國立臺灣大學醫學院

國際三校農業生技與健康醫療碩士學位學程

主題性統整報告

GIP-TRIAD Master's Degree in Agro-Biomedical Science

College of Medicine

National Taiwan University

Comprehensive Report

產食動物抗生素抗藥性：

法國、日本及台灣之防疫一體分析

Antibiotic Resistance in Food-Producing Animals:

A One Health Analysis Across France, Japan, and Taiwan

邱于珍

Yu-Chen Chiu

指導教授：丁詩同 博士

Advisor: Shih-Torng Ding Ph.D.

中華民國 114 年 7 月

July 2025

doi:10.6342/NTU202503336

Acknowledgements

This report was conducted under the affiliation of GIP-TRIAD, a master's program jointly offered by National Taiwan University, the University of Bordeaux, and the University of Tsukuba.

Supervision was provided by Shih-Torng Ding, Chia-Lang Hsu, Thibault Stalder, Kazuya Morikawa, Patricia Thébault, and Pascal Sirand-Pugnet. Technical support was provided by Maaya Sasaki, Margaux Gaschet, and Chien-Ying Chiou. The Mandarin report title was advised by Huei-Yin Suen from the National Animal Industry Foundation in Taiwan.

Supporting teams include the High-Throughput Genomics and Big Data Analysis Core, Department of Medical Research at National Taiwan University Hospital, INSERM Resinfit UMR 1092 at the University of Limoges, and the Institute of Medicine Laboratory of Microbiology at the University of Tsukuba.

Biological samples used in this report were provided by the Musashino Seed Co., Ltd. Niihari Breeding & Research Station in Japan and the Clevicus cattle farm in France. Publicly available sequencing data were retrieved from the European Nucleotide Archive, submitted by the Centre for Genomic Epidemiology at the National Food Institute, Technical University of Denmark.

Gratitude is extended to all individuals and institutions who supported this project, including my family and friends, all the people I met during the two years of this journey, and myself for continuing to explore my passion as a cancer survivor.

doi:10.6342/NTU202503336

摘要

抗生素抗藥性（AMR）為影響防疫一體（One Health）架構之重要議題。本報告視產食動物之抗生素使用為人為因素，以全面的觀點探討其傳播抗生素抗藥性之可能性。研究方法包括分析法國、日本及台灣之產食動物抗生素使用習慣及實地樣本之抗藥性分析。結果顯示相較於日本及台灣，法國具有較強力度之法規及較少之抗生素使用。相似趨勢亦發現於廢水宏基因體定序分析，與日本及台灣之集群相比，法國樣本之集群表現出較為獨立之趨勢。依據前述之研究結果，同時擁有 β-內醯胺類抗生素及多黏菌素抗性之抗藥性基因被提議作為追溯產食動物抗藥性來源之指標基因。抗生素敏感性試驗更進一步的被用於探討產食動物相關樣品分離出之菌株抗藥性機制。自法國肉牛農場採集之動物及環境樣本篩選分離出之革蘭氏陰性菌菌株用於探討以質體為媒介之抗藥性傳播，而自日本蔬菜農場採集之含產食動物糞便肥料樣本篩選分離出之革蘭氏陽性菌菌株則被用於探討其可能之 β-內醯胺類抗生素抗藥性。ESKAPEE 病原體所屬之菌屬在抗藥性傳播中可能扮演的生態棲位也同時於本研究中探討。產食動物抗生素使用及抗生素抗藥性之間存在由多樣因子構成之關聯，現今之相關研究因而有所侷限，多元之研究方法因此被建議使用以彌補研究之不足。本報告提議以功能性宏基因體定序分析作為填補現今產食動物抗生素抗藥性研究缺口之方法，從而連接防疫一體及全球意識，提升抗生素抗藥性監測效能。整體而言，本研究提供關於產食動物抗生素抗藥性的全面見解，旨在守護所有生物與環境的健康與福祉，並為未來抗生素抗藥性提供可能之研究方向。

關鍵字：抗生素抗藥性、防疫一體、產食動物、人為因素、宏基因體、抗生素敏感性試驗

doi:10.6342/NTU202503336

Abstract

Antimicrobial resistance (AMR) is a serious concern that affects the One Health sectors. This report examines antibiotic use in food-producing animals as an anthropogenic factor, identifying its potential contribution to resistance spread by analyzing antibiotic use patterns and real-life sample resistance profiling in France, Japan, and Taiwan to provide a comprehensive perspective. The results indicated stricter policies and lower levels of antibiotic use in France compared to Japan and Taiwan. Similar trends were discovered in the wastewater metagenomic analyses, with samples from France forming more distinct clusters than the other two countries. Genes resistant to both beta-lactam and polymyxin are proposed as potential markers to track animal-originated sources. Phenotypic profiling was further performed to explore strain-specific, animal-originated resistance mechanisms by identifying plasmid-mediated resistance in Gram-negative isolates from France and possible beta-lactam resistance in Gram-positive isolates from Japan, alongside the consideration of possible ecological roles played by ESKAPEE-associated genera in resistance dissemination. The relationship between food-producing animal antibiotic practices and AMR is shaped by complex factors, which pose limitations for current studies. A multi-method approach is therefore recommended to complement existing constraints. Functional metagenomics is proposed as a potential solution to address current gaps in food-producing animals' AMR research to bridge One Health and global efforts toward improved AMR surveillance. Overall, this study provides insights about AMR related to food-producing animals with the aim of protecting the health and welfare of all living beings, and offers possible directions for future AMR research.

Keywords: Antimicrobial Resistance (AMR), One Health, Food-Producing Animals, Anthropogenic Factors, Metagenomics, Antibiotic Susceptibility Testing

doi:10.6342/NTU202503336

Nomenclature

AMR	Antimicrobial Resistance
WHO	World Health Organization
FAO	Food and Agriculture Organization of the United Nations
WOAH	World Organisation for Animal Health
EU	European Union
ENA	European Nucleotide Archive
ARG	Antibiotic Resistance Gene
mOTU	Molecular Operational Taxonomic Units
FDR	False Discovery Rate
TMM	Trimmed Mean of M-values
PCA	Principal Component Analysis
ESKAPEE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, *Escherichia coli*
PLSDB	The Plasmid Database
CARD	The Comprehensive Antibiotic Resistance Database
GSP	Glutamate Starch Phenol Red Agar
KBA	*Klebsiella* Blue Agar
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight
DNA	Deoxyribonucleic Acid
MIC	Minimum Inhibitory Concentration
ECOFF	Epidemiological Cut-Off Value
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
MSA	Mannitol Salt Agar
UK	United Kingdom
USA	United States of America
ESBL	Extended-Spectrum Beta-Lactamase

doi:10.6342/NTU202503336

Table of Contents

doi:10.6342/NTU202503336

List of Figures

doi:10.6342/NTU202503336

List of Tables

doi:10.6342/NTU202503336

Chapter 1

Introduction

1.1. Global Threat of Antimicrobial Resistance (AMR)

Global food and health systems face escalating threats from various linked concerns, with AMR as one of the critical threats. AMR not only limits the treatment of infectious diseases but also impairs food safety, animal health, and environmental integrity, posing a serious risk to global health and sustainable food production[1]. AMR is defined as the ability of microorganisms to resist the effects of medications that once successfully treated them. AMR is a natural process occurring as time evolves and is accelerated by human activities[2]. This resistance makes infections harder or even impossible to treat, weakening the foundations of both human and veterinary medicine[3].

This report focuses on antibiotics, a subset of antimicrobials that contain active substances with antibacterial effects, as the focus of this resistance study. While antibiotics have long been used to treat infections in both humans and animals, their widespread application has contributed to the accelerated emergence and spread of resistant strains[2].

1.2. One Health Framework and AMR Dissemination

Global organizations such as the WHO, FAO, and WOAH have highlighted the need to combat AMR through a One Health approach that holistically monitors the issue across human, animal, and environmental domains[4]. AMR does not stay limited to one sector. It can surpass the sector's boundaries and spread through direct contact, food chains, and

doi:10.6342/NTU202503336

environmental vectors, including water systems contaminated by animal excrement[5-7]. An indicative example is the worldwide spread of resistance to the last-resort antibiotic, colistin, encoded by the *mcr-1* gene. This antibiotic resistance gene (ARG), which emerged from animals, has spread worldwide through mobile genetic elements, such as plasmids, and is now found in human pathogens within clinical settings[8-11]. Another example is the prevalence of ESKAPEE across the One Health sectors. ESKAPEE, including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, and *Escherichia* coli, are a group of clinically critical pathogens that possess multi-drug resistance, and there is evidence showing that these pathogens appear in the animal sector with similar ARG patterns to those in the human sector that cause treatment difficulties due to resistance to standard antibiotics[12,13]. These examples, among others, emphasize the important role the animal reservoir plays in the antibiotic resistance crisis.

While One Health is often seen as the point where human, animal, and environmental health intersect, it also embraces an extra layer of concept, one that brings together science, surveillance, policy, and economic concerns to combat the crisis. Understanding these different layers of meaning is essential for addressing AMR holistically and for designing interventions that are both effective and sustainable across sectors[14].

1.3. Concern of AMR in Food-Producing Animals

Food-producing animals are especially concerning due to their substantial use of antibiotics. Approximately 70% of antimicrobials used worldwide are designated for animals raised for food production[15]. Particularly in cases when antibiotics are administered not only therapeutically but also for growth promotion and prophylaxis, these factors increase the chance of resistance selection and dissemination[16].

doi:10.6342/NTU202503336

Research has demonstrated that limiting antibiotic usage in food-producing animals lowers antibiotic-resistant bacteria in the animals and potentially in human populations[17]. Multiple countries worldwide are attempting to reduce the use of antibiotics in food-producing animals by approaches such as policy regulations. However, quantifying the impacts of such regulations has hardly been conducted[18]. Therefore, further investigating whether antibiotic use in food-producing animals affects other One Health sectors may yield meaningful and previously unexplored insights.

Policies tackling the crisis of AMR may differ across countries due to variations in involved stakeholders, regulatory structures, and available resources[19]. Given the potential impact of antibiotic use regulation on AMR trends, comparing differences in regulatory frameworks and corresponding AMR patterns across countries could help assess whether a correlation exists.

1.4. Surveillance of AMR

Surveillance of AMR has become increasingly necessary. Although surveillance systems for AMR in humans and animals are well established in numerous countries, environmental AMR surveillance is still notably lacking[20]. This gap has been acknowledged at the international level, with regions such as the EU initiating efforts to build environmental surveillance networks to strengthen a more balanced One Health monitoring framework[21].

Metagenomic sequencing enables the characterization of resistomes without the need for cultivation, hence facilitating the identification of ARGs from both culturable and unculturable microbes[22]. Yet, this technique is strongly dependent on existing annotated databases, constraining its capacity to identify novel or uncharacterized genes[23-25]. On the

doi:10.6342/NTU202503336

other hand, phenotypic approaches, while conventional, are essential for validating resistance performance and uncovering transmission mechanisms[26].

Despite growing efforts, the mechanisms that explain how resistance spreads are still only partially comprehended. A wide range of contributing factors, ranging from genetic mechanisms to ecological and human behavioral patterns, complicates the determination of a single cause[6,7]. As a result, utilizing multi-method approaches is crucial to comprehensively understand resistance dynamics. Culture-based methods are essential for identifying specific resistant bacterial species and clarifying their phenotypic characteristics, whereas the metagenomic approach facilitates the study of resistomes on a broader genomic scale that covers both culturable and unculturable bacteria in complex environments. The integration of these methods provides a more thorough comprehension of AMR and helps with the development of concentrated interventions[6,27,28].

1.5. Case Selection: France, Japan, and Taiwan

However, surveillance at the global scale still faces challenges. Countries with skewed or sparse data are suspected of being able to distort regional or continental assessments in this report[29], highlighting the need for targeted comparative studies between selected nations. A more focused perspective enables a thorough understanding of how particular practices and policies affect AMR. At the same time, there is still a limited understanding of how veterinary antibiotic use affects resistance trends in countries that follow different policies, farming systems, and public health approaches[16,30].

Given these factors, this study focuses on France, Japan, and Taiwan. Three countries with contrasting regulatory frameworks and surveillance practices regarding veterinary antibiotic use. These differences provide a valuable basis for comparing how regulation influences resistance patterns in the One Health framework.

doi:10.6342/NTU202503336

1.6. Study Objective and Scope

For this report, we hypothesize that different countries will exhibit different AMR trends due to different policies and usage patterns of antibiotics in food-producing animals across countries. This research attempts to investigate the possible contribution of antibiotic use in food-producing animals (in this study defined as cattle, pigs, and chickens) to the dissemination of AMR in the One Health sectors through multidisciplinary approaches, including regulatory and usage review, metagenomic resistome profiling of wastewater, and phenotypic analysis of cultured bacterial isolates. Here, the use of veterinary antibiotics is considered a key anthropogenic factor that contributes to the emergence and spread of resistance. This study brings together different types of data, lab-based findings, and policy materials, with the support of affiliated international programs. It proposes tracking ARGs particularly associated with veterinary antibiotics, especially those used in feed additives, utilizing functional metagenomics as a possible approach. The study ultimately supports discovering surveillance gaps, understanding resistance dissemination pathways, and suggesting responsible and sustainable applications to reduce the use of antibiotics in food-producing animal systems.

doi:10.6342/NTU202503336

Chapter 2

Food-Producing Animal Antibiotic Use in France, Japan, and Taiwan

Understanding the patterns of veterinary antibiotic usage is crucial for assessing its possible influence on AMR within a One Health framework[31]. Veterinary antibiotic use can be divided into two main purposes: growth promotion/feed conversion efficacy and disease control. The latter consists of prophylactic, therapeutic, and metaphylactic interventions[32]. This chapter investigates country-level differences in food-producing animal policies on the use of antibiotic feed additives for growth promotion, along with the range of authorized antibiotics and annual sales data for disease control. Incorporating both regulatory and utilization viewpoints, this study aims to provide a comprehensive overview of the various potential anthropogenic factors contributing to resistance from the food-producing animal sector.

2.1. Policies Regarding Antibiotics in Animal Feed

This section focuses on national feed additive policies to evaluate the application of antibiotics as growth promoters in food-producing animals. Antibiotics used in animal feed to promote feed efficiency or weight gain are categorized as feed additives in Japan and Taiwan[33-35]. In contrast, France, following EU legislation, forbids the utilization of antibiotics in feed for growth promotion[36].

