributed. Species were homogeneously mixed in farms with at least two species, meaning that the probability of contact among species was homogeneous regardless of/when species were segregated by barns (e.g., commercial swine farms are housed in barns with limited changes of direct contact with cattle). The within-farm dynamics consist of mutually exclusive health states (i.e., an individual can only be in one state per discrete time step) for animals of each species (bovines, swine, and small ruminants). These health states (hereafter, "compartments") include susceptible (S), exposed (E), infectious, (I), and recovered (R), defined as follows:

(i) Susceptible: animals that are not infected and are susceptible to infection.
(ii) Exposed: animals that have been exposed but are not yet infected.
(iii) Infectious: infected animals that can successfully transmit the infection.
(iv) Recovered: animals that have recovered and are no longer susceptible.

Our model considers birth and death, which is used to update the population of each farm. The total population is calculated as $N = S + E + I + R$. The number of individuals within each compartment transitions from $S\beta \rightarrow E, 1/ \rightarrow I,\ 1/\gamma \rightarrow R$ according to the following Equations 1-5:

$$\frac{dS_i(t)}{dt} = u_i(t) - v_i(t) - \frac{\beta S_i(t) I_i(t)}{N_i} \quad (1)$$

$$\frac{dE_i(t)}{dt} = \frac{\beta S_i(t) I_i(t)}{N_i} - v_i(t) E_i(t) - \frac{1}{\sigma} E_i(t) \quad (2)$$

$$\frac{dI_i(t)}{dt} = \frac{1}{\sigma} E_i(t) - \frac{1}{\gamma} I_i(t) - v_i(t) I_i(t) \quad (3)$$

$$\frac{dR_i(t)}{dt} = \frac{1}{\gamma} I_i(t) - v_i(t) \quad (4)$$

Transmission depends on infected and susceptible host species, as reflected by the species-specific FMD transmission coefficient β (Table 1).

Births are represented by the number of animals born alive $u_i(t)$ that enter the S compartment on the farm i at the time t according to the day-to-day records; similarly, $v_i(t)$ represent the exit of the animals from any compartment due to death at the time t. The transition from E to I is driven by $1/\sigma$, and the transition from I to R is driven by $1/\gamma$; these values are drawn from the distribution generated from each specific species according to the literature (Table 2). The dynamics of within-farms are depicted in Figure 1.

2.3.2 Kernel transmission dynamics

Spatial transmission can occur through various mechanisms, including airborne transmission, contact between animals over fence lines, and the sharing of equipment between farms (24, 25). In this model, we included all these effects by fitting local spread using a spatial transmission kernel. This kernel assumes that the likelihood of transmission decreases as the distance between farms increases, with transmission beyond 40 km not being considered. The probability PE at time t describes the likelihood that a farm becomes exposed and is calculated as follows:

$$PE_j(t) = 1 - \prod_i \left(1 - \frac{I_i(t)}{N_i} \varphi e^{-\alpha d_{ij}}\right) \quad (5)$$

where j represents the uninfected population and ad_{ij} represents the distance between farm j and infected farm i, with a maximum of 40 km. Given the extensive literature on distance-based FMD dissemination and a previous comprehensive mathematical simulation study (26), distances above 40 km were not considered. Here, $1 - \frac{I_i(t)}{N_i} \varphi e^{-\alpha d_{ij}}$ represents the probability of transmission between farms i and j scaled by infection prevalence of farm i, $\frac{I_i}{N_i}$, given the distance between the farms in kilometers (Figure 2). The parameters φ and α control the shape of the transmission kernel; $\varphi = 0.044$ which is the probability of transmission when $d_{ij} = 0$, and $\alpha = 0.6$ control the steepness with which the probability declines with distance (24, 25). The exposure probability over distance is depicted in Figure 2.

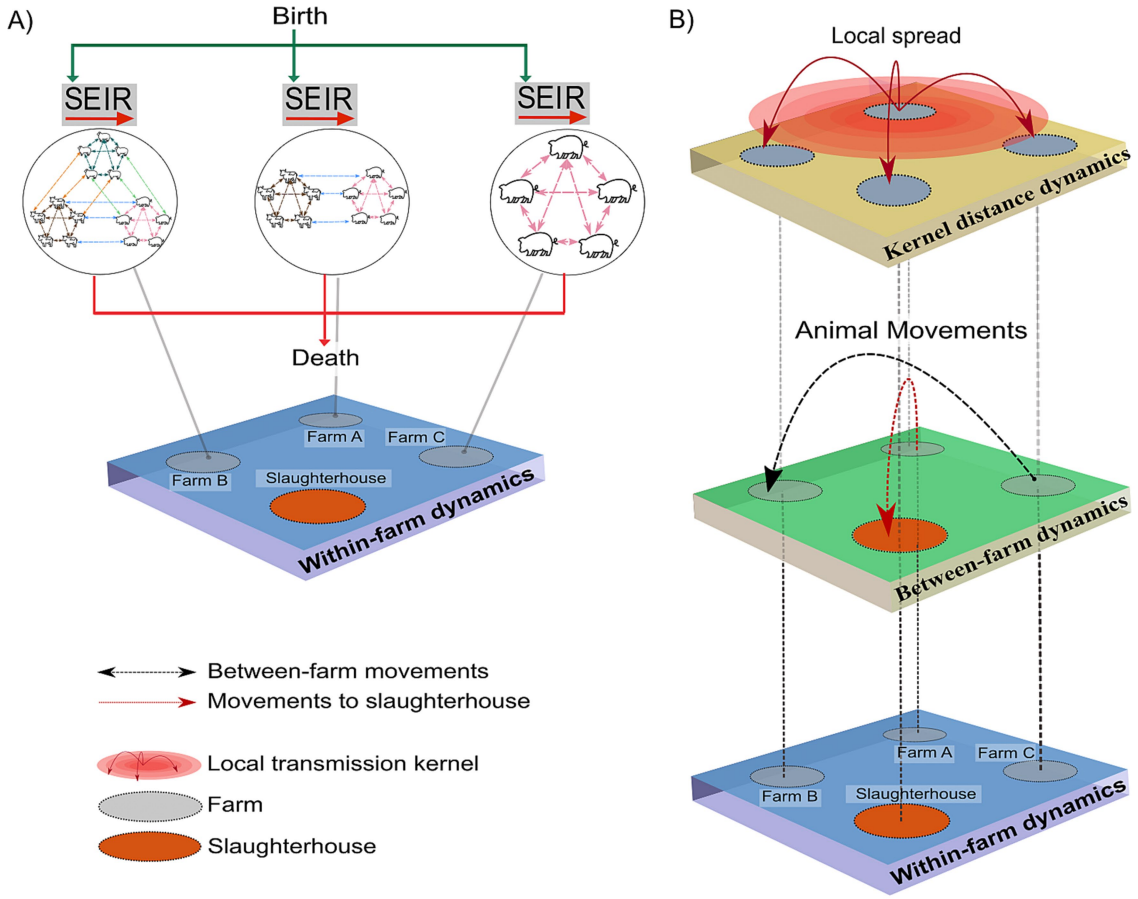

FIGURE 1
Schematic of state transitions during within-farm and between-farm dynamics. **(A)** Within-farm dynamics: green arrows indicate the introduction of animals (births) into the susceptible (S) compartment at farm *i* at time *t*. Each circle indicates a farm with single or multiple species with specific host-to-host transition parameters (σ, γ); dashed lines represent interactions within and between host species. The red arrows represent the removal of animals (deaths) regardless of infection status. **(B)** Between-farm dynamics: the layer represents the number of animals moved (batches; *n*) from the farm of origin (*i*) to a destination farm (*j*) at time t (indicated by the black dashed arrows). Animals moved to the slaughterhouse were removed from the simulation regardless of their infection status and are indicated by red dashed arrows. The kernel distance dynamics represent the spatial transmission distances.

