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SÃO PAULO STATE UNIVERSITY (UNESP)
SCHOOL OF VETERINARY MEDICINE AND ANIMAL SCIENCE

**RESISTOME COMPOSITION AND DISTRIBUTION IN BROILER
CHICKEN PRODUCTION RAISED UNDER CONVENTIONAL AND
ANTIBIOTIC-FREE SYSTEMS**

EMANOELLI APARECIDA RODRIGUES DOS SANTOS

Botucatu – SP
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my greatest source of inspiration
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ABBREVIATIONS LIST

AFS - Antibiotic-free system
ABPA – Brazilian Association of Animal Protein
AMR – Antimicrobial Resistance
ANVISA – National Health Surveillance Agency
ARGs – Antimicrobial Resistance genes
CA – clean area
CARD – Comprehensive Antibiotic Resistance Database
CE – Cecum
CEUA - Ethics Committee on Animal Use
CS - Conventional system
DA – Dirty area
DNA - Deoxyribonucleic acid
EFSA - European Food Safety Authority
ESBL - Extended-spectrum β -lactamase
EU – European Union
Evs – Extracellular vesicles
FAO - Food and Agriculture Organization of the United Nations
FDA – Food and Drug Administration
FDR - False discovery rate
FP – Final product
GMO – Genetically modified
HGT – Horizontal gene transfer
HF – Human feces
IS – Insertion sequence
LB – Lysis buffer
MAPA – Ministry of Agriculture, Livestock, and Supply
MGEs – Mobile genetic elements
MLS - Macrolide-lincosamide-streptogramin
NAAU – No Antibiotics No Anticoccidials
OECD – Organization for Economic Co-operation and Development

oriT – Origin of transfer

PAHO – Pan American Health Organization

PAN-BR AGRO – National action plan for prevention and control of antimicrobial resistance in agriculture

PcoA - Principal coordinate analysis

PH – Poultry house

rRNA - Ribosomal RNA

RNA - Ribonucleic acid

SARG - Structured ARG Reference Database

SENASA – National Service for Agri-food Health and Quality

smDNA-FISH – single-molecule DNA fluorescence in situ hybridization

SS - Saline solution

WHO – World Health Organization

WOAH - World Organization for Animal Health

WGS – Whole genome sequence

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CHAPTER 3

Comparative taxonomic and functional characterization of microbiota and resistome in broiler feed from conventional and antibiotic-free production systems

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ABSTRACT

Broiler production systems, due to their scale of production and intensity, constitute an ecological interface for the dissemination of antimicrobial resistance genes (ARGs). This thesis was structured into two chapters and investigated the dynamics of resistome circulation across the broiler production chain under conventional (CS) and antibiotic-free systems (AFS) in southeastern Brazil. In Chapter 1 we evaluated the distribution, diversity, and persistence of ARGs along the broiler production chain. A total of 120 samples were collected over two years from 32 commercial broiler batches (CS, n = 16; AFS, n = 16) across eight vertically integrated farms (four CS and four AFS). Sampling included poultry house environments, cecal contents, slaughterhouse surfaces, final products (carcass rinsates), and fecal samples from farm and slaughterhouse workers. Total DNA was extracted using the MagicPure® Stool and Soil Genomic kit, and shotgun metagenomic libraries were prepared and sequenced on an Illumina NextSeq platform. After quality filtering and trimming, host-associated sequences were removed by mapping against *Gallus gallus* (GRCg6a) and *Homo sapiens* (GRCh38) reference genomes. ARG profiling was conducted with ARGs-OAP v2.3, and abundance was normalized as gene copies per cell. Statistical analyses included Wilcoxon tests with FDR correction, alpha diversity indices, beta diversity based on Bray–Curtis and Jaccard distances, ordination analyses (PCoA and NMDS), and PERMANOVA. A total of 1,349 ARG subtypes belonging to 27 resistance classes were detected across the production chain, with macrolide–lincosamide–streptogramin (MLS), beta-lactam, aminoglycoside, and tetracycline resistance genes predominating in both systems. Although 77% of ARG subtypes were shared between systems, the conventional system exhibited greater ARG richness, whereas the antibiotic-free system showed greater evenness, indicating a more uniform resistome structure. Beta-diversity analyses revealed differences between systems ($p < 0.001$). Clinically relevant genes, including *erm(C)*, *optrA*, *fexA*, *tet(X5)*, and *TEM-117*, were exclusively detected in CS, while *mecA* and *vanYG1* were identified in AFS. Core ARGs were detected across all production stages, indicating continuous circulation independent of

