

Non-antibiotic alternative strategies in poultry:  
Evaluating organic farming systems and integrated  
vaccination-nutrition strategies

By  
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## Abstract

Poultry health, human health and economic losses caused by pathogenic zoonotic bacteria including *Salmonella* remain a major concern, despite ongoing prevention and control efforts. The use of antibiotics for prophylaxis, treatment and growth promotion has led to selection and dissemination of antibiotic-resistant bacteria which significantly impacts human health, animal health as well as the environmental health. To mitigate antimicrobial resistance (AMR), poultry producers have increasingly adopted non-antibiotic strategies including organic farming practices to satisfy the consumer demands, vaccination programs, and nutritional interventions. However, it is not clear to which extent organic farming systems reduce antibiotic resistance compared to conventional counterparts. Furthermore, interactions between *Salmonella* vaccines and nutritional dietary components such as SQMFe® iron (an organic iron with a complexation process that enables time- and tissue-specific release of this critical nutrient) has not been characterized in poultry. This thesis addresses these knowledge gaps through two studies. Chapter 1 presents a global comparison of antimicrobial resistance trends between organic and conventional farming systems across food animal products and environment and how various regulatory policies impact AMR prevalence across multiple geographical regions. Chapter 2 examines the interactive effects of *Salmonella* vaccination and supplemental iron (SQMFe®) on the poultry cecal microbiota composition. The results show that organic farming systems have lower AMR prevalence compared to conventional counterparts. Additionally, SQM® Iron enriched fermentative taxa such as *Pygmaibacter* and *Odoribacter*, while reducing *Streptococcus*. Vaccination alone promoted short-chain fatty acid (SCFA)-producing members of the *Bacillota* phylum. However, when vaccination was combined with SQM® Iron, several beneficial taxa were suppressed and *Staphylococcus* was markedly increased, suggesting that co-management strategies can produce unexpected and potentially counterproductive outcomes. These findings indicate that integrated approaches including organic farming, vaccination and micronutrient supplementation can reduce AMR and selectively remodel the poultry gut microbiome. This work has implication for nutrient utilization, developing targeted policy to control AMR and zoonotic pathogens while supporting animal

health.

## Introduction

Poultry meat is an important source of animal protein, with consumption projected to rise 15% by 2032 due to increased demand from population growth, urbanization, and increasing purchasing power in low and middle income countries (LMICs) (OECD and Food and Agriculture Organization of the United Nations, 2021). This rising demand has led to increased adoption of intensive farming practices, with the aim of reducing production costs (Gržinić *et al.*, 2023). However, this intensification has led to widespread use of antimicrobials in poultry production for disease prevention, treatment and growth promotion (Farkas *et al.*, 2025). This has made the industry a major contributor to global antimicrobial resistance (AMR). Antibiotic use is the major driver of AMR, with about 73% of antibiotics used in livestock production (Van Boeckel *et al.*, 2019), leading to implications for both animal and human health.

Poultry production acts as one of the control points of both AMR and zoonotic foodborne pathogens. Why poultry? Poultry production is one of the highest antimicrobial-consuming sectors with 148 mg/population correctional unit (PCU) (Van Boeckel *et al.*, 2015). In addition, consumption of undercooked poultry products can lead to transmission of zoonotic foodborne pathogens such as *Salmonella* spp. and *Campylobacter* spp., posing direct public health threats (Chlebicz and Śliżewska, 2018; O'Bryan, Ricke and Marcy, 2022; Shaji, Selvaraj and Shanmugasundaram, 2023). Lastly, poultry litter serves as a reservoir for disseminating resistant bacteria and antimicrobial resistance genes (ARGs) into the environment (Nhung, Chansiripornchai and Carrique-Mas, 2017; Hedman, Vasco and Zhang, 2020; Lopes *et al.*, 2024; Singh *et al.*, 2025). Therefore, both pathogen control and antibiotic usage are critical for protecting animal and public health.

Recently, there has been increased worldwide adoption of non-antibiotic alternative strategies in poultry farms to reduce antibiotic usage while reducing pathogenic bacteria. In 2021, the USDA made reducing pre-harvest *S. enterica* in poultry a top priority (USDA, 2021) and called for new approaches beyond the standard stringent cleaning protocols and drug

administration programs that rely on the continued efficacy of a very limited number of antibiotics. Two distinct mitigation approaches have emerged to address AMR and pathogenic bacteria in poultry production: nutritional strategies, and organic farming which promotes both food safety and animal health.

Nutritional interventions targeting iron metabolism offer a promising strategy to mitigate food-borne pathogens like *Salmonella*. Iron is a micronutrient required for essential biological processes in hosts, including respiration, gene regulation, and DNA biosynthesis (Cronin *et al.*, 2019; Tan *et al.*, 2021). In pathogens like *Salmonella*, iron availability directly influences pathogenicity, virulence, and tissue adhesion, with up to 7% of its genome differentially regulated in response to iron bioavailability (Cunrath and Palmer, 2021). During infection, *Salmonella* acquires iron in the inflamed gut using siderophores, specialized iron-chelating molecules that enable it to scavenge iron from the host (Julien *et al.*, 2019). Meanwhile, the host's immune system attempts to restrict iron availability as a defense mechanism, influencing the duration and severity of infection (Hennigar and McClung, 2016; Julien *et al.*, 2019; Iatsenko *et al.*, 2020). This interaction of iron between host and pathogen presents an opportunity for controlling iron bioavailability, thus limiting *Salmonella* colonization while maintaining host nutrition. Excess dietary iron can exacerbate this dynamic by increasing gut inflammation, intestinal permeability, bacterial translocation, and disrupting beneficial microbial communities (Garrett, Nunnery and Mc Naughton, 2019; Wellawa *et al.*, 2022), potentially worsening pathogenic infections (Kortman *et al.*, 2012).

To overcome this challenge, encapsulated organic iron supplementation offers a solution by enabling controlled, site-specific release within the gastrointestinal tract, optimizing iron bioavailability in the small intestine, the primary absorption site, while limiting availability to pathogens in the cecum and colon (Garrett, Nunnery and Mc Naughton, 2019; Iatsenko *et al.*, 2020; Jing *et al.*, 2022). Previous studies have focused on iron bioavailability and pathogen burden, with limited investigation into how encapsulated iron influences the cecal microbial community, a key determinant of both nutrient utilization and pathogen resistance. More importantly, the interactions between iron supplementation and other control interventions,

particularly *Salmonella* vaccination, remain uncharacterized. This lack of understanding regarding the interplay between nutritional, immunological, and microbial factors hinders the development of effective integrated control strategies, as it remains unclear whether combining encapsulated iron with vaccination will produce synergistic or antagonistic effects for *Salmonella* control.

While nutritional interventions like encapsulated iron offer targeted solutions at the biochemical level, system-wide production modifications provide an alternative pathway for AMR mitigation. Organic farming systems use different practices to address antimicrobial resistance and pathogen control. While the definition of "organic" varies across countries, regions, and regulatory bodies - from USDA standards in the United States, to EU Organic Regulations in Europe, and various national frameworks elsewhere (Seufert, Ramankutty and Mayerhofer, 2017), most organic certification standards agree on core principles, including prohibition of antimicrobial use for growth promotion or routine prophylaxis, compliance to high animal welfare standards, provision of outdoor access, and reliance on preventive health management through improved housing conditions, lower stocking densities, and enhanced biosecurity measures (Ager *et al.*, 2023). However, some heterogeneity exists in specific requirements, with variations in permissible therapeutic antibiotic use, minimum outdoor access provisions, maximum stocking densities, and feed composition standards across regulatory frameworks (Lund and Algers, 2003). These management practices create distinct production environments that differ from conventional systems. Organic broilers typically experience longer production cycles, are raised at lower stocking densities that reduce contact rates and disease transmission pressure and have access to outdoor environments that may enhance immune system development while increasing exposure to environmental pathogens and wildlife disease vectors (Rodenburg *et al.*, 2008; Lindgren *et al.*, 2014; Louton *et al.*, 2019). Previous studies examining AMR prevalence between organic and conventional poultry operations have reported reduced occurrence of antibiotic-resistant *Escherichia coli* and *Campylobacter* isolates in organic systems, though the magnitude of these differences varies across studies depending on geographic region, specific bacterial species, and particular management practices used

(Sapkota *et al.*, 2007; Miranda *et al.*, 2008; Luangtongkum *et al.*, 2009).

These observed reductions in AMR prevalence suggest that, by reducing routine antimicrobial use and enhancing animal health through environmental and management practices, organic systems may achieve lower resistance levels compared to conventional production. However, the relationship between organic production and resistance is more complex than simple antibiotic exclusion as resistant bacteria can persist in organic environments through contamination from previous land use, wildlife reservoirs, and horizontal gene transfer within environmental bacterial communities (Schwaiger, Schmied and Bauer, 2008; Smith-Spangler *et al.*, 2012). Moreover, the regulatory and practical variability described above, combined with differences in baseline disease ecology and management practices across geographic regions, means that the magnitude and consistency of AMR reduction in organic systems may vary across different contexts. Comprehensive analysis that accounts for geographic variation, temporal trends, specific bacterial pathogens and regulatory frameworks is therefore necessary to fully characterize the AMR-reducing potential of organic production.

This thesis integrates epidemiological surveillance of AMR with mechanistic studies of control strategies, linking broad patterns of resistance with specific interventions designed to reduce both AMR and foodborne pathogens in poultry production. The first chapter addresses the organic farming research gap through a synthesis of global AMR prevalence data from surveys across five continents and livestock hosts, including poultry. This epidemiological analysis examines whether organic farming effectively reduces AMR prevalence compared to conventional systems, while identifying how these effects vary across temporal trends, geographical regions, antimicrobial classes, bacterial species, and regulatory frameworks. The second chapter investigates the interactive effects of *Salmonella* vaccination and encapsulated iron (SQM Iron) supplementation on cecal microbiome composition in conventional broiler chickens using 16S rRNA gene sequencing. By characterizing microbial community structure and diversity across different iron supplementation treatments (Control, FeSO<sub>4</sub>, and SQM iron) and vaccination status (*Salmonella*-vaccinated and non-vaccinated), this study determines whether these interventions produce synergistic or antagonistic effects on *Salmonella* control

and gut health, providing insights into how integrated management strategies influence the poultry gut microbiome. Together, these complementary approaches address both the epidemiological evidence needed to inform AMR and food safety policy decisions and the mechanistic understanding required to optimize intervention strategies that simultaneously address antimicrobial resistance and foodborne pathogen control in poultry production.

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## Chapter 1

### Global trends in antimicrobial resistance on organic and conventional farms

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Global trends in antimicrobial resistance on organic and conventional farms

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### **1.1. Abstract**

The important hypothesis that organic livestock management reduces the prevalence of antimicrobial resistance is either fiercely supported or bitterly contested. Yet, empirical evidence supporting this view remains fragmentary, in part because relationships between antimicrobial use and drug resistance vary dramatically across contexts, hosts, pathogens, and country-specific regulations. Here, we synthesize global policies and definitions of 'organic' and ask if organic farming results in notable reductions in the prevalence of antimicrobial resistance when directly examined alongside conventional analogs. We synthesized the results of 72 studies, spanning 22 countries and five pathogens. Our results highlight substantial variations in country-specific policies on drug use and definitions of 'organic' that hinder broad-scale and generalizable patterns. Overall, conventional farms had slightly higher prevalence of antimicrobial resistance (28%) relative to organic counterparts (18%), although we found significant context-dependent variation in this pattern. Notably, environmental samples often exhibited high levels of resistance to medically important drugs, underscoring the need for more stringent and consistent policies to control antimicrobial contaminants in the soil. Taken together, these results emphasize the challenges inherent in understanding links between drug use and drug resistance and the critical need for global standards governing organic policies and greater investment in viable alternatives for managing disease in livestock.

## 1.2. Introduction

Antimicrobial resistance (AMR) is becoming an increasingly urgent global health crisis that disproportionately affects developing nations (Antimicrobial Resistance Collaborators, 2022; Ikhimiukor *et al.*, 2022). Mounting evidence indicates that the use (and overuse) of antimicrobials in livestock is generally linked with the rise of drug-resistant infections — in both animals and humans (Arason *et al.*, 2006; Bell and MacLean, 2018). Globally, the majority (~73%) of all antibiotics are used in animals conventionally raised for food (Van Boeckel *et al.*, 2019b), with over 45 mg/population correction unit (PCU) in cattle, 148 mg/PCU and 172 mg/PCU in chickens and pigs, respectively (Van Boeckel *et al.*, 2015b). A key objective, therefore, is to understand how the use of antimicrobials in livestock shapes the prevalence, identity, and transmission of antimicrobial resistance. Answers to this central, but challenging, question govern practices in policy decision-making, food safety, and public health.

'Organic' and 'non-conventional' farming practices have been proffered as means to reduce the use of antimicrobials in livestock, therefore reducing the selective pressures that promote the evolution of drug resistance (Witte, 2000; Mann *et al.*, 2021). Indeed, mounting evidence suggests that organic farming practices can reduce the occurrence of pathogenic outbreaks and the presence of genes that carry and spread antimicrobial resistance (Sapkota *et al.*, 2011; Mie *et al.*, 2017; Gücükoğlu *et al.*, 2020). These patterns suggest a potential win-win for animal welfare, conservation, and public health. Mechanistic insight into how, when, and where organic farming practices substantially and reliably reduce the prevalence and spread of antimicrobial resistance could be used to formulate scalable solutions for conventional farming practices with benefits for both agriculture and public health.

However, our understanding of the extent to which organic farming practices can successfully reduce the emergence and spread of AMR remains fragmentary — and hotly debated (Alali *et al.*, 2010; Quintana-Hayashi and Thakur, 2012; Buntenkoetter *et al.*, 2014; Hansson *et al.*, 2021). Empirical evidence linking specific farming practices to patterns of antimicrobial resistance is hard to establish, in part because the relationship between antimicrobial use and drug resistance varies dramatically across contexts, depending on

environmental conditions, livestock hosts, and pathogens. Additionally, pronounced differences in country-specific regulations undermine efforts to develop universal standards, exhaust consumer confidence, and weaken economic efficiency. These critical gaps in knowledge represent a major impediment in policy and management decisions and hamper investment in the research and development needed to generate viable and scalable non-pharmacological alternatives for managing disease in livestock.

As a first step in this endeavor, we begin by synthesizing global differences in policies and definitions of ‘organic.’ Broadly speaking, conventional livestock production focuses on technologies for increased productivity, such as high-yielding breeds, modern feeding techniques and veterinary health products, and (synthetic) fertilizers and pesticides. In contrast, organic practices broadly focus on reducing the antimicrobials in livestock by integrating cultural, biological, nutritional, and mechanical methods to ensure environmentally safe and residue-free foods, along with improved animal welfare standards (Alimentarius, 2017; Hosain, Kabir and Kamal, 2021; Åkerfeldt *et al.*, 2021; Rodrigues da Costa and Diana, 2022). In general, organic production strives to provide animals with a more spacious and enriched environment, access to an outdoor range, and limited group sizes, all of which ostensibly improve animal health and reduce the need for medications, including antimicrobials. However, a central obstacle to identifying the more specific broad-scale and generalizable differences between ‘organic’ and ‘conventional’ farming practices resides in the lack of a global or even regional consensus about standard practices.

Once we have synthesized the thinking about differences between organic and conventional farming practices, we conduct a literature review to examine where and when organic farming results in notable reductions in the prevalence of antimicrobial resistance over the range of contexts in which it has been directly examined alongside conventional analogs.

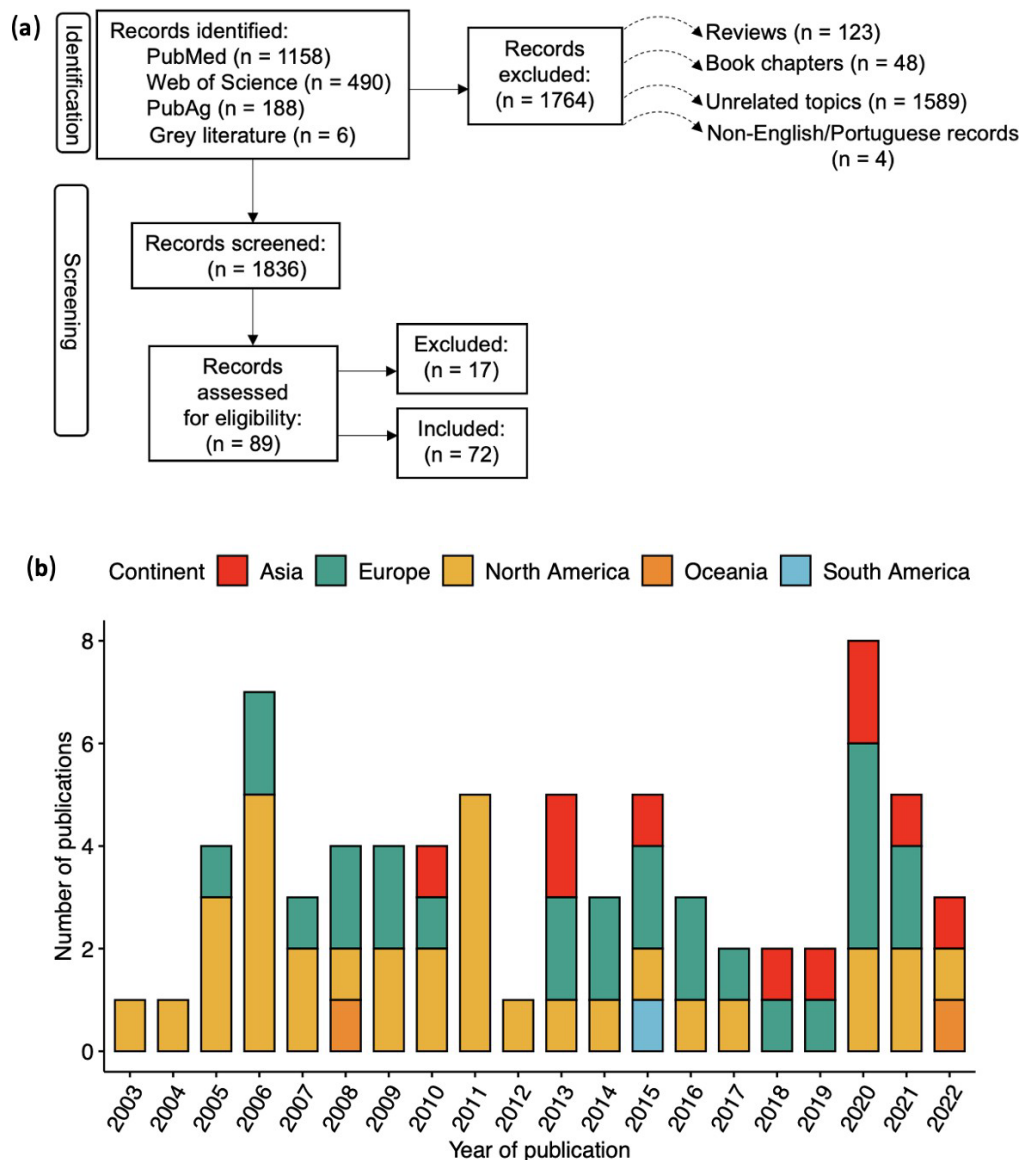
### **1.3. Methods**

#### *1.3.1. Literature search strategy*

Our initial goal was to examine studies that directly compared patterns of antibiotic resistance from organic and conventional farms within the same region (i.e., US: state; Africa:

province; Canada: province; UK: county) and livestock species (e.g., cow, chicken). However, these initial search criteria were too restrictive and yielded only sixty-four studies. Therefore, we expanded the search to include studies that reported antimicrobial resistance from organic farms without directly comparing their patterns alongside conventional counterparts.

We conducted literature searches for studies published between 2000-2022 using three electronic databases (PubMed, Web of Science, and PubAg). We used the following search terms, which we modified slightly for each database. Note, for brevity, we show abbreviated terms (e.g., “livestock names” reflects individual searches for sheep, goats, chickens, etc., and the name of the pathogen reflects individual searches for each pathogen – i.e., *Campylobacter*, *E. coli*, *Salmonella*, etc. In the PubMed search, for example, we used the following terms: livestock name AND product OR livestock production OR livestock farm OR name of pathogen OR antimicrobial AND resistant OR agriculture OR conventional OR organic AND agriculture. The full search terms are provided in the Supplementary Materials. Finally, references from other literature reviews (Yang *et al.*, 2019, 2019) and all other studies were screened for inclusion. Our search produced 1,836 hits (Fig.1a).