2.4 FMD spread and control actions

We first simulated an initial silent spread over ten days. This procedure yielded a wide range of initial outbreak scenarios, depicted in Figure 2, before implementing control actions. Next, we outline four different control actions scenarios considered outlined by the Brazilian Ministry of Agriculture and Livestock (27).

The baseline control scenario named hereafter as "*base*": The following measures are considered: (i) depopulation of infected farms, (ii) emergency vaccination of all farms in the infected and buffer zones, (iii) a 30-day animal movement standstill, and (iv) the establishment of three distinct control zones around infected farms, with radii of 3 km (infected zone), 7 km (buffer zone), and 15 km (surveillance zone) (see Supplementary Figure S5), in which control actions vary as described below. These measures aim to prevent the further spread of the disease by enforcing biosecurity protocols and conducting regular inspections of animals and farms within these zones.

Depopulation of infected farms involves the removal of all animals from farms located within the infected zone(s). The daily depopulation capacity was set to four farms for this study, which was aligned with the maximum capacity observed in Rio Grande do Sul (personal communication with Dr. Fernando Groff). Farms with higher animal populations were prioritized for depopulation. Once depopulated, these farms are no longer considered in the simulation. If the daily depopulation capacity was insufficient to cover all identified infected farms within a day, those farms were scheduled for depopulation on the following day or as soon as possible, respecting the maximum capacity constraints.

2.4.1 Emergency vaccination

Bovine farms are vaccinated within infected and buffer zones. The daily maximum capacity was ten farms in the infected zone(s) and ten farms in the buffer zone(s) (personal communication Dr. Fernando Groff.) We simulated the delay in starting vaccination, set to 7 days post-FMD detection. Farms not vaccinated within a day due to the limited vaccination capacity were vaccinated on the subsequent day(s). Here, we do not assume any particular type of vaccine or FMD sorotype. Additionally, vaccine effectiveness was 90% in 15 days (28, 29). For details about the implementation of emergency vaccination, see Supplementary material control actions.

TABLE 1 The distribution of each host-to-host transmission coefficient (β) per animal^{-1} day^{-1}.

Infected species	Susceptible taxon	Transmission coefficient (β), shape and distribution (minimum, mode, maximum)	Reference
Bovines	Bovines	PERT (0.18, 0.24, 0.56)	Calculated from the 2000–2001 FMD outbreaks in the state of Rio Grande do Sul (22)
Bovines	Swine	PERT (0.18, 0.24, 0.56)	Assumed
Bovines	Small ruminants	PERT (0.18, 0.24, 0.56)	Assumed
Swine	Bovines	PERT (3.7, 6.14, 10.06)	Assumed (60)
Swine	Swine	PERT (3.7, 6.14, 10.06)	(60)
Swine	Small ruminants	PERT (3.7, 6.14, 10.06)	Assumed (60)
Small ruminants	Bovines	PERT (0.044, 0.105, 0.253)	Assumed (61)
Small ruminants	Swine	PERT (0.006, 0.024, 0.09)	(62)
Small ruminants	Small ruminants	PERT (0.044, 0.105, 0.253)	(61)

TABLE 2 The within-farm distribution of latent and infectious FMD parameters for each species.

FMD parameter	Species	Mean, median (25th, 75th percentile) in days	Reference
Latent period, σ	Bovines	3.6, 3 (2, 5)	(63)
	Swine	3.1, 2 (2, 4)	(63)
	Small ruminants	4.8, 5 (3, 6)	(63)
Infectious period, γ	Bovines	4.4, 4 (3, 6)	(63)
	Swine	5.7, 5 (5, 6)	(63)
	Small ruminants	3.3, 3 (2, 4)	(63)

The time unit is days.

2.4.2 Traceability

We utilized contact tracing to identify farms that had direct contact with infected farms within the past 30 days. These farms underwent surveillance, including clinical examination and detection. Farms testing positive during traceback were categorized as detected infected farms.

2.4.3 Movement standstill

A 30-day restriction on animal movement across all three control zones was implemented, prohibiting any incoming or outgoing movements. The control zones were lifted, and a standstill was maintained until depopulation was complete.

We assumed that 10% of the infected farms were identified when control measures began, specifically after ten days of initial spread from the introduction of the index case. For example, if there were 100 infected farms, only ten would be detected. If the calculated number of detected farms falls below one, we will round up to one detected farm. The detection parameter was set arbitrarily due to the lack of empirical data on the percentage of infected farms at which the surveillance system can reliably detect cases. After the first detection, the detection rate in the subsequent days is influenced by two primary factors: the total number of farms within the control zones and the number of infected farms. Notably, when there are fewer farms under surveillance but a higher number of infected farms, the likelihood of detection increases. Additionally, infected farms located outside the control zones are also included in the pool of farms subject to detection. For more details, refer to Supplementary Figure S6.

2.4.4 Alternative control scenarios

Base x 2: In this scenario, the daily number of vaccination increased to 40 farms and the depopulation to eight farms. *Base x 3:* This scenario differed to included 60 farms vaccinated daily and 12 farms depopulated. *Depopulation*. This scenario differed from the baseline control scenario by increasing the depopulation of infected farms to 12, and vaccination was not used.

2.5 Model outputs

Our simulations tracked the number of animals in each health compartment and the number of infected farms at each time step. The epidemic trajectories were used to calculate the geodetic distances in km between the seeded index infections and the subsequent infections. In addition, we determined the probability of distance-dependent transmission by calculating the cumulative empirical spatial distribution. We utilized a generalized additive (GAM) model to plot the relationship between the number of infected farms and the epidemic duration across different scenarios, as well as a one-way analysis of variance (ANOVA) with a Tukey post-hoc test to compare scenarios. This enabled us to explore potential nonlinear relationships between the variables, effectively capturing complex patterns that might exist in the data. In addition, a mixed-effects regression model was fitted to describe the relation between days working on control action and the initial number of infected farms controlled by each scenario.

2.6 Sensitivity analysis

We used a combination of Latin hypercube sampling (LHS), developed by McKay (30), and the partial rank correlation coefficient (PRCC) technique to perform a local sensitivity

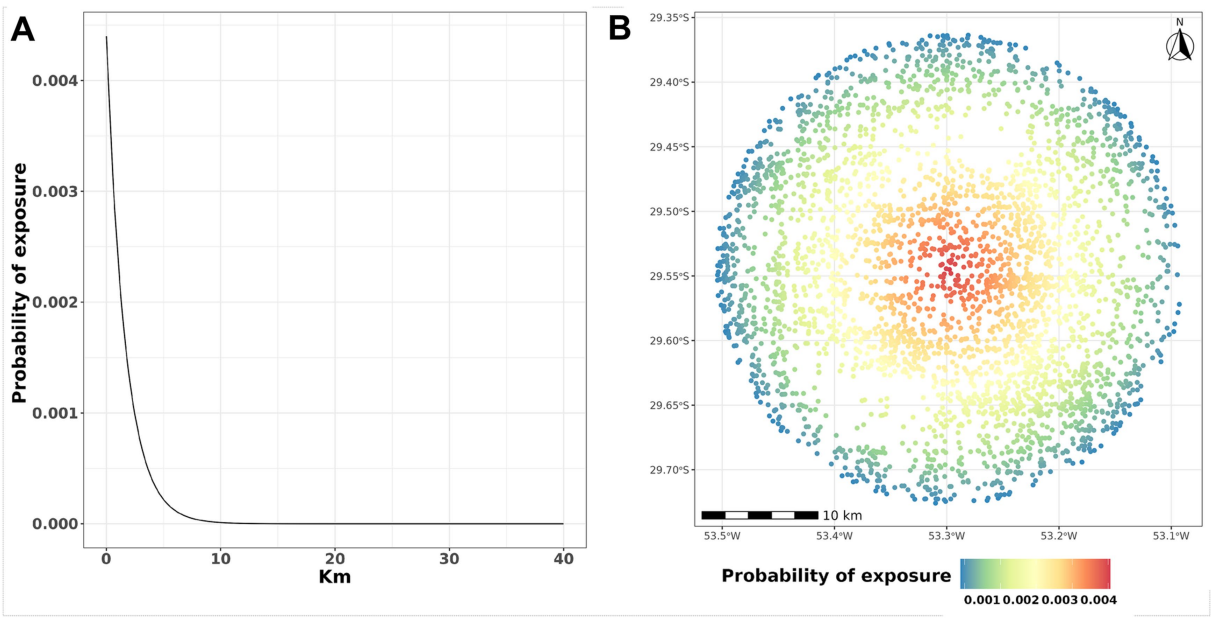

FIGURE 2
Probability of exposure and distances. **(A)** The y-axis represents the probability of exposure *PE* while the x-axis represents the distance in km. **(B)** Representation of farm locations. The color of the dot represents the probability of *PE* exposure; in this example, the infected farm is located in the center of the radius.