antimicrobial use. Human fecal samples showed similar environmental resistomes, and workers from CS harbored higher abundances of tetracycline, beta-lactam, and MLS resistance genes, demonstrating occupational exposure as a relevant driver of ARG dissemination. Chapter 2 assessed feed as an ecological niche for microbial and genetic dissemination within broiler production systems. Sixteen feed samples (eight per system) were collected during winter 2024 and summer 2025 and analyzed by shotgun metagenomics following the same DNA extraction and sequencing workflow applied in Chapter 1. Microbial community profiles were dominated by Proteobacteria, Actinobacteria, and Firmicutes in both systems. Conventional feeds were enriched in *Yaniella*, *Aureimonas*, and *Lactobacillus*, which are associated with industrial and process-derived contamination, whereas antibiotic-free feeds were dominated by environmental and biofilm-associated genera such as *Sphingomonas*, *Corynebacterium*, and *Streptomyces*. Although alpha diversity did not differ significantly between systems, antibiotic-free feeds exhibited more evenly structured communities. Beta-diversity analyses revealed clear system-level segregation ($p < 0.01$). A total of 283 ARGs belonging to 27 resistance classes were detected in feed. The conventional system was dominated by *bacA*, whereas antibiotic-free feeds showed significantly greater ARG richness and functional diversity, with enrichment in MLS, phenicol, and aminoglycoside resistance genes. Efflux pump genes (*MFS*, *mdtK*) and biocide resistance determinant *qacG* were ubiquitous, suggesting co-selection mediated by disinfectants and metals. Seasonal effects moderately influenced microbial structure but exerted a limited impact on the resistome. Altogether, the results demonstrate that antimicrobial resistance (AMR) in broiler production systems is governed by multifactorial pressures involving antimicrobial use, environmental exposure, feed microbiology, and occupational contact. Although antibiotic-free systems reduce ARG abundance and diversity, they do not eliminate the persistent resistome. This thesis highlights feed as an underexplored yet relevant ecological interface in the AMR network and reinforces the need for integrated surveillance strategies to mitigate antimicrobial resistance across the food production chain.

Key words: ARGs, food safety, public health

CHAPTER 1

1 INTRODUCTION

In recent years, broiler chicken production has stood out as one of the main activities in livestock production, meeting the growing demand for low-cost animal protein and playing a key role in food safety and the economic development of several countries (FAO, 2023). Brazil holds a prominent position in this scenario, being the third largest producer with a total production of 14,972 million tons, of which 35.36% is destined for export, and a *per capita* consumption of 45.5 Kg/person (ABPA, 2025). To sustain this high level of production, modern poultry production systems have achieved notable productivity due to advances in genetic selection, nutrition, management, and precision farming technologies, combined with improvements in biosecurity, vaccination, and stress reduction (MARMELSTEIN et al., 2024; YIMING & ZIJIA, 2025).

These factors, along with enhanced pathogen control, traceability, certification programs, and automation tools, have contributed to better animal welfare and higher production efficiency. The recent incorporation of artificial intelligence and microbiome monitoring has emerged as a promising and expanding approach, offering new opportunities to optimize production processes and disease prevention strategies (DEPURU et al., 2024; ABREU FILHO et al., 2025). Also, the historical use of antibiotics as growth promoters to improve feed conversion, performance, and disease prevention has played a major role in sustaining intensive production systems (MULCHANDANI et al., 2023). This practice, although effective in the short term, has raised serious concerns about its long-term consequences for public health and the environment.

Antimicrobial resistance (AMR) has emerged as a serious global threat, with a direct link between the intensive use of antibiotics in animal production and the rise in the number of resistant bacteria. AMR undermines the effectiveness of essential medical treatments, turning common infections into potentially fatal conditions (TIEDJE et al., 2023). This situation directly calls for the implementation of more sustainable practices and strict regulations in the livestock sector.