**Figure 1. (a) Overview of PRISMA-based literature search results and categorization of studies. (b) After literature search and screening, 72 studies were included in this review.** These studies examined 109 farms and 61,299 bacterial isolates. The vast majority (46%) of these studies were in North America (n = 33), 36% were in Europe (n = 26), 14% were in Asia (n = 10), while Oceania and South America contributed to 3% (n = 2) and 1% (n = 1), respectively.

### 1.3.2. Study selection

We reviewed all full English-language and Portuguese-language articles that directly compared patterns of antimicrobial resistance (AMR) from chicken, turkey, cattle, and pigs, and compared environmental samples from organic and conventional farms in a given geographic region. After screening the reference lists, we excluded reviews, unrelated topics, and book chapters (Fig. 1a). Following the search, all records were exported to Endnote's web citation manager (The EndNote Team, 2013), and duplicates were removed. The records were then

exported to a spreadsheet and organized by title, doi, authors, journal, year of publication, and abstract. Finally, the titles and abstracts were screened against the inclusion criteria.

The studies that met the eligibility criteria were retrieved in full text and were thoroughly reviewed. Seventy-two studies met our inclusion criteria (Fig. 1a). We attributed the reduction in sample size to two conditions: 1) our search terms covered general antimicrobial resistance and antimicrobial susceptibility topics and 2) our search focused only on articles written in English. As a way of assessing data quality in our review, we excluded records that did not clearly identify farm types as organic or conventional, gave no geographic information on study location, provided unclear resistance rates, or involved imported products. Data extraction results were stratified according to country name, antimicrobial resistance results, farm type, and pathogens.

### 1.3.3. *Statistical analysis*

All data analyses were conducted in R version 4.2.0 (R core Team, 2022) and QGIS version 3.24.0-Tisler (QGIS Development Team, 2022). To examine differences in the prevalence of AMR on organic and conventional farms, we used generalized linear models (GLMs) with quasibinomial distributions and log link functions (Crawley, 2013). We built candidate models starting with the full model with all combinations of main effects among relevant biological and methodological factors, while avoiding overfitting. Specifically, to examine overall changes in the prevalence of AMR across the 19-year time frame included in this review, the full model included farm type (organic vs. conventional), country, and study year and their interaction as fixed effects. Then, to examine more fine-scale differences in the prevalence of AMR, the full model examined effects of farm type, host, pathogen, country, and their interactions. We excluded antimicrobial type because the large number of drugs covered in these studies led to overfitting the models.

Following Burnham and Anderson, we compared candidate models using Akaike's information criterion and  $\Delta AIC$  (the difference in AIC values for the focal model and the model with the lowest AIC, i.e., the 'winning' model) (Burnham and Anderson, 2004). We conducted model selection analyses using the `aictab` function in the R package `AICcmodavg` (Mazerolle,

2023). We also calculated the Akaike weight ( $w$ ), which further quantifies the probability that a model is the most appropriate model relative to the candidate models.  $\Delta AIC$  less than two and a higher  $w$  generally indicates that a model has substantial support while a suite of best models with low weights ( $w \sim 0$ ) indicates that no single variable plays a substantial role in mediating infection dynamics (Burnham and Anderson, 2004).

Using the Anova function in the R package car (Weisberg and Fox, 2011), we assessed significance of the effects using Wald  $\chi^2$  statistics for the winning model. We also evaluated model fits with visual diagnostics, quantile-quantile plots, and residual-versus-predictor plots (Dunn and Smyth, 2018; Schweinberger, 2022). Note, the MIC (mean inhibitory concentration) values and MIC breakpoints were not used in this analysis due to challenges in accessing consistent data from the surveys. Moreover, variations in methodologies and criteria used by these laboratories and differing epidemiological contexts posed a challenge to analyze. Therefore, we focused on AMR prevalence estimates as a more feasible and consistent measure of resistance across studies. To report resistance in foodborne pathogens, we calculated the pooled prevalence of resistance from each pathogen-drug resistance rate to report AMR in foodborne pathogens (Van Boeckel et al., 2019b; Schar et al., 2021) using the formula below:

$$\text{Pooled prevalence} = \sum \frac{\text{Number of isolates resistant}}{\text{Total number of isolates tested}}$$

#### 1.4. Results

We identified 1,833 unique academic studies published between 2000 and 2022, as well as six grey literature studies (e.g., WHO website, with reports on point prevalence of antimicrobial resistance). After the references were screened, 1,744 academic studies and all six grey literature publications were removed (Fig. 1a). After assessing the remaining 89 references for eligibility, 17 studies were excluded, leaving 72 studies that met the inclusion criteria. Geographically, 46% (n=33) were from North America, 36% (n=26) from Europe, 14% (n=10) from Asia, and 3% (n=2) and 1% (n=1) from Oceania and South America, respectively (Fig. 1b). All surveys covered antimicrobials classified as critically important and highly

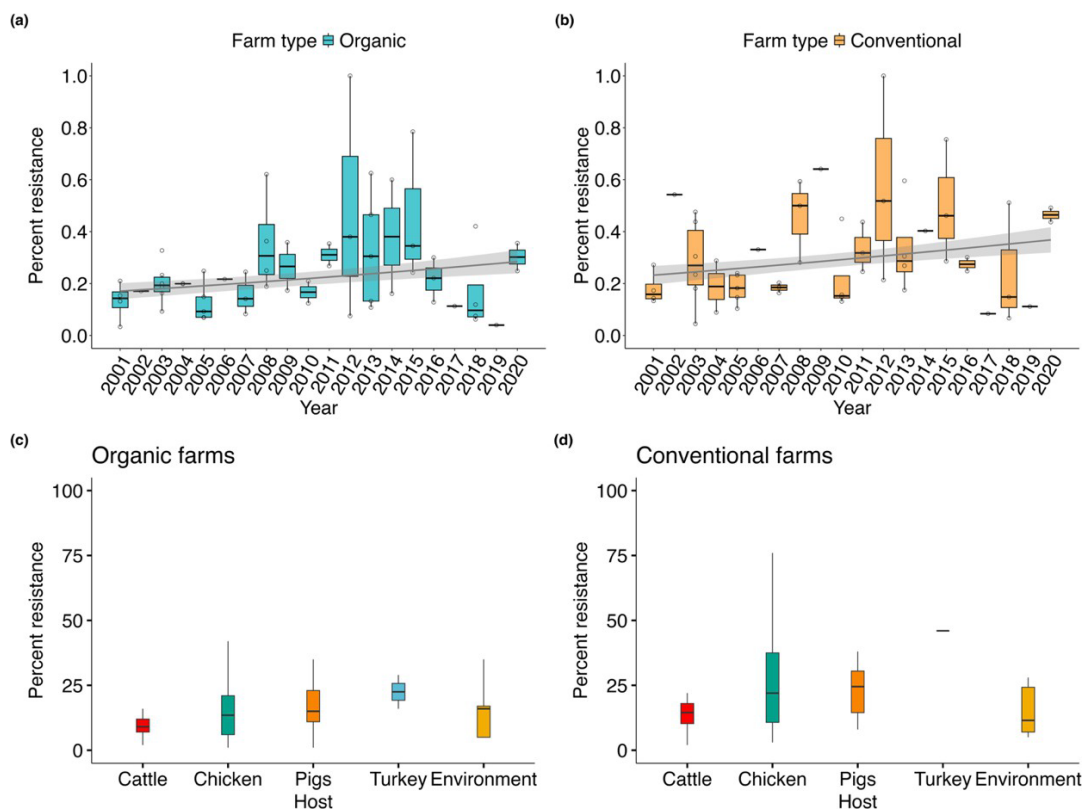
important for human medicine by the World Health Organization.

These 72 studies contributed 77 unique data points, with five studies examining multiple sample types or production stages within the same investigation. Samples were obtained from chickens (n=37, 48.1%), cattle (n=16, 20.8%), pigs (n=16, 20.8%), environmental sources (n=6, 7.8%), and turkeys (n=2, 2.6%). When categorized by production system, poultry accounted for the majority (n=40, 51.9%), followed by pig production (n=19, 24.7%), dairy (n=14, 18.2%), and beef (n=4, 5.2%). Within poultry, samples included slaughter-ready birds (chickens n=16, turkeys n=2), retail meat (n=9), broilers (n=6), layers (n=3), eggshells (n=2), embryos (n=1), and litter (n=1). Cattle samples were predominantly from lactating dairy cows (n=9), with additional samples from slaughter-ready animals (n=2), retail meat (n=2), young cattle (n=1), calves (n=1), adult cows (n=1), manure (n=1), and soil (n=1). Pig samples were primarily from slaughter-ready animals (n=12), with limited representation of sows (n=2), weaners (n=1), finishers (n=1), and environmental sources including manure (n=1), water (n=1), and resting areas (n=1) (Supplementary Table 3. Due to differences in region-specific regulations for 'organic' policies (Supplementary Table 1) and context dependencies, we first discuss the overall patterns. Then, we highlight key differences across host-, pathogen-, and region-specific contexts. For all drug acronyms, see Supplementary Table 2.

For all drug acronyms, see Supplementary Table 2.

#### *1.4.1. Overall trends in AMR across organic and conventional farms*

Overall, conventional farms had higher a prevalence of mean antimicrobial resistance (28%) relative to organic farms, (18%), (Binomial GLM main effect of farm type (GLM),  $\chi^2 = 3403$ ,  $df = 1$ ,  $p < 0.0001$ ). However, between 2001 and 2020, the percentage of antimicrobial resistance increased on both organic and conventional farms. The percentage of AMR on organic farms increased from 17% (CI: 16.5-18.5%) to 28% (CI: 26-30%), while the percentage of resistance on conventional farms increased from 23% (CI: 21-25%) to 36% (CI: 33.5-38.5%,  $\chi^2 = 15,429$ ,  $df = 17$ ,  $p < 0.0001$ , Fig. 2a and 2b).



**Figure 2. Temporal trends in the prevalence of antimicrobial resistance from different hosts (including environment samples) on organic and conventional farms.** Note: 12 studies were excluded from this analysis because they did not report the year data were collected. (a) Surveys spanning organic farms ( $n = 56$ ) and (b) conventional farms ( $n = 53$ ). Sixty surveys with complete sampling dates included in the analysis. Globally, the prevalence of antimicrobial resistance was slightly lower on organic farms (18%) relative to conventional farms (28%). However, antimicrobial resistance appears to be increasing on both organic and conventional farms. From 2001 – 2020, the prevalence of antimicrobial resistance in isolates ( $n = 29,417$ ) from organic farms increased from 17% (CI: 16.5-18.5%) to 28% (CI: 26-30%), Fig. 2a), while the prevalence of drug resistance in isolates ( $n = 31,882$ ) from conventional farms increased from 23% (CI: 21-25%) to 36% (CI: 33.5-38.5%, Fig. 2b). Examining host-specific patterns indicates that the prevalence of antimicrobial resistance from cattle, chicken, pigs, and turkey isolates was higher on conventional farms as compared to organic farms. However, antimicrobial resistance was higher in environmental samples collected from organic farms compared to conventional farms (Fig. 2c and 2d). Data represent the median  $\pm$  the first and third quartile ranges.

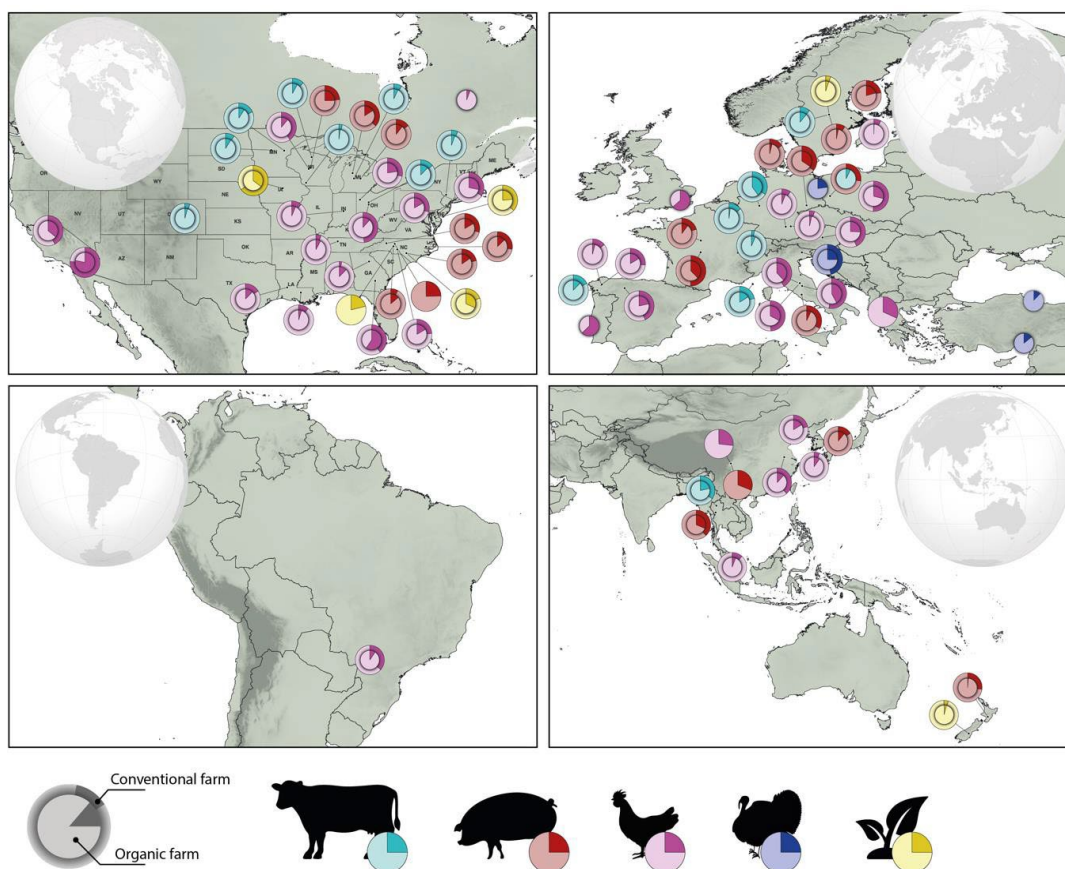
#### 1.4.2. Host-specific patterns

Looking across all geographic regions, resistance patterns were highly variable across different host classes, with overall resistance higher in hosts from conventional farms. Conventional farms reported a higher AMR prevalence in isolates from cattle, chicken, pigs, and turkey. For instance, in isolates from cattle, the prevalence of AMR was 14.5% on conventional farms and 9% on organic farms (Fig 2c and 2d). For chicken isolates, AMR prevalence was higher on conventional farms, 22% compared to organic farms, 13.5%. Similar

trends were reported for other hosts. For example, resistance was higher on conventional pig farms, 24.5 % than on organic farms 15% (Fig. 2c and 2d), and on conventional turkey farms, 46% as compared to organic farms, 22.5% . For environmental samples, the *median* prevalence of AMR in environmental isolates was slightly higher on organic farms, 16% relative to conventional farms, 11.5% (Fig. 2c and 2d). These patterns, however, were highly variable across geographical regions.

#### 1.4.3. *AMR patterns across broad geographic regions*

Examining region-specific variation in the prevalence of antimicrobial resistance may help identify areas where specific management practices warrant more attention (i.e., 'hot spots'). For example, in parts of the US, the prevalence of AMR was 64% (n = 135 isolates) and 35% (n = 135 isolates) in environmental isolates from conventional and organic farms, respectively. Countries with low antimicrobial usage in food production animals, like Sweden and New Zealand, reported low AMR prevalence from environmental samples. For instance, Sweden reported 5% (n = 725 isolates) AMR prevalence on both organic and conventional farms, while New Zealand reported 5% (n = 814 isolates) AMR prevalence on conventional farms and 3.8% (n = 814 isolates) on organic farms (Fig. 3).



**Figure 3. Global patterns of antimicrobial resistance in isolates collected from organic and conventional farms.** Studies spanned four hosts (cattle, pigs, chicken, and turkey) and environmental samples collected from conventional and organic farms throughout North America, Europe, Asia, Oceania, and South America. Pie charts show the prevalence of antimicrobial resistance on conventional farms (outer pie chart,  $n = 66$ ) relative to their organic counterparts (inner pie chart,  $n = 69$ ). Geographic regions with a single pie (i.e., outer pie only) represent areas lacking data from organic farms.

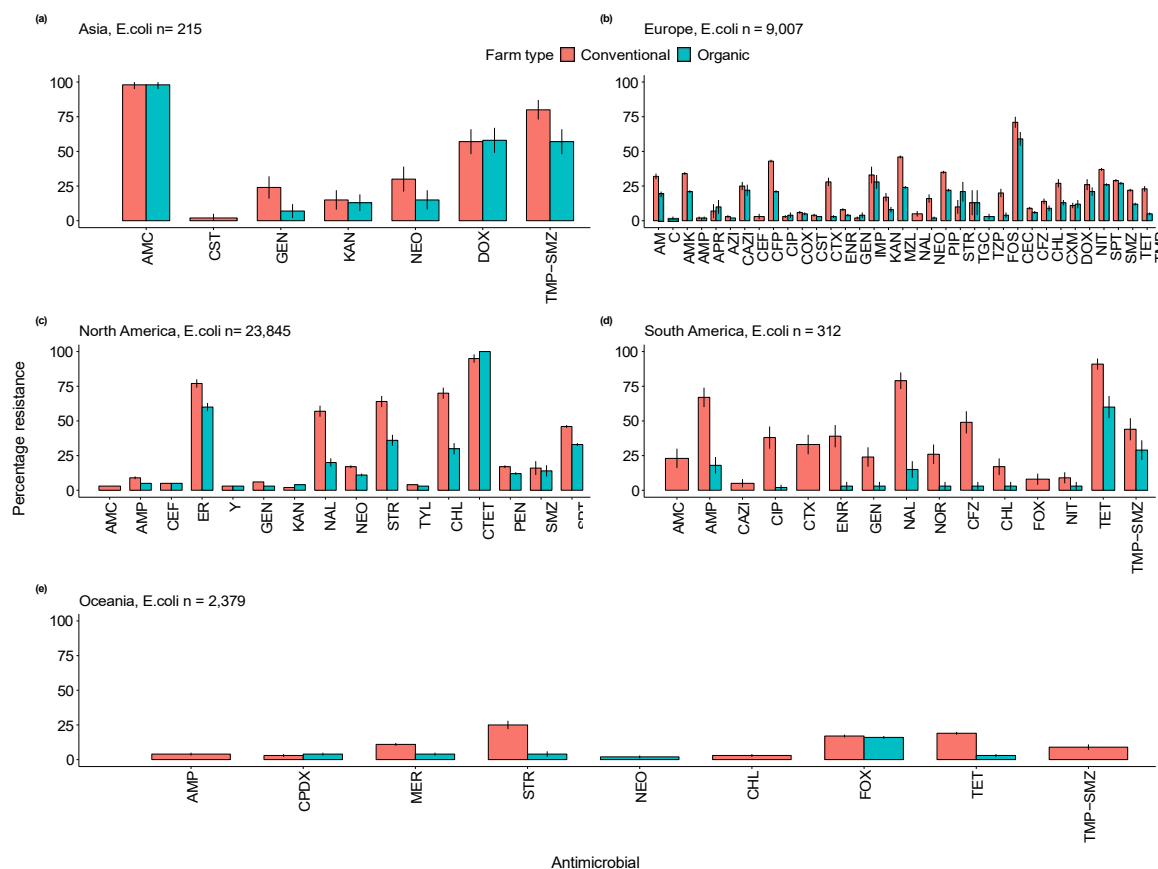
Patterns for other hosts were also highly variable across broad geographic scales. However, except for chickens, the prevalence of AMR was relatively similar across organic and conventional farms (Fig. 3). For chickens, patterns from organic farms were particularly notable. In Georgia, USA, the prevalence of AMR in isolates from chickens was marginally higher on organic farms than on conventional farms: 40% ( $n = 60$  isolates) on conventional farms and 59% ( $n = 60$  isolates) on organic farms. In California, USA, the prevalence of AMR in chicken isolates was notably high on both farm types: 78% ( $n = 132$  isolates) on organic farms and 75% ( $n = 132$  isolates) on conventional farms. These results were primarily driven by drug-resistant *Campylobacter* spp, discussed in detail below.