France banned the use of antibiotics as growth promoters in 2006[37]. Unless properly justified, further regulations prohibit the use of antibiotics for preventive and

doi:10.6342/NTU202503336

metaphylactic reasons in 2022[32,36]. Only therapeutic applications can be used and are categorized as medicated feed[36].

Japan and Taiwan implement a positive list approach, allowing solely the use of legally permitted active substances as feed additives. France, on the other hand, uses a negative list strategy, prohibiting antimicrobials, especially those considered crucial for human health[38]. All veterinary antibiotics sold within the EU require authorization evaluation. The regulatory approaches and allowed substances are presented in Table 1. Over the past decade, both Japan and Taiwan have gradually removed certain antibiotics from their feed additive lists, including active substances from critical drug classes such as macrolides, aminoglycosides, and tetracyclines. Alongside broader drug classes, some particular active substances that present potential public health hazards were also removed[39-48] (Figure 1).

It is worth noting that Japan classifies approved antibiotics in their chemically specific forms, such as different salts or derivatives with the active substances, while Taiwan lists only the active substances. The variation of listing style may affect how antibiotic usage is monitored and regulated, presumably indicating a more detailed or managed approach in Japan. Further investigation would be needed to assess whether this detail has practical implications for usage patterns or resistance development. For this analysis, only active substances were considered.

Based on these findings, further research could explore whether Japan and Taiwan exhibit more similar antibiotic resistance patterns in the environment compared to France, as their regulatory frameworks on antibiotic feed additives are more closely aligned.

doi:10.6342/NTU202503336

Country	Regulatory Approach	Currently Allowed Active Substances
France (EU)	Negative List	Prohibited
Japan	Positive List	Avilamycin, Enramycin, Flavomycin, Bacitracin, Salinomycin, Lasalocid, Semduramicin, Monensin, Nosiheptide, Bicozamycin, Narasin
Taiwan	Positive List	Avilamycin, Enramycin, Flavomycin, Nosiheptide, Tiamulin

Table 1. Food-producing animal antibiotic regulations for growth promotion. Last verified on April 8th, 2025.

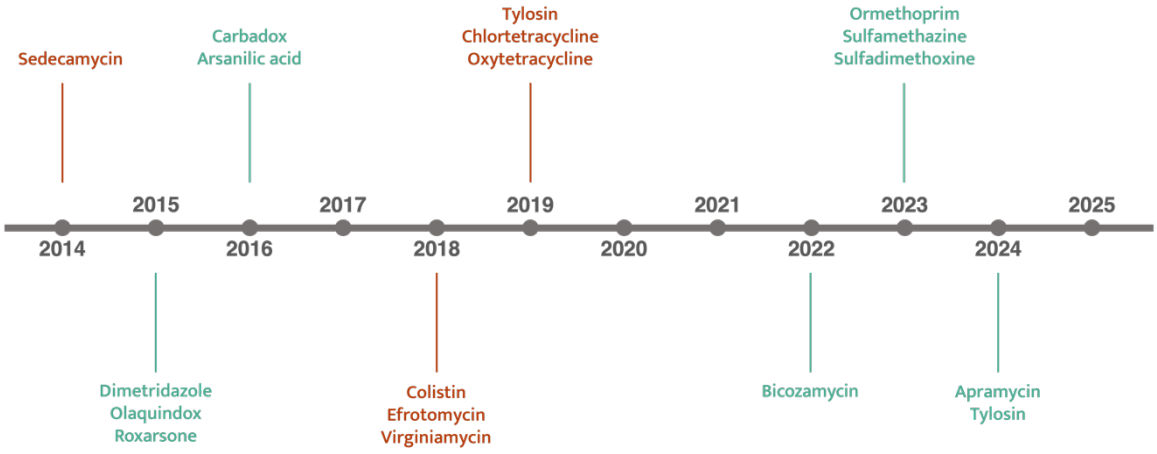


Figure 1. Timeline of eliminated allowed-to-use antibiotics over the past decade. Active substances eliminated in Japan are labeled in red, while in Taiwan are labeled in green. The figure was created with Microsoft PowerPoint.

doi:10.6342/NTU202503336

2.2. Authorized Antibiotic Products

Materials and Methods

This section compares currently authorized veterinary antibiotics used for disease control across Japan, Taiwan, and France. Antibiotic authorization data were collected and analyzed from each country's official database[49-52]. In line with the scope of this study, food-producing animals here for analysis were defined as cattle, pigs, and chickens. Data was retrieved from each country's official veterinary drug database and filtered accordingly. The active substance(s) in each product were then matched with known antibiotics listed in AntibioticDB to obtain its corresponding drug class. Analysis was performed in Python (version 3.13.1).

According to the original data sources, the accuracy of the content cannot be entirely assured due to possible limitations or data gaps. Therefore, the databases used in this section were released for informational purposes only. All data were retrieved on February 10, 2025.

France

Drug authorization data were retrieved from the IRCP Veterinary Drug Database to represent the result from France. Products used for cattle, pigs, and chickens were identified by filtering the "espèces cibles" column using the keywords "bovins," "poule," "vache," and "veau." The active substances listed in the "substances actives" column were translated from French to English using DeepL Translator. The translated names were then matched against the "Drug Name" field in the AntibioticDB database to determine their corresponding drug classes.

doi:10.6342/NTU202503336

Japan

Drug authorization data were retrieved from the official Veterinary Medicinal Products database to represent the result from Japan. The dataset was filtered to include only products listed under the categories of "抗生物質製剤" (antibiotic formulations) and "合成抗菌剤" (synthetic antimicrobial agents) within the "製剤区分" (formulation category) field. Approved drugs for cattle, pigs, and chickens were identified by screening the "効能効果" (efficacy) column for the presence of "鶏," "豚," and "牛." The "主成分" (main ingredient) field was translated using DeepL Translator, and the translated entries were matched with the AntibioticDB database to determine their corresponding drug class.

Taiwan

Drug authorization data were retrieved from the Taiwan Veterinary Drug License Query platform to represent the results from Taiwan. Only entries with an effective permit status ("有效證") were included, and further filtering was done based on the presence of the keywords "牛" (cattle), "豬" (pigs), or "雞" (chickens) within the "效能(適應症)" (efficacy) field. The active ingredients listed in the "成分" (ingredient) column were translated using DeepL Translator, and their corresponding drug classes were determined by matching them with the AntibioticDB database. To ensure consistency across countries, veterinary antibiotics authorized solely for feed additive purposes in Taiwan were excluded from the analysis, as the databases for Japan and France do not contain comparable information.

These steps ensured a consistent and comparative dataset across the three countries, aligned with the study's focus on food-producing animals and their potential role in antimicrobial resistance dissemination.

doi:10.6342/NTU202503336

Findings and Implications

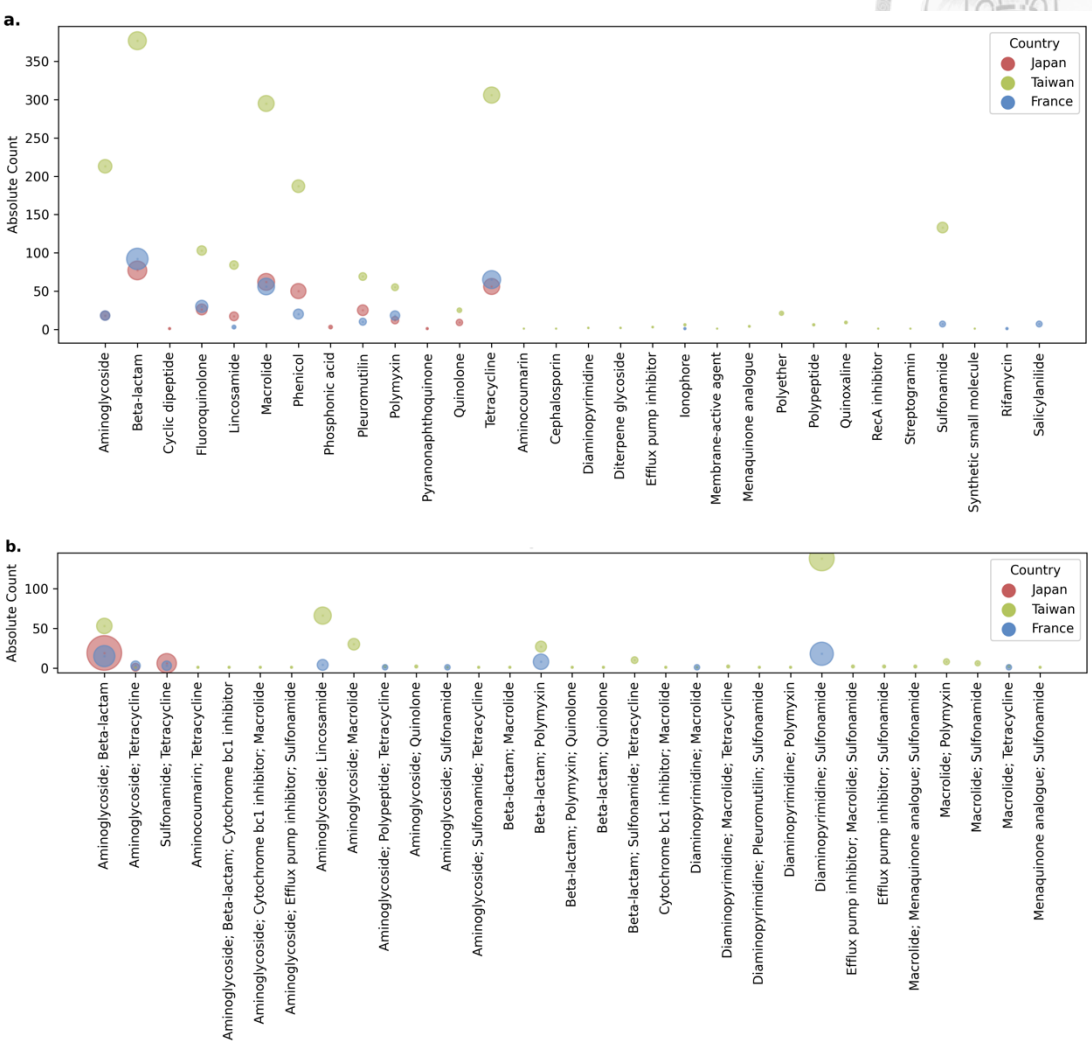


Figure 2. Bubble plot presenting the number of authorized food-producing animal antibiotic products in the three countries with distinct country colors. Antibiotic products are categorized by their belonging drug class by columns with two subfigures: a. products belonging to a single drug class; and b. products belonging to multi-drug classes. The rows display the absolute counts of products in the belonging drug class. The size of the bubble represents its relative abundance. The relative abundance cannot be compared between single- and multi-drug classes plots.

Results show that Taiwan has a substantially larger number of authorized veterinary antibiotics, with 2,272 products, more than 5 times the number of products in Japan and France, both having 383 products. Taiwan also shows a greater diversity, with more

doi:10.6342/NTU202503336

antibiotic drug classes allowed for food-producing animals. Japan, on the other hand, has the least diversity and fewer combination antibiotic products (Figure 2).

Based on the findings, future studies could consider whether the increased abundance and variety of authorized veterinary antibiotics correspond with Taiwan's broader and more diverse resistance profiles in environmental samples.

2.3. Food-Producing Animal Antibiotic Sales

Materials and Methods

To assess real-world antibiotic usage, national sales data for veterinary antibiotics were collected and compared between France and Japan[53,54]. For meaningful cross-country comparisons, drug classes and years that were not mutually available in both countries were excluded to ensure consistency in temporal trend analysis. Taiwan is excluded from this analysis due to a lack of available data. A series of linear regression models with interaction terms was applied to determine annual differences in antibiotic sales trends by country and by drug class.

In France, annual sales volumes of veterinary antibiotics were reported separately for cattle, pigs, and poultry. These figures were aggregated for each antimicrobial drug class. A normalization step was applied to account for species-specific differences in body weight, using the following equation:

$$\text{Sales}_{\text{class}} = \left(\frac{\text{Body Weight Treated}_{\text{class}}}{\text{Total Body Weight Treated}} \right) \times \text{Total Sales}$$

In Japan, antibiotic sales were disaggregated by beef cattle, dairy cattle, pigs, broilers, and layers. The sales data across these animal categories were combined for each drug

doi:10.6342/NTU202503336

class and converted from kilograms to tonnes, matching the reporting format used for France.

To evaluate whether temporal trends in antibiotic use differed between the two countries, time-series multiple linear regression models with interaction terms were fitted. These models aimed to assess whether the trajectory of antibiotic sales, measured in tonnes, changed differently over time in France compared to Japan.

The dataset included annual national sales volumes by drug class and country from 2011 to 2022. Each record reflected a unique combination of drug class, country, and year. The regression models treated year as a continuous predictor and country as a binary categorical variable, with Japan serving as the reference category. The model specification was as follows:

$$\text{Sales}_{it} = \beta_0 + \beta_1 \cdot \text{Year}_t + \beta_2 \cdot \text{Country}_i + \beta_3 \cdot (\text{Year}_t \times \text{Country}_i) + \epsilon_{it}$$

In this model, the term Sales_{it} represents the annual antibiotic sales in tonnes for drug class i at year t. The coefficient β_0 serves as the intercept, denoting the baseline sales volume in Japan in 2011. The coefficient β_1 captures the annual change in antibiotic sales for Japan, while β_2 reflects the baseline difference in sales between France and Japan in 2011. The interaction term β_3 estimates whether France's trend in antibiotic sales diverges significantly from that of Japan. Lastly, ϵ_{it} accounts for random error in the model and is assumed to follow a normal distribution with constant variance.

The interaction coefficient β_3 was of primary interest, as it indicates whether the trend in antibiotic sales over time in France diverged from Japan's. Hypothesis testing for the model coefficients was conducted using *t*-tests, with a significance level set at $\alpha = 0.05$.

doi:10.6342/NTU202503336

All statistical analyses were performed in R (version 2024.12.0 + 467). The models were implemented using the lm() function, with tidy summaries extracted via the broom package. Panels with significant interaction effects were annotated with asterisks to denote *p*-value thresholds ($p < 0.05$: *, < 0.01: **, < 0.001: ***), facilitating the interpretation of differences in antibiotic sales trends across countries.

Findings and Implications

Both France and Japan reported declining overall veterinary antibiotic sales from 2011 to 2022. However, as the interaction effect in the regression model ($p < 0.05$) demonstrates, France noticed a significantly greater decrease in overall sales volumes (Figure 3).