analysis. LHS is a stratified Monte Carlo sampling method without replacement that provides unbiased estimates of modeling output measures subject to combinations of varying parameters. The PRCC approach can be used to classify how output measures are influenced by changes in a specific parameter value while linearly accounting for the effects of other parameters (31). As input model parameters, we selected the following categories and interspecies interactions: β bovine to bovine, β bovine to swine, β bovine to small ruminants, σ bovine, γ bovine, β swine to swine, β swine to bovine, β swine to small ruminants, σ swine, γ swine, β small ruminants to small ruminants, β small ruminants to bovine, β small ruminants to swine, σ small ruminants, and γ small ruminants. In total, 15 parameters were used to classify the monotone relation of infection status with our input variables to classify model sensitivity. The inputs include one farm where the initial conditions varied across 10,000 simulations over the LHS space. A positive PRCC indicates a positive relationship with the number of infected animals, whereas a negative PRCC indicates an inverse relationship with the number of infected animals; however, the magnitude of PRCC does not necessarily indicate the importance of a parameter (32).

2.7 Software

The language software used to develop the MHASpread model and create graphics, tables, and maps was R v. 4.1.1 (33) and Python v. 3.8.12, R utilizing the following packages: sampler (34), tidyverse (35), sf (36), brazilmaps (37), doParallel (38), lubridate (39) and Python v. 3.8.12 with the following packages: Numpy (40), Pandas (41), and SciPy (42). This model is available in both R and Python versions.

3 Results

3.1 Initial spread and detection

Initially, we explore the variation in initial infection trends within the first ten days (Figure 3). The median number of infected farms was 52.5 (IQR: 26.75 to 78.25, maximum 123), of which the majority were swine farms with a median of 43.5 (IQR: 22.25 to 64.75, maximum 105), compared to bovine 43 (IQR: 22 to 64, maximum 85) and small ruminants with 20.5 (IQR: 10.75 to 30.25, maximum 42).

3.2 Distances from the initial outbreak

Of the 284,396 unique simulated FMD events, the distance from seeded infection to the secondarily infected farm within the first ten days exhibited a median of 4.78 km (IQR: 2.64 Km to 7.98 Km, maximum 6.88 Km) (Figure 4A). Furthermore, we observed a linear increase in the distance to which FMD disseminated (Figure 4B).

3.3 Effectiveness of control measures

All control scenarios were effective in eliminating all outbreaks within 120 days of the start of control measures. However, effectiveness was significantly different (ANOVA, p-value <0.05), except when we compared *depopulation* with the *base x 3* scenario. In general, the most effective alternative scenarios were *base x 3* and *depopulation*. The most notorious differences in means of infected farms by scenario were between the *base* and *depopulation* scenarios with mean differences of 2.36 (95% CI: 2.22 to 2.49), followed by *base x 3* and *base* with 2.26 (95% CI: 2.13 to 2.39) and

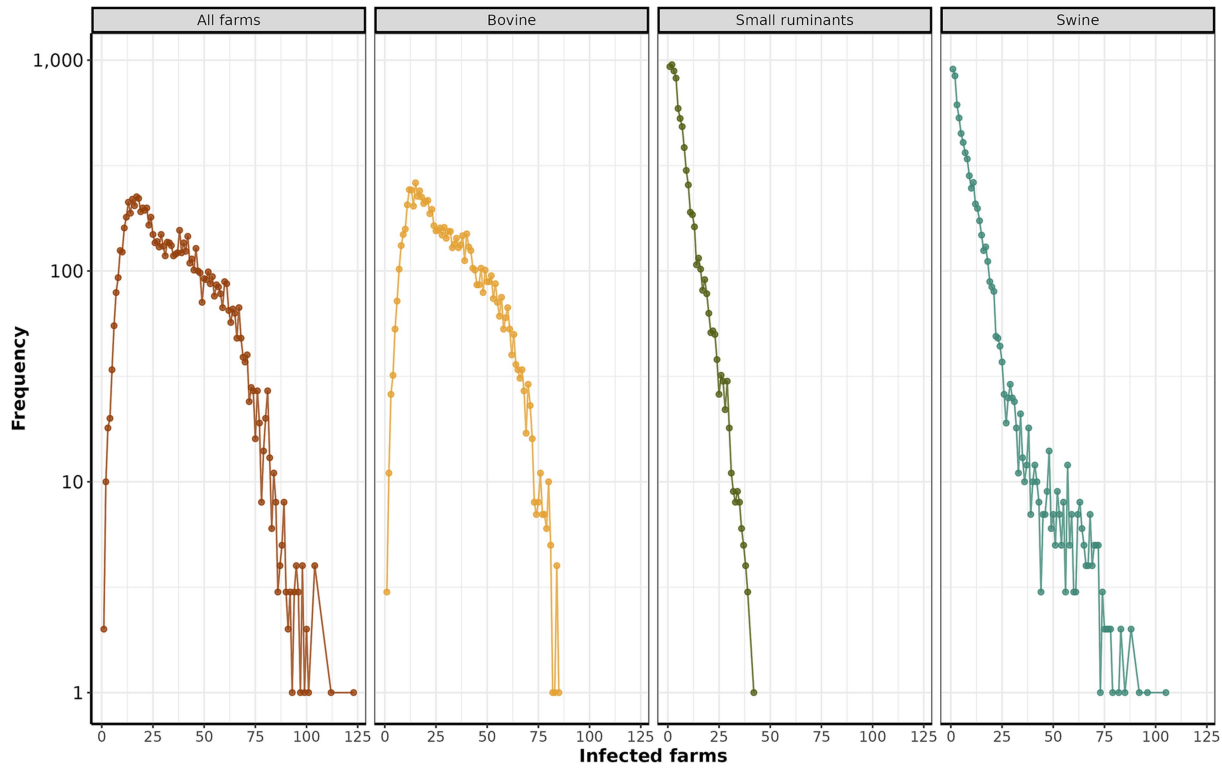

FIGURE 3
Distribution of the initial infected farms. The y-axis depicts the logarithm (base 10) of the frequency of infected farms across all simulations. The x-axis represents the number of infected farms.

base x 2 and *base* with 1.35 (95% CI: 1.22 to 1.49) (Supplementary Figure S8). In addition, we used a generalized additive model (GAM) to visualize the course of simulated epidemics over time. Notably, scenario *base x 3* consistently exhibited lower prevalence over time when benchmarked with *depopulation*, *base x 2*, and *base* scenarios (Figure 5).