#### 1.4.4. Region-specific patterns in foodborne pathogens

Studies included in our review presented patterns of AMR prevalence in five pathogens sampled from a total of 61,299 isolates: *Escherichia coli*, *Salmonella* spp, *Campylobacter* spp, *Enterococcus*, and *Staphylococcus aureus*. These isolates were sampled from organic farms and conventional farms in Asia (n = 1,164 isolates), Europe (n = 11,759 isolates), North America (n = 44,979 isolates), Oceania (n = 3,085 isolates), and South America (n = 312 isolates).

#### 1.4.5. Asia

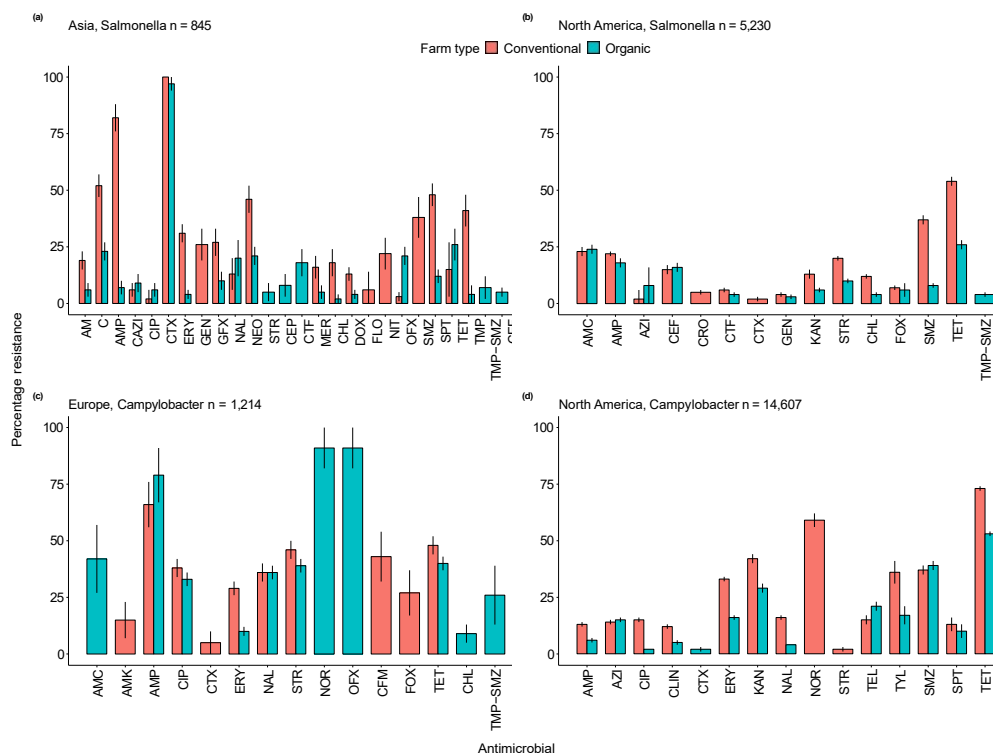
Across Asia, the prevalence of AMR was relatively high and similar across both organic and conventional farms, regardless of pathogen type. *Escherichia coli* isolates were highly resistant (98%, CI: 95-100%) to amoxicillin-clavulanic acid on both organic (n = 105 isolates) and conventional farms (n = 110 isolates, Fig. 4a). A similar trend was observed for erythromycin-resistant *Salmonella*, 100% (n = 171 isolates) on conventional farms and 98% (CI: 96-100%, n = 165) on organic farms (Fig. 5a). Moreover, *E. coli* and *Salmonella* also showed high resistance to other critically important antimicrobials like erythromycin, regardless of farm management type. In contrast, *Enterococcus* isolates exhibited lower resistance to ciprofloxacin on organic farms, 12% (CI: 10-13%, n = 104) versus 48% (CI: 45-53%) on conventional farms (Fig. 6a).



**Figure 4. Patterns of antimicrobial resistance in *E. coli*.** The AMR prevalence is shown for the number of isolates (n) examined on organic and conventional farms in each geographic region. (a) Asia, n = 215, (b) Europe, n = 9,007, (c) North America, n = 23,845, (d) South America, n = 312, (e) Oceania, n = 2,379. Data represent the mean  $\pm$  95% confidence intervals. The grey shading indicates antimicrobials classified as critically important; the unshaded region indicates highly important antimicrobials. For drug acronyms, see supplementary table 2. The figure present data with more than 10 isolates.

#### 1.4.6. South America (Brazil)

In South America, conventional farms reported higher resistance in *E. coli* isolates. However, conventional farms, reported moderate resistance patterns to most antimicrobials tested against *E. coli* — except for ampicillin, nalidixic acid, and tetracycline, which reported AMR prevalence of 67% (CI: 65-73%, n = 132 isolates), 79% (CI: 75-84%, n = 132 isolates) and 49% (CI: 45-55%, n = 132 isolates), on conventional farms as compared to 18% (CI: 15-22%, n = 51 isolates), 32% (CI: 28-35%, n = 32 isolates) and 14% (CI: 10-16%, n = 14 isolates) on ampicillin, nalidixic acid, and tetracycline on organic farms respectively (Fig. 4d).

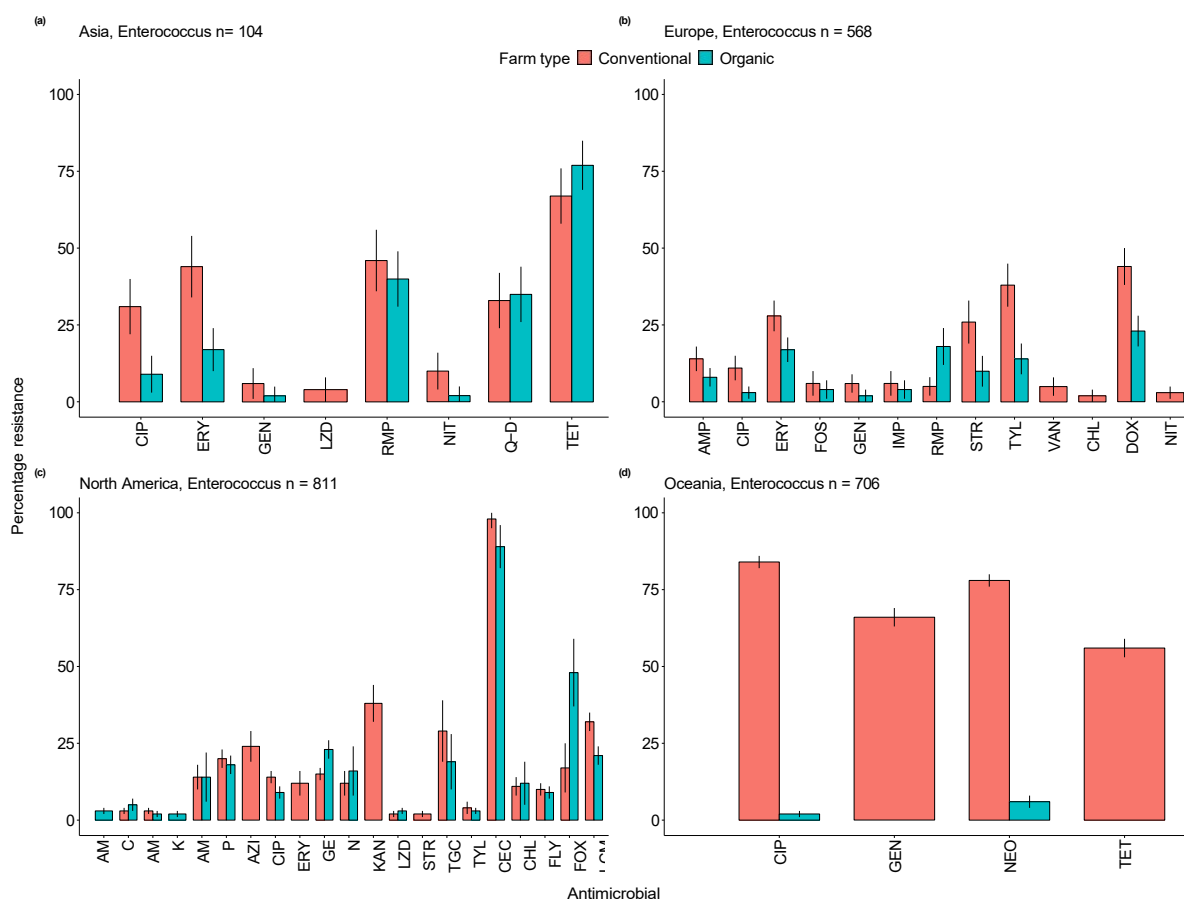


**Figure 5. Patterns of antimicrobial resistance in Salmonella and Campylobacter.** The prevalence of antimicrobial resistance is shown for the number of isolates (n) examined on organic and conventional farms in each geographic region. (a) Asia, salmonella, n = 845, (b) North America, salmonella, n = 5,230 (c) Europe, campylobacter, n = 1,214, (d) North America, campylobacter, n = 14,607. Data represent the mean  $\pm$  95% confidence intervals. The grey shading indicates antimicrobials classified as critically important; the unshaded region indicates highly important antimicrobials. For drug acronyms, see supplementary table 2. The figure present data with more than 10 isolates.

#### 1.4.7. European Union and the United Kingdom

Across Europe, and on both conventional and organic farms, we found high levels of resistance to quinolones, like norfloxacin and ofloxacin. These drugs are considered ‘critically important antimicrobials’ and were restricted in 2009 by the EU. For example, the prevalence of resistance in *Campylobacter* was 91% (CI: 82-100%, n = 43 isolates) on both conventional and organic farms (Fig. 5c). However, ampicillin-resistant *Campylobacter* was slightly higher on organic farms 79% (CI: 67-91%, n = 43 isolates) relative to conventional farms 66% (CI: 56-76%, n = 41 isolates). For *Enterococcus*, 100% (n = 36 isolates) from conventional farms were cefoxitin-resistant (Fig. 7a). In contrast, resistance to erythromycin was relatively low on both conventional farms, 6% (CI: 1-8%, n = 284 isolates, Fig.7a) and organic farms 6% (CI: 1-8%, n = 284 isolates). Intriguingly, we found that the prevalence of rifampicin-resistant *Enterococcus* was higher on organic farms, 19% (CI: 15-22%, n = 134 isolates) as compared to conventional

farms 5%, (CI: 3-7%, n = 134 isolates, Fig 6b).



**Figure 6. Patterns of antimicrobial resistance in *Enterococcus*.** The prevalence of antimicrobial resistance is shown for the number of isolates (n) examined on organic and conventional farms in each geographic region. (a) Asia, *enterococcus*, n = 104, (b) Europe, *enterococcus*, n = 568, (c) North America, *enterococcus*, n = 811, (d) Oceania, *enterococcus*, n = 706. Data represent the mean  $\pm$  95% confidence intervals. The grey shading indicates antimicrobials classified as critically important; the unshaded region indicates highly important antimicrobials. For drug acronyms, see supplementary table 2. The figure present data with more than 10 isolates.

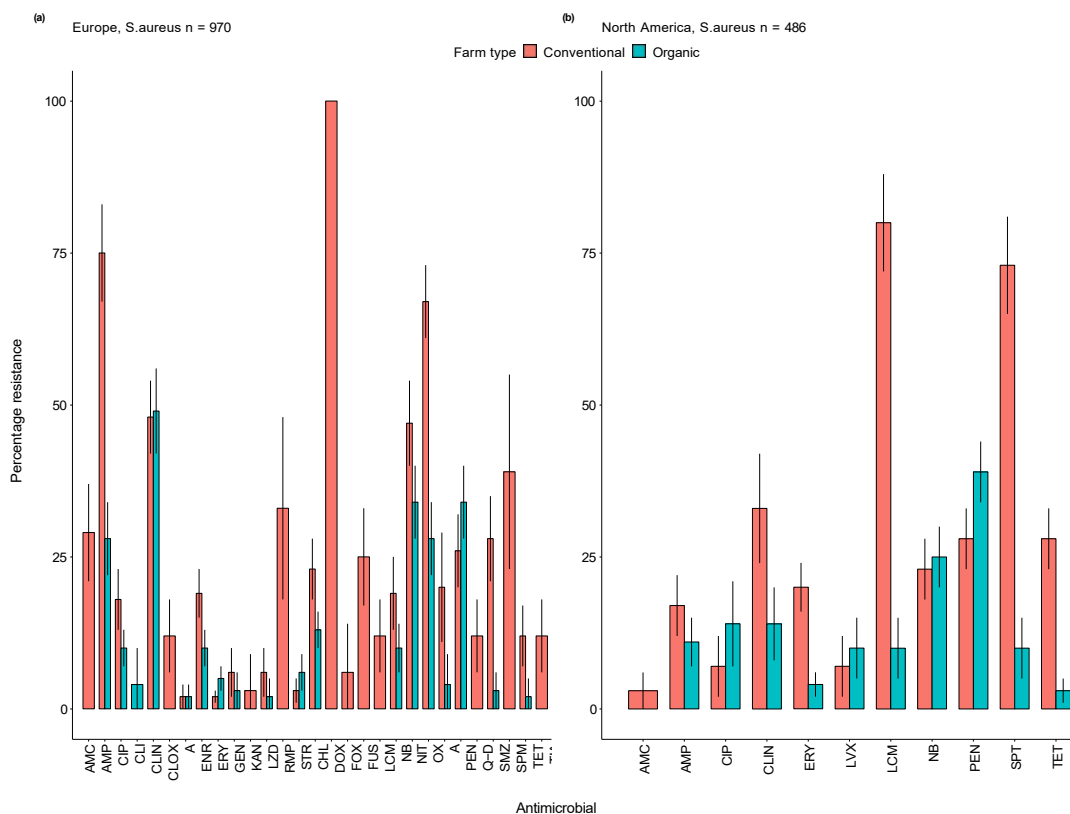
#### 1.4.8. North America

Across North America, *E. coli* showed high levels of resistance to some critically important antimicrobials like erythromycin, and resistant levels were similar on both conventional and organic farms. For example, the prevalence of erythromycin-resistant *E. coli* was 74% (CI: 70-76%, n = 789 isolates, Fig. 4c) on conventional farms and 65% (CI: 62-67%, n = 789 isolates, Fig. 4c) on organic farms. We also found higher resistance to lincomycin and spectinomycin in *Staphylococcus aureus* isolates on both conventional farms 74%, (CI: 72-77%, n = 554 isolates) and organic farms 68%, (CI: 62-74%, n = 418 isolates, Fig.7b). We found similar trends for penicillin resistance in *E. coli* isolates on both organic farms 100%, (n =

2,346 isolates) and conventional farms 96%, (CI: 94-98%, n = 3764 isolates). Conversely, the prevalence of AMR *Salmonella* isolates was relatively low. For example, levels of azithromycin-resistant *Salmonella* were 12% (CI: 8-14%, n = 511 isolates) on organic farms and 10% (CI: 8-12%, n = 511 isolates) on conventional farms. Additionally, levels of gentamicin-resistance were even lower: 3% (CI: 2-4%, n = 118 isolates) on organic farms and 5% (CI: 4-6%, n = 118 isolates) on conventional farms. *Enterococcus* isolates showed high levels of resistance to Lincomycin 96%, CI: 92-97%, n = 42 isolates) on conventional and 75%, (CI: 72-78%, n = 42 isolates, Fig. 6c) on organic farms.

#### 1.4.9. Oceania

Given the extremely low sample sizes, results from Oceania warrant further investigation. We include them here, in part to highlight the paucity of data and the variable levels of resistance. For example, the prevalence of ampicillin-resistant *E. coli* was 4% (CI: 2-6%, n = 375 isolates) on conventional farms, while the level of neomycin resistance was 3% (CI: 2-5%, n = 375 isolates) on organic farms (Fig. 4e). In contrast, *Enterococcus* isolates showed high levels of resistance to ciprofloxacin 84%, (CI: 82-86%, n = 353 isolates), neomycin 74%, (CI: 70-73%, n = 353 isolates), and gentamicin 67%, (CI: 65-69%, n = 353 isolates Fig. 6d) on conventional farms. We found no data for organic farms in this region.



**Figure 7. Patterns of antimicrobial resistance in *S. aureus*.** The AMR prevalence is shown for the number of isolates (n) examined on organic and conventional farms in each geographic region. (s) Europe, *S. aureus*, n = 970, (b) North America, *S. aureus*, n = 486. Data represent the mean  $\pm$  95% confidence intervals. The grey shading indicates antimicrobials classified as critically important; the unshaded region indicates highly important antimicrobials. For drug acronyms, see supplementary table 2. The figure present data with more than 10 isolates.

## 1.5. Discussion

Our findings suggest that overall, antimicrobial resistance (AMR) was *slightly* lower in organic livestock production systems relative to their conventional counterparts, while also revealing significant context-dependent variation in this pattern. Specifically, the prevalence of AMR was 18% on organic farms and 28% on conventional farms. However, the substantial region- and country-specific variations in regulations and policies governing organic farming obfuscate broad-scale and generalizable patterns on how organic farming practices affect AMR. Countries have taken markedly different approaches to the guidelines and regulatory agencies that govern the use of antimicrobials in both conventional and organic livestock production. Further, these regulations are often overlooked or met with strong resistance.

For example, the European Union (EU) banned the use of antimicrobials for growth

promotion (APGs) in livestock production systems in 2006 (Nunan, 2022), and the United States followed suit in 2017 (AccessScience Editors, 2017; Smith, 2019). These policy changes, however, resulted in a ‘repackaging’ of both labeling and marketing practices for these products, characterizing them as ‘prophylactic therapeutics’ instead of ‘growth promoters’ (Armbruster and Roberts, 2018; Smith, 2019). Moreover, the US and countries across Europe reported an *increase* in the use of antimicrobials for prophylactic purposes *after* the ban of APGs (Van Boeckel *et al.*, 2015b; Tiseo *et al.*, 2020). Thus, these well-intentioned policy changes backfired due, in part, to maneuvering to recharacterize the nature of the antimicrobials — a legal but questionable work-around that remains unaddressed.

More stringent regulations on drug use in livestock matter not just for conventional farming but also for organic farming, which seeks to limit but not always eliminate antimicrobial usage in production animals. In the US, use of antimicrobials is prohibited for organic livestock, while the European regulation for organic dairy herds allows a maximum of three treatments with antimicrobials per cow per year (Sato, Bartlett and Saeed, 2005; Sjöström *et al.*, 2020; US Department of Agriculture, 2022). Denmark, the United Kingdom, and Norway adopted their own regulations, imposing more stringent prohibitions on the use of antimicrobials for growth promotion and requiring supervision by a veterinarian for the use of a limited number of antimicrobials (Jensen and Hayes, 2014; Løes *et al.*, 2017). Outside the EU and US, however, conventions governing policies on organic production can be more variable within a country and can become even more challenging to standardize on a national level (Supplementary Table 1). For instance, in Canada, regulations can vary both within and across provinces for products that are distributed and sold solely within those regions (Government of Canada, 2020).

These differences underscore the need for a more comprehensive and global governance framework to review the science underpinning policies and regulations for organic livestock production systems. Such advances are critical to improving investment in research and development to provide viable, non-pharmacological alternatives for disease management in livestock and to move toward global standards, policies, and regulations for organic livestock production systems. These directives are essential to understanding how, when, or where

specific practices of 'organic' livestock production reduce the prevalence of AMR. R&D investment is crucial to identifying practical and scalable alternative solutions for farmers who depend on antimicrobials to prevent outbreaks and maintain herd health.

The high levels of drug resistance found in environmental samples further emphasize the need to regulate antimicrobial use and contamination across livestock production systems. In particular, the similarly high levels of AMR from environmental isolates on organic and conventional farms (Fig. 2c and 2d) warrant further attention. While more detailed studies are needed to address the mechanistic underpinnings of these results, at least three key factors could play an important role. First, transit times between conventional and organic management practices or from previous land use patterns can substantially impact environmental contaminants. The studies included here likely differ in the timing of the transition from conventional to organic farming, especially given country- and region-specific variations in regulations on transition time. For instance, the US National Organic Program standards allow for a three-year transition period from conventional to organic management in livestock systems (Oregon tilth, 2019; USDA, 2019), whereas the UK allows a two-year transition period (Organic Research Centre, 2023). We were unable to find specific regulations on transition timeframes for other countries.