France exhibited steady declines in all major antibiotic categories at the drug class level, whereas Japan showed divergent patterns, with certain antibiotic drug classes declining and others either stable or increasing. These results imply that France implemented stronger or more effective policies that reduce the use of antibiotics in practice, while Japan's cuts could have been drug class-specific or selective.

The results from France possibly indicate the success of governmental action plans, Écoantibio, implemented since 2011[53]. The Écoantibio plans consist of different stages, from plan 1 of a quarter of the reduction of antibiotics in animals in 2011, plan 2 of half of the reduction in colistin in food-producing animals in 2016, to the currently implementing plan 3 targeting the reduction of antibiotics in companion animals[53,55]. This is a promising result to demonstrate that governmental actions do impact the national antibiotic usage patterns and may further ease the AMR burden across the One Health sectors.

doi:10.6342/NTU202503336

These patterns could assist future research to determine whether France's more consistent and lower antibiotic consumption corresponds with a lower resistance burden in environmental samples as compared to Japan. Lack of publicly accessible veterinary drug sales data in Taiwan makes comparable assessments for Taiwan difficult and points out a major data gap for future surveillance actions.

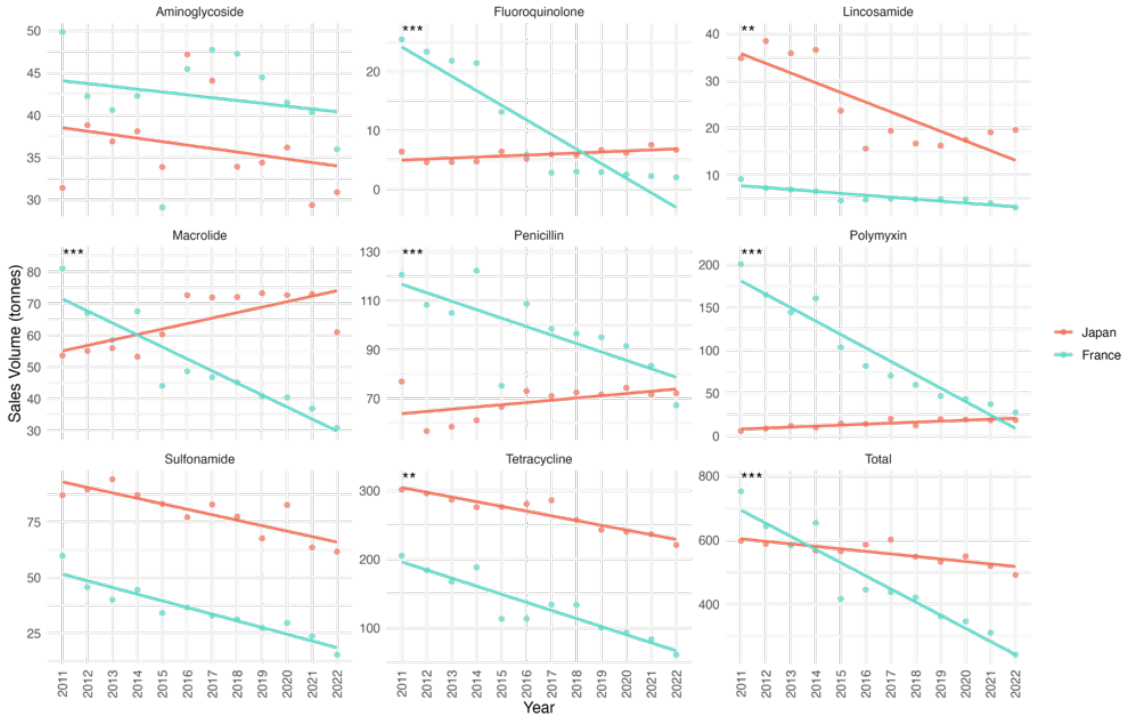


Figure 3. Linear regression plots with interaction terms for different antibiotic drug classes and their total values. Drug classes marked with an asterisk (*) in the top-left corner indicate statistically significant regression differences between the two countries. Significance thresholds are denoted as follows: $p < 0.01$ (**) and $p < 0.001$ (***).

2.4. Quantifying the Impact of Antibiotic Use on AMR

Other than antibiotic policy and usage patterns in food-producing animals, various additional factors could potentially impact resistance dissemination, such as the density of animals per unit land area and the biomass of the animals[56]. Mathematical modeling is a theoretical approach to estimate the influence of such factors on AMR dissemination.

doi:10.6342/NTU202503336

However, there remain significant knowledge gaps regarding the extent to which these factors contribute to the correlation[57,58]. This section focuses on one such correlated factor, animal density per unit land area within a country, to estimate how the inclusion or exclusion of this variable might influence the modeling output.

Materials and Methods

To calculate the density of animals per unit area, data on the number of cattle, chickens, and pigs, as well as the total land area of each country, were retrieved from government publications[59-62]. The 2021 data were selected as all three countries, France, Japan, and Taiwan, had available records for this year for the three animal types.

The retrieved number of heads of animals was organized by their respective country and animal type. The number of heads was then divided by the area of the country to obtain the density value. These density values were subsequently compared across countries for each animal type.

Findings and Implications

Results show that France has the highest density of cattle, Taiwan has the highest density of chickens, pigs, and all three animals combined, while Japan consistently remains in the middle in terms of animal density (Figure 4). For chickens and pigs, the variations between Taiwan and the other two countries are largely driven by land area differences. Taiwan is over ten times smaller, while the differences in animal counts remain under fourfold. In the case of cattle density, the number of cattle plays a more crucial role. France has over 100 times more cattle than Taiwan and almost 25 times more than Japan.

doi:10.6342/NTU202503336

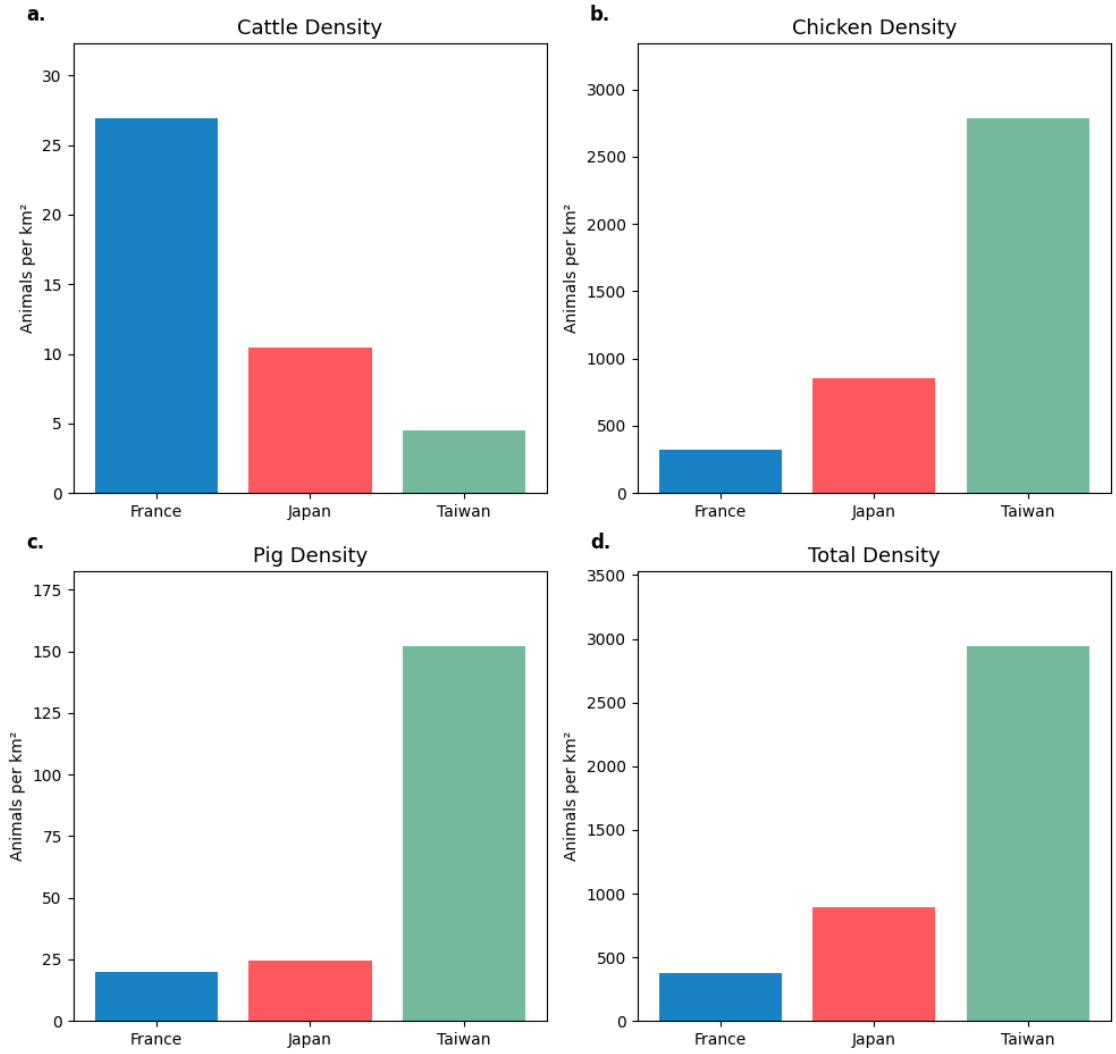


Figure 4. Box plots of food-producing animal density in France, Japan, and Taiwan, categorized by animal type: a. Cattle; b. Chicken; c. Pig; d. Total, with cattle, chicken, and pig combined. Animal density values are not comparable across subfigures, as the y-axis scales differ in each panel.

In the theoretical model, inputting various independent variables for a single country, including antibiotic policy, usage patterns, and animal density, will yield the output dependent variable representing the possible impact of AMR dissemination from food-producing animals in that country. Each independent variable is multiplied by a coefficient that represents its degree of influence on the spread of resistance. Here, we apply a multiple linear regression model using the animal density result as an example (Figure 4). The model equation is as follows:

doi:10.6342/NTU202503336

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \varepsilon$$

In this model, Y represents the possible impact of AMR dissemination from food-producing animals in the country. β_0 is the Y intercept, β_1 serves as the coefficient for antibiotic policy, β_2 as the coefficient for antibiotic usage pattern, and β_3 as the coefficient for animal density. X_1 is the independent variable for antibiotic policy, X_2 for antibiotic usage pattern, and X_3 for animal density. Lastly, ϵ accounts for random error in the model and is assumed to follow a normal distribution with constant variance.

The inclusion or exclusion of animal density as an independent variable might greatly impact the modeling result, as the total food-producing animal density in Taiwan is over five times higher than in France. One thing to keep in mind is that the degree of AMR impact also depends on the coefficient value, which needs to be carefully studied and determined.

This example provides a conceptual idea of how different factors might influence the modeling output, and detailed studies and interpretations are required to build a solid and convincing model that can reflect AMR patterns observed in the real world.

doi:10.6342/NTU202503336

Chapter 3

Metagenomic Profiling of the Resistome in Wastewater from France, Japan, and Taiwan

The regulatory frameworks and trends in veterinary antibiotic usage across France, Japan, and Taiwan provide essential context for understanding potential selection pressures driving AMR in each country. However, policy implementation and usage statistics alone cannot fully capture the ecological consequences of antibiotic use, nor the extent to which ARGs are circulating. To evaluate the downstream impact of antimicrobial use on environmental reservoirs of resistance, metagenomic surveillance offers a powerful and culture-independent approach[6,20,63].

Wastewater represents an integrative matrix that reflects microbial signatures, providing a source for AMR monitoring. It is more accessible than other biological materials, requiring minimal infrastructure, which enables large-scale surveillance, even in low-resource settings[6,22]. This chapter presents a comparative metagenomic analysis of urban wastewater samples from the three countries to explore national AMR trends reflecting the human sector and examine the possibility of linking the observed trends to anthropogenic factors studied in the previous chapter.

Materials

The ARGprofiler pipeline was employed to process wastewater metagenomic datasets retrieved from the ENA[63,64]. This study utilized selected steps of the pipeline, including dataset retrieval from ENA, read trimming and quality control, and mapping and alignment of reads against annotated databases[65,66]. Normalized fragment count values

doi:10.6342/NTU202503336

were utilized to analyze the abundance. Two samples from Japan were excluded from this chapter due to outlier behavior, characterized by their separation from the main cluster of Japan samples and the presence of abnormally high or low expression of certain ARGs, possibly influenced by sampling site-specific factors. The trends associated with these outliers are presented in Appendix 1.

3.1. Comparative Resistome Structure Based on ARG Abundance

Methods

This section aims to evaluate whether different approaches to analyzing ARG abundance at the gene and drug class level yield consistent clustering trends among countries. Fragment count values in both of the following analyses were normalized by the TMM method, followed by $\log_2$-transformation, as recommended for metagenomic data to correct for library size differences, and to stabilize variance[67-70]. The normalization result is presented in Appendix 2. In the gene-level analysis, hierarchical clustering was performed using Euclidean distance and complete linkage on differentially abundant ARGs (FDR < 0.05, edgeR quasi-likelihood *F*-test) (Figure 5). Separately, PCA analysis was used to visualize the variance structure in drug class-level ARG abundance across samples (Figure 6). In this PCA analysis, ARGs annotated with resistance to multiple drug classes were grouped into a single “multi-drug” category. The result of an analysis with original multi-drug groups is presented in Appendix 3.

Findings and Implications

Both analyses revealed closer resistome similarity between Japan and Taiwan, with France forming more distinct clusters. This trend aligns with previous studies reporting continental patterns in ARG profiles and supports the hypothesis from the previous chapter that Japan and Taiwan may exhibit more similar resistance trends due to

doi:10.6342/NTU202503336

similarities in their veterinary antibiotic policies[63]. Additionally, the inclusion of metal resistance genes and other non-antibiotic ARGs noticeably modified the hierarchical clustering results, as shown in Appendix 4, suggesting that co-selection mechanisms beyond antibiotic use may shape national resistome profiles.

While wastewater provides a comprehensive reservoir of ARGs, its capacity to accumulate ARGs from various origins makes it difficult to trace the source of specific genes. The factors contributing to observed continental differences in resistome profiles remain unclear and require further investigation. In the next section, this study focuses on identifying potential resistance signatures in wastewater that may be linked specifically to food-producing animal antibiotic usage.