3.3.1 Control actions duration

The median number of days of control actions implemented for the *base* scenario was 22 (IQR: 17 to 29, maximum 109). While *base x 2* had a median of 16 (IQR: 14 to 19, maximum 51), *base x 3* with a median of 15 (IQR: 13 to 17, maximum 43) *depopulation* had 14 days (IQR: 13 to 17, maximum 45) (Figure 6). In addition, we describe the similarities and disparities between the mean number of days control actions were active, meaning at least one outbreak response action was still ongoing. The comparison between *depopulation* and *base*, *base x 3* and *base*, and *base x 2* and *base* revealed substantial disparities in the group means: -9.77 (95% CI: to 10.04 to −9.49), −9.83 (95% CI: −10.10 to −9.56), and −8.09 (95% CI: −8.36 to −7.81), respectively. The complete statistics are depicted in Supplementary Figure S9. Our finding indicates a positive relationship between time working in control action and the number of initially infected farms. We found a linear relationship in which, on average, for each additional infected farm at the beginning of the control actions, the number of days working on control actions is expected to increase by approximately 1.59 days (GLM, p-value <0.05).

3.3.2 Vaccination

In the *base* scenario, the daily median of vaccinated animals was 1,928 (IQR: 1,562 to 3,567, maximum: 20,740). In the *base x 2* scenario, the median increases to 3,959.32 (IQR: 3,067.75 to 5,865.56, maximum: 25,877). Similarly, in the *base x 3* scenario, the median increases to 5,947 (IQR: 4,157 to 8,384, maximum: 25,006). In the initial 30 days, there was a significant increase in the number of vaccinated animals, and after that, the amount of vaccine continued to increase on a reduced step demand (Figure 7).

3.3.3 Depopulation

We analyzed the daily average number of depopulated animals over time. Scenario *base x 2* and *depopulation* showed the highest cumulative mean with 3,071 (IQR: 1,767 to 3,768, maximum: 4,120) and 2,159.09 (IQR: 1,314 to 3,000, maximum: 3,830), respectively. Following closely were the *base* and *base x 3* scenarios, with means of 2,139 (IQR: 1,798 to 2,541, maximum: 3,039) and 1,151.93 (IQR: 500 to 2,799, maximum: 4,398), respectively. The *depopulation* scenario consistently showed the highest count of affected animals, followed by *base x 3*, *base x 2*, and *base*, particularly for bovine and small ruminants (Figure 8). However, in the case of swine, scenarios *base* and *base x 2* exhibited a higher incidence than other species.

3.4 Sensitivity analysis

We evaluated the sensitivity of 15 model parameters with weights ranging from −0.86 to 0.72; this sensitivity indicated a limited influence of model parameters on the number of simulated secondary infections. Specifically, the latent period σ had a negative impact on the number of secondary infections, and the Infectious period γ had a positive influence

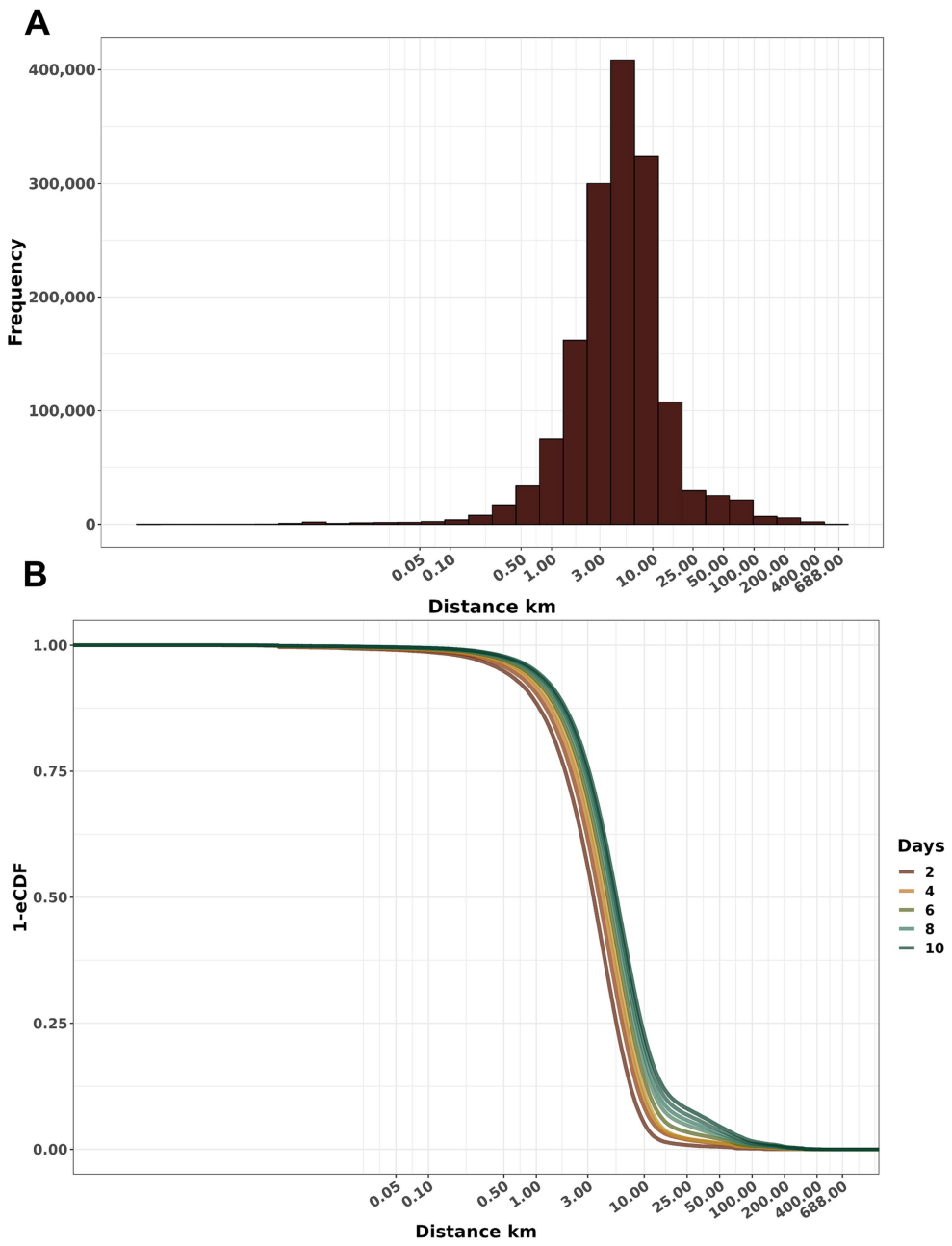

FIGURE 4
Distribution of the cumulative distance of dissemination. **(A)** Frequency histogram of secondary infections at varying distances from the seeded infection. **(B)** Empirical cumulative distribution function (1-ECDF) probability of infection according to distances from the initial outbreak within the days after the disease introduction. Both x-axes are a log10 scale.

on the number of secondary infections; both σ and γ have significant results (p-value <0.05) overall simulated species. The complete sensitivity analysis results are depicted in Supplementary Figure S10.

4 Discussion

This study aimed to develop an FMD multiscale, multispecies stochastic model that explicitly incorporates species-specific transmission interaction. Our model was used to simulate the spread of FMD among cattle, buffalo, swine, and small ruminants of Rio Grande do Sul, Brazil, and to examine the effectiveness of countermeasure scenarios. Bovine farms were the most infected species, followed by swine and small ruminants, mostly because of the higher number of cattle farms, and the connectivity of the swine contact network. Most secondary infections spread within 25 km, showing that disease transmission by proximity plays an important role in the spread dynamics. Our simulations demonstrated that tripling the number of daily depopulated and the vaccination eliminated epidemic trajectories within 15 days, which required 5,947 animals to be vaccinated and the depopulation of 2,139 animals.