Transition times matter because animal manure can increase drug residues in the environment and allow organisms to select for higher antimicrobial resistance via genes or plasmids — mobile genetic elements that can transfer resistance within and across species (Ruuskanen *et al.*, 2016; Zalewska *et al.*, 2021). Recent advances in molecular and gene sequencing technologies have increased our awareness that plasmids can transfer among bacteria as well as to other species, largely through horizontal gene transfer among microbes in the gut microbiome, leading to rapid transfer of multi-drug resistance in various hosts. Contaminated soil can function as a potential reservoir leading to high AMR prevalence on organic farms (Shterzer and Mizrahi, 2015; Hiltunen, Virta and Laine, 2017; Gurmessa *et al.*, 2021; Vinayamohan, Pellissery and Venkitanarayanan, 2022). Future studies focused on quantitative risk analyses are needed to help identify approaches to mitigate environmental

contamination of antimicrobials on both organic and conventional farms. Examining different transition times or soil management practices appears to be an important first step.

Environmental contamination of antimicrobials also may contribute to the notably high prevalence of AMR on organic poultry farms (Fig. 3). Across some parts of the US, the prevalence of AMR in poultry was slightly higher on organic farms compared to conventional farms. A similar trend also was reported on organic farms in the UK and Portugal, but we are unable to compare the results to conventional farms in these two countries due to lack of data. While organic practices and regulations are, again, highly variable across different regions, access to outdoor grazing may expose chickens to contaminants in the soil, including antimicrobials or drug-resistant microbes or insects, which also can serve as reservoirs for drug resistance (Bailey *et al.*, 2020). Exposure to these antimicrobial contaminants in soils may increase the prevalence of AMR in these insect reservoirs and thus in poultry.

Our results highlight that drug resistance in *Campylobacter* spp. may warrant particular attention to organic poultry systems. For example, 60-80% of global *Campylobacter* cases originate from poultry products, and 400-500 million cases are reported globally every year (Igwaran and Okoh, 2019). Annually in the US, approximately 310,000 cases of *Campylobacter* are potentially untreatable due to resistance to azithromycin and ciprofloxacin, two important anti-*Campylobacter* antibiotics (Yang *et al.*, 2019). For *Campylobacter*, the prevalence of resistance to quinolones was notably high on organic farms in Europe. Yet, quinolones are considered critically important antimicrobials and have been restricted for use in livestock and humans in the EU since 2018 (Bausch and Bonkat, 2022). We also found high levels of resistance to drugs considered critically and highly important to human medicine in Asia, Europe, and North America and across a wide gradient of organic and conventional management practices (Fig. 4b, 5a, 5b, 5c, and 5d). Given the public health concerns related to *Campylobacter*, our results join others in calling for greater regulations in these components of livestock management (Balta *et al.*, 2021; Ocejo *et al.*, 2021).

Another factor that could play a key role in the differences observed in AMR across global regions is the variance in organic policy with respect to the use of livestock manure as a

feed source. The use of manures from various sources on organic crops differs depending on the specific regulatory framework (e.g., US regulations allow use of manures on organic crops from conventional Confined Animal Feeding Operations (CAFOs), as opposed to EU regulations, which limit the use of manures from “industrial” animal operations). Thus, environmental contamination of antimicrobials may be more prevalent on organic land certified under regulatory frameworks that allow regular use of these industrial manures sourced from conventional farms that routinely use antimicrobials. For example, the use of conventional poultry manure is commonplace in organic grain production in the US, including the production of feed for organic poultry operations.

Our study had several limitations. First, the variation in organic livestock management practices and regulations at national and regional levels may limit the generalizability of our results. Second, despite a comprehensive literature search with broad search terms, our study yielded a relatively limited number of studies ( $n = 72$ ) and very few studies from low and middle-income parts of the world (e.g., Africa [ $n = 0$ ], Oceania [ $n = 2$ ], and South America [ $n = 1$ ]). Third, our search criteria included studies written in English and Portuguese. This language limitation may have caused us to miss other studies written in other languages. While we recognize and regret this common limitation, additional language searches are beyond the scope of this current study. In addition, AMR point prevalence surveys use various methodologies for susceptibility testing and thus results are *relative* though not quantitative *per se*. For example, the studies included here that report the prevalence of AMR to spectinomycin and lincomycin used binary metrics (excluding intermediate resistance), which may over- or underestimate the prevalence of resistance.

Given these limitations, results presented here should be interpreted with caution as they capture only a small snapshot of the true state of organic farming practices and global patterns of AMR. Indeed, our review and synthesis serve, in large part, to highlight these discrepancies and the paucity of data required to understand links between AMR use in livestock and broader patterns of AMR. The important hypothesis that organic practices for livestock production reduce the prevalence of antimicrobial resistance is often taken at face

value. Yet, as we show here, data to address this hypothesis are largely lacking (as evidenced by the small sample size produced from our literature search). Moreover, rigorous and large sample sizes are especially needed to test this hypothesis because relationships between antimicrobial use and drug resistance vary dramatically across contexts, differing between hosts, pathogens, and country-specific regulations. The similarities in patterns of AMR prevalence across broad geographic regions with markedly different practices for regulating drug usage suggest that in some cases organic livestock practices have marginally reduced the prevalence of AMR. In other cases (e.g., free-range poultry), however, organic farms suffer from a high prevalence of AMR that warrants further investigation.

The trends presented here are consistent with previous research indicating high multidrug resistance in *E.coli* and *Salmonella* found in livestock (Haque *et al.*, 2022; Racewicz *et al.*, 2022). Given a projected 14% increase in consumer demand for meat products by 2030 according to Food and Agriculture Organization (FAO) projection (Food and Agriculture Organization (FAO) Agricultural Outlook, 2022), AMR in livestock will continue to increase unless substantial management changes are implemented. Traditional interventions like stringent cleaning, antibiotics, and vaccines are critical for managing herd health and treating disease. In isolation, however, these costly and reactive approaches aimed at limiting pathogen proliferation can concomitantly select for more virulent and resistant variants and ultimately ease their spread. The growing threat of antimicrobial resistance and consumer demands to reduce the use of antimicrobials in livestock emphasizes the need to leverage non-pharmacological approaches to prevent and manage disease (Van Boeckel *et al.*, 2017; Björkman *et al.*, 2021; European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA), 2021; Konwar *et al.*, 2022; Murugaiyan *et al.*, 2022).

Our results underscore the need for multidisciplinary global approaches combining organic farming practices and non-pharmacological interventions to reduce routine antibiotics use. In addition, research on interventions like bacteriophages and probiotics and increased surveillance of antimicrobial resistance have shown promising results in reducing AMR

(Jansen, Knirsch and Anderson, 2018; Ikhimiukor *et al.*, 2022). Furthermore, a collaboration among stakeholders (i.e., farmers, researchers, and policymakers in the animal health sector) could help disseminate information and best practices that can help reduce AMR in livestock production. Unfortunately, the industry continues to move in the opposite direction, particularly with the rapidly expanding trend toward growth of corporate-owned livestock farms that control both the dietary and pharmaceutical regimes of the animals (Moyer, 2016). The lack of regulations and transparency in these practices prevent a clear understanding of when and in what quantities antibiotics are provided in feed, for instance.

Future studies could help formulate scalable solutions for conventional farming practices with benefits for both agriculture and public health. Our review indicates that a key focal area includes a better understanding of how transition times and soil properties influence the prevalence, viability, and retention of pathogens — and the genes that harbor AMR (Moyer, 2016; Verma, Jadoun and Bhadauria, 2019). Taken together, these results emphasize the inherent challenges to understanding links between drug use, livestock production practices, and drug resistance in livestock. Greater understanding of how, when, and where antimicrobials can be reduced in livestock production systems (e.g., by adopting *some* organic-based practices) without a concomitant increase in disease outbreaks would greatly enhance efforts to reduce the evolution of drug resistance and extend the shelf life of these powerful biomedical tools. As our synthesis highlights, we are far from reaching such an understanding. We hope that by pointing out these challenges, our study catalyzes future empirical research to address these gaps in knowledge.

### **1.6. Acknowledgements**

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### **1.7. Funding**

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### **1.8. Data availability statement**

All data generated or analyzed from this study are available in Zenodo public repository:  
<https://zenodo.org/record/7600391#.Y9v6TezMK3I> (E. Ager *et al.*, 2023)

### **1.9. Competing Interests**

Authors declare no competing interests.

## 1.10. Supplemental Materials

### a) PubMed search terms:

#### *Livestock:*

( "goats" [MeSH Terms] OR "goat" [TW] OR "goats" [TW] OR "capra" [TW] OR "caprine" [TW] OR "caprines" [TW] OR "cattle" [MeSH Terms] OR "cow" [TW] OR "cows" [TW] OR "cattle" [TW] OR "bovine" [TW] OR "bos" [TW] OR "sheep, domestic" [MeSH terms] OR "sheep" [TW] OR "lamb" [TW] OR "ovis aries" [TW] OR "poultry" [MeSH Terms] OR "poultry" [TW] OR "turkey" [TW] OR "chicken" [TW] OR "duck" [TW] OR "geese" [TW] OR "domestic fowl" [TW] OR "Sus scrofa" [MeSH terms] OR "swine" [TW] OR "sow" [TW] OR "pig" [TW] OR "pigs" [TW] OR "livestock" [MeSH Terms] OR ("livestock" [TW] AND "meat" [TW]) OR ("livestock" [TW] AND ("dairy" [TW] or "milk" [TW])) OR "beef" [TW] OR "meat production" [TW] OR "dairy production" [TW] OR "dairy farm\*" [TW] OR pork [TW] OR ("broiler" [TW] AND ("chick" [TW] OR "flock" [TW])) OR ("layer" [TW] AND ("chick" [TW] OR "flock" [TW])) OR "egg" [TW] OR "eggs" [TW] OR "cheese" [TW] OR "milk" [TW] OR "cheese" [MeSH Terms] OR "Milk" [MeSH Terms] OR "cultured milk products" [MeSH Terms] )

#### *Bacteria:*

( "campylobacter"[MeSH Terms] OR "campylobacter"[TW] OR "campylobacters"[TW] OR "escherichia coli"[MeSH Terms] OR "e coli"[TW] OR "escherichia coli"[TW] OR "salmonella"[MeSH Terms] OR "salmonella"[TW] OR "salmonellas"[TW] OR "salmonellae"[TW] OR "staphylococcus"[MeSH Terms] OR "staphylococcus"[TW] OR "staphylococcu"[TW] OR "methicillin resistant staphylococcus aureus" [TW] OR "MRSA" [TW] OR "staphylococcus aureus" [TW] OR "enterococcus"[MeSH Terms] OR "enterococcus"[TW] OR "enterococcu"[TW])

#### *Antibiotic Resistance:*

( "drug resistance, microbial" [MeSH terms] OR ("antimicrobial" [TW] AND "resistan\*" [TW]) OR ("antibacterial" [TW] AND "resistan\*" [TW]) OR ("antibiotic" [TW] AND "resistan\*" [TW]) OR ("drug" [TW] AND "resistan\*" [TW]) AND (antibiotic [tw] OR antibiotics[tw])) OR

"antimicrobial susceptibility" [TW] OR "antibacterial susceptibility" [TW] OR "antibiotic susceptibility testing" [TW] OR "antimicrobial susceptibility patterns" [TW] OR "antimicrobial stewardship" [MeSH Terms] OR "antimicrobial stewardship" [TW]

OR "microbiological profile" [TW] OR "microbiological profiles" [TW] OR "microbiological profiling" [TW] OR "phylogenetic profile" [TW] OR "phylogenetic profiles" [TW] OR "phylogenetic profiling" [TW] )

Organic/Conventional Farming:

( "agriculture" [MeSH Terms] OR ("organic" [TW] AND "agriculture" [TW]) OR ("organic" [TW] AND "farm\*" [TW]) OR ("conventional" [TW] AND "agriculture" [TW]) OR ("conventional" [TW] AND "farm\*" [TW]) OR (("agriculture" [TW] OR "farm\*") AND ("with antibiotic\*" OR "without antibiotic\*" OR "antimicrobial free")) OR "organic livestock" [TW] OR ("organic" [TW] AND "dair\*" [TW]) OR ("conventional" [TW] AND "dair\*" [TW]) OR ("organic" [TW] AND "flock\*" [TW]) OR ("conventional" [TW] AND

"flock\*" [TW]) OR ("organic" [TW] AND "herd\*" [TW]) OR ("conventional" [TW] AND "herd\*" [TW]) )

**b) Web of Science search terms:**

*Livestock:*

livestock OR cattle OR cow\* OR bovine\* OR beef OR herd OR milk OR cheese OR sheep\* OR lamb\* OR goat\* OR pig\* OR swine OR Sow OR meat OR pork OR chick\* OR flock OR

poultry OR egg\* OR broiler OR turkey\* OR geese

*Bacteria:*

bacteri\* OR "Escherichia coli" OR "E. Coli" OR Salmonella\* OR Staphylococcus OR MRSA OR Campylobacter OR Enterococcus OR "enteric bacteria"

*Antibiotic Resistance:*

(antimicrob\* NEAR/2 resistan\*) OR (antibiotic\* NEAR/2 resistan\*) OR (drug NEAR/2 resistan\*) OR (antibacter\* NEAR/2 resistan\*) OR (antimicrob\* NEAR/2 susceptib\*) OR (antibacter\* NEAR/2 susceptib\*) OR (antibiotic NEAR/2 susceptib\*)

*Organic/conventional farming:*

(conventional NEAR/2 farm\*) OR “conventional agriculture” OR (farm NEAR/2 antibiotic\*) OR (farm NEAR/2 antimicrob\*) OR (conventional NEAR/2 livestock) OR (conventional NEAR/2 dairy) OR (conventional NEAR/2 poultry) OR (conventional NEAR/2 egg\*) OR (organic NEAR/2 farm\*) OR (organic NEAR/2 agriculture) OR (farm NEAR/2 antibiotic\*) OR (organic NEAR/2 livestock) OR (organic NEAR/2 dairy) OR (organic NEAR/2 poultry) OR (organic NEAR/2 egg\*)

**c) Pub Ag search terms:**

((antimicrobial OR antibiotic OR drug) AND (resistance OR resistant OR susceptibility)) AND ((conventional OR organic) AND (agriculture OR farming OR dairy OR livestock OR poultry))

**d) Grey literature search terms:**

(Antimicrobial resistance OR antibiotic resistance OR drug resistance) AND (bacteria species) AND (conventional\* OR organic\*)

We did not add geographic location as a search parameter because geographic location often does not work well as a keyword.

The grey literature searched includes:

- a) National Antibiotic Monitoring Program (USDA)
- b) NARMS Reports/Summaries | NARMS interactive data
- c) FAO Antimicrobial Resistance
- d) WHO GLASS database
- e) ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals
- f) Antimicrobial resistance surveillance in Europe 2022 - 2020 data

**Portuguese search terms**

a) *Web of Science*

("frango\*" OR "granja\*" OR "galinha\*" OR "galo\*" OR "ave" OR "aves" OR "aviário\*" OR "gado" OR "boi\*" OR "vaca\*" OR "bovino\*" OR "gado" OR "rebanho" OR "leite\*" OR "queijo\*" OR "ovelha\*" OR "cordeiro\*" OR "cabra\*" OR "suíno\*" OR "porco" OR "porcos" OR "leitão" OR "leitões" OR "leitoa\*" OR "carne\*" OR "ovo\*" OR "peru\*" OR "ganso\*") AND ("produção" OR "produções" OR "criação" OR "criações" OR "fazenda\*" OR "agricultura\*" OR "bactéria\*" OR "Campylobacter" OR "Escherichia coli" OR "E. coli" OR "Salmonella" OR "Campylobacter" OR "Staphylococcus aureus" OR "Enterococcus" OR "antimicrobial\*" OR "antibiótico\*" OR "antibacteriano\*") AND ("Resistência\*" OR "resistente\*" OR "convencional" OR "convencionais" OR "orgânica" OR "orgânicas")

b) *Pub ag*

((antimicrobiano OR antibiótico OR droga) AND (resistência OR resistente OR suscetibilidade)) AND ((convencional OR orgânico) AND (agricultura OR pecuária OR laticínios OR produção OR aves))

c) *PubMed*

The PubMed search was done using the terms used in the PubMed English search then filtered for Portuguese articles.

Supplementary Table 3. Number of included studies showing author information, publication year, host species sampled, age/production stage, and production system type. A total of 72 unique studies with five studies examining multiple sample types or production stages.

study ID	author	year	host	age	production system
1	Alali, Walid Q., et al.	2,010	Chicken	Slaughter ready	Poultry
2	Bailley et al	2,020	Chicken	Broiler	Poultry
3	Bombyk, R. A. M., et al.	2,008	Cattle	Lactating	Dairy
4	Boron, Lenaart et al	2,016	Chicken	Retail meat	Poultry
5	Boutet Philippe et al.	2,005	Cattle	Lactating	Dairy
6	Bunner, Christine A., et al.	2,007	Pigs	Slaughter ready	Pig
7	Buntenkoetter, Vitus, et al.	2,014	Pigs	Slaughter ready	Pig
8	Cui et al.	2,005	Chicken	Retail meat	Poultry
9	Cui, Lulu, et al.	2,021	Chicken	Embryo	Poultry

study ID	author	year	host	age	production system
10	Economou et al.	2,015	Chicken	Slaughter ready	Poultry
11	Fraqueza, M. J., et al	2,014	Chicken	Slaughter ready	Poultry
12	Gebreyes et al.	2,006	Pigs	Slaughter ready	Pig
13	Gucukoglu, et al	2,020	Chicken	Slaughter ready	Poultry
14	Halbert, Lisa W., et al	2,006	Cattle	Lactating	Dairy
15	Han, Feifei, et al.	2,009	Chicken	Retail meat	Poultry
16	Hansson, Ingrid, et al.	2,021	Chicken	Slaughter ready	Poultry
17	Hayashi and Thakur	2,012	Environment	Resting area	Pig
17	Hayashi and Thakur	2,012	Pigs	Slaughter ready	Pig
18	Helbert et al.	2,006	Cattle	Lactating	Dairy
19	Horsny et al.	2,015	Turkey	Slaughter ready	Poultry
20	Kassem et al.	2,017	Chicken	Layer	Poultry
21	Keelara, Shivaramu, et al.	2,013	Environment	Water	Pig
21	Keelara, Shivaramu, et al.	2,013	Pigs	Slaughter ready	Pig
22	Kempf et al.	2,017	Pigs	Slaughter ready	Pig
23	Kenji et al.	2,005	Cattle	Lactating	Dairy
24	Kilonzo-Nthenge et al	2,015	Chicken	Slaughter ready	Poultry
25	Kim et al.	2,018	Chicken	Slaughter ready	Poultry
26	Kim et al.	2,021	Chicken	Broiler	Poultry
27	Koga, Vanessa L., et al.	2,015	Chicken	Retail meat	Poultry
28	LeJEUNE, JEFFREY T.	2,004	Cattle	Retail meat	Beef
29	Lee, Soo-Kyoung, et al.	2,013	Chicken	Eggshells	Poultry
30	Lestari et al.	2,009	Chicken	Broiler	Poultry
31	Li, Ruichao, et al.	2,013	Chicken	Slaughter ready	Poultry
31	Li, Ruichao, et al.	2,013	Pigs	Slaughter ready	Pig
32	Luangtongkum et al.	2,006	Chicken	Slaughter ready	Poultry
33	Mazurek, Justyna, et al.	2,013	Cattle	Retail meat	Beef
33	Mazurek, Justyna, et al.	2,013	Pigs	Sows	Pig
34	Miranda et al	2,007	Chicken	Retail meat	Poultry
35	Miranda, J. M., et al.	2,009	Cattle	Slaughter ready	Beef
36	Miranda, J. M., et al.	2,008	Chicken	Retail meat	Poultry
37	Miranda, J. M., et al.	2,009	Cattle	Lactating	Dairy

study ID	author	year	host	age	production system
38	Mitchaothai, J., & Srikijkasemwat, K.	2,022	Pigs	Sows	Pig
39	Morley, Paul S., et al	2,011	Cattle	Slaughter ready	Beef
40	Much et al	2,019	Chicken	Slaughter ready	Poultry
41	Mughini-Gras, Lapo, et al.	2,020	Turkey	Slaughter ready	Poultry
42	Musa et al.	2,020	Chicken	Slaughter ready	Poultry
43	Musa, Laura, et al.	2,021	Chicken	Slaughter ready	Poultry
44	Nulsen, M. F., M. B. Mor, and D. E. B. Lawton.	2,008	Pigs	Slaughter ready	Pig
45	Omega et al.	2,022	Environment	Soil	Dairy
46	Osterberg et al.	2,016	Pigs	Slaughter ready	Pig
47	Pesciaroli, Michele, et al	2,020	Chicken	Slaughter ready	Poultry
48	Rama et al.	2,022	Chicken	Slaughter ready	Poultry
49	Ray et al	2,006	Cattle	Calf (less than 6 months)	Dairy
50	Rhodes, Sarah, et al.	2,021	Pigs	Slaughter ready	Pig
51	Roesch, M., et al.	2,006	Cattle	Adult (more than 5 years)	Dairy
52	Rollo et al.	2,010	Pigs	Slaughter ready	Pig
53	SM, Jajere et al	2,020	Chicken	Layer	Poultry
54	Sanchez et al	2,020	Chicken	Broiler	Poultry
55	Sapkota, Amy R., et al.	2,014	Chicken	Broiler	Poultry
56	Sapkota, Amy R., et al.	2,011	Environment	Litter	Poultry
57	Schwaiger et al.	2,008	Chicken	Layer	Poultry
58	Schwaiger et al.	2,010	Chicken	Eggshells	Poultry
59	Siemon et al.	2,007	Chicken	Broiler	Poultry
60	Sjostrom et al.	2,020	Cattle	Young (less than 2 years)	Dairy
60	Sjostrom et al.	2,020	Environment	Manure	Dairy
61	Soonthornchaikul, Nantika, et al	2,006	Chicken	Retail meat	Poultry
62	Suriyasathaporn	2,010	Cattle	Lactating	Dairy
63	Tadesse et al	2,011	Pigs	Finisher	Pig
64	Tamang et al.	2,015	Pigs	Weaner	Pig

study ID	author	year	host	age	production system
65	Tenhagen et al.	2,018	Cattle	Lactating	Dairy
66	Thakur et al.	2,005	Pigs	Slaughter ready	Pig
67	Thibodeau, Alexandre, et al	2,011	Chicken	Slaughter ready	Poultry
68	Tikofsky et al.	2,003	Cattle	Lactating	Dairy
69	Zhang, Jiayi, et al.	2,011	Chicken	Retail meat	Poultry
70	Zwonitzer et al..	2,016	Environment	Manure	Pig
71	Alvarez-Fernandez, Elena, et al.	2,013	Chicken	Retail meat	Poultry
72	Incili eta al.	2,019	Chicken	Slaughter ready	Poultry