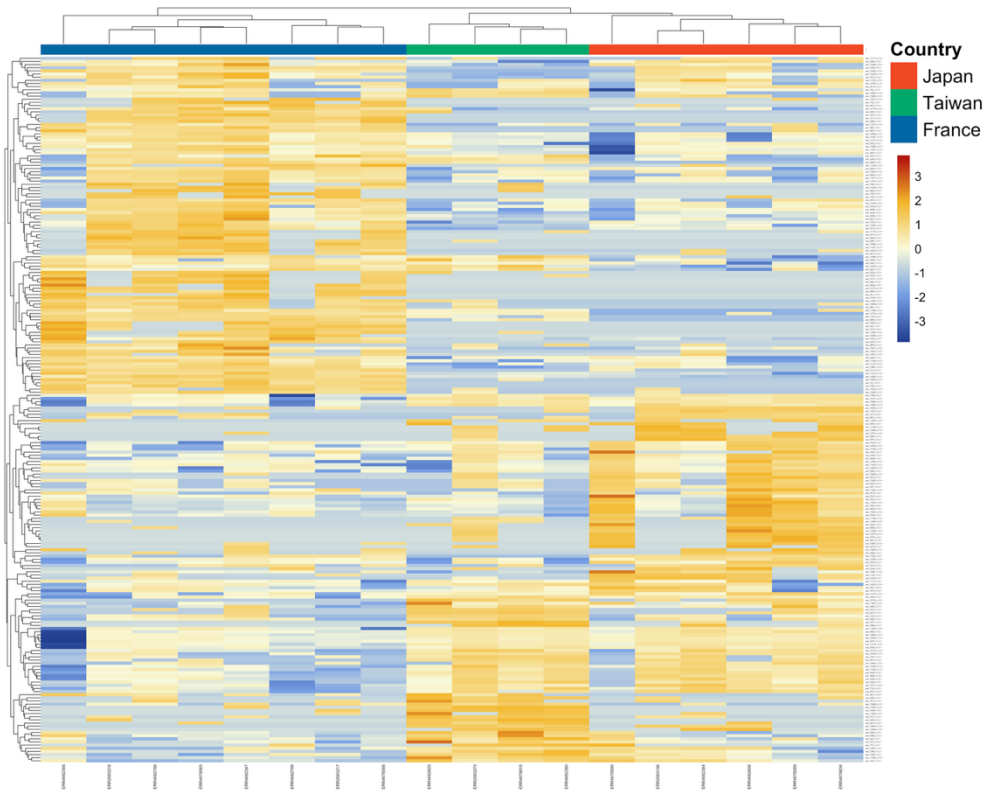


Figure 5. Heatmap showing the ARGs (rows) with significantly differential abundance across countries (FDR < 0.05). Samples (columns) were clustered based on the Euclidean distance using ARG abundance profiles. Samples from different countries are distinguished by color-coding.

doi:10.6342/NTU202503336

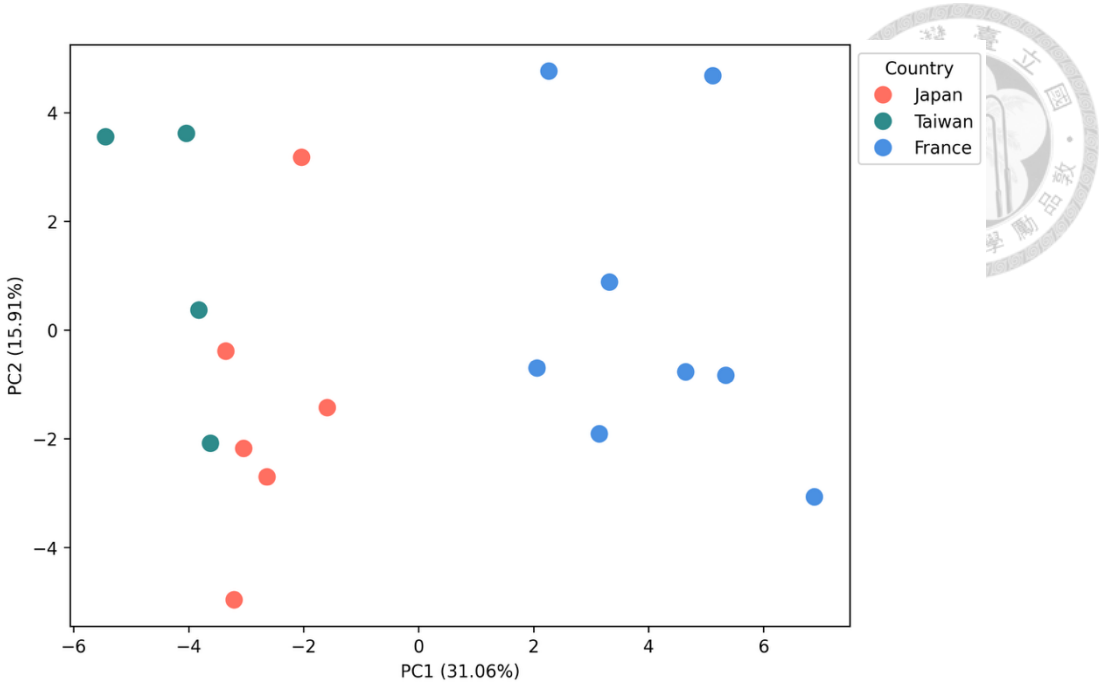


Figure 6. PCA plot based on ARG abundance, grouped by their belonging resistance drug class.

3.2. Potential Signatures of Food-Producing Animal Contribution to the Wastewater Resistome

Methods

To explore the possible influence of veterinary antibiotic use on AMR dissemination, this section further investigates ARG classes in wastewater that might be potential indicators to trace back to the source of food-producing animal antibiotic use. To find suitable indicators for this purpose, several criteria have to be met: the use of the group of antibiotic products has to be specific to animals to distinguish animal-originated sources; the usage pattern of the group of antibiotic products has to differ between countries to correlate and compare the significant differences observed in the wastewater results; and the corresponding ARG drug class has to be present in the PanRes annotated database. Beta-lactam and polymyxin combination drugs were considered as a potential indicator that fits all the required criteria for food-producing animal source tracking, as they are used in veterinary products in France and Taiwan but are absent in Japan and rare

doi:10.6342/NTU202503336

in human clinical settings (Figure 2, Table 2). Clinical use of colistin drugs in the three countries is detailed in Appendix 5. Novel ARGs conferring resistance to both beta-lactam and polymyxin have been recently found[71]. These ARGs are annotated in PanRes, allowing for comparative quantification in the wastewater metagenomes. Targeted ARG fragment counts were divided by the total ARG fragment counts from each sample to obtain normalized proportions and proportions between countries.

Antibiotics Combination	France	Taiwan
Ampicillin + Colistin	5	23
Cloxacillin + Colistin	2	0
Amoxicillin + Colistin	1	0
Penicillin G + Colistin	0	4

Table 2. Amount of authorized antibiotic products under the "beta-lactam + polymyxin" drug class combination in France and Taiwan.

Findings and Implications

Results indicate that Taiwan had a significantly higher relative abundance of beta-lactam/polymyxin ARGs compared to Japan ($p = 0.0048$, Mann-Whitney U test). France showed a slightly higher proportion than Japan, though not statistically significant (Figure 7). This may reflect the fact that such combination antibiotics are absent in Japan and have broader availability in Taiwan. However, without access to veterinary antibiotic sales data from Taiwan, it is difficult to assess the usage and its potential impact. Additionally, this result represents only one possible hypothesis. As multi-drug resistance can develop cumulatively over time rather than solely from simultaneous selective pressures[72], we cannot conclusively determine that the observed trend is driven by the specific antibiotic combinations currently in use. Further study is required to understand

doi:10.6342/NTU202503336

their resistance mechanisms. Nonetheless, this observation could serve as a valuable direction for future research aiming to better understand the ecological dynamics and resistance development influenced by veterinary antibiotic practices.

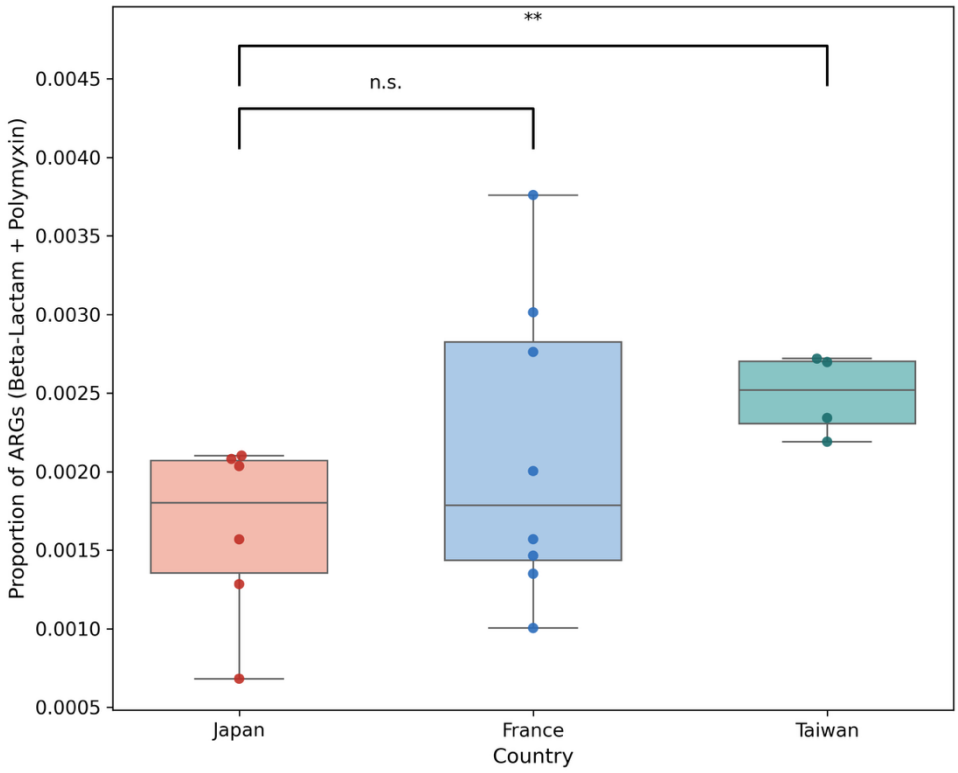


Figure 7. Box plot showing the relative abundance of ARGs conferring resistance to beta-lactam and polymyxin across countries. Significance thresholds are denoted as $p < 0.01$ (**) and not significant (n.s.).

3.3. Prevalence of ESKAPEE Pathogen-Associated Genera

The previous chapter of the metagenomic analysis focused on the resistome with ARGs potentially carried by various organismal hosts, not limited to bacterial species, to provide a broad overview of each country's resistance landscape. However, it is also important to consider the microbial taxa most likely to carry and spread resistance. Resistance dissemination is often facilitated by certain bacterial groups with a higher tendency to harbor ARGs[27]. In this context, the genera associated with ESKAPEE pathogens, organisms of high clinical concern due to their multidrug resistance

doi:10.6342/NTU202503336

capabilities, offer valuable insight into possible differences in dissemination risk across countries[73,74].

Methods

This section presents the relative abundance of ESKAPEE-associated genera in wastewater samples from the three countries. These data may provide insights into whether differences in taxonomic composition contribute to varying resistance trends across countries and potentially reflect resistance mechanisms identified through the culture-based methods discussed in the following section.

The genus-level mOTU data were extracted, and the count of mOTUs corresponding to ESKAPEE-associated genera was divided by the total number of mOTU counts in the certain sample. This normalization step provided relative abundance values.

Findings and Implications

In all three countries, the order of abundance among genera was consistent. However, Japan and Taiwan exhibited higher relative abundance for certain genera (Figure 8).

Comparing the relative abundance of certain bacteria across countries, especially ones known to be effective vectors of ARGs, could provide context on which settings pose a higher risk for AMR dissemination. This approach could serve as a complementary tool for risk assessment and guide AMR surveillance efforts under the One Health framework.

Future research could explore if higher relative abundances of specific ESKAPEE-associated genera in Japan and Taiwan correlate with a greater potential for resistance dissemination, based on the findings of this study. Integrating ARG and taxonomic profiling may improve the ability to link environmental resistance patterns to specific anthropogenic sources.

doi:10.6342/NTU202503336

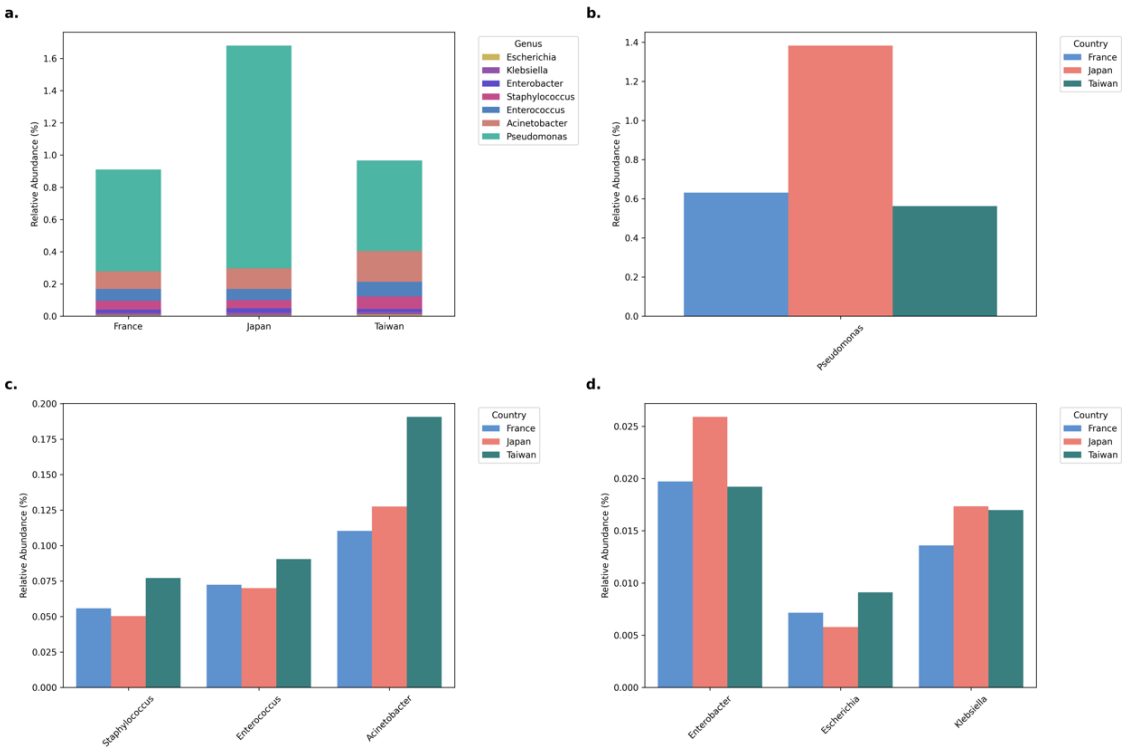


Figure 8. Relative abundance of ESKAPEE pathogen-associated genera in France, Japan, and Taiwan. a. The stacked bar plot represents the combined data from all seven ESKAPEE pathogens, with the most abundant genus positioned at the top of each bar and others arranged in descending order of abundance; b. Relative abundance of the genus *Pseudomonas*; c. Relative abundance of the genera *Staphylococcus*, *Enterococcus*, and *Acinetobacter*; d. Relative abundance of the genera *Enterobacter*, *Escherichia*, and *Klebsiella*. Relative abundance values are not comparable across subfigures, as the y-axis scales differ in each panel.

doi:10.6342/NTU202503336

Chapter 4

Phenotypic Characterization of Antibiotic Resistance in Animal-Associated Bacteria

Although metagenomics provides a comprehensive overview of the resistome in a sample, studies of the various mechanisms of resistance in a specific bacterial species are still crucial, since some mechanisms may be more common or clinically relevant than others[27]. Based on their functional expression of resistance, phenotypic profiling could help with the identification of resistant strains of interest, allowing targeted studies on their mechanisms. By confirming the functional relevance of identified ARGs, phenotypic insights can enhance metagenomic findings[6].