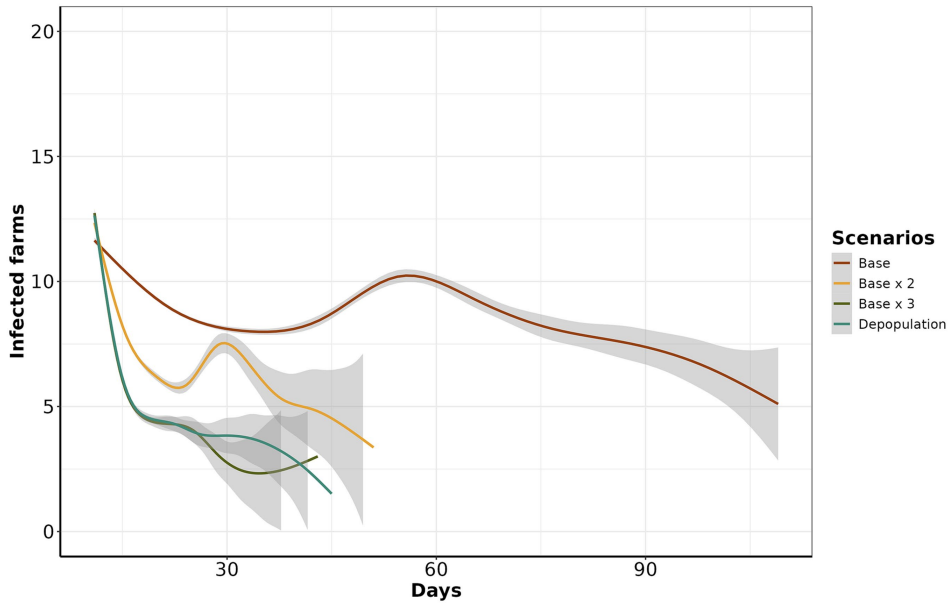

FIGURE 5
Estimated number of infected farms from day 11 to 120. The y-axis represents the number of infected farms, while the x-axis represents the day of simulation of control actions. The color line corresponds to each scenario.

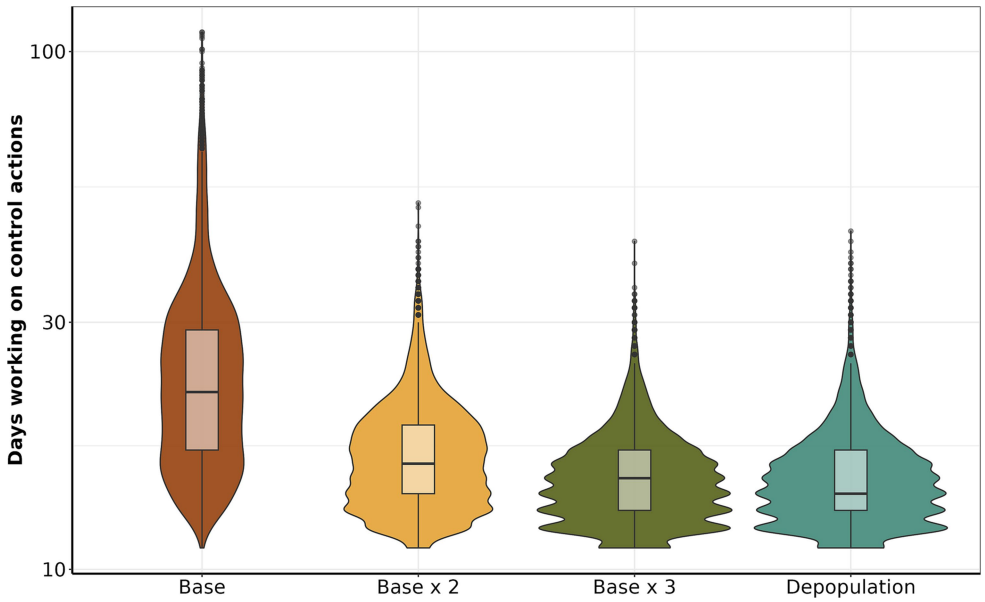

FIGURE 6
Comparison of the number of days working on control actions. The y-axis shows the total number of days dedicated to each control action, the x-axis presents the scenarios.

Within ten days of introduction, the range of infected farms varied from 1 to 123, with the majority of simulations (90.12%) resulting in fewer than 50 infected farms. Our study also revealed that when FMD infected swine, the epidemic sizes were significant (Figure 3). This risk is of particular relevance to areas of dense swine populated with commercial swine production, which are typically vertically integrated, which means such farms move a significant number of swine facilitation long-distance spread (43–45). Our study demonstrated that the number of farms initiating control actions has a linear impact on the duration of these actions, regardless of the implemented scenario. Specifically, each additional infected farm extended control actions by an average of 1.6 days. Consequently, enhancing the sensitivity of foreign animal disease detection is crucial for optimizing the effectiveness of control strategies (46). Therefore, we argue that improving the timing of detections and optimizing the response and management of outbreaks are pivotal to ensuring effective control. The scenario *base x 3*

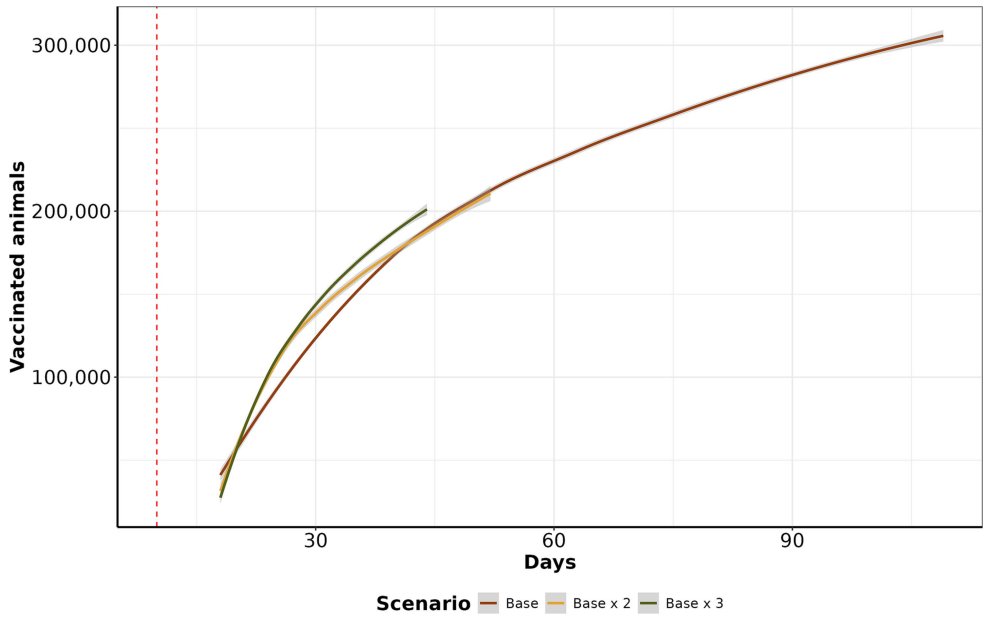

FIGURE 7
Vaccination curve by scenario. The y-axis represents the cumulative average of vaccinated animals per day, in the log10 scale. The x-axis shows the day of vaccination. The red dashed line represents when control actions were initiated after 15 days of initial the other control actions.