Supplementary tables can be found with the publication at doi: 10.1038/s41598-023-

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## Chapter 2

### **Diet–Vaccine Interactions: SQM® Iron and *Salmonella* Vaccination Shape Poultry Gut Microbiota**

A version of this chapter is submitted for publication in *Applied and Environmental Microbiology* (in review)

Diet–Vaccine Interactions: SQM® Iron and *Salmonella* Vaccination Shape Poultry Gut Microbiota

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## 2. Abstract

Vaccines to prevent *Salmonella* are gaining rapid adoption in U.S. poultry production, yet their interactions with other emerging management strategies remain unclear. We investigated how a live-attenuated *Salmonella* vaccine (AviPro® Megan® Vac 1) and micronutrient management via a polysaccharide-complexed iron supplement (SQM® Iron) interact to shape the cecal microbiota of broiler chickens. Ross 708 female broilers were assigned to a 2 × 3 factorial design (vaccination × iron supplementation: control, FeSO<sub>4</sub>, SQM® Iron at 60 ppm) in a 49-day feeding trial, with five replicate pens per treatment and seven birds per pen. Vaccinated birds received AviPro® Megan® Vac 1 on days 0 and 14. On day 49, cecal contents were collected from one bird per pen (n = 5 per treatment) for 16S rRNA gene sequencing (V4 region). Data were analyzed using QIIME2 and R (P ≤ 0.05). While alpha diversity was unaffected, treatments significantly altered microbial composition. SQM® Iron enriched fermentative taxa such as *Pygmaibacter* and *Odoribacter*, while reducing *Streptococcus*. Vaccination alone promoted short-chain fatty acid (SCFA)-producing members of the *Bacillota* phylum. However, when vaccination was combined with SQM® Iron, several beneficial taxa were suppressed and *Staphylococcus* was markedly increased, suggesting that co-management strategies can produce unexpected and potentially counterproductive outcomes. These findings illuminate the potential of integrated strategies, combining immune stimulation with precision micronutrient supplementation, to improve poultry health and food safety. However, these results also underscore the intricate microbial trade-offs that must be carefully navigated to avoid unintended consequences in modern production systems.

## 1.1. Introduction

Non-typhoidal *Salmonella* (NTS) continues to impose significant public health and economic burdens in the United States, causing an estimated 1.35 million infections, 26,000 hospitalizations, and 400 deaths annually, with poultry products accounting for over 23% of these cases (CDC 2025; Balasubramanian et al. 2019; Kim et al. 2024). Despite longstanding investments in sanitation, vaccination, and antimicrobial interventions, these strategies have not sustainably reduced *Salmonella* prevalence in poultry or infection rates in humans (O'Bryan et al. 2022). As global demand for affordable poultry products increases, especially in major export markets like the U.S., the need for integrative, non-pharmacological control strategies is increasingly urgent (USDA 2021).

Nutritional management represents a promising complementary approach to vaccination for improving both poultry health and food safety outcomes. Among nutritional factors, iron metabolism is particularly compelling given its essential role in host biological processes, such as respiration, gene regulation, and DNA biosynthesis, while simultaneously being critical for pathogen virulence and colonization (Cunrath & Palmer 2021; Hennigar & McClung 2016). However, iron supplementation in poultry is complex: excess unabsorbed iron can promote gut inflammation and dysbiosis, while insufficient iron impairs performance (Garrett et al. 2019; Wellawa et al. 2022).

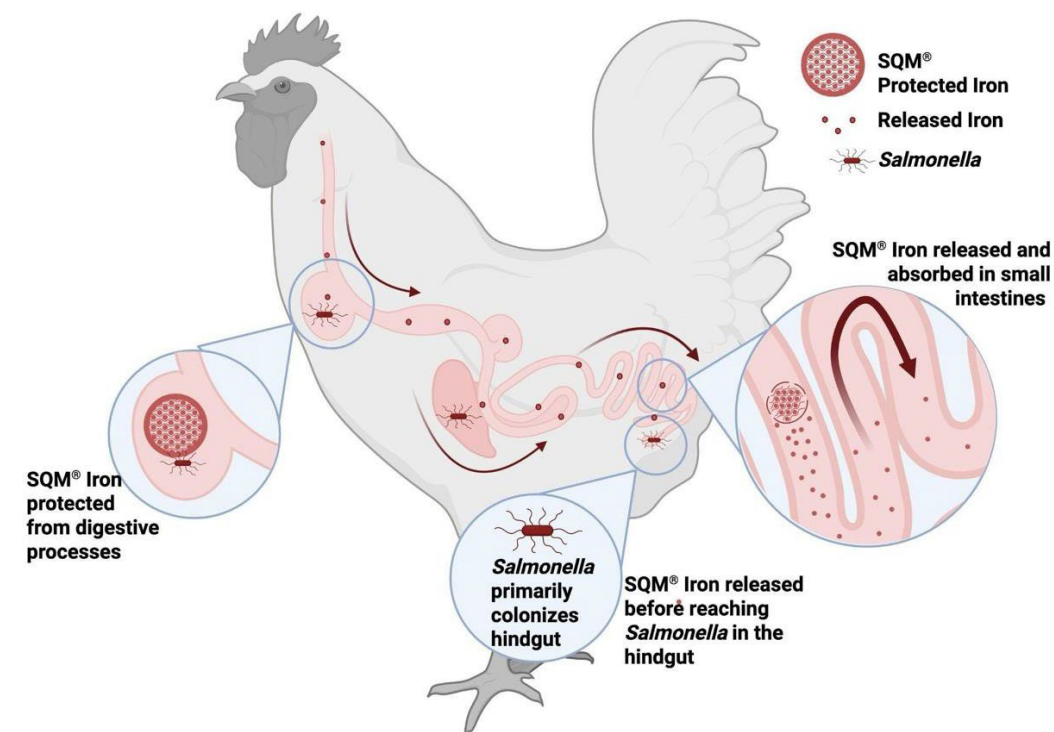
To overcome these challenges, time-release encapsulation technologies, such as SQM® Iron, offer a targeted solution by balancing host iron requirements with pathogen control. SQM® Iron is an FDA-approved polysaccharide-iron complex that uses PolyTransport® technology to chelate iron ions with polysaccharide ligands. Because more than one biopolymer chain in SQM® Iron can bind to a single iron atom, this compound is classified as a complex under FDA rules. This technological innovation ensures controlled, site-specific release within the gastrointestinal tract (Jing et al. 2022). This time-release mechanism stabilizes iron during digestion, preventing premature interactions in the upper gut and improving bioavailability in the small intestine, the primary site of iron absorption in monogastric animals like poultry.

By maximizing absorption where it is most needed and minimizing unabsorbed iron

reaching the ceca, a key site for *Salmonella* colonization, SQM® Iron addresses both nutritional and food safety goals (Figure 1). This targeted delivery approach offers a practical pathway to harmonize production performance with reduced pathogen risk in modern poultry systems. By improving nutrient utilization while minimizing iron availability to pathogens, it holds particular promise for simultaneously optimizing bird health and limiting *Salmonella* proliferation.

However, despite their promise, these strategies have yet to be widely adopted, partly because their interactions with other management practices, especially vaccination and microbiome dynamics, remain poorly understood. *Salmonella* vaccines are routinely used in poultry production, but their interplay with iron metabolism and gut microbiota, both crucial to preventing pathogen colonization, has not been fully explored (Beck et al. 2025; Borey et al. 2022). Here, we focus on interactions with a live-attenuated *Salmonella* vaccine, AviPro® Megan® Vac 1. This product is administered to enhance the immune response against and reduce colonization of *Salmonella* serovars Typhimurium, Enteritidis, and Heidelberg within the chicken digestive tract and internal organs (Lyimu et al. 2023). Additionally, this vaccine serves as an immunological aid to reduce the colonization of *Salmonella typhimurium* and *Salmonella enteritidis* in eggs. Unlike inactivated vaccines, live-attenuated vaccines could *potentially* have a direct effect on the microbiome, especially in response to dietary treatments (Beck et al. 2025; Liu et al. 2024). Bridging this knowledge gap is critical for designing integrated approaches to promote both food safety and flock health.

This study, therefore, takes a first step toward evaluating these dynamics by examining the combined effects of vaccination and SQM® Iron supplementation on the cecal microbiome in female broiler chickens, providing foundational insight into how integrated nutritional management strategies could reshape microbial communities to enhance food safety and animal health. By evaluating the individual and combined effects of vaccination and nutritional iron modulation, our goal is to identify synergistic or antagonistic effects relevant to integrated strategies to control *Salmonella*.



**Figure 1. Overview of SQM® Iron.** Time-release encapsulation technologies improve the bioavailability of iron supplements by ensuring that they remain in a non-absorbable form until they reach the physiological site of maximum absorption. Complexation of organic trace minerals (such as iron) to organic ligands (such as polysaccharide biopolymers) improves bioavailability to the animal. How? By stabilizing the charge state of the mineral, thereby reducing the likelihood of the mineral engaging in undesirable reactions that would render it insoluble and unavailable to the host. Phytic acid presents a classic example: a storage form of phosphorus in plants, it is notorious for its ability to form insoluble complexes with a wide variety of nutrients, including trace minerals, macro minerals, and amino acids. Minerals already complexed to soluble/degradable ligands do not interact with phytic acid in the stomach where phytic acid precipitation reactions are most likely to occur and can later be released from the ligand at the site of absorption by endogenous and exogenous carbohydrases, which are abundant in the small intestine. This protection of trace minerals increases the proportion of ingested iron that is absorbed by the host, not the pathogen. Created in BioRender. Dittoe, D. (2025) <https://BioRender.com/t5n4y0x>.

## 1.2. Materials and methods

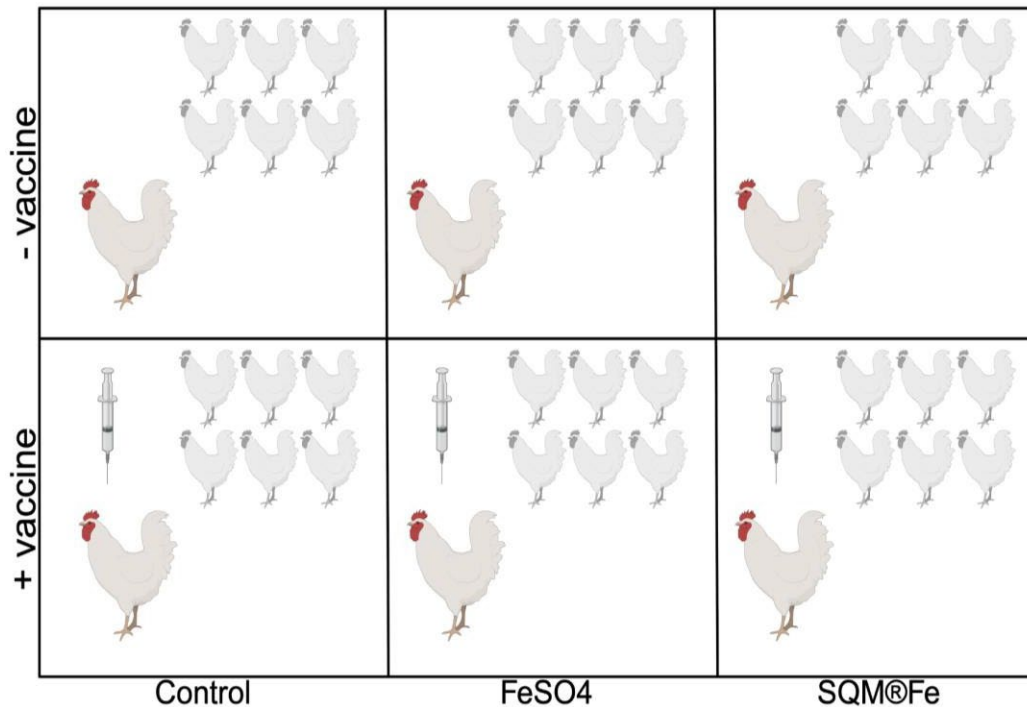
### 1.2.1. Experimental design

To investigate the interaction between iron supplementation and vaccination on the cecal microbiome of chickens, we conducted a 49-day feeding trial using day-old Ross 708 female broilers (N = 210) in a 2 × 3 factorial design (Figure 2). Treatments included two vaccination groups (vaccinated or non-vaccinated with AviPro® Megan® Vac 1) and three iron sources (control without supplemental iron, FeSO<sub>4</sub>, or SQM® Iron from QualiTech, Inc.), with all iron sources included at 60 ppm. Vaccinated birds received AviPro® Megan® Vac 1 via spray application (1.0 mL per 100 chicks) on day 0, with a booster administered via drinking water on

day 14, per manufacturer guidelines. To prevent cross-contamination, vaccinated and non-vaccinated birds were housed in separate sections of the facility, maintaining a minimum distance of 20 feet.

Upon arrival at the Virginia Diversified Research facility (VDRC), Harrisonburg, VA, USA, birds were individually weighed and randomly assigned to treatment groups using a randomized complete block design, with five replicate pens per treatment and seven birds per pen. Birds were housed in 1.5 × 1.5 m floor pens bedded with fresh wood shavings (0.074 m<sup>2</sup> per bird) under standard lighting conditions, with ad libitum access to water and mash-form feed provided through a three-phase feeding program: starter (day 0–14), grower (day 14–28), and finisher (day 28–49). The feed was formulated by Hooze Consulting Service, LLC, (Eagle Mountain, UT) according to industry standards and guidelines established by the National Research Council to meet or exceed nutrient requirements for chickens (Dale 1994). Each dietary phase was formulated from a common basal diet with iron source adjustments according to treatment (Supplementary Table S1). At day 49, one bird per pen was selected for cecal sampling to assess the effects of vaccination and iron supplementation on gut microbial composition through metagenomic analysis.

The animal care and use procedures adhered to the Guide for the Care and Use of Agricultural Animals in Research and Teaching and were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC protocol #B00000944-AM008) (FASS 2010). All experimental procedures, including vaccination, dietary supplementation, and sample collection were designed to minimize animal discomfort and distress and followed established standards for poultry care and husbandry. Birds were monitored daily for general health and welfare, with veterinary oversight throughout. Housing conditions, feeding regimens, and handling protocols complied with institutional animal care policies and federal regulations to ensure the humane treatment of animals throughout the study.



**Figure 2.** Overview of experimental design. Ross 708 female broilers were allocated across six treatment groups in a fully crossed  $2 \times 3$  factorial design (vaccination status: vaccinated vs. unvaccinated; supplementation: control,  $\text{FeSO}_4$ , SQM® Iron). Birds were housed in five replicate pens per treatment group with each pen containing seven birds (35 birds per treatment group, 210 birds total). Birds received feed and water *ad libitum* throughout the study. Cecal samples were collected on day 49. Due to logistical constraints, we sampled one bird per pen (hence each treatment had 5 replicates per treatment,  $n = 5$  birds per each treatment).

### 1.2.2. DNA extraction and quality assessment

Total genomic DNA was extracted from cecal content samples to enable downstream 16S rRNA gene amplification and sequencing. The DNA from cecal samples (0.5 g) was isolated and purified using the QIAamp PowerSoil Pro kit (Qiagen catalog #47016) following the manufacturer's protocol exactly, with final elution performed in 100  $\mu\text{L}$  of the elution buffer. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (model ND-1000), with acceptable samples exhibiting 260/280 ratios between 1.8 and 2.0 and 260/230 ratios above 1.5. Following quality assessment, all DNA samples were normalized to 10  $\text{ng}/\mu\text{L}$  using the kit elution buffer to ensure consistent template concentrations for subsequent PCR amplification.

### 1.2.3. 16S rRNA amplicon sequencing

High-throughput sequencing of the 16S rRNA gene was performed to characterize the

cecal microbiome composition. The V4 hypervariable region was amplified using dual-indexed primers following the protocol described by (Kozich et al. 2013). PCR reactions were performed in 25  $\mu$ L volumes containing 19  $\mu$ L AccuPrime Pfx SuperMix (Invitrogen catalog #12344040), 1  $\mu$ L each of 10  $\mu$ M forward and reverse primers, 2  $\mu$ L template DNA (10 ng/ $\mu$ L), and 2  $\mu$ L DMSO. Thermocycling conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, with final extension at 72°C for 5 min. PCR products were verified by 1.5% agarose gel electrophoresis, then normalized using SequelPrep Normalization Plates (Invitrogen A1051001) with 20  $\mu$ L elution buffer. Normalized amplicons were pooled and quantified by qPCR using the KAPA Library Quantification Kit (Roche KAPA code: KK4824) and Qubit 1x dsDNA HS Assay Kit (Illumina). The final library was diluted to 6 pM containing 10% PhiX control (Illumina FC-110-3001) and sequenced on an Illumina MiSeq platform using V2 chemistry (Illumina MS-102-2003) to generate paired-end 250 bp reads.

#### *1.2.4. Sequencing output, preprocessing, and taxonomic assignment*

Quality filtering and denoising of demultiplexed sequencing data yielded a total of 617,853 paired-end reads with a median of 13,846 (IQR: 9,137–26,942) per sample. The FASTQ files were processed using the DADA2 plugin within QIIME2 (v2024.2.0) via the denoise-paired command, which performs quality filtering, denoising, merging of paired-end reads, and chimera removal in a single step (Callahan et al. 2016). Specific parameters used for this step included trimming the first 8 bases from forward reads and 29 bases from reverse reads and truncating forward and reverse reads at 232 and 156 bases, respectively. Representative sequences were taxonomically classified using QIIME2's feature-classifier classify-sklearn command with a pretrained naive Bayes classifier trained against the Silva 138 99% clustered reference sequence database, using a confidence threshold of 0.7 (Quast et al. 2013; Yilmaz et al. 2014; Bokulich et al. 2018). This initial dataset underwent further processing to ensure data quality, including the removal of reads classified as mitochondrial or chloroplast sequences and exclusion of low-abundance amplicon sequence variants (ASVs) that appeared in fewer than 0.001% of samples (Silva et al. 2022; Quast et al. 2013; Yilmaz et al. 2014;

Bokulich et al. 2018).