This chapter employed culture-based approaches that could be suitable for studying individual resistance trends. This approach not only strengthens the resolution of resistance source tracking but also offers functional insights into resistance mechanisms and transmission potential, which are qualities that are essential in understanding the spread of AMR within a One Health framework. This chapter presents analyses of antibiotic resistance phenotypes and potential mechanisms among bacteria isolated from animal-associated sources in France and Japan, with a different focus on Gram-negative and Gram-positive species, respectively. Based on previous studies, Gram-negative and Gram-positive bacteria are composed of different structures, which lead to different resistance responses[75,76]. With different targets on Gram-negative and positive bacteria, the combined findings could contribute to a broader understanding of resistance persistence, mobility, and risk in the animal-environment interface.

doi:10.6342/NTU202503336

4.1. Resistance in Gram-Negative Bacteria from France

Horizontal gene transfer is a well-known mechanism that enables bacteria to transfer ARGs across species[8]. Certain bacteria are particularly prone to acquiring and disseminating ARGs via plasmids, which are more mobile element able to transfer between bacteria of the same or different species[77,78]. This characteristic increases their potential to spread AMR across One Health sectors.

This section aims to explore the ecological potential for plasmid-mediated resistance transmission. The hypothesis is that certain "keystone strains" may bring disproportionate influence on AMR spread by facilitating the horizontal spread of plasmid-borne resistance. These keystone strains are often generalist bacteria capable of thriving in diverse habitats, which increases their ecological impact[8]. When such strains belong to genera phylogenetically related to ESKAPEE pathogens, which are organisms already of high clinical concern, the potential risk of transferring resistance to clinically important pathogens becomes even more significant.

Determination of Antibiotics to Be Used for Selection and Susceptibility Testing

Materials and Methods

The goal of this section of the study is to select bacterial strains that contain resistance plasmids. To achieve this goal, we utilized phenotypic culturing pre-enriched with antibiotics, plasmid extraction, and antibiotic susceptibility testing to select strains that are present with plasmids and resistant to multiple antibiotics. The determination of which antibiotics to utilize in the study is therefore crucial, as it determines the type of resistance the selected strains possess. The corresponding antibiotic drug class that ARGs are resistant to and are found on plasmids is thus considered.

doi:10.6342/NTU202503336

The META archive from the Plasmid Database (PLSDB) was utilized[79]. The database provides plasmid sequencing data. Values of taxonomy species information (mash_neighbor_identification) from the "typing" file, with ARGs (gene_symbol) and resistance drug class (antimicrobial_agent) from the "amr" file, were mapped with the corresponding "NUCCORE_ACC" value. Mapped results that were absent with the drug class values were further mapped with the data from the Comprehensive Antibiotic Resistance Database (CARD)[80]. Data was then mapped with the AntibioticDB database to get the corresponding drug class[52]. Mapped and cleaned data were then pivoted to get the counts of the ARG under the bacterial genus, including *Acinetobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Pseudomonas*. The pivoted data was then filtered to get the top 20 most abundant ARGs among all of the genera considered. A bipartite network graph was constructed using Python (version 3.13.1) with the libraries pandas, numpy, networkx, and matplotlib.

Findings and Implications

The 20 most abundant ARGs in PLSDB under the *Acinetobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Pseudomonas* genera include *aadA*, *aadA1*, *aadA2*, *APH(3'')-Ib*, *APH(3')-Ia*, *APH(6)-id*, *blaTEM-1*, *ble*, *dfrA12*, *dfrA14*, *dfrA17*, *mph(A)*, *mrx*, *aac(6')-Ib-cr5*, *aac(6')-Ib-cr6*, *qnrS1*, *sul1*, *sul2*, *floR*, and *tet(A)*. The corresponding resistance drug classes of these ARGs include aminoglycoside, beta-lactam, bleomycin, diaminopyrimidine, macrolide, quinolone, fluoroquinolone, sulfonamide, sulfone, phenicol, and tetracycline (Figure 9).

In the genera *Escherichia* and *Klebsiella*, there are visibly more and thicker edges compared to the other genera. ARGs in plasmids are more abundant in these two genera, which could be the potential explanation, but other reasons, such as the database

doi:10.6342/NTU202503336

containing more studies from the two genera, might also be the potential reason for this phenomenon.

With the analysis from the PLSDB, it can serve as a reference for this study to determine the antibiotic to be used in experiments such as pre-enrichment and MIC. With the most abundant ARGs found in the plasmid database, it might increase the probability of screening strains with plasmid resistance.

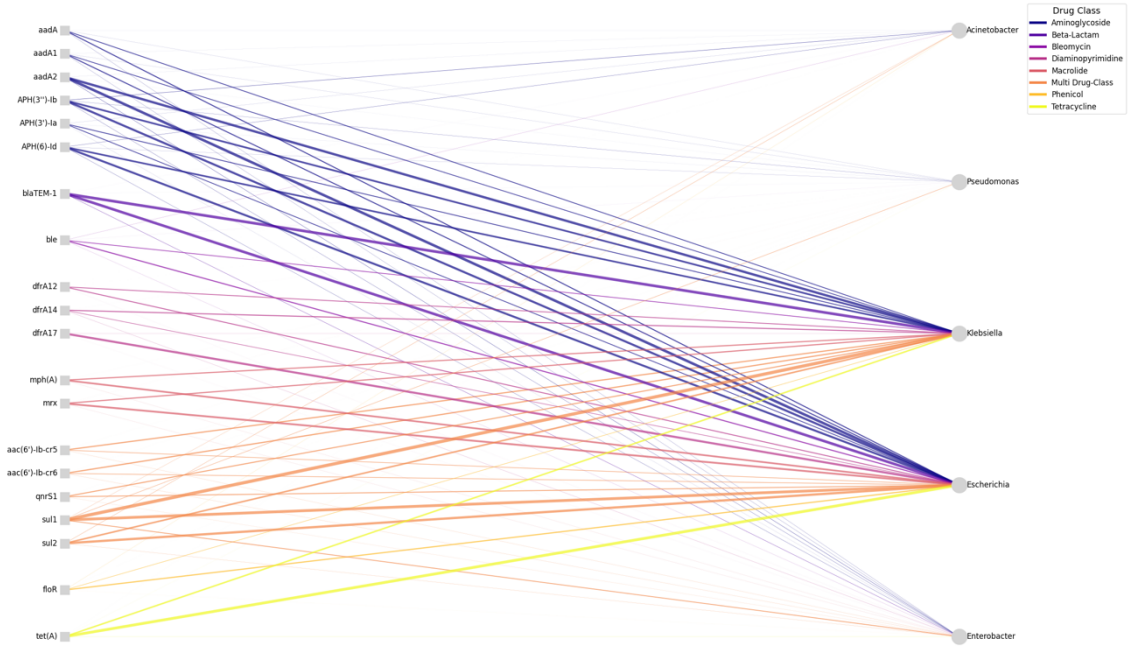


Figure 9. Bipartite network graph of the top 20 most abundant ARGs among the genera *Acinetobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Pseudomonas* in the PLSDB database. The left column displays the names of the top 20 most abundant ARGs, while the right column displays the bacterial genera. The edges in the middle are colored with the ARG's corresponding drug class, with the color legend displayed at the top right corner of the figure. The thickness of the edges represents the weight of the counts of the ARGs under the corresponding genus. The orders of the ARGs and genera in this graph from top to bottom are the same in the text in the first paragraph of this subsection from the beginning to the end. ARGs that are resistant to more than one drug class are grouped as "Multi Drug-Class" for the edges.

doi:10.6342/NTU202503336

Isolation of Bacterial Strains from Samples

Materials and Methods

Samples, including animal samples (nasal and oral swabs, feces) and environmental samples (drinking water, hay, and dust), were collected from a cattle farm for meat consumption purposes near Limoges, France. A total of 147 samples were collected.

Samples from the same batch and category were further pooled together and incubated overnight in 10 separate Mueller-Hinton broths containing antibiotics commonly known to be related to resistance on plasmids, including ceftazidime, ceftolozane, chloramphenicol, ciprofloxacin, colistin, ertapenem, gentamicin, sulfamethoxazole, tetracycline, and trimethoprim, respectively. The 10 pre-enrichment broths were then diluted to a uniform McFarland, pooled together, and plated on selective media. The steps were to pre-select bacterial strains that have the possibility of possessing resistance plasmids.

Selective media, including CHROMagar *Acinetobacter*, Glutamate Starch Phenol Red Agar (GSP), *Klebsiella* Blue Agar (KBA), and MacConkey Agar, were utilized for targeted isolation to enrich for Gram-negative bacteria in ESKAPEE-associated genera, including *Acinetobacter spp.*, *Enterobacter spp.*, *Escherichia spp.*, *Klebsiella spp.*, and *Pseudomonas spp.*[81-84]. These bacterial groups are well-documented in literature as frequently containing resistance plasmids[85-88].

The Bruker MALDI-TOF Biotyper was utilized for species identification. Strains that were confidently identified as gram-negative bacteria would be preserved in the -80°C freezer as collection stocks.

doi:10.6342/NTU202503336

Findings and Implications

Among the 840 isolates that underwent the identification process, 409 strains were confidently identified as gram-negative bacteria and were further preserved in the -80°C freezer as collection stocks (Figure 10).

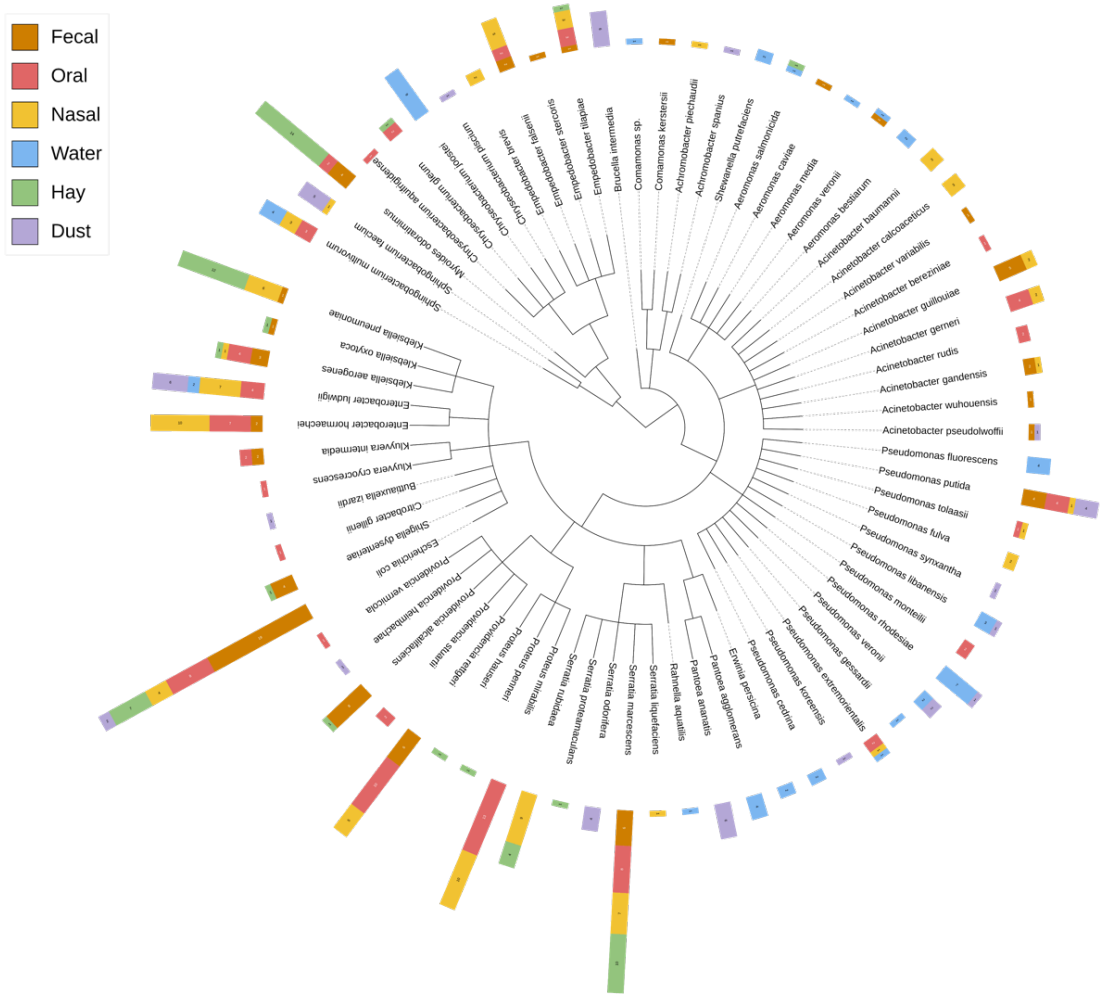


Figure 10. Total bacterial strains that were stocked from the cattle farm sampling isolation as a collection, displayed in a phylogenetic tree plot. The outer bars represent the number of colonies collected in the species, and bar colors represent the type of sample origin. The figure was created with iTOL Interactive Tree of Life[89].