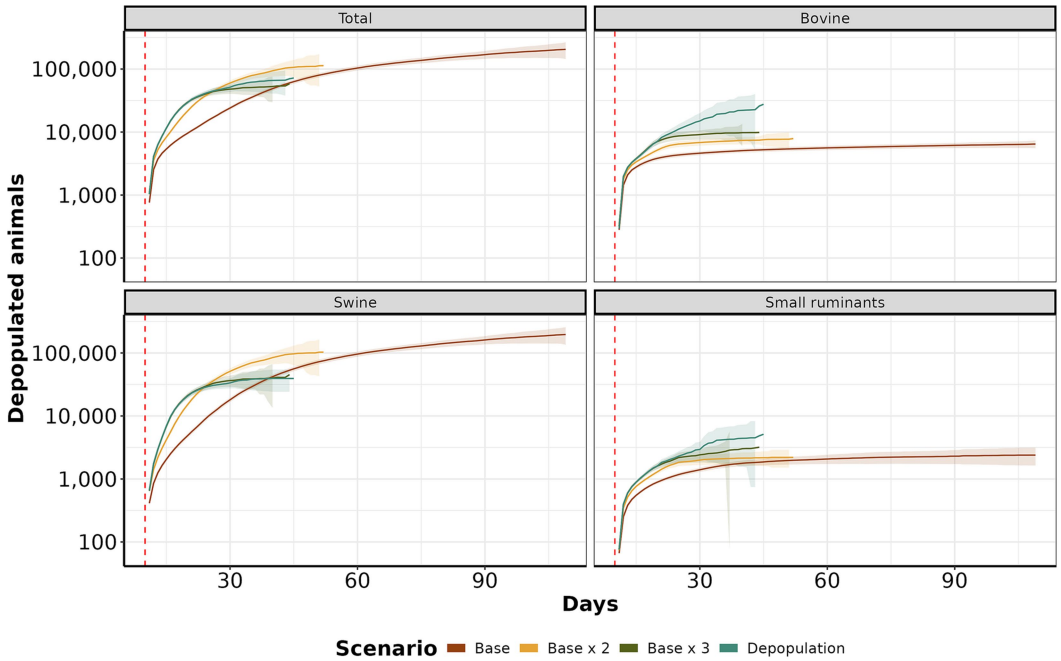

FIGURE 8
Depopulation curves by scenario. The y-axis shows the daily cumulative average of depopulated animals. The x-axis represents the day. The red dashed line color represents each control scenario.

demonstrated the best performance compared to the other proposed scenarios, requiring a median of 15 days to eliminate the outbreak (Figure 4). For comparison, the *base* scenario had a median duration of 22 days, and the *base x 2* scenario had a median duration of 16 days. Due to the large number of vaccines administered in the *base x 3* scenario, averaging 5,947 per day, compared to 1,928 per day in the *base* scenario and 3,959 per day in the *base x 2* scenario. Finally, the depopulation of 12 farms daily was successful in mitigating outbreaks; however, this scenario poses a significant challenge for official services.

Emergency vaccination presents an alternative to preemptive culling policies but may also limit the accuracy of surveillance systems in detecting infected farms, as it may mask clinical signs (47, 48). When

examining the median number of vaccinated animals at the end of the control action scenarios, the *base* scenario had the highest median of vaccinated animals at 305,105, followed by *base x 2* with 210,786 and *base x 3* with 202,471 vaccinated animals. Interestingly, despite the higher vaccination rates, the final average number of vaccinated animals was lower in scenarios with increased vaccination rates. This occurs because increasing vaccinations reduces the duration of outbreaks, ultimately resulting in fewer doses at the end of control actions. Our findings align with studies from Australia, Canada, New Zealand, and the United States, where emergency vaccination was correlated with a reduction in the number of infected farms and a decrease in outbreak duration (49–53). Examining the cumulative number of depopulated animals, we demonstrated that the *depopulation* scenario was more effective in controlling epidemics than the *base* and *base x 2* scenarios. The primary reason for this effectiveness was the high intensity of farm depopulation per day. Our analysis of the daily average number of depopulated animals over time reveals that the *base x 2* scenario had the highest mean cumulative count, with 3,071 animals culled, followed closely by the *depopulation* scenario, with 2,159 animals. The *base* scenario had a mean of 2,139 animals, while the *base x 3* scenario had 1,151 animals. When examining depopulation by species, the *depopulation* scenario consistently recorded the highest number of culled animals, particularly for bovine and small ruminants. In contrast, the *base* and *base x 2* scenarios revealed a higher prevalence of culled animals in swine than other species. The main reason for this is that prolonged outbreaks tend to affect more pig farms. Despite fewer pig farms than cattle farms, the number of animals per pig farm is significantly higher (Supplementary Figure S1). While depopulating was an effective countermeasure to contain highly contagious diseases like FMD (51, 54, 55), culling healthy animals raises ethical concerns. It also increases economic losses due to reduced production and farmer compensations (56). Conversely, other studies have proposed alternatives to ring depopulation, for instance, Seibel et al. (16) simulated target density strategy and showed its advantages in combating FMD since the number of healthy animals depopulated was lower than traditional total ring depopulation while the time to eliminate the outbreaks were similar. Besides, we emphasize the significance of timing when initiating depopulation for FMD control to prevent a disease outbreak across all farms in the area and potential outward spread (57). Moreover, prolonged delays in culling can lead to recurrent outbreaks in previously controlled areas, and under specific atmospheric conditions, there exists a risk of long-distance airborne spread (55).

4.1 Limitations and further remarks

Since there are no recent FMD outbreaks in the study region, we used data from the most recent FMD outbreak in Rio Grande do Sul (2000 and 2001) to extract parameters while utilizing the literature for the remaining parameters. Our sensitivity analysis did not identify any number of infected animals. Thus, our model has an acceptable level of robustness. FMD virulence, infectivity, and transmission can vary among strains (1, 11). Even though the most recent outbreaks in the State of Rio Grande do Sul were serotypes O and A (22), we cannot rule out the possibility that other strains were introduced and exhibited different dissemination patterns. Future work could include transmission scenarios with strains circulating in neighboring countries.

Additionally, other important between-farm transmission routes, such as vehicles and farm-staff movements, which have been previously associated with FMD dissemination (58, 59), were not included in our model. If such indirect contact networks are considered, the results would likely change, and model realism would be improved (51). We assumed 100% compliance with the restriction of between-farm movement from infected farms and farms directly linked to infected farms and the restriction of movement into and from control zones; we also assumed that the disposal of depopulated animals eliminated any possibility of further dissemination. Nevertheless, real-world compliance with the control actions was not examined or considered. Our model can also provide a distribution of expected FMD epidemics for any current or future control actions listed in the Brazilian control and elimination plan (27). Nevertheless, because our results are based on population data and between-farm movement data from Rio Grande do Sul, the interpretation of our findings should not be extrapolated to other regions. However, since the MHASpread model, infrastructure is highly flexible and can be easily extended to other Brazilian states and other countries.

5 Conclusion

In summary, we have shown the importance of including species-specific propagation dynamics in FMD transmission models designed to assist decision-makers in planning control and mitigation strategies for FMD. We have shown that a quick response in initiating control actions on a lower number of infected farms is crucial to reduce the necessary duration of control actions. We found that increasing depopulation capacity was sufficient to eliminate outbreaks without vaccination. Eliminating infected or likely-infected animals is an optimal strategy for preventing further epidemics, but culling large numbers of healthy animals raises welfare concerns. Regardless of which species in which FMD was introduced, the median distance over which the disease spread was within 25 km, a finding that could explain the effectiveness of the simulated countermeasures within the control areas used for FMD response. Our model projections, along with the necessary software, are available to local animal health officials. Thus, our model can be used as a policy tool for future responses to FMD epidemics through computer-based preparedness drills and capacity building and during emergency responses to FMD epidemics by providing rules of thumb generated from simulated control scenarios.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

NC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. FL: Resources, Validation, Writing – review & editing. AM: Funding acquisition, Software, Writing – review & editing. VM: Funding acquisition, Writing – review & editing. CT: Software, Writing – review & editing. FM: Software, Writing – review & editing. GM:

Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1468864/full#supplementary-material

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Utilising a livestock model for wildlife health planning

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Health planning provides a structure for the application of epidemiological data to managed populations with the intention of maximising health and identifying targets for intervention. Whilst this is established practice in livestock health, such schemes are rarely applied to free-living wild animal populations. The health of wildlife is important for a variety of reasons including conservation, human health, and ecosystem health, and so it is recommended that a formalised health planning approach be adopted for wildlife, based upon advantages of livestock health schemes identified here. Six key strengths of livestock herd health plans are identified in that these plans are: (1) Outcome driven, (2) Structured and repeatable, (3) They can incorporate both health and welfare considerations and in doing so, establish multidisciplinary management teams, (4) Evidence-based allowing for the prioritisation of key risk factors, (5) Encompassing of both population and individual metrics, and (6) Offer the opportunity for accreditation schemes. The benefits highlighted have implications for both wildlife management and research agendas where the structured format of the health plans will highlight knowledge gaps. Challenges are acknowledged, and it is recognised that livestock health planning cannot simply be copied across to a wildlife context. However, the strengths identified are great enough that it is recommended that wildlife population health planning is developed for active management of individual populations, learning lessons from existing plans.