After these quality control steps, 90,086 high-quality reads remained, classified into 893 unique ASVs across 30 samples. To standardize sequencing depth for diversity comparisons, all samples were rarefied to 2,906 sequences per sample, which retained the maximum number of samples while excluding two low-count technical replicates. Rarefaction curves plateaued at this sequencing depth (Supplementary Figure S1), confirming sufficient coverage for detecting bacterial diversity across treatment groups. The most abundant sequence from each ASV was used as the representative sequence for further taxonomic classification and analysis. On average, samples contained 195 ASVs post-rarefaction.

All downstream statistical analyses and visualizations were performed in R using phyloseq, ggplot2 and vegan packages (Mac version 4.4.2) (R Core Team 2021; McMurdie & Holmes 2013; Oksanen et al. 2019). Sample metadata underwent additional filtering via a custom R script (utils/metadata\_utils.R), which excluded four samples (CN223 as a suspected outlier and CN414, CN415, CN416 as technical replicates) prior to final analyses.

#### 1.2.5. *Alpha diversity*

To assess within-sample microbial diversity, we calculated two metrics: the number of observed ASVs (richness) and Shannon diversity using QIIME 2 (v2024.5) (Bolyen et al. 2019). To evaluate the individual and interactive effects of vaccination and iron supplementation on Shannon diversity (Shannon 1948; Kruskal & Wallis 1952; Pielou 1966), we applied the Scheirer–Ray–Hare test, an extension of Kruskal-Wallis for a two-dimensional non-parametric analogue of the two-way ANOVA (Scheirer et al. 1976). This approach was selected due to the non-normal distribution of Shannon values, making parametric alternatives inappropriate.

#### 1.2.6. *Beta diversity*

We quantified between-sample diversity using Bray–Curtis dissimilarity (Bray & Curtis 1957), calculated in QIIME 2 (v2024.5) (Bolyen et al. 2019). We applied non-metric multidimensional scaling (NMDS) based on both Bray–Curtis dissimilarity (relative abundance) and Jaccard  $\beta$ -diversity (presence/absence) to visualize differences in bacterial community composition across samples. NMDS was performed using the metaMDS() function in the *vegan*

package (version 2.8-0) (Real & Vargas 1996; Kruskal & Wallis 1952; Oksanen et al. 2019), providing an ordination of community composition without assumptions about underlying taxonomic relationships. Dimensionality optimization was conducted by comparing stress values across 1–6 dimensions, selecting a 4D NMDS solution (stress = 0.095) for its balance between interpretability and model fit (Supplementary Fig S2). For Bray–Curtis, stress decreased from 0.344 (1D) to 0.095 (4D), and for Jaccard, from 0.283 (1D) to 0.081 (4D), representing reductions of 72.4% and 71.4%, respectively. A four-dimensional solution was selected for both metrics based on stress values below 0.10, substantial improvement over lower-dimensional models, and consistent algorithm convergence.

To examine the effects of vaccination, iron supplementation, and their interaction on beta diversity, we applied permutational multivariate analysis of variance (PERMANOVA) on both Bray–Curtis and Jaccard  $\beta$ -diversity matrices using the `adonis2` function from the *vegan* package with 999 permutations (Oksanen et al. 2019). PERMANOVA models estimated Pseudo- $F$  values and  $p$ -values for each main effect and interaction term (Lozupone et al. 2011; Lozupone et al. 2007; Hamady et al. 2010; McDonald et al. 2018). Pairwise differences between treatment groups were further evaluated using `pairwise.adonis()` with Bonferroni correction for multiple testing (Crawley 2012). These statistical results complemented visual patterns observed in NMDS ordinations and confidence ellipses, supporting interpretation of treatment effects on microbial community structure.

Finally, to examine specific taxonomic differences in microbial composition across treatment groups, we employed ANCOM-BC2 (Analysis of Compositions of Microbiomes with Bias Correction 2) using the ANCOMBC package (version 2.8.1). This general framework for multigroup differential abundance analysis incorporates covariate adjustments and interaction effects. ANCOM-BC2 also accounts for both sample-specific and taxon-specific biases, the latter of which is particularly important due to variable sequencing efficiencies that can lead to preferential detection of certain taxa. This bias correction improves control of the false discovery rate (FDR) and enhances the interpretability of log-fold change estimates (Lin & Peddada 2024). Here, genus-level taxonomic abundance data were exported from QIIME2 (level-6) and

subjected to quality control filtering. Genera were retained only if they had valid NCBI taxonomic classifications, and taxonomic names were updated using the NCBI Taxonomy Database to reflect current scientific nomenclature. Samples with fewer than 1,000 total sequence counts were excluded to ensure adequate sequencing depth. Genera present in fewer than 10% of samples were removed to focus the analysis on prevalent taxa. After filtering, the final dataset included 84 validated genera across 29 samples.

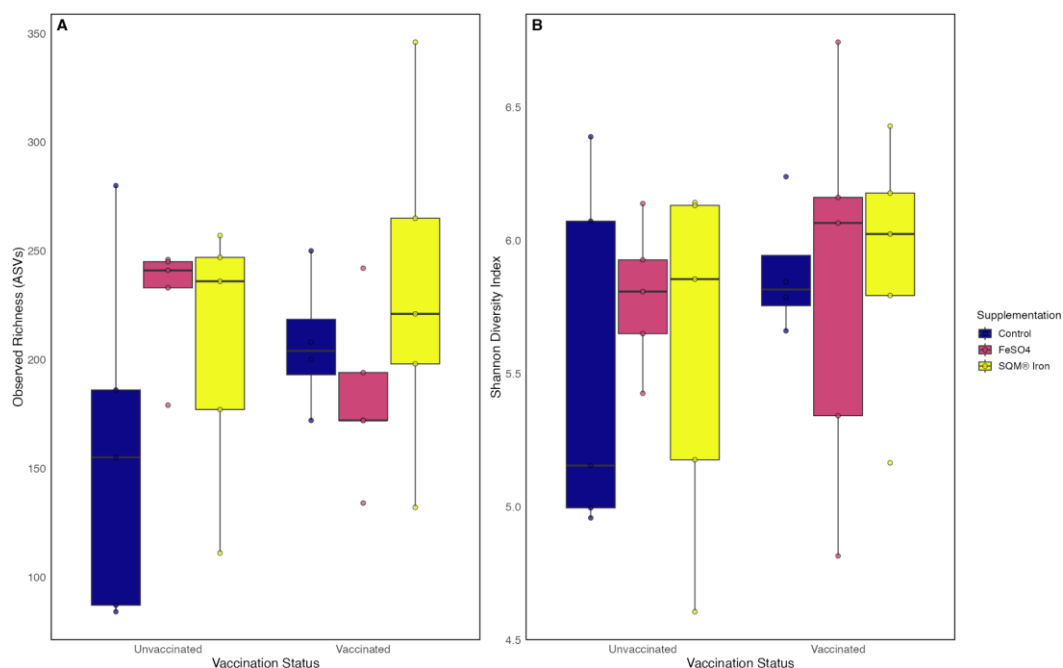
#### *1.2.7. Differential Abundance Analysis*

Differential abundance testing was conducted using ANCOM-BC2 that tested for the main effects of vaccination status (vaccinated vs. unvaccinated), iron supplementation (control, FeSO<sub>4</sub>, SQM® Iron), and their interaction (Lin & Peddada 2024). Statistical significance was determined using the Benjamini-Hochberg procedure to correct for multiple testing, with a false discovery rate threshold of  $q < 0.05$ . Effect sizes were interpreted based on  $\log_2$  fold change thresholds, with values less than 0.5 considered small, values between 0.5 and 1.0 considered medium, and values  $\geq 1.0$  considered large, consistent with commonly accepted standards for biological relevance. To enhance statistical robustness, the analysis incorporated prevalence filtering ( $\geq 10\%$ ), library size filtering ( $\geq 1,000$  counts), and structural zero detection. In total, 29 samples were analyzed, comprising 84 genera after filtering. The factorial design included three iron treatment groups and two vaccination statuses, resulting in 252 individual coefficient tests (84 genera  $\times$  3 effects).

### **1.3. Results**

#### *1.3.1. Alpha diversity*

Overall, vaccination and iron supplementation influenced cecal microbial communities in distinct ways. Alpha diversity, measured by observed richness and Shannon diversity, did not differ significantly across vaccination or supplementation treatments, nor was there evidence of an interaction effect (Figures 3A, 3B; Tables 1, 2; Scheirer–Ray–Hare test, all  $p > 0.05$ ).



**Figure 3.** Alpha diversity of gut microbiota across vaccination and iron supplementation treatments. (A) Observed richness (amplicon sequence variants, ASVs) and (B) Shannon diversity index in poultry cecal contents. Data represent a  $2 \times 3$  factorial design with vaccination status (vaccinated vs. unvaccinated) and iron supplementation (control, FeSO<sub>4</sub>, SQM® Iron). Neither vaccination nor iron supplementation produced statistically significant main effects on microbial diversity metrics, and the interaction term was non-significant (all  $p > 0.05$ ), indicating that iron supplementation effects on gut microbial diversity are independent of vaccination status. Boxplots show median, interquartile range, and individual data points ( $n = 5$  birds per treatment group, one bird sampled per pen across five replicate pens per treatment).

**Table 1.** Statistical analysis of observed richness (ASVs) across vaccination and iron supplementation treatments. Results from Scheirer-Ray-Hare test (non-parametric two-way ANOVA) examining the effects of vaccination status and iron supplementation on microbial community richness in poultry cecal contents. The analysis included 29 samples across six treatment groups in a  $2 \times 3$  factorial design. Effect = source of variation; df = degrees of freedom; Sum Sq = sum of squares; H = test statistic; p-value = probability value). No significant main effects or interactions were detected (all  $p > 0.05$ ).

Effect	df	Sum Sq	H	p-value
Vaccination	1	88.86	1.226	0.268
Iron supplementation	2	19.51	0.269	0.874
Vaccination × Iron supplementation	2	8.74	0.121	0.942
Residual	23	1908.4	—	—
Total	28	2025.51	—	—

**Table 2.** Statistical analysis of Shannon diversity across vaccination and iron supplementation

treatments. Results from Scheirer-Ray-Hare test (non-parametric two-way ANOVA) examining the effects of vaccination status and iron supplementation on microbial community diversity in poultry cecal contents. The analysis included 29 samples across six treatment groups in a 2 × 3 factorial design. Effect = source of variation; df = degrees of freedom; Sum Sq = sum of squares; H = test statistic; p-value = probability value). No significant main effects or interactions were detected (all  $p > 0.05$ ).

Effect	df	Sum Sq	H	p-value
Vaccination	1	0.21	0.003	0.957
Iron supplementation	2	106.98	1.477	0.478
Vaccination × Iron supplementation	2	245.42	3.388	0.184
Residual	23	1675.60	—	—
Total	28	2028.21	—	—

### 1.3.2. Beta diversity (Bray-Curtis, community composition)

Beta diversity analyses revealed significant differences in microbial community composition across treatments. Both vaccination and iron supplementation significantly affected community structure, with significant interaction effects. For Bray–Curtis  $\beta$ -diversity, both vaccination and iron supplementation produced statistically significant main effects (PERMANOVA; vaccination:  $F = 2.65$ ,  $R^2 = 0.07$ ,  $p = 0.001$ ; iron supplementation  $F = 3.43$ ,  $R^2 = 0.18$ ,  $p = 0.001$ ), with significant interactive effects ( $F = 2.36$ ,  $R^2 = 0.13$ ,  $p = 0.001$ ) (Table 3). Post-hoc pairwise PERMANOVA comparisons with Bonferroni adjustment for multiple comparisons identified a significant shift in microbial community composition between the vaccinated animals receiving standard feed (controls) and those supplemented with SQM® Iron ( $F = 5.67$ ,  $p_{adj} = 0.05$ ), suggesting that SQM® Iron supplementation exerts a measurable impact on the gut microbiota of vaccinated birds. In contrast, no other treatment comparisons reached significance after adjustment for multiple testing, underscoring the specificity of the SQM® Iron effect.

More specifically, NMDS ordination (Figure 4A) provided a clear visualization of treatment-driven community separation. These findings indicate that the impact of iron

supplementation on microbial community structure depended on vaccination status. Post-hoc pairwise PERMANOVA comparisons with Bonferroni adjustment for multiple comparisons did not identify significant pairwise differences in the cecal microbiome across treatments for specific taxa ( $p_{adj} > 0.05$ ).

**Table 3.** Permutational Multivariate Analysis of Variance (PERMANOVA) testing the effects of vaccination and iron supplementation on microbial composition using Bray-Curtis dissimilarity. Significant effects were set at  $p \leq 0.05$  with all main effects and interactions showing significant differences in community composition.

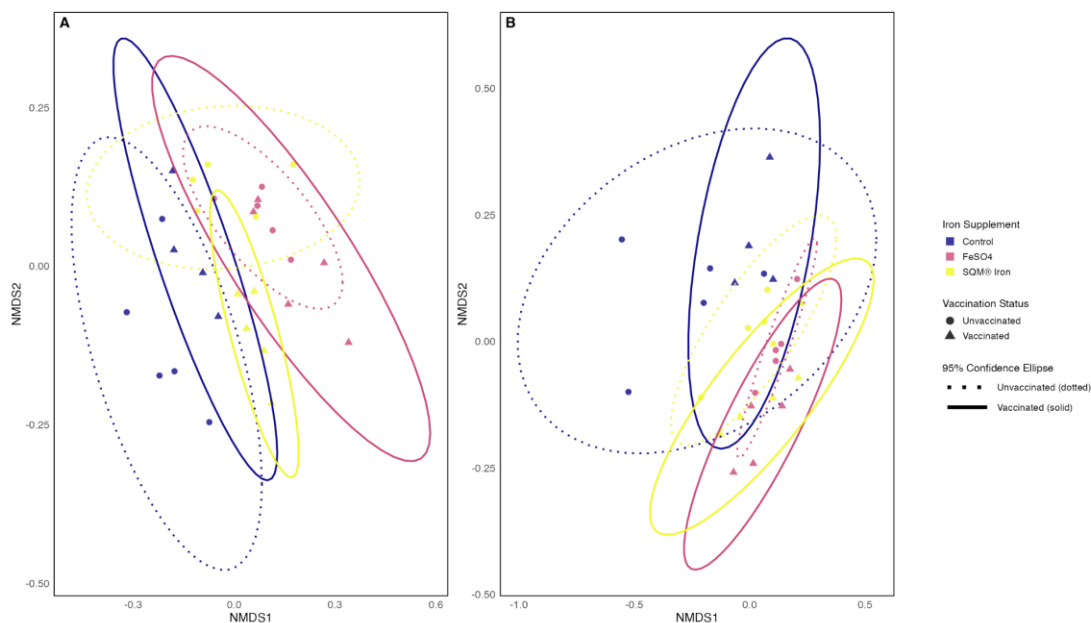
Effect	df	Sum Sq	R <sup>2</sup>	F	p-value
Vaccination <sup>1</sup>	1	0.2328	0.071	2.65	0.001
Iron supplementation <sup>2</sup>	2	0.6032	0.184	3.43	0.001
Vaccination × Iron supplementation	2	0.4143	0.127	2.36	0.001
Residual	23	2.0221	0.618	—	—
Total	28	3.2723	1.000	—	—

<sup>1</sup>Birds were vaccinated with live-attenuated *Salmonella* vaccine (AviPro® Megan® Vac 1).

<sup>2</sup>Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) and ferrous sulfate as the iron sources

### 1.3.3. Jaccard $\beta$ -diversity (Presence/Absence)

Jaccard  $\beta$ -diversity analyses highlighted significant differences in the presence or absence of taxa in the cecal microbiome across treatments. For Jaccard  $\beta$ -diversity, both vaccination and iron supplementation produced statistically significant main effects (PERMANOVA; vaccination:  $F = 1.73$ ,  $R^2 = 0.05$ ,  $p = 0.002$ ; iron supplementation  $F = 2.03$ ,  $R^2 = 0.13$ ,  $p = 0.001$ ), with significant interactive effects ( $F = 1.71$ ,  $R^2 = 0.106$ ,  $p = 0.001$ ) (Table 4). Jaccard  $\beta$ -diversity was calculated based on rarefied ASVs presence/absence data and visualized using NMDS (4D solution, stress = 0.081). Recall, unlike Bray–Curtis analyses (Figure 4A), Jaccard  $\beta$ -diversity metrics reflect differences in the presence or absence of taxa rather than their relative abundance. These findings indicate that the impact of iron supplementation on the presence and absence of different taxa depended on vaccination status (Figure 4B).



**Figure 4. Beta diversity of gut microbiota across vaccination and iron supplementation treatments.** NMDS ordination plots show microbial community dissimilarity patterns across treatment groups, with individual samples represented as points colored by iron supplementation treatment (Control, FeSO<sub>4</sub>, SQM® Iron) and shaped by vaccination status (circles = unvaccinated, triangles = vaccinated). Confidence ellipses (95%) indicate within-group variation for each treatment combination. **(A)** Bray-Curtis dissimilarity based on rarefied ASV abundance data (4D NMDS solution, stress = 0.095). Analysis revealed significant effects of both vaccination and iron supplementation on community structure ( $F = 2.24$ ,  $R^2 = 0.07$ ,  $p = 0.001$  and  $F = 3.19$ ,  $R^2 = 0.199$ ,  $p = 0.001$ , respectively), with a significant interaction ( $F = 2.14$ ,  $R^2 = 0.266$ ,  $p = 0.001$ ) indicating that iron supplementation effects depend on vaccination status. Post-hoc pairwise PERMANOVA with Bonferroni adjustment identified a significant difference between vaccinated controls and SQM® Iron supplemented birds ( $F = 5.67$ ,  $p_{adj} = 0.045$ ). **(B)** Jaccard dissimilarity based on ASV presence/absence patterns (4D NMDS solution, stress = 0.081). Analysis showed significant main effects of vaccination and iron supplementation ( $F = 1.73$ ,  $R^2 = 0.054$ ,  $p = 0.002$  and  $F = 2.03$ ,  $R^2 = 0.126$ ,  $p = 0.001$ , respectively) and a significant interaction ( $F = 1.71$ ,  $R^2 = 0.106$ ,  $p = 0.001$ ). Post-hoc pairwise comparisons showed no significant differences after Bonferroni correction (all  $p_{adj} > 0.05$ ).

**Table 4. Permutational Multivariate Analysis of Variance (PERMANOVA) testing the effects of vaccination and iron supplementation on the presence or absence of specific microbes using Jaccard  $\beta$ -diversity (presence or absence).** Significant effects were set at  $p \leq 0.05$  with all main effects and interactions showing significant differences in community composition.

Effect	df	Sum Sq	R <sup>2</sup>	F	p-value
Vaccination <sup>1</sup>	1	0.3414	0.054	1.73	0.002
Iron supplementation <sup>2</sup>	2	0.7985	0.126	2.03	0.001
Vaccination × Iron supplementation	2	0.6749	0.106	1.71	0.001
Residual	23	4.5336	0.714	—	—
Total	28	6.3485	1.000	—	—

<sup>1</sup>Birds were vaccinated with live-attenuated *Salmonella* vaccine (AviPro® Megan® Vac 1).

<sup>2</sup>Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) and ferrous sulfate as the iron sources

#### 1.3.4. *Differential abundance analysis*

Bacteroidota remained the dominant phylum across all treatment groups, several low-abundance taxa exhibited notable changes in response to vaccination, iron supplementation, and their interaction. Although relatively few of these changes were statistically significant (*q-values* > 0.05), likely due to limited sample sizes, the corresponding effect sizes suggest biologically meaningful shifts in microbial community structure.