All of the bacterial genera that we targeted, including *Acinetobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Pseudomonas*, are present in the collection. Under these

doi:10.6342/NTU202503336

genera, we also have collections of some ESKAPEE pathogens, including *Acinetobacter baumannii*, *Enterobacter hormaechei*, *Enterobacter ludwigii*, *Escherichia coli*, and *Klebsiella pneumoniae*. The *Acinetobacter* and *Klebsiella* genera in the collection are present with both pathogenic and non-pathogenic bacteria, which could be good candidates for researching how generalist bacteria spread their resistance plasmids to pathogenic bacteria.

The genera *Enterobacter*, *Escherichia*, and *Klebsiella* all belong to the Enterobacteriaceae family. Further study can focus on whether strains in between the Enterobacteriaceae family have a higher plasmid transfer rate compared to more phylogenetically distant strains, such as between the Enterobacteriaceae family and *Acinetobacter* or *Pseudomonas*. These ecological studies could help determine which strains have a higher chance of spreading AMR and possibly identify the keystone strains.

Extraction and Identification of Plasmids

Materials and Methods

Plasmid extraction and agarose gel electrophoresis were conducted on the 40 *Escherichia coli* collections to verify the presence of plasmids. 1 mL of overnight bacterial culture was centrifuged and resuspended in Solution I containing RNase A, Tris-HCl, EDTA, lysozyme, and glucose for resuspension. Solution II, containing NaOH, SDS, and EDTA, was further added for lysis. After, Solution III containing sodium acetate was added for neutralization. The supernatant then underwent phenol:chloroform extraction to remove residual proteins. After, the aqueous phase was extracted and mixed with isopropanol to precipitate the DNA. The pellet was then washed with cold ethanol to remove salts and residual solvents. The purified DNA pellet was dried and resuspended in TE buffer. Gel electrophoresis was then conducted in 0.8% agar with TAE buffer. 25 μL of the sample solution with 5 μL of the gel loading dye (purple (6X), no SDS).

doi:10.6342/NTU202503336

Findings and Implications

Among the 40 *Escherichia coli* collections tested, 21 display visible plasmid bands on gels (Figure 11). Some tested colonies displayed identical plasmid band patterns and originated from the same sample source. Therefore, we suspected that some collected colonies are clones. The hypothesis requires further testing with other experimental approaches. Gel electrophoresis result images of the 40 tested collections are detailed in Appendix 6.

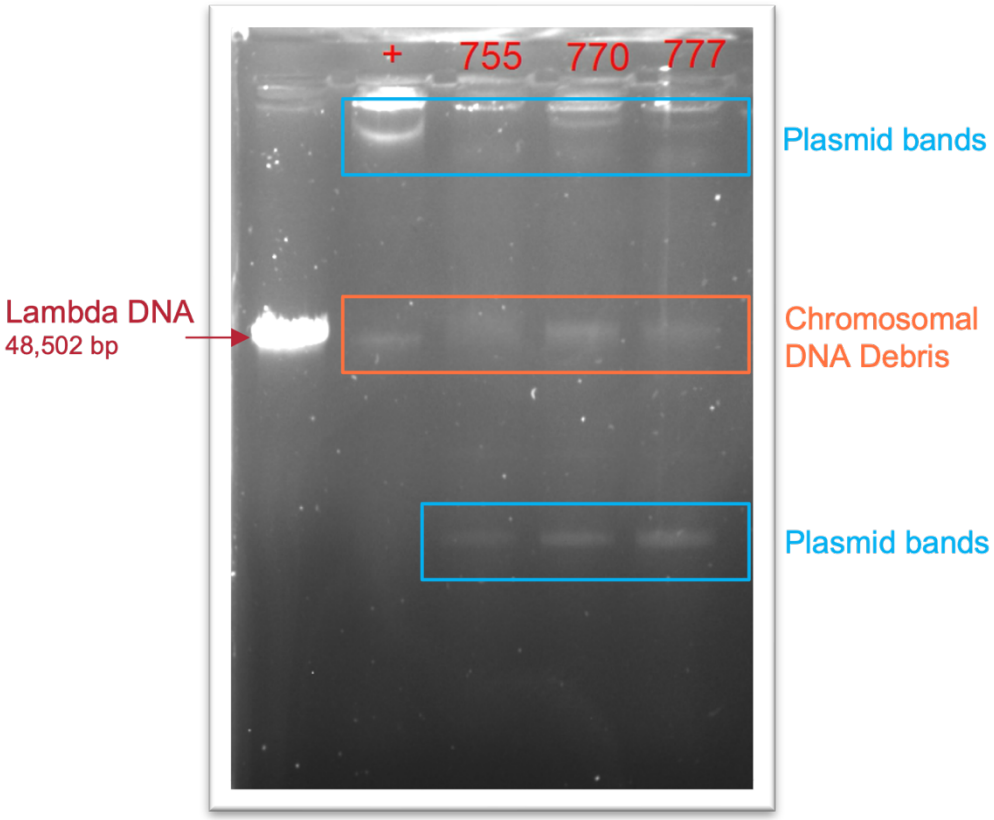


Figure 11. An example of the plasmid extraction result of *Escherichia coli* collections, displayed on an agarose gel. The collections with the IDs 755, 770, and 777 are shown in the three lanes on the rightmost part of the gel. The positive control of *Escherichia coli* containing a 65 kb plasmid is shown on the second lane, counting from the left. The Lambda DNA in the leftmost lane serves as a marker, as the chromosomal DNA debris resulting from the plasmid extraction process consistently aligns with its band.

doi:10.6342/NTU202503336

One thing to keep in mind about this procedure is that the absence of plasmid bands does not guarantee the sample is free of plasmids. Plasmid bands might not be visible due to various reasons, including the plasmid being too large to be displayed on the gel and the technical issues that occur during the extraction process. *Klebsiella pneumoniae* collections also underwent the extraction procedure, but the extraction results on the gel were not as visible as those from *Escherichia coli*. Therefore, we suspect that different bacterial species, or even strains, can yield different extraction quality results using the same extraction protocol. The extraction process might need to be adjusted based on different target species.

Antibiotic Susceptibility Testing

Materials and Methods

Antibiotic susceptibility testing was carried out using the minimum inhibitory concentration (MIC) via the broth microdilution method for the 21 *Escherichia coli* collections that displayed visible plasmid bands on gel to observe the resistance pattern between strains with plasmids. Ciprofloxacin, gentamicin, trimethoprim, sulfamethoxazole, amoxicillin, and cefoxitin were selected to be tested. The MIC results were interpreted by the epidemiological cut-off value (ECOFF) guideline, which is a guideline known to be suitable for AMR surveillance[28]. The ECOFF for sulfamethoxazole is currently lacking due to insufficient data on EUCAST. Based on the current *Escherichia coli* MIC distribution for sulfamethoxazole on the EUCAST Antimicrobial Wild Type Distributions of Microorganisms[90], we set an approximate, unofficial value of 32 mg/L to distinguish wild-type and non-wild-type *Escherichia coli* for sulfamethoxazole. The distribution of he current Escherichia coli MIC distribution for sulfamethoxazole on the EUCAST Antimicrobial Wild Type Distributions of Microorganisms is visualized in Appendix 7. With the final step of resistance screening,

doi:10.6342/NTU202503336

we can expect to filter strains with our criteria and select desired strains for precise sequencing to check for ARGs on plasmids.

Findings and Implications

Among the 21 *Escherichia coli* collections that displayed visible plasmid bands on gel, 20 are non-wild type to at least one tested antibiotic, and 1 is wild type to all tested antibiotics. The broth microdilution results of the tested collections are detailed in Appendix 8. With the MIC result paired with the previous plasmid extraction result, it is even more convincing that there are possible clones in our sample collections, as some collections display identical plasmid band position and resistance pattern for the six tested antibiotics. To avoid over-representation from cloned samples, the result from this subsection filtered out suspected clones and only kept one representative clonal collection using the following criteria to categorize suspected clones: the suspected clonal collections have to be from the same sample type, and have to at least have identical plasmid band position or resistance pattern based on our experimental results.

According to our self-built criteria for filtering clonal strains, 13 collections from *Escherichia coli* are suspected to be non-clonal strains (Figure 12). Amoxicillin, sulfamethoxazole, and trimethoprim are the most common multi-drug non-wild type combination of the 13 strains and originate from the fecal and oral sample types. Amoxicillin and sulfamethoxazole are the most common antibiotics that are found to be non-wild type when looking into the individual antibiotics. All the tested *Escherichia coli* strains are non-wild type to gentamicin based on the broth microdilution result.

The results indicate that the animal-originated samples contain *Escherichia coli* strains that are accompanied by plasmids and are resistant to antibiotics. The same experimental methodologies can be applied to other bacterial species in the collection or even to samples collected from other habitats. With this filtered information, we can select the

doi:10.6342/NTU202503336

representable strains for further genomic sequencing and ecological experiments to obtain a detailed genomic profile and understand how plasmids facilitate resistance spread under the One Health framework.

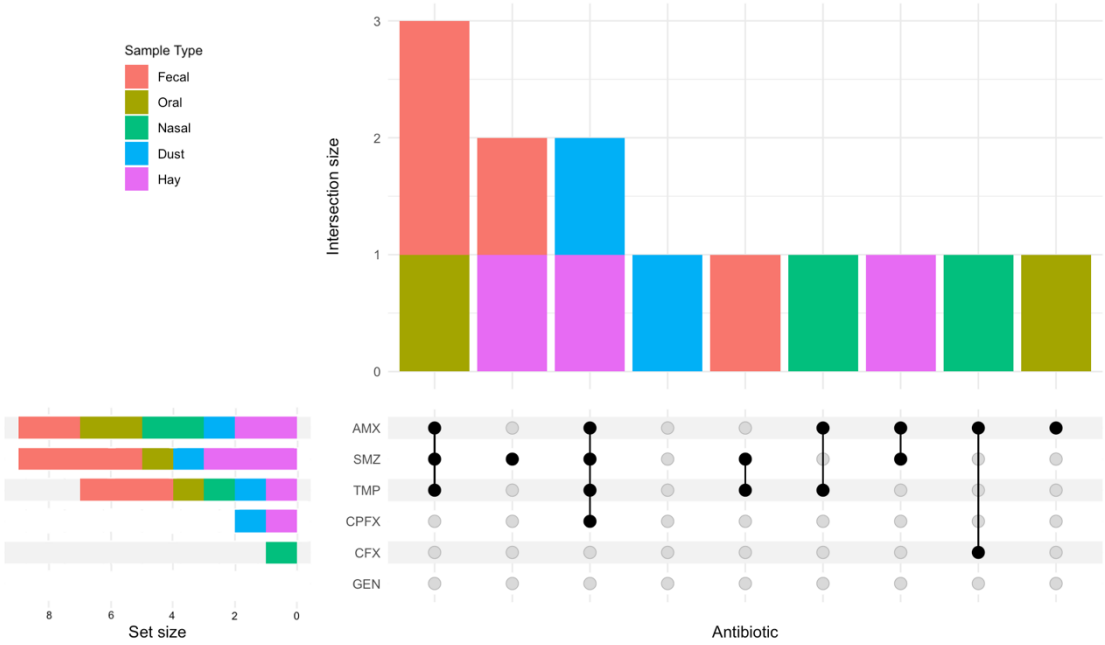


Figure 12. UpSet plot of the MIC results with 6 different antibiotics tested from 13 suspected non-clonal *Escherichia coli* strains originating from the French cattle farm samples. The black dots in every column of the "Antibiotic" panel represent the antibiotic combination to which the strain is non-wild type. Each row represents a single antibiotic tested in the MIC experiment. The abbreviations represent the following antibiotics. AMX: amoxicillin, SMZ: sulfamethoxazole, TMP: trimethoprim, CPFX: ciprofloxacin, CFX: cefoxitin, GEN: gentamicin. The "Intersection size" stacked bar chart represents the number of strains and their original sample type grouped by colors that displayed the corresponding non-wild type combination from the column of the "Antibiotic" panel. The "Set size" bar chart represents the number of strains and their original sample type grouped by colors that are non-wild type to the single antibiotic listed on each row of the "Antibiotic" panel.

Concluding Remarks

In this section, we utilized *Escherichia coli* strains to demonstrate the process to filter and select suitable strains for further studies (Figure 13). The identification of isolates

doi:10.6342/NTU202503336

carrying plasmids within high-risk genera may support the idea that certain keystone strains play a crucial role in the spread of resistance. Strains confirmed to carry both plasmids and resistance traits may serve as useful models for future studies examining how AMR is disseminated at the molecular level. Furthermore, the same species carrying plasmids and resistance traits that are consistently present in both animal and environmental samples might indicate that these are the crucial strains facilitating the cross-habitat transmission of resistance. Investigating these strains may provide insights into the mechanisms and processes involved in the spread of resistance between environmental and animal reservoirs.