KEYWORDS

evidence-based practice, wildlife health planning, wildlife management, applied epidemiology, health outcomes, multidisciplinary teams, wildlife health and welfare, knowledge gaps

1 Introduction

Epidemiological studies play a key role in the development of animal disease control measures (1) and have therefore been crucial in the development of herd health plans (HHPs) for livestock (2). With increasing access to electronic patient records, epidemiologists have recently been able to make evidence-based recommendations with respect to the management of companion animals through projects such as Vet Compass (3). Whilst there are numerous epidemiological studies into wild animal populations, methodologies are rare for converting research into applied surveillance schemes as management tools. True surveillance differs from simply monitoring, in that the former has a requirement for health data to contribute towards plans for risk mitigation (4). It is therefore clear that more could be done to capitalise on current wildlife research in order to aid health management.

Whilst wildlife health is a popular area for research, studied for a variety of reasons, for this work to have practical application there is a need for research to lead to action (5, 6). Wildlife health may be studied for the benefit of the wild animals themselves (conservation), but other reasons for focussing on this include the potential knock-on impacts to human health (zoonotic transmission), livestock (food security), or the

environment. From a One Health perspective, wildlife health has important implications for pandemic prevention (7). Poor wildlife health therefore has potential implications for conservation, ecosystem function, food security or public health (8), and so where this occurs it is likely that interventions would be required. Interventions would be expected to have an evidence base, ideally showing not just an impact on a designated risk factor, but a pathway to influencing the overall health of a population. Active health planning for a population therefore needs to be able to identify and measure factors of proven relevance to overall population health.

Whilst there is some debate around the definition of health in the human field (e.g., (9, 10)) it has long been established that this is more than merely the absence of disease. This definition is not consistently paralleled in wildlife studies with the meaning of health often assumed (11), or with a focus on a single pathogen. Ryser-Degiorgis (12) argues that whilst there may well be relevance in focussing on a single health component, population health is often multifactorial. When talking about a population's health it is therefore important to have a clear understanding of what a good health outcome would look like. If the research interest is driven by risk of a zoonotic pathogen transmitting to humans, then it is likely to be appropriate for studies to focus on a single pathogen, whereas a multifactorial approach may well be favoured if the viability of the study population itself is of concern.

The current picture of wildlife health research only partially satisfies the requirements for the sort of applied health surveillance which can lead to meaningful interventions. This observation agrees with Stephen (13) in calling for a renewed approach to wildlife health, with a focus on providing decision-makers with tools for action. This direction of travel appears typical of a general trend in epidemiological research with Frérot et al. (14) describing an increased focus within the literature on "health," as opposed to single pathogen and "control," implying responsive actions.

Adapting existing methods from livestock health planning may offer a framework for developing wildlife health planning based on established protocols. This paper sets out the case for taking this approach, based around six key reasons, and makes recommendations as to how to develop wildlife population health planning. For each reason given, the role of that factor in livestock health planning is described, along with a discussion of the applicability to wildlife health.

2 Rationale for translating a herd-health planning approach to wildlife

2.1 Outcome driven

No health planning approach will succeed without a clearly defined outcome and whilst many surveillance schemes may be managed by external participants, a HHP is typically constructed around a farmer's goals for their enterprise (15). Dairy HHPs are built around maximising milk quality and production, whilst beef and sheep equivalents will predominantly focus on meat, with economic improvements being seen as central to such approaches (16). These defined goals are essential for structuring the plan. Booker et al. (17) highlight the need for such goals as a starting point for health plans if a structured epidemiological approach is to be taken.

Analysis of the application of dairy HHPs has shown a clear positive relationship between the presence of tailored goals within a plan, and the active participation of stakeholders (18). Kristensen and Jakobsen (19), when discussing what they refer to as farmers perceived to be "irrational," highlight the necessity for the involvement of farm owners (the stakeholders) in the setting of farm goals, and livestock health planning has increasingly moved towards this tailored approach.

In a wildlife context, the selection of defined outcomes will rarely be as straightforward as it is for a dairy herd and will need to be specific to local issues. Factors that may be considered important could include the ability of a population to maintain and transmit zoonotic infections or pathogens of livestock importance, the population's ecosystem services, or something as fundamental as the continued existence of a population. Setting meaningful goals for wildlife health is almost certain to require the input of local stakeholders who have a good knowledge both of the populations themselves, but also of the local challenges (20–22). In practice therefore, it is important that there be careful consideration of the most appropriate methods for engaging local communities both to capitalise on their existing understanding, and to better understand their future needs (23).

The importance of incorporating clearly agreed outcomes into livestock health planning has been recognised, and there is clear merit in using this as a foundation for a wildlife model as without them both structure and participation are likely to be disadvantaged.

2.2 Structured and repeatable

Having established defined outcomes, the remainder of a livestock HHP will consider how best to achieve them, usually through a multi-level approach. The desired outcomes will be monitored, as well as risk factors (see below) that influence these outcomes. The approach incorporates what Cook (24) refers to as "top-level" indicators and a "drill-down" techniques. Within this system a series of key performance areas are identified which relate to the overall outcome. For example, a dairy HHP focussed on farm milk output may have top-level reporting for animal nutrition, infectious disease, cow mobility (all impacting animals' ability to access and convert energy), reproductive health, youngstock rearing (both considering the next generation of milk producers), and milk quality itself. This list is of course not exhaustive. Each of these top-level categories is impacted by a huge range of different factors, but if the herd is performing well in one of these areas then it is an inefficient use of time to be investigating the risk factors in detail. However, when a category is performing sub-optimally, managers can "drill down" to those risk factors. Interventions are then targeted at risk-factors that appear to be impacting those selected population outcomes.

Resources for investigating wildlife health are notoriously stretched (12) and a structure such as this which only prioritises investigation and intervention into areas where there is a functional deficit would seem to be an efficient use of time and budget. Clearly identifying the most appropriate top-level categories would require careful thought, and there would be a need for research in order to create an evidence base for such plans. The clear advantages of such an approach for wildlife would include the transferability and repeatability of the approach allowing it to be locally adapted and implemented by a range of different individuals.

2.3 Incorporation of both health and welfare criteria within a multidisciplinary team

It is common to encounter the phrase "health and welfare" with no clear boundary established between those two different measures. This is true within a wildlife context as much as anywhere else and the two terms have their own definitions and specialists. The result of this can be siloed teams working independently of each other; inefficient in terms of both resources and the sharing of ideas. Livestock HHPs recognise that both health and welfare parameters contribute towards farm production outcomes and so incorporate both areas within typical plans. In livestock systems, a tendency has been noted for welfare investigations to focus too heavily on those welfare concerns relating to production diseases (25), and whilst this is unlikely to be a concern in a non-production context, being alert for study biases is a useful lesson.

There is therefore an opportunity to incorporate both wildlife health and wildlife welfare into wildlife health planning, thus improving both efficiency and a cohesion between practitioners. The process of developing models for dairy herd health planning has coincided with the evolution of multidisciplinary teams for managing on-farm health (26). Cook (24) talks about a change in style within veterinary practice over recent decades, moving from the "physician" model through to the "facilitator" model, whereby veterinary involvement in health has become about bringing together multidisciplinary teams rather than simply treating individual animals. Both Cook (24) and Kelly et al. (26) partially attribute these shifts to an increasing tendency to focussing on production (outcome) and population level management with larger herds.