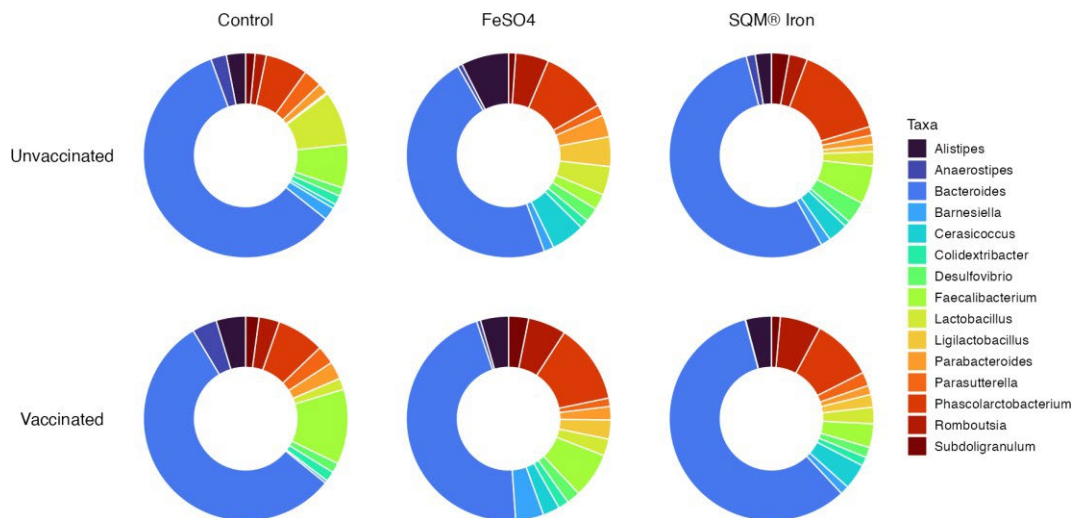
At the phylum level, both vaccination and iron supplementation influenced the relative abundances of several minor microbial lineages (Figure 5). Although none of these changes reached statistical significance (ANCOM-BC2; *q-values* > 0.05), the relatively large effect sizes suggest that these low-abundance (rare) phyla may be responsive to both immunological and nutritional interventions. These changes were largely driven by Cyanobacteriota, Campylobacterota, Thermodesulfobacteriota, and Verrucomicrobiota. Vaccination had notable effects on several microbial taxa, though none reached statistical significance. At the phylum level, vaccination was associated with a large decrease in Verrucomicrobiota (−1.61, *q* > 0.05), with this phylum being approximately 3 times more abundant in unvaccinated birds. Two other phyla showed medium effect sizes: Cyanobacteriota (+0.86) was more abundant in vaccinated birds, while Actinomycetota (+0.53) also showed increased abundance with vaccination, though neither change reached statistical significance (*q-values* > 0.05). (Supplementary Table S2). The interaction between vaccination and SQM® iron drove additional shifts, including increases in Verrucomicrobiota (+1.44) and Thermodesulfobacteriota (value not reported), alongside reductions in Cyanobacteriota (−1.39) and Campylobacterota (−1.00) (Supplementary Table S3).



**Figure 5. Relative abundance of bacterial phyla in the gut microbiota of poultry across vaccination and iron supplementation treatments.** Donut plots display the mean relative abundance of classified bacterial taxa at the phylum level, stratified by vaccination status (rows) and iron supplementation treatment (columns). Data represent classified taxa only, with unclassified phyla excluded and remaining phyla rescaled to 100% within each treatment group. Bacteroidota dominate the microbial community across all treatment groups, comprising the majority of the gut microbiota, while other phyla contribute smaller but variable proportions. Colors represent individual phyla as shown in the legend, with all detected and classified phyla included in the visualization.

At the genus level, vaccination, iron supplementation, and their interaction produced distinct shifts in microbial composition, with iron supplementation, particularly SQM® Iron, exerting the most pronounced effects (Figure 6). These changes were observed across both dominant and rare taxa, with several reaching statistical significance.

Vaccination alone tended toward minor enrichments in several genera within the Bacillota phylum, including *Merdibacter*, *Thomasclavelia*, *Ligilactobacillus*, *Pseudoflavonifractor*, and *Catenibacillus*. Although none of these changes reached statistical significance ( $q$ -values > 0.05), the effect sizes were relatively large, suggesting biologically meaningful shifts (Supplementary Table S4).



**Figure 6. Relative abundance of dominant bacterial genera in the gut microbiota of poultry across vaccination and iron supplementation treatments.** Donut plots display the mean relative abundance of the most prevalent classified bacterial taxa at the genus level, stratified by vaccination status (rows) and iron supplementation treatment (columns). Data represent the top 15 most abundant genera based on overall prevalence, with unclassified genera excluded and displayed genera rescaled to 100% within each treatment group. *Bacteroides* is the most abundant genus across all treatment groups, followed by other key genera including *Faecalibacterium*, *Phascolarctobacterium*, and *Lactobacillus*. Notable treatment-specific variations are observed in the relative abundances of minor genera. Colors represent individual genera as shown in the legend, with only the top 15 most prevalent genera shown.

Iron supplementation alone also produced formulation-specific shifts in microbial composition. These effects were particularly evident among rare taxa, suggesting that iron availability influences not only dominant genera (Figure 6) but also less abundant members of the microbiome with potential functional relevance (Table 5). *Pygmaibacter* and *Odoribacter* were the only genera consistently enriched by both iron formulations. For *Pygmaibacter*, the increase was stronger in SQM® Iron-treated birds (+2.23) compared to FeSO<sub>4</sub> (+1.94), while *Odoribacter* showed a slightly higher response to FeSO<sub>4</sub> (+1.25) relative to SQM® Iron (+1.15). In other words, the model predicts that *Pygmaibacter* was ~4 times more abundant with SQM® Iron relative to controls. Additionally, FeSO<sub>4</sub> supplementation significantly increased *Ligilactobacillus*, *Butyricimonas*, and *Odoribacter*, while significantly decreasing *Bacillus* (Table 5). SQM® Iron significantly elevated *Catenibacillus*, *Oderibacter*, and *Desulfovibrio*, and significantly reduced *Sutterella*, *Streptococcus*, *Anaerofilum*, and *Oscillospira* (Table 6).

**Table 5. Differentially abundant genera between FeSO<sub>4</sub> supplementation and control treatments.** Results from ANCOMB2 analysis identifying genera with significant abundance differences ( $q < 0.05$ ) between iron sulfate (FeSO<sub>4</sub>) supplemented birds and control birds across vaccination groups. Taxon = genus name; lfc = log<sub>2</sub> fold change (positive values indicate higher abundance in FeSO<sub>4</sub> treatment, negative values indicate higher abundance in control); se = standard error of log<sub>2</sub> fold change; p-value = uncorrected probability value; q-value = false discovery rate adjusted p-value; Effect Size = magnitude classification (Small, Medium, Large based on |lfc| thresholds); Direction = interpretation of fold change direction. Analysis controlled for vaccination status and identified 4 genera with significant differential abundance: *Ligilactobacillus*, *Butyricimonas*, and *Odoribacter* were significantly more abundant in FeSO<sub>4</sub>-supplemented birds, while *Bacillus* was significantly more abundant in control birds.

Genus	Log <sub>2</sub> F C	SE	Effect Size	p-value	q- value	Direction
<i>Ligilactobacillus</i>	2.456	0.386	Large	0.000005	0.000	Higher in FeSO <sub>4</sub>
<i>Pygmaibacter</i>	1.936	-	Large	1.000000	1.000	Higher in FeSO <sub>4</sub>
<i>Bacillus</i>	-1.628	0.214	Large	0.000628	0.012	Higher in Control
<i>Butyricimonas</i>	1.334	0.344	Large	0.001338	0.019	Higher in FeSO <sub>4</sub>
<i>Odoribacter</i>	1.251	0.314	Large	0.000621	0.012	Higher in FeSO <sub>4</sub>

The interaction between vaccination and iron supplementation revealed unique microbial shifts not observed under either treatment alone. No genus-level changes from the interaction reached statistical significance ( $q$ -values  $> 0.05$ ), but, again, the effect sizes suggest potentially biologically meaningful shifts in microbial function and composition. Several SCFA-producing genera, including *Anaerostipes* ( $-2.64$ ), *Catenibacillus* ( $-1.75$ ), and *Faecalibacterium* ( $-1.29$ ), were markedly reduced in birds receiving both interventions, suggesting a potential suppressive interaction that may dampen fermentative and anti-inflammatory microbial functions. In contrast, the combined treatment enriched *Barnesiella* ( $+1.38$ ), *Lactobacillus* ( $+1.48$ ), *Staphylococcus* ( $+2.66$ ), and *Streptococcus* ( $+1.20$ ).

Together, these findings suggest that vaccination and iron supplementation may exert synergistic or additive effects on specific gut microbiota members, highlighting their potential for targeted microbial modulation strategies.

**Table 6. Differentially abundant genera between SQM® Iron supplementation and control treatments.** Results from ANCOMB2 analysis identifying genera with significant abundance differences ( $q < 0.05$ ) between SQM® Iron supplemented birds and control birds across vaccination groups. Taxon = genus name; lfc = log<sub>2</sub> fold change (positive values indicate higher abundance in SQM® Iron treatment, negative values indicate higher abundance in control); se = standard error of log<sub>2</sub> fold change; p-value = uncorrected probability value; q-value = false discovery rate adjusted p-value; Effect Size = magnitude classification (Small, Medium, Large based on |lfc| thresholds); Direction = interpretation of fold change

direction. Analysis controlled for vaccination status and identified 7 genera with significant differential abundance: *Catenibacillus*, *Odoribacter*, and *Desulfovibrio* were significantly more abundant in SQM® Iron-supplemented birds, while *Sutterella*, *Streptococcus*, *Anaerofilum*, and *Oscillospira* were significantly more abundant in control birds.

Taxon	Log2FC	SE	Effect Size	p-value	q-value	Direction
<i>Pygmaibacter</i>	2.232	0.092	Large	0.026221	0.147	Higher in SQM® Iron
<i>Catenibacillus</i>	1.203	0.142	Large	0.000062	0.002	Higher in SQM® Iron
<i>Sutterella</i>	-1.178	0.112	Large	0.000002	0.000	Higher in Control
<i>Odoribacter</i>	1.145	0.320	Large	0.001700	0.016	Higher in SQM® Iron
<i>Streptococcus</i>	-1.143	0.293	Large	0.001300	0.014	Higher in Control
<i>Desulfovibrio</i>	0.895	0.263	Medium	0.003000	0.024	Higher in SQM® Iron
<i>Anaerofilum</i>	-0.680	0.105	Medium	0.000350	0.006	Higher in Control
<i>Oscillospira</i>	-0.577	0.092	Medium	0.000760	0.011	Higher in Control

#### 1.4. Discussion

This study examined the effects of vaccination, iron supplementation, and their interaction on the composition of the poultry cecal microbiome, revealing distinct shifts at both the phylum and genus levels. While Bacteroidota remained dominant across all treatment groups, several low-abundance taxa exhibited notable changes in response to the interventions, suggesting biologically meaningful effects despite the absence of statistical significance.

The effects of vaccines were largely driven by Cyanobacteriota, Campylobacterota, Thermodesulfobacteriota, and Verrucomicrobiota. Although none of these changes reached statistical significance ( $q$ -values > 0.05), the effect sizes were relatively large, indicating the potential for biologically meaningful shifts. Verrucomicrobiota, for example, was ~3 times more abundant in unvaccinated birds. Additionally, vaccination alone was associated with notable shifts in several genera belonging to the Bacillota phyla, which dominates the poultry gut and includes many short-chain fatty acid producers and immune-modulating taxa. Notably, *Merdibacter*, *Thomasclavelia*, *Ligilactobacillus*, *Pseudoflavonifractor*, and *Catenibacillus* were all enriched in vaccinated birds, suggesting that these taxa may be part of a core

immunoresponsive microbiome (Clavijo & Flórez 2018; Mbaye et al. 2023; Diaz Carrasco et al. 2019; Goris & Braune 2024). Several of these genera have been previously identified in poultry ceca and feces, and are suspected to contribute to fermentation, nutrient metabolism, and mucosal barrier support. The consistent enrichment of these Bacillota genera in vaccinated birds supports the hypothesis that immune stimulation selectively promotes taxa involved in gut homeostasis and host–microbiota interactions.

Iron supplementation produced more pronounced and statistically significant changes at the genus level. *Odoribacter* showed significant responses to both iron treatments ( $q = 0.012$  for  $\text{FeSO}_4$ ,  $q = 0.016$  for SQM® Iron), with a slightly stronger response to  $\text{FeSO}_4$  (+1.25) compared to SQM® Iron (+1.15). *Pygmaibacter* demonstrated a large but non-significant increase with SQM® Iron treatment (+2.23,  $q = 0.147$ ) compared to a non-significant response to  $\text{FeSO}_4$  (+1.94,  $q = 1.0$ ), resulting in approximately 4.69-fold higher abundance with SQM® Iron supplementation. This pattern suggests that certain genera may be particularly responsive to iron availability, with some showing preferential responses to SQM® Iron.

Other genera showed formulation-specific responses. *Merdibacter*, *Butyricimonas*, and *Ligilactobacillus* were significantly enriched in birds receiving  $\text{FeSO}_4$  supplementation, while *Catenibacillus*, *Cerasicoccus*, *Desulfovibrio* and *Phascolarctobacterium*, and were significantly elevated in SQM® Iron-treated birds. In contrast, *Streptococcus* was significantly reduced in SQM® Iron-treated birds ( $-1.12$ ,  $q = 0.001$ ). Thus, *Streptococcus* was twice as abundant in controls relative to those birds receiving SQM® Iron. These findings suggest a potentially important suppressive effect on taxa associated with opportunistic infections. Together, these findings indicate that iron supplementation promotes fermentative and metabolically active taxa, with SQM® Iron exerting stronger effects on some potentially beneficial genera while also reducing certain taxa of concern.

The combined effects of vaccination and iron supplementation revealed unique microbial shifts that were not observed under either treatment alone. Most notably, several SCFA-producing genera (Cao et al. 2023; Liu et al. 2021; Goris & Braune 2024), including *Anaerostipes* ( $-2.64$ ), *Catenibacillus* ( $-1.75$ ), and *Faecalibacterium* ( $-1.29$ ), were markedly

reduced in birds receiving both vaccination and SQM® Iron. The interaction between vaccination and iron treatment revealed complex but statistically non-significant effects on the microbial community. These interactive effects appeared to suppress some potentially beneficial genera while enriching others, including *Barnesiella* (+1.38 SQM, +2.55 FeSO<sub>4</sub>), *Lactobacillus* (+1.48 SQM, +0.31 FeSO<sub>4</sub>), *Staphylococcus* (+2.66 SQM, +1.35 FeSO<sub>4</sub>), and *Streptococcus* (+1.20 SQM, 0 FeSO<sub>4</sub>). While *Barnesiella* and *Lactobacillus* are generally considered beneficial, the increased abundance of *Staphylococcus* (6.33 times more abundant with SQM, 2.56 times with FeSO<sub>4</sub> relative to vaccinated controls) and *Streptococcus* (2.30 times more abundant with SQM relative to controls) raises concerns, as these genera include species linked to spoilage and opportunistic infections, though the lack of statistical significance limits the strength of these conclusions. These results underscore the complexity of host–microbiota dynamics and highlight the need to evaluate not only the individual effects of nutritional and immunological interventions but also their interactions, which may produce synergistic or antagonistic outcomes with implications for poultry health and productivity.

Our results extend these observations by showing that SQM® Iron supplementation influences microbial communities in a vaccine-dependent manner, suggesting a synergistic potential for combined nutritional and immunological interventions. Key next steps include determining how SQM® Iron directly influences the dynamics of *Salmonella* and whether these effects vary across different live attenuated vaccines. While our work provides a snapshot of vaccine–iron–microbiome interactions, longitudinal trials will be essential to assess the durability of these effects and their dependence on the timing and specific vaccine regimen (Borey et al. 2021; Lyimu et al. 2023; Gloanec et al. 2023). Replication in larger cohorts, including both infected and uninfected birds, will be necessary to confirm these patterns and link them to pathogen load, bird performance, and immune responses. Under commercial conditions, such studies will be critical for translating microbiome shifts into measurable gains in health and food safety, particularly given the complex interactions observed when treatments are combined. The distinct SQM® Iron–associated taxa identified here, along with the SCFA-producing taxa promoted by vaccination, offer promising leads for designing next-generation

strategies that combine vaccination with targeted nutritional or probiotic interventions.

The observation that vaccination and iron supplementation exert independent effects on certain taxa underscores the presence of multiple, distinct pathways through which the gut microbiota can be shaped. SQM® Iron's unique influence demonstrates that not only the inclusion, but also the formulation, of micronutrients plays a decisive role in determining microbial outcomes. Moreover, the synergistic modulation observed when vaccination and SQM® Iron were combined points to the potential power of integrated strategies, a principle called for by the broader poultry community (Celi et al. 2017) as well as policy makers (National Advisory Committee on Microbiological Criteria in Foods (NACMCF) 2024; USDA 2021; T Nair et al. 2018). Collectively, these findings reinforce the concept that coupling immune stimulation with targeted micronutrient supplementation can unlock new opportunities for optimizing gut health. Ultimately, integrating vaccination with precision nutritional strategies such as SQM® Iron supplementation may offer a scalable, non-antibiotic pathway to enhance poultry health, welfare, and performance while also improving food safety and reducing *Salmonella* risk across the production chain.

### **1.5. Acknowledgements**

Steven Ricke provided useful discussions on the role of iron in poultry and non-pharmacological methods for reducing poultry-borne *Salmonella*. We are grateful for the expert team at Virginia Diversified Research and for Dr. Paul Simoni for assistance with computational analyses.

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### **1.7. Institutional Review Board Statement**

The animal study protocol was approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC protocol #B00000944-AM008)

### **1.8. Conflicts of Interests**

We disclose a potential conflict of interest: one of the co-authors (Josh Jedanza) is employed by the company (QualiTech, LLC) that manufactures SQM® Iron, which was used in this study. This affiliation did not influence the study design, data collection, analysis, or interpretation of the results. All other authors declare no competing interests.

### **1.9. Authors' Contributions**

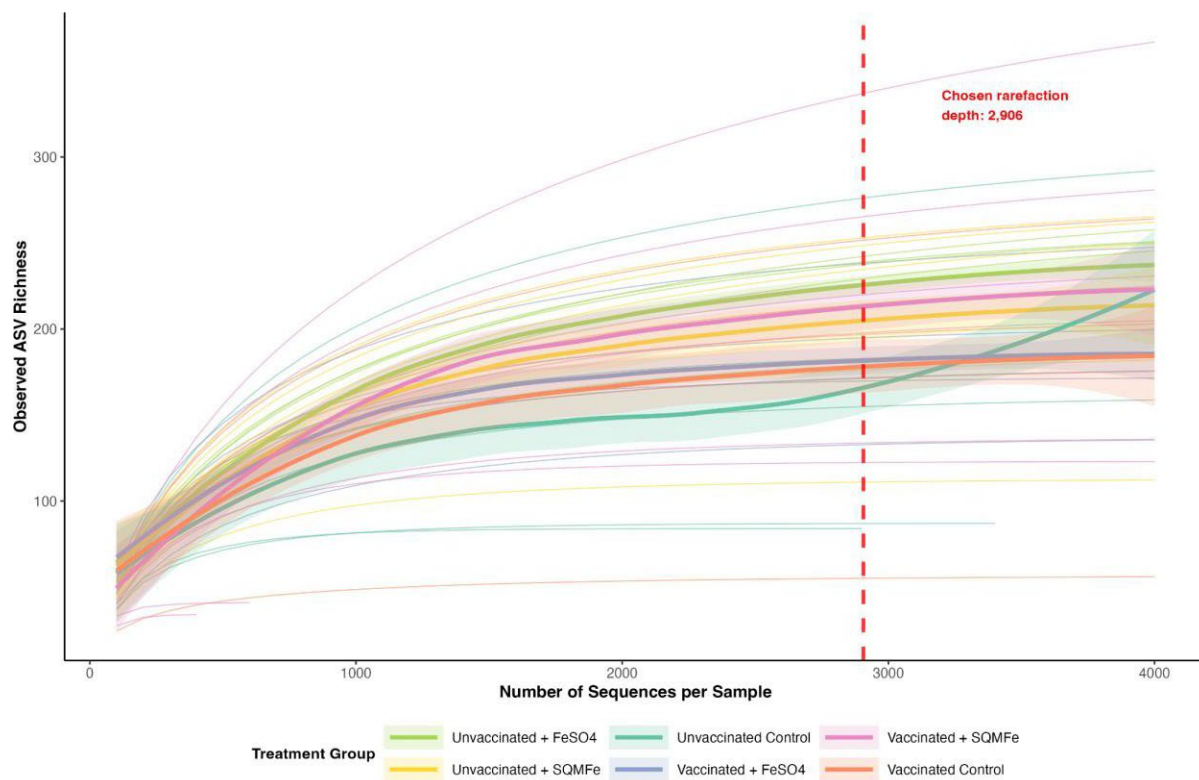
Conceptualization, E.O.A, C.A.N and J.L.H; Funding acquisition, J.L.H; Investigation, J.L.H; Methodology, E.O.A, C.A.N, J.L.H, J.B, E.N, D.K.D and J.L.H; Resources, J.J and J.L.H; Software, J.L.H; Supervision, J.L.H; Visualization, E.O.A, M.S and D.K.D; Writing – original draft, E.O.A and C.A.N; Writing – review & editing, E.O.A, C.A.N, J.B, E.N, D.K.D and J.L.H. All authors have read and agreed to the published version of the manuscript.