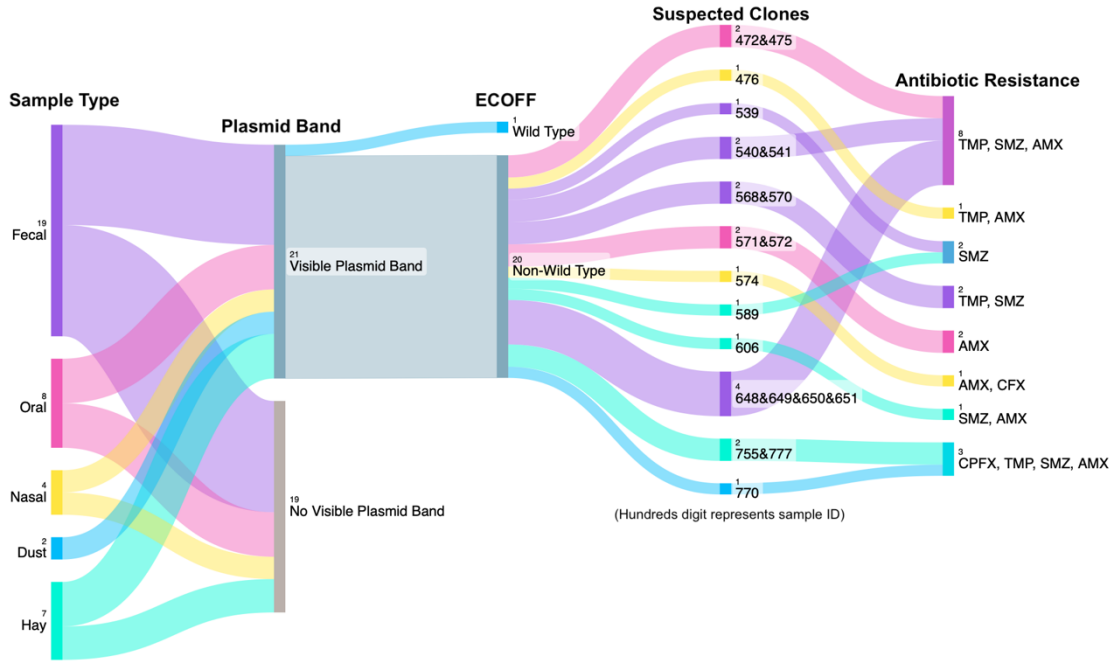


Figure 13. Overview of the categorizing processes for the 40 *Escherichia coli* collections visualized through a Sankey diagram. The flow of the stages goes from left to right. The titles of the stages are labeled on the uppermost nodes in each stage. The color of the flows and nodes represents the original sample type, except for the "Visible Plasmid Band" node and flow. The abbreviations represent the following antibiotics. AMX: amoxicillin, SMZ: sulfamethoxazole, TMP: trimethoprim, CPFX: ciprofloxacin, CFX: cefoxitin, GEN: gentamicin. The figure was created with SankeyMATIC.

doi:10.6342/NTU202503336

4.2. Resistance in Gram-Positive Bacteria from Japan

Studies have shown that manure used for crops has the potential to pose threats to the One Health sectors regarding the issue of AMR, especially the spread of ARGs to cropland soil and food crops[91,92]. Based on the observations, manure containing resistance to antibiotics might cause serious concerns in the One Health framework. Therefore, the objective of this section of the study is to explore whether crop fertilizer containing manure feces from food-producing animals has antibiotic resistance potential.

Materials and Methods

To evaluate AMR in livestock-associated bacteria with direct contact to the environment and potential food-related transmission pathways, manure samples containing cattle feces were collected from a seed company that produces vegetable crops in Japan. The study prioritized the isolation of Gram-positive bacteria, where the ESKAPEE pathogens *Enterococcus faecium* and *Staphylococcus aureus* belong. The two pathogens are noted for their environmental persistence and ability to engage in horizontal gene transfer through structured communities such as biofilm that promote close cell-to-cell contact and exhibit resistance to beta-lactam antibiotics[93-96].

Selective media favoring Gram-positive ESKAPEE pathogen *Staphylococcus aureus* growth, Mannitol Salt Agar (MSA) added with cefoxitin, were used for initial culture[97]. Isolates were tested using disk diffusion methods with beta-lactam antibiotics, including cefazolin, cefmetazole, cefoxitin, and oxacillin. Taxonomy of the isolated strains was then identified via the Bruker MALDI-TOF Biotyper.

Findings and Implications

Identified species included *Niallia circulans* (formerly *Bacillus circulans*), *Siminovitchia fordii* (formerly *Bacillus fordii*), and *Paenibacillus lautus*. Over half of the

doi:10.6342/NTU202503336

antibiotic disks tested showed no measurable inhibition zone, which may suggest potential resistance (Table 3). However, due to the lack of established interpretive criteria for these species-antibiotic combinations in the present disk diffusion standards, definitive conclusions about resistance status cannot be made.

	N. circulans (1)	*N. circulans* (2)	*S. fordii*	*P. lautus*	*N. circulans* (3)	*N. circulans* (4)	*N. circulans* (5)	*S. aureus* (+)
Oxacillin	0	0	0	0	0	0	0	27
Cefoxitin	0	0	0	15	0	0	0	27
Cefmetazole	0	0	0	13	0	0	0	32
Cefazolin	17	24	12	15	23.5	21.5	20	41

Table 3. Disk diffusion result of the isolated colonies. The columns represent the identified bacterial strains. The "(+)" symbol denotes a positive control strain for susceptibility. Multiple colonies were identified as *N. circulans*. To differentiate these colonies, numerical labels in parentheses (e.g., (1), (2), etc.) were assigned. The rows represent the antibiotic disks used in the test. The numerical values in the table indicate the diameter of the inhibition zones, measured in millimeters. The susceptibility of the strains to the tested antibiotics could not be determined due to the lack of established interpretive guidelines for the species analyzed.

This limitation reflects a broader challenge in assessing phenotypic resistance in environmental samples, where non-clinical bacterial species are often not covered by existing guidelines, which once again proves the importance of utilizing multiple methods to complement their limitations. Nevertheless, the findings suggest that Gram-positive resistant bacteria may be present in livestock manure. The rich organic matter and dense microbial population in manure could create micro-ecological niches that support genetic exchange, especially in soils that have been described as resistance hotspots[86]. *Niallia circulans*, a clinical pathogen commonly found in the environment[98], may serve as a manure model for exploring Gram-positive resistance mechanisms in the context of One Health. Altogether, these observations suggest that manure could act as a long-term reservoir for resistant bacteria that persist in the environment.

doi:10.6342/NTU202503336

Chapter 5

Conclusion and Discussion

5.1. Human: Decision Maker for Anthropogenic Factors

In the chapter discussing food-producing animal antibiotic policy and usage, distinct differences were observed among the three countries. Japan and Taiwan demonstrated a closer alignment in their regulatory frameworks when compared to France, a trend that aligns with earlier observations regarding continental differences in ARG profiles[63]. Although various factors may play a role in this difference, it would be beneficial for subsequent investigations to examine if changes in regulatory design have a direct impact on resistome composition in humans, animals, and the environment in targeted countries.

The ongoing changes in the approval of antibiotics within the feed sector present a unique and opportune moment to examine the relationship between regulatory modifications and their ecological impacts. Unlike the human health sector, where reductions in antibiotic use are often slow due to clinical demand, the feed additive landscape allows more frequent policy adjustments, which can be tracked and analyzed over time.

Our analysis of authorized veterinary antibiotics revealed substantial variation in the diversity and abundance of approved drug classes. Taiwan had a notably broader range of authorized antibiotics for food-producing animals, which may serve as an important factor when assessing resistance risk. Comparing which drugs are used exclusively in Taiwan or used at significantly higher levels than in other countries may offer insight into country-specific resistance trends.

doi:10.6342/NTU202503336

Nevertheless, regulation and authorization alone are insufficient for fully evaluating resistance development. Including actual antibiotic usage data offers a more reliable foundation for assessment. In this regard, the lack of publicly available veterinary antibiotic usage data from Taiwan presents a major limitation. This is not an isolated case: a previous study reported that not many countries had established routine veterinary antibiotic usage reporting systems[29]. Among those that had, our study identified that only EU member states and seven other countries, including Canada, Chile, Japan, South Korea, New Zealand, the UK, and the USA, had updated reports available at least through 2022.

It is promising to see that global recognition and advocacy for the One Health approach have motivated national governments to implement measures. In 2025, the Taiwanese government initiated the "One Health Framework Antibiotic Resistance Surveillance System" project, marking a significant change in emphasis on addressing AMR across various sectors, including clinical, environmental, and agricultural areas[99]. The project also incorporates wastewater surveillance as part of its environmental monitoring strategy. While Taiwan already has robust systems in place for clinical antibiotic usage surveillance, the infrastructure for monitoring veterinary antibiotic usage is still being developed. The inclusion of publicly accessible veterinary antibiotic usage data would be an important step toward enabling more comprehensive cross-national research on food-producing animal contributions to AMR.

5.2. Environment: Wastewater Samples as Surveillance Model

Urban wastewater is one type of environmental sample that is increasingly acknowledged to be suitable for broad AMR surveillance due to its cost-effectiveness and capacity to capture microbial signatures from a wide range of diverse human

doi:10.6342/NTU202503336

populations[6,63]. Combining metagenomic sequencing with wastewater analysis facilitates a genome-level investigation of resistance, providing crucial insights into the presence and diversity of ARGs, as well as the potential identification of emerging resistance trends[63].

While wastewater-based metagenomics presents several advantages, it also has notable drawbacks. The precision and thoroughness of ARG detection are greatly impacted by the extent of the annotated reference database's completeness. The absence of ARGs, particularly novel or poorly characterized ARGs, makes detection impossible through this method, thereby constraining the analytical scope[25].

This limitation was evident in the study, particularly when assessing links between ARGs and anthropogenic drivers like food-producing animal antibiotic usage. We hypothesized that ARGs corresponding to currently approved feed additives might serve as useful indicators for tracing agricultural influence. However, it appears that some gene types are either lacking or not adequately represented in the PanRes database utilized by ARGprofiler for this analysis, making it challenging to accurately identify feed-related ARGs.

Moreover, the shared application of antibiotics in both human and veterinary medicine makes it challenging to pinpoint the exact source of selection for ARGs. This study explored veterinary antibiotic products that combine various drug classes of antibiotics, a mix that rarely occurs in clinical practice. It was believed that genes able to confer resistance to more than one drug class might serve as clearer indicators of the role veterinary practices play in AMR dissemination. However, the annotations related to multi-drug resistance may not be fully comprehensive or could be absent from the database, adding a dimension of uncertainty. It is crucial to recognize that multi-drug resistance can develop through a range of mechanisms and exposure patterns, rather than

doi:10.6342/NTU202503336

exclusively from simultaneous selective pressure[72]. Therefore, the idea of utilizing multi-drug class antibiotic products as an indicative link between veterinary factors and the wastewater samples representing human populations requires further justification.

Another limitation is the absence of detailed metadata for the wastewater samples utilized in this study. Although information regarding the country, city, geographic coordinates, and collection date was provided, more specific contextual details were absent, such as wastewater type, local industry, or temperature. This made it difficult to fully interpret certain anomalies, such as the two excluded samples from Japan (ERR4678591 and ERR4678592), which shared identical metadata with two other samples (ERR4678589 and ERR4678590) but demonstrated distinct outlier behavior. It is hypothesized that unrecorded environmental or infrastructural factors contributed to these differences.

In addition, this study observed that certain ARGs identified by ARGprofiler did not correspond to any entries in the given annotation database, indicating a possible gap between sequence detection and gene identification. This issue requires additional dialogue with the developers of the tool to verify the accuracy of the mapping and annotation processes.

This study demonstrates the value of wastewater metagenomics as a viable surveillance method for AMR. Nonetheless, identifying the sources of ARGs in complex pooled samples remains a significant challenge. Recent investigations have sought to address this limitation by integrating machine learning models that can predict the origins of ARGs[100]. This offers a possible path for future investigation into the environmental spread of resistance associated with antibiotic use in food-producing animals, framed within a One Health perspective.

doi:10.6342/NTU202503336

5.3. Animal: Phenotypic Profiling of Originated Strains

Though phenotypic profiling by itself seems outdated for identifying antibiotic resistance, it is still quite essential in complementing modern research methods[27,28]. In this study, targeted isolation of particular bacterial groups, such as Gram-negative and Gram-positive bacteria, as well as direct observation of their antibiotic susceptibility profiles, was conducted by culture-based phenotypic approaches.

The studies from Japan and France both employed culture-based isolation, MALDI-TOF species identification, and antibiotic resistance testing. Their areas of focus, however, reflected different aspects of the AMR landscape.

The France study focused on Gram-negative bacteria and their possibility for plasmid-mediated resistance transfer. Species such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* are frequently found to harbor conjugative plasmids that facilitate the efficient horizontal transfer of ARGs. Their cellular structure, including outer membranes and type IV secretion systems, enables plasmid transfer across diverse taxa and environments. These traits set up Gram-negative ESKAPEE bacteria as crucial carriers of high-risk ARGs, including ESBLs and carbapenemases[85,101].

The Japan study focused on Gram-positive bacteria isolated from cattle feces-included manure. Species such as *Staphylococcus aureus* and *Enterococcus faecalis* are less commonly associated with plasmid-driven resistance dissemination at the same scale. Rather, they flourish in niches favorable to biofilm development. Biofilms protect against antibiotics and desiccation while facilitating local gene exchange and the persistence of resistance[93,96].

This variation emphasizes ecological specialization: clinically important Gram-negative ESKAPEE bacteria thrive in dynamic habitats defined by genetic exchange and

doi:10.6342/NTU202503336

population turnover, while Gram-positive ESKAPEE bacteria have less ARG mobility but still can thrive in persistence via surface colonization and biofilm stability. With different methods facilitating the dissemination of AMR across sectors, distinct surveillance and mitigation strategies for the two bacterial groups might need to be applied.

Even though ESKAPEE pathogens are the group of bacterial species that cause serious concern in terms of AMR, other groups of bacteria, such as the generalist bacteria that are mentioned in the Gram-negative bacteria study, could be a group of keystone strains that are less well-studied compared to the ESKAPEE pathogens[102]. As a consequence, we discovered limitations in conducting the same studies on the bacterial species that are less studied. In the Gram-negative study conducted in France, the plasmid extraction protocol might yield different results based on the bacterial species. Therefore, the protocol might need to be adjusted for other species in the future. When comparing the MIC results with the ECOFF database, we also noticed the limitation that the ECOFF is missing in some species or antibiotics, such as the lack of ECOFF of sulfamethoxazole for *Escherichia coli*. A similar limitation was also found in the Gram-positive study in Japan, where there are no reference guidelines for the identified bacterial species that are more commonly found in the environment and are less clinically relevant.

Collectively, the results display the combined threats of genetic mobility and environmental persistence in the dissemination of AMR. Concurrent and distinctive monitoring designed for Gram-negative and Gram-positive bacteria for comprehending the ecological dynamics of resistance evolution and for informing One Health interventions might be beneficial to capture more precise resistance trends. Based on the limitation of a lack of susceptibility or cut-off value guidelines for less clinically known strains, it demonstrates that resistance phenotyping research is still crucial in the future,

doi:10.6342/NTU202503336

as releasing a guideline for a bacterial species or genus requires a large amount of experimental data from various conditions to provide indicative values[28].

5.4. Solution: Functional Metagenomics as One Health Gap-Filler

The study reveals a major gap in assessing how reduced antibiotic use in food-producing animals affects the spread of resistance within the One Health framework. While Japan, Taiwan, and France utilize different approaches to minimize the impacts of veterinary antibiotics, current surveillance and analytical methods limit the capacity to track alterations in resistance dissemination in the environment.

This study suggests that the persistent utilization of antibiotic feed additives as growth promoters in Japan and Taiwan has a unique opportunity to serve as anthropogenic markers for monitoring the spread of resistance across One Health sectors. This proposition is supported by multiple reasons: (1) these regulations have been recently amended, creating a time-sensitive opportunity to observe the dynamics of resistance evolution in real-world contexts; (2) a natural negative control exists in France, where such practices have been prohibited; (3) the remaining or recently withdrawn antibiotics are typically specific to veterinary medicine and very rarely utilized in human clinical settings; and (4) many of these compounds represent relatively novel classes of antibiotics.

Additionally, authorized multi-substance veterinary drugs, particularly those combining drug classes not typically co-formulated for human use, may contribute to the emergence of multi-drug resistance. Investigating whether such combinations play a role in resistance development could provide insight into both ecological and clinical implications. Previous studies have shown that novel antibiotics are equally vulnerable to resistance evolution as traditional ones, emphasizing the importance of monitoring both established and emerging compounds[71].

doi:10.6342/NTU202503336

Although Japan's Food Safety Commission has published the "Feed Additives Evaluation Reports," which assess the impact of additives on food safety and human health[103], there remains limited evaluation of their environmental implications. Given our findings that the environment can serve as a reservoir for ARGs, the evaluation of environmental AMR impact represents an important area for future research.

This study recommends applying functional metagenomics to assess the environmental impact of feed antibiotic use[104]. Environmental samples, including urban wastewater, livestock wastewater, manure, feces, and surrounding water bodies, should be collected from each country. Metadata, including collection site, sample type, date, temperature, and humidity, must be thoroughly documented. Prior research has demonstrated that climate change and population density can affect AMR patterns, highlighting the necessity of including environmental variables in the analysis of resistance dissemination[105]. This would facilitate further factor analysis to assess the relative degree environmental or anthropogenic variables influence AMR dissemination. This approach also requires collaboration with researchers worldwide to collect and build a global resistance profile for novel antibiotics.

Collected samples will undergo total DNA extraction and purification, followed by fragmentation into suitable sizes for library building. The fragments would thereafter be cloned into plasmid vectors. Transformed clones will be transformed into bacterial host strains and then undergo antibiotic resistance screening on agar media supplemented with targeted feed antibiotics or pharmaceutical combinations such as ampicillin and colistin.

Resistant colonies would be isolated and sequenced using both short-read and long-read sequencing, enabling reconstruction of full-length metagenomic contigs. Detected ARGs would be annotated against known resistance databases to assess novelty and

doi:10.6342/NTU202503336

function. The resulting gene profiles could be compiled into a custom reference database focusing on feed antibiotic-related resistance markers (Figure 14).

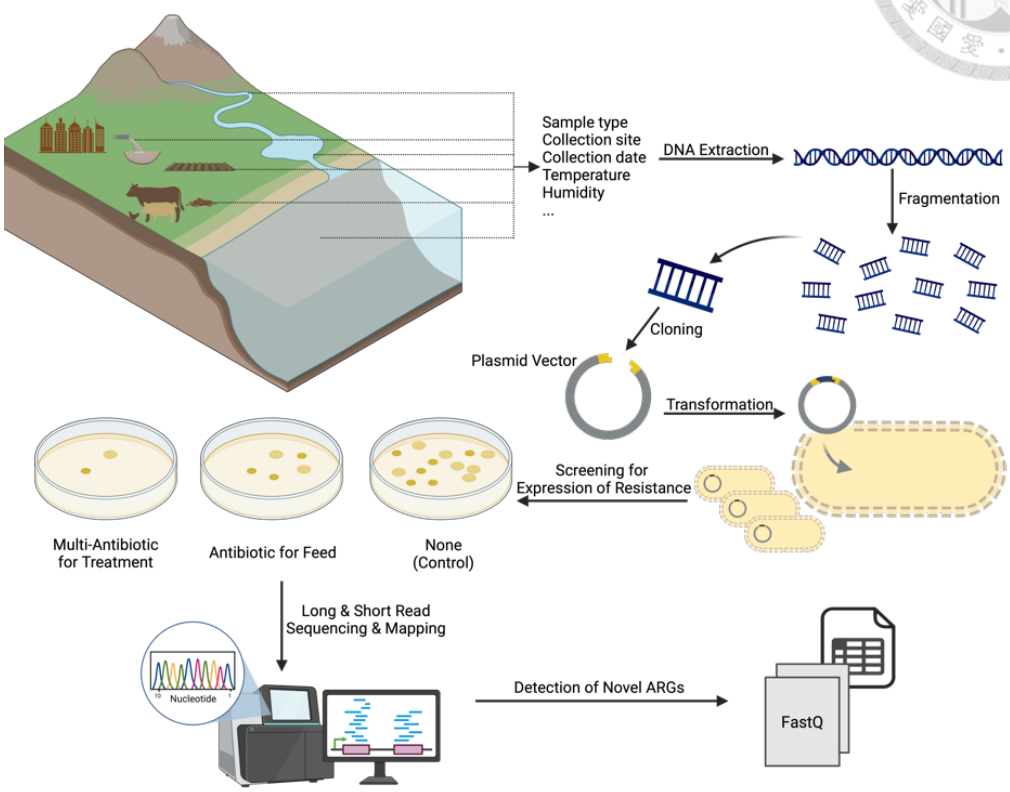


Figure 14. Graphical illustration of the proposed functional metagenomics workflow for novel food-producing animal-related ARGs screening. The figure was created with BioRender.com.

This method could facilitate systematic screening for ARGs associated with food-producing animal antibiotic usage, clarifying the interaction between human-induced factors and environmental variables in shaping resistance dynamics. With extensive metadata gathered, the same samples could also function as a valuable surveillance resource for comparing and analyzing the relative impact of environmental versus anthropogenic variables on AMR spread. If keystone strains mentioned in chapter 4 are identified in the future, they could also serve as an indicator to monitor how resistance originating from food-producing animals spreads across the One Health sectors. All these suggested approaches combined would greatly enhance the comprehension of AMR from a One Health viewpoint and facilitate the development of targeted solutions.

doi:10.6342/NTU202503336

5.5. Conclusion

AMR is a global threat that requires both One Health and international collaborative efforts. Coordination across human, animal, and environmental sectors is required. Among these sectors, the food-producing animal is a key contributor that requires effective surveillance.

This study examined how France, Japan, and Taiwan address AMR through veterinary antibiotic policy and usage, revealing factors that may influence resistance dynamics. With the limitations observed in each section of studies, it points out the importance of utilizing a multi-disciplinary approach to tackle and surveillance AMR in food-producing animals. We also proposed strategies and models to guide future research and monitoring efforts.

While countries face different agricultural, ecological, and regulatory circumstances that shape their approaches to AMR, the shared objective remains the same: reducing antimicrobial resistance in food-producing animals. A core principle that should be kept in mind while applying all strategies is the importance of improving animal welfare through better housing, nutrition, and health care as a foundation for minimizing antibiotic reliance and reducing resistance spread[106].

In conclusion, addressing AMR in food-producing animals will require sustained global and local efforts. The application of tools such as functional metagenomics can be anticipated to help enhance our understanding of resistance trends and inform more targeted actions.

doi:10.6342/NTU202503336

References

1. Szoke, Z., Fauszt, P., Mikolas, M., David, P., Szilagyi-Tolnai, E., Pesti-Asboth, G., Homoki, J.R., Kovacs-Forgacs, I., Gal, F., Stundl, L., et al. (2025). Comprehensive analysis of antimicrobial resistance dynamics among broiler and duck intensive production systems. Sci Rep *15*, 4673. 10.1038/s41598-025-89432-z.
2. World Health Organization (2023). Antimicrobial resistance. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.
3. Salam, M.A., Al-Amin, M.Y., Salam, M.T., Pawar, J.S., Akhter, N., Rabaan, A.A., and Alqumber, M.A.A. (2023). Antimicrobial Resistance: A Growing Serious Threat for Global Public Health. Healthcare (Basel) *11*. 10.3390/healthcare11131946.
4. World Health Organization (2024). One Health priority research agenda for antimicrobial resistance in the WHO European Region. WHO/EURO:2024-9510-49282-73655.
5. Zhang, T., Nickerson, R., Zhang, W., Peng, X., Shang, Y., Zhou, Y., Luo, Q., Wen, G., and Cheng, Z. (2024). The impacts of animal agriculture on One Health-Bacterial zoonosis, antimicrobial resistance, and beyond. One Health *18*, 100748. 10.1016/j.onehlt.2024.100748.
6. Larsson, D.G.J., and Flach, C.F. (2022). Antibiotic resistance in the environment. Nat Rev Microbiol *20*, 257-269. 10.1038/s41579-021-00649-x.
7. Andersson, D.I., Balaban, N.Q., Baquero, F., Courvalin, P., Glaser, P., Gophna, U., Kishony, R., Molin, S., and Tønjum, T. (2020). Antibiotic resistance: turning evolutionary principles into clinical reality. FEMS Microbiol Rev *44*, 171-188. 10.1093/femsre/fuaa001.
8. Castañeda-Barba, S., Top, E.M., and Stalder, T. (2024). Plasmids, a molecular cornerstone of antimicrobial resistance in the One Health era. Nat Rev Microbiol *22*, 18-32. 10.1038/s41579-023-00926-x.
9. Kuo, S.C., Huang, W.C., Wang, H.Y., Shiau, Y.R., Cheng, M.F., and Lauderdale, T.L. (2016). Colistin resistance gene mcr-1 in Escherichia coli isolates from humans and retail meats, Taiwan. J Antimicrob Chemother *71*, 2327-2329. 10.1093/jac/dkw122.
10. Kuo, S.-C., and Lauderdale, T.-L. (2017). Plasmid-Mediated Colistin Resistance. Infection Control Journal *27*, 29-33. 10.6526/ICJ.2017.104.
11. Rhouma, M., Madec, J.Y., and Laxminarayan, R. (2023). Colistin: from the shadows to a One Health approach for addressing antimicrobial resistance. Int J Antimicrob Agents *61*, 106713. 10.1016/j.ijantimicag.2023.106713.
12. Oyenuga, N., Cobo-Díaz, J.F., Alvarez-Ordóñez, A., and Alexa, E.A. (2024). Overview of Antimicrobial Resistant ESKAPEE Pathogens in Food Sources and Their Implications from a One Health Perspective. Microorganisms *12*. 10.3390/microorganisms12102084.
13. Xu, C., Kong, L., Gao, H., Cheng, X., and Wang, X. (2022). A Review of Current Bacterial Resistance to Antibiotics in Food Animals. Front Microbiol *13*, 822689. 10.3389/fmicb.2022.822689.
14. World Health Organization, Food and Agriculture Organization of the United Nations, United Nations Environment Programme, and World Organisation for Animal Health (2023). A one health priority research agenda for antimicrobial resistance. CC BY-NC-SA 3.0 IGO.

doi:10.6342/NTU202503336

15. Ritchie, H., and Spooner, F. (2024). Large amounts of antibiotics are used in livestock, but several countries have shown this doesn't have to be the case. https://ourworldindata.org/antibiotics-livestock.
16. Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., and Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. Proc Natl Acad Sci U S A *112*, 5649-5654. 10.1073/pnas.1503141112.
17. Tang, K.L., Caffrey, N.P., Nóbrega, D.B., Cork, S.C., Ronksley, P.E., Barkema, H.W., Polachek, A.J., Ganshorn, H., Sharma, N., Kellner, J.D., and Ghali, W.A. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. Lancet Planet Health *1*, e316-e327. 10.1016/s2542-5196(17)30141-9.
18. Van Boeckel, T.P., Glennon, E.E., Chen, D., Gilbert, M., Robinson, T.P., Grenfell, B.T., Levin, S.A., Bonhoeffer, S., and Laxminarayan, R. (2017). Reducing antimicrobial use in food animals. Science *357*, 1350-1352. 10.1126/science.aao1495.
19. Birgand, G., Castro-Sánchez, E., Hansen, S., Gastmeier, P., Lucet, J.C., Ferlie, E., Holmes, A., and Ahmad, R. (2018). Comparison of governance approaches for the control of antimicrobial resistance: Analysis of three European countries. Antimicrob Resist Infect Control *7*, 28. 10.1186/s13756-018-0321-5.
20. Bengtsson-Palme, J., Abramova, A., Berendonk, T.U., Coelho, L.P., Forslund, S.K., Gschwind, R., Heikinheimo, A., Jarquín-Díaz, V.H., Khan, A.A., Klümper, U., et al. (2023). Towards monitoring of antimicrobial resistance in the environment: For what reasons, how to implement it, and what are the data needs? Environ Int *178*, 108089. 10.1016/j.envint.2023.108089.
21. EU-JAMRAI. Surveillance. https://eu-jamrai.eu/surveillance/#.
22. Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., et al. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat Commun *10*, 1124. 10.1038/s41467-019-08853-3.
23. Berglund, F., Österlund, T., Boulund, F., Marathe, N.P., Larsson, D.G.J., and Kristiansson, E. (2019). Identification and reconstruction of novel antibiotic resistance genes from metagenomes. Microbiome *7*, 52. 10.1186/s40168-019-0670-1.
24. Liu, Z., Klümper, U., Liu, Y., Yang, Y., Wei, Q., Lin, J.G., Gu, J.D., and Li, M. (2019). Metagenomic and metatranscriptomic analyses reveal activity and hosts of antibiotic resistance genes in activated sludge. Environ Int *129*, 208-220. 10.1016/j.envint.2019.05.036.
25. Gschwind, R., Ugarcina Perovic, S., Weiss, M., Petitjean, M., Lao, J., Coelho, L.P., and Ruppé, E. (2023). ResFinderFG v2.0: a database of antibiotic resistance genes obtained by functional metagenomics. Nucleic Acids Res *51*, W493-w500. 10.1093/nar/gkad384.
26. Corona, F., and Martinez, J.L. (2013). Phenotypic Resistance to Antibiotics. Antibiotics (Basel) *2*, 237-255. 10.3390/antibiotics2020237.
27. Manaia, C.M. (2017). Assessing the Risk of Antibiotic Resistance Transmission from the Environment to Humans: Non-Direct Proportionality between Abundance and Risk. Trends Microbiol *25*, 173-181. 10.1016/j.tim.2016.11.014.

doi:10.6342/NTU202503336

28. Kahlmeter, G., and Turnidge, J. (2022). How to: ECOFFs-the why, the how, and the don'ts of EUCAST epidemiological cutoff values. Clin Microbiol Infect *28*, 952-954. 10.1016/j.cmi.2022.02.024.
29. Mulchandani, R., Wang, Y., Gilbert, M., and Van Boeckel, T.P. (2023). Global trends in antimicrobial use in food-producing animals: 2020 to 2030. PLOS