Given the changes that have been seen in livestock health planning, it can be expected that the development of multidisciplinary teams for managing the health of wildlife populations would better facilitate interactions between disciplines and incorporate metrics of both health and welfare in their own rights. Currently the number of projects incorporating both health and welfare in wildlife populations is low.

2.4 Evidence-based approach

The top-level system that has been described here is built upon a body of livestock health research. In cases where the top level figures for clinical mastitis, for example, in a herd are considered unacceptably high, then identification of the pathogens involved may be a possible next step. Bacteria, such as *Escherchia coli*, are considered to be of environmental origin as opposed to being transmitted cow to cow, and so interventions targeting contamination of the environment would be favoured over addressing cow to cow transmission (27). Or in cases of poor mobility, investigations into the anatomical location of pathologies will lead investigators towards likely causes and therefore the interventions with the greatest chance of success (28). In both examples there is a chain of established evidence linking interventions to risk factors to outcomes.

The key advantages of this approach are that it does not require continual analysis of all potential risk factors (which would be labour intensive, expensive, and probably unrealistic), and it ensures that any data that is being collected and analysed is related to a defined health objective and is therefore meaningful. Whilst these are advantageous in livestock health work, these advantages are magnified in wildlife where observation and sampling is likely to be far more difficult. Without a structured approach to health planning in wildlife currently there are a whole range of analyses carried out, but the association between each analysis and an overall goal is rarely evidenced. By utilising this approach, risk factor analysis could be implemented to ensure that factors forming part of a surveillance scheme were those that were truly associated with desired outcomes. Common approaches in livestock health consider not just the presence or absence of a component, but also implement intervention thresholds [e.g., (29)], and this may be something for the future once the initial stages of wildlife health planning are established. As well as merely identifying those key measurements required, this then means that the value of interventions can be better understood and investments justified.

This does present a problem for translating livestock HHP approaches to wildlife as in the majority of cases the causal relationships between desired outcomes and risk factors have not been proven. Whilst this clearly offers a challenge, it does also mean that the proposed structure of a wildlife health plan would effectively become scaffolding for research priorities. This could be of use to both funding bodies as they will be able to see immediately a pathway to impact for proposed research, and to early career researchers looking to establish meaningful projects.

2.5 Encompassing both population and individual metrics

Population health statistics come in two forms; those that consider the population as the unit of interest, or those that collate data on individuals. The difference between these two approaches is often not highlighted, but both have advantages and disadvantages. Population-based measures may be much more useful when the desired health outcome from a population is, for example, an environmental impact. In such cases, what is important is not the proportion of individuals carrying out the desired activity, but the overall impact of the population. With increasing sizes in dairy herds, some movement has been seen towards health measures that reflect a population outcome without information about the individuals, for example the use of bulk tank milk samples (aggregate data) for disease testing (30). In such cases the reported statistic gives useful information about the final output, but the result could be swayed by one extremely heavily infected individual, and it would not be possible to distinguish this from low-level infections throughout the herd.

Livestock HHPs routinely use both population and individual measures and therefore can be used as a template for how to handle both. Understanding the origin of the data and being familiar with what interpretations can and cannot be made on the basis of a measurement are clearly key to being comfortable utilising both types of data. Health measures based on population parameters are utilised in both livestock and human health but are rare in wildlife health studies where population health tends to be described as a collection of individual health statistics. Aggregate data, for some observations, may be easier to obtain in a wildlife setting than individual samples, and so a recognition of how to incorporate such data in wildlife health planning would be a valuable lesson learned from livestock HHPs.

2.6 Accredited schemes

The final consideration mentioned here is that of the use of livestock health plans as essential components of accreditation schemes. This may not be the way in which every wildlife health practitioner wants to go, but there is the possibility of being able to use a structured wildlife health plan as evidence for the success of wildlife projects. In the United Kingdom, farm assurance schemes have increased the uptake of HHPs with benefits including increased milk quality and control of antimicrobial residues (31). There is therefore evidence in livestock that health planning has played a role in improving the health of farmed animals, and that participation has often been driven by the requirements of assurance schemes (32).

The formal structure of livestock HHPs, were it to be adopted for wildlife, could therefore have the dual benefits of incentivising participation from wildlife managers, and providing confidence for funding agencies. Financing wildlife health interventions can be very challenging, and being able to evidence to investors that their money will be used in an effective manner has great value (33). Environmental accreditation schemes have the potential to become very complex and participation in them should not be allowed to be the central determinant of the desired health outcomes of a wildlife health plan. However, if managed carefully formal wildlife health plans could be integrated into accreditation schemes with benefits in terms of participation, funding and recognition.

3 Discussion

The factors laid out above highlight the advantages of adopting a structured approach to wildlife health planning, building upon existing techniques for livestock health planning. Basing the wildlife model on existing livestock health structures offers the opportunity to reflect on current practices, learning lessons from what works well. Key advantages highlighted include structure, a framework for linking metrics (and interventions) to population outcomes, and a system which would highlight research needs.

Whilst it would be naïve to suggest that planning tools from livestock health could simply be copied directly across to a wildlife population, the broad principles described here would appear valuable enough to justify the effort involved in adapting processes. The current model of livestock HHPs was not struck upon immediately and is one which has evolved over time. A logical next step would therefore be to bring together a range of livestock practitioners and wildlife health professionals in order to highlight the most appropriate building blocks for a wildlife planning model, and to identify knowledge gaps that are important to address.

The requirement for clearly agreed wildlife health outcomes from the outset of this process stands out as key from these discussions as this feeds into so many of the other factors discussed. It is difficult to envision a scenario where health targets can be established for a population without integration of stakeholder inputs. Seeing wildlife population health as a context-specific set of goals rather than a rigid state will help to make health management plans more adaptable. Whilst a need will remain for single-issue health investigations, a model for tailored wildlife health planning will be essential for a move towards a more holistic view of health for wildlife species.

Livestock health planning and evidence based veterinary medicine have offered platforms for both veterinary practitioners and epidemiologists to contribute to wider teams managing livestock health. It is therefore strongly recommended that these principles now be extended to wildlife health.

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

SP: Conceptualization, Writing – original draft, Writing – review & editing.

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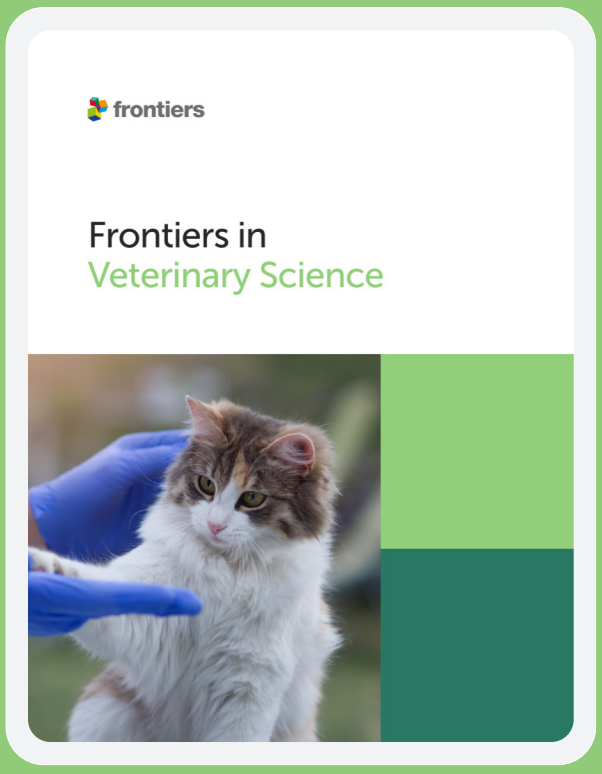

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