The intensive use of antibiotics in poultry production can lead to environmental issues, such as alterations in microbial diversity in the environment, potentially causing ecological imbalance, and the spread of micropollutants, including antibiotic/antimicrobial resistance genes (ARGs) (SHEN et al., 2025; RANSIRINI et al., 2025). Due to these practices, critical points have emerged in broiler chicken

production, such as areas prone to contamination by antibiotics and resistant microorganisms (SCICCHITANO et al., 2024; SZOKE et al., 2025). In this context, it is essential to identify hotspots and clarify how antibiotic use can impact everything from the production environment to the final product.

Due to the need for a One Health approach, public policies on antimicrobial use have been implemented in different regions of the world to reduce selective pressure that can favor the emergence and dissemination of resistant microorganisms, in that way protecting public health and preserving the efficacy of antimicrobials. The Organization for Economic Co-operation and Development (OECD) and the World Health Organization (WHO) emphasized the importance of a global and cross-sectoral approach to tackling AMR, promoting integrated policies that encompass human, animal, and environmental health (JAMES et al., 2025). In Brazil, efforts have been made to monitor AMR through plans and initiatives aimed at controlling antibiotic use in animal production. The National Health Surveillance Agency (ANVISA) and the Ministry of Agriculture, Livestock, and Food Supply (MAPA) have implemented regulations to restrict the indiscriminate use of antibiotics in livestock (BRASIL, 2023a; BRASIL, 2023b). Additionally, programs governed by the Pan American Health Organization (PAHO) and the European Union seek to restrict use and promote more sustainable and responsible antimicrobial management practices (WHO, 2025).

Alternative production systems, such as antibiotic-free, organic, and non-GMO poultry farming, came out as strategies to mitigate the spread of antimicrobial resistance and improve food sustainability. These systems ban the use of antibiotics and anticoccidials throughout the production cycle, relying instead on preventive health management measures, including probiotics, prebiotics, organic acids, phytogenic additives, and strict biosecurity protocols. Beyond reducing selective pressure, such systems align with global consumer demands for safer, more transparent, and welfare-oriented food production (SIVAPIRUNTHEP et al., 2025; ZHOU et al., 2025). However, the microbiological and ecological consequences of shifting from conventional to antibiotic-free systems remain poorly understood.

Official AMR data are typically related to zoonotic pathogens and indicator microorganisms that can reflect phenotypic assessment and the presence of ARGs in animal production environments (EFSA, 2024). The main zoonotic and indicators bacteria associated with poultry are *Salmonella* spp., *Campylobacter jejuni*, *Campylobacter coli*, *Escherichia coli*, and *Enterococcus* spp. Similarly, literature

1 extensively elucidates the AMR situation in isolates of these four species originating
2 from poultry (COLL et al., 2024; MORASI et al., 2022; RAMA et al., 2024; RAWAT et
3 al., 2024). However, as with culture-dependent phenotypic assessments, the
4 identification of antibiotic resistance markers is essential for monitoring and controlling
5 the spread of ARGs. Genomic studies investigating the impact of antibiotics on
6 microbial ecology reveal that exposed habitats may exhibit altered microbial diversity
7 and an increased abundance of resistance genes. (CAO et al., 2020). The whole-
8 genome sequencing (WGS) of microorganisms presents in different habitats, including
9 soil, water, and of chickens' gut, provides data on the presence of these ARGs.
10 However, unless an epidemiological study is conducted, it is not possible to map the
11 ecological dissemination routes of these genetic elements and thus identify possible
12 hotspots present in the production environment (REN et al., 2021).

13 In this context, understanding the distribution of ARGs across different broiler
14 production systems is essential for formulating strategies and interventions to control
15 the spread of resistance within and beyond animal production systems, as well as for
16 understanding how alternative production systems may contribute to AMR mitigation.

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2 OBJECTIVES

To evaluate the occurrence and distribution of antimicrobial resistance genes (ARGs) in broiler chickens raised under conventional and antibiotic-free systems, across various production stages, and to consider the interactions of these genes and elements in humans, animals, and environments.

2.1 Specific objectives

To characterize the taxonomic profile of microbial communities associated with the different production systems (conventional and antibiotic-free) and the distinct stages of the production chain.

To compare the diversity and abundance of microbial communities and ARGs between the two systems, identifying potential hotspots of resistance and genetic transfer.