### **1.10. Data Availability Statement**

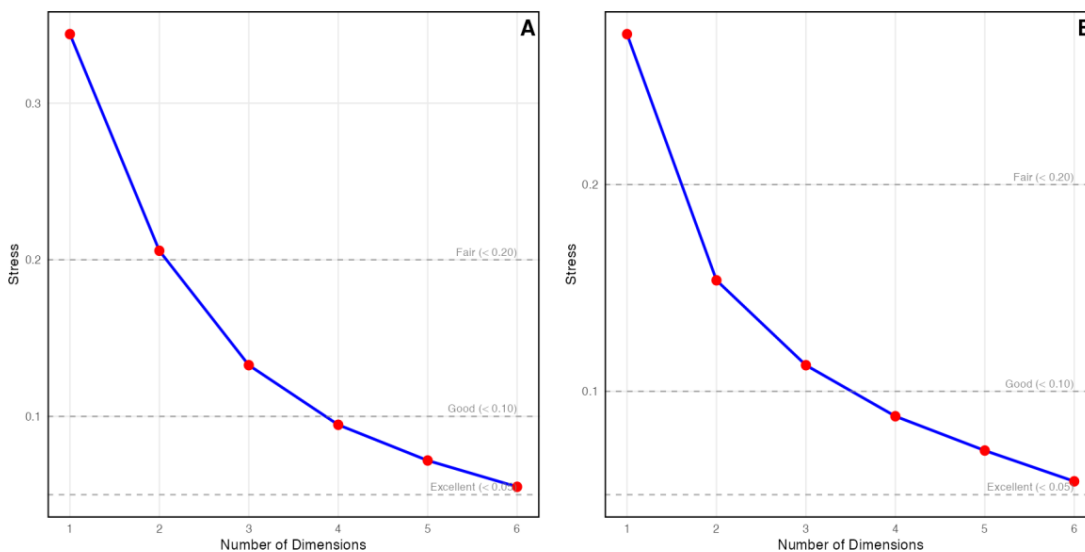
All data and analysis code have been deposited in GitHub [<https://github.com/jhite-eco->

epi/SQM-Iron-Avipro-Megan-Vac-1-Vaccine-Poultry-Microbiome]. The 16S rRNA raw sequence reads generated in this study have been deposited into the NCBI Sequence Read Archive (SRA) database and are publicly available under accession number PRJNA1299482.

### 1.11. Supplemental Figures and Tables



**Supplementary Figure S1.** Rarefaction curves of all samples. Number of observed ASVs plotted against sequencing depth (number of reads). Each line represents one sample ( $n = 30$ ).



**Supplementary Figure S2: NMDS stress analysis for beta diversity dimensionality optimization.**

Stress values plotted against number of dimensions for (A) Bray-Curtis dissimilarity based on ASV abundance data and (B) Jaccard dissimilarity based on ASV presence/absence data. Both analyses used rarefied data (2,906 sequences per sample) from 29 samples across six treatment groups. Horizontal dashed lines indicate stress quality thresholds: excellent (< 0.05), good (< 0.10), and fair (< 0.20). Blue lines show stress reduction with increasing dimensions, with red points marking specific dimensional solutions. Both distance metrics support 4-dimensional NMDS solutions with good stress values (Bray-Curtis: 0.095; Jaccard: 0.088), providing adequate representation of beta diversity patterns for visualization while maintaining interpretability. The 4D solutions were selected for ordination plots based on the balance between model fit (stress < 0.10) and dimensional parsimony.

**Supplementary Table S1. Composition and nutritional values of experimental diets for Ross 708 female broilers supplemented with different iron sources, as-fed basis (g/kg).** The study used a three-phase feeding program spanning 49 days, with nutritionally equivalent diets formulated for starter (0-14 days), grower (14-28 days), and finisher (28-49 days) phases.

	Starter (0-14 d)		Grower (14-28 d)		Finisher (28-49 d)	
Ingredients	SQM® Iron <sup>1</sup>	FeSO <sub>4</sub> <sup>2</sup>	SQM® Iron	FeSO <sub>4</sub>	SQM® Iron	FeSO <sub>4</sub>
Corn	663.7	663.7	671.13	671.13	658.59	658.59
Soybean meal (47.9% CP)	237.26	237.26	183.61	183.61	151.28	151.28
DDGS <sup>3</sup> (27% CP)	40	40	80	80	120	120
Meat & bone meal (46% CP)	40	40	40	40	40	40
Soybean oil	-	-	4.72	4.72	13.41	13.41
Limestone	4.66	4.66	4.4	4.4	4.12	4.12
Dicalcium phosphate	4.59	4.59	2.96	2.96	1.16	1.16
Salt	4.27	4.27	4.09	4.09	3.91	3.91
Choline chloride (60%)	1.03	1.03	0.94	0.94	0.76	0.76
Vitamin premix <sup>4</sup>	0.5	0.5	0.5	0.5	0.5	0.5
SQM® Iron premix <sup>5</sup>	1.26	-	1.26	-	1.26	-
FeSO <sub>4</sub> premix <sup>6</sup>	-	1.26	-	1.26	-	1.26
L-Lysine HCl	0.85	0.85	3.33	3.33	2.95	2.95
DL-Methionine (99%)	1.68	1.68	2.24	2.24	1.57	1.57
L-Threonine (98.5%)	-	-	0.64	0.64	0.31	0.31
Phytase <sup>7</sup>	0.18	0.18	0.18	0.18	0.18	0.18
Total	1000	1000	1000	1000	1000	1000
Analyzed iron content (mg/kg)	143.88	143.88	118.77	118.77	93.06	93.06

Calculated nutrients						
Crude protein (%)	19.5	19.5	18.4	18.4	17.76	17.76
ME poultry (kcal/kg)	3027	3027	3080	3080	3135	3135
Calcium (%)	0.85	0.85	0.8	0.8	0.75	0.75
Available phosphorus (%)	0.42	0.42	0.4	0.4	0.38	0.38
Sodium (%)	0.22	0.22	0.22	0.22	0.22	0.22
SID <sup>a</sup> Lysine (%)	0.93	0.93	1	1	0.9	0.9
SID <sup>a</sup> Methionine (%)	0.47	0.47	0.51	0.51	0.43	0.43
SID <sup>a</sup> Met+Cys (%)	0.73	0.73	0.75	0.75	0.68	0.68
SID <sup>a</sup> Threonine (%)	0.65	0.65	0.65	0.65	0.59	0.59
SID <sup>a</sup> Valine (%)	0.85	0.85	0.78	0.78	0.74	0.74

<sup>1</sup>SQM® Iron = Polysaccharide iron complex (SQM® Iron, QualiTech). <sup>2</sup>FeSO<sub>4</sub> = Ferrous sulfate. <sup>3</sup>DDGS = Dried Distillers Grains with Solubles. <sup>4</sup>The vitamin premix provided the following quantities of vitamins per kilogram of diet: vitamin A, 4,248-4,744 IU; vitamin D<sub>3</sub>, 1,500 IU; vitamin E, 10.7-12.2 IU; vitamin B<sub>12</sub>, 7.8 µg; biotin, 0.04-0.08 mg; menadione, 0.75 mg; thiamine, 0.66-0.78 mg; riboflavin, 3.0-3.3 mg; pantothenic acid, 5.4-5.9 mg; pyridoxine, 1.1-1.4 mg; niacin, 22.2-24.6 mg; folic acid, 0.33-0.36 mg. <sup>5</sup>The SQM® Iron trace mineral premix contained (per kg of diet): zinc (from zinc sulfate), 120 mg; manganese (from manganese sulfate), 120 mg; iron (from SQM® Iron), 60 mg; copper (from copper sulfate), 16 mg; iodine (from calcium iodide), 1.25 mg; selenium, 0.3 mg. <sup>6</sup>The ferrous sulfate trace mineral premix contained (per kg of diet): zinc (from zinc sulfate), 120 mg; manganese (from manganese sulfate), 120 mg; iron (from ferrous sulfate), 60 mg; copper (from copper sulfate), 16 mg; iodine (from calcium iodide), 1.25 mg; selenium, 0.3 mg. <sup>7</sup>Quantum Blue Phytase. <sup>8</sup>SID = Standardized ileal digestible.

### Supplementary Table S2: Phylum-level differential abundance analysis for vaccination effects.

Results from ANCOM-BC2 analysis examining phylum-level abundance differences between vaccinated and unvaccinated groups with significance threshold set at  $q < 0.05$ . Taxon = phylum name; Log2FC = log2 fold change (positive values indicate higher abundance in vaccinated animals, negative values indicate higher abundance in unvaccinated animals); SE = standard error of the log2 fold change estimate; p-value = raw p-value from statistical test; q-value = false discovery rate adjusted p-value; Effect Size = magnitude classification (Small, Medium, Large based on |Log2FC| thresholds); Direction = interpretation of fold change direction relative to vaccination status. Analysis identified eight phyla with varying responses to vaccination, with effect sizes ranging from small to large, but none reaching statistical significance after multiple testing correction. Verrucomicrobiota showed the largest vaccination effect (Log2FC = -1.613, Large effect) with higher abundance in unvaccinated animals, while Cyanobacteriota and Actinomycetota demonstrated medium-sized positive responses to vaccination. The remaining five phyla (Thermoplasmata, Bacteroidota, Thermodesulfobacteriota, Campylobacterota, and Pseudomonadota) showed small effect sizes with mixed directional responses, suggesting subtle phylum-level microbiome shifts associated with vaccination status.

Taxon	Log2FC	SE	p-value	q-value	Effect Size	Direction
Verrucomicrobiota	-1.613	0.692	0.031000	0.248	Large	Higher in Unvaccinated
Cyanobacteriota	0.858	0.635	0.190000	0.758	Medium	Higher in Vaccinated

Actinomycetota	0.531	0.795	0.511000	0.882	Medium	Higher in Vaccinated
Thermoplasmatota	-0.308	0.608	0.621000	0.882	Small	Higher in Unvaccinated
Bacteroidota	-0.302	0.612	0.626000	0.882	Small	Higher in Unvaccinated
Thermodesulfobacteriota	-0.161	0.642	0.804000	0.882	Small	Higher in Unvaccinated
Campylobacterota	0.131	0.873	0.882000	0.882	Small	Higher in Vaccinated
Pseudomonadota	0.108	0.631	0.865000	0.882	Small	Higher in Vaccinated

**Supplementary Table S3: Phylum-level vaccination × SQM® iron interaction effects analysis.**

Results from ANCOM-BC2 analysis examining interaction effects between vaccination status and SQM® iron supplementation at the phylum level with significance threshold set at  $q < 0.05$ . Taxon = phylum name; Log2FC = log2 fold change for the interaction term (positive values indicate vaccination effects are stronger in SQM® iron group, negative values indicate vaccination effects are weaker in SQM® iron group compared to control); SE = standard error of the interaction effect estimate; p-value = raw p-value from statistical test; q-value = false discovery rate adjusted p-value; Effect Size = magnitude classification (Small, Medium, Large based on |Log2FC| thresholds); Direction = interpretation of how vaccination effects differ between SQM® iron and control groups. Analysis identified eight phyla with varying interaction patterns, with effect sizes ranging from small to large, but none reaching statistical significance after multiple testing correction. Verrucomicrobiota showed the largest positive interaction effect (Log2FC = 1.438, Large effect), indicating vaccination effects are substantially stronger in the SQM® iron treatment group. Conversely, Cyanobacteriota and Campylobacterota demonstrated large negative interaction effects (Log2FC = -1.391 and -1.004, respectively).

Taxon	Log2FC	SE	p-value	q-value	Effect Size	Direction
Verrucomicrobiota	1.438	1.194	0.243247	0.809	Large	Vaccination effect stronger in SQM®
Cyanobacteriota	-1.391	0.913	0.141085	0.809	Large	Vaccination effect weaker in SQM®
Campylobacterota	-1.004	1.179	0.404306	0.809	Large	Vaccination effect weaker in SQM®
Thermodesulfobacteriota	-0.893	0.984	0.373398	0.809	Medium	Vaccination effect weaker in SQM®
Actinomycetota	-0.599	1.164	0.611672	0.923	Medium	Vaccination effect weaker in SQM®
Thermoplasmatota	0.391	0.967	0.692591	0.923	Small	Vaccination effect stronger in SQM®
Pseudomonadota	0.197	0.881	0.825247	0.943	Small	Vaccination effect stronger in SQM®
Bacteroidota	-0.065	0.938	0.945241	0.945	Small	Vaccination effect weaker in SQM®

**Supplementary Table S4: Genus-level differential abundance analysis for vaccination effects.**

Results from ANCOM-BC2 analysis examining genus-level abundance differences between vaccinated

and unvaccinated groups with significance threshold set at  $q < 0.05$ . Taxon = genus name; Log2FC = log2 fold change (positive values indicate higher abundance in vaccinated animals, negative values indicate higher abundance in unvaccinated birds); SE = standard error of the log2 fold change estimate; p-value = p-value from statistical test; q-value = false discovery rate adjusted p-value; Effect Size = magnitude classification (Small, Medium, Large based on |Log2FC| thresholds); Direction = interpretation of fold change direction relative to vaccination status. Analysis identified six genera with large effect sizes showing consistently higher abundance in vaccinated animals, though none reached statistical significance after multiple testing correction. *Merdibacter* showed the largest vaccination effect (Log2FC = 1.784), followed by *Thomasclavelia* (Log2FC = 1.659) and *Mailhella* (Log2FC = 1.537).

Taxon	Log2FC	SE	p-value	q-value	Effect Size	Direction
<i>Merdibacter</i>	1.784	0.491	0.036000	0.287	Large	Higher in Vaccinated
<i>Thomasclavelia</i>	1.659	0.589	0.010000	0.240	Large	Higher in Vaccinated
<i>Mailhella</i>	1.537	0.523	0.009000	0.240	Large	Higher in Vaccinated
<i>Pseudoflavonifractor</i>	1.506	0.533		1.000	Large	Higher in Vaccinated
<i>Catenibacillus</i>	1.472	0.491	0.020000	0.240	Large	Higher in Vaccinated
<i>Ligilactobacillus</i>	1.027	0.384	0.015000	0.240	Large	Higher in Vaccinated

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## General Discussion

This thesis examined alternative non-antibiotic alternative strategies for controlling antimicrobial resistance in poultry at two complementary scales: farm-level management practices and host microbiome interventions. By combining data synthesis of global AMR patterns with experimental microbiome analysis, this work reveals why resistance persists despite targeted interventions and highlights the ecological complexity that must be addressed for effective management in agricultural systems.

In Chapter 1, we compared AMR prevalence between organic and conventional farms to evaluate to what extent does organic farming reduce AMR as compared to conventional counterparts. Previous research has suggested that organic farming should substantially reduce resistance prevalence because of elimination of antibiotic usage in most organic farms (Lund and Algers, 2003; Smith-Spangler *et al.*, 2012; Van Boeckel *et al.*, 2015), the extent of this reduction remained unclear. AMR prevalence was lower in organic farms (18%) compared to conventional farms (28%), demonstrating benefits from antibiotic restriction. However, the persistent 18% prevalence in organic farms revealed that direct antibiotic use is not the sole driver of resistance development and transmission. Regional variation in our data supported this interpretation, despite organic certification, some organic operations exhibited AMR prevalence rates comparable to or exceeding those of conventional farms in the same regions. This finding suggests that resistance can spread through environmental pathways that operate independently of on-farm antibiotic management practices. For example, wildlife, migratory birds, and aerosol vectors are known to transport resistant bacteria between nearby farms (Gwenzi *et al.*, 2021; Rodó *et al.*, 2024), while resistance genes persist in soil for 5-10 years following antibiotic withdrawal (Rilling *et al.*, 2025; Wang *et al.*, 2025), a period that far exceeds organic certification's two to three-year transition standards depending on the jurisdiction (Seufert, Ramankutty and Mayerhofer, 2017). Farms transitioning to organic production may therefore inherit resistance reservoirs from prior management practices that cannot be eliminated through antibiotic withdrawal alone. This work highlight that resistance management

requires coordinated regional strategies that address environmental transmission pathways.

In Chapter 2, we examined whether SQMFe® iron supplementation combined with *Salmonella* vaccination could enhance pathogen resistance through microbiome modulation. Iron supplementation alone enriched beneficial genera including *Pygmaibacter* and *Odoribacter*, which produce butyrate. It also reduced potentially pathogenic *Streptococcus*. Vaccination stimulated *Lactobacillus* taxa. The combination of SQMFe® iron supplementation and vaccination, however, produced both synergistic and antagonistic effects. This likely occurred because vaccine-induced iron sequestration created conditions that favored harmful bacterial taxa (Ramírez et al., 2022). These indirect effects extended to microbial populations that conventional studies typically ignore. Methanogenic archaea comprising less than 1% of the chicken cecal microbial community yet consuming substantial amounts of hydrogen that would otherwise inhibit fermentation (St-Pierre and Wright, 2013). Their reduction under iron supplementation suggested that nutritional interventions alter metabolic networks, not just specific bacterial populations. Similarly, Cyanobacteria sequences traditionally dismissed as contamination showed patterns reflecting dietary components rather than true gut colonizers (Rychlik, 2020). These transient populations, likely ingested with feed or water, provide insights into digestive processes that microbiota analysis alone would miss.

One central limitation was sampling only one bird per pen across five replicate pens. This approach aligns with standard poultry microbiome studies that sample 1 to 12 birds per pen. It avoids pseudoreplication when multiple birds come from identical pen environments (McKinnon Reish, Dewey and Kirschman, 2024). The low sample size, however, limits statistical power to detect subtle treatment effects. Our findings still establish a framework. They show that SQMFe® iron and vaccination can interact both synergistically and antagonistically. These unexpected ecological dynamics require investigation with larger sample sizes.

Future studies should address these limitations. Larger sample sizes with multiple birds per treatment group will improve statistical power. Longitudinal sampling at key time points (days 0, 7, 14, 21, 35, 49) will capture how vaccination-iron interactions develop throughout the

production cycle. Endpoint measurements alone miss this temporal complexity. Dose-response studies testing SQMFe® concentrations from 40 to 80 ppm with varied vaccination timing will help. They can determine whether antagonistic effects at 60 ppm can be avoided through optimization.

Live *Salmonella* challenge studies are essential. They will confirm whether microbiome changes translate to actual protection against colonization. Advanced methodologies will provide deeper mechanistic insights. Shotgun metagenomics can identify species-level differences, antimicrobial resistance genes, virulence factors, and metabolic pathways. Tracking iron metabolism markers at multiple time points will clarify how iron bioavailability shapes microbiome composition. These markers include serum iron, transferrin saturation, soluble transferrin receptor, hepcidin expression in liver, ferroportin-1 in duodenal enterocytes, and tissue iron content.

In conclusion, this thesis demonstrates that antimicrobial resistance and pathogen control in poultry are multi-scale ecological problems. Farm-level interventions alone cannot eliminate AMR, and microbiome interventions can have unintended consequences, producing both beneficial and harmful effects.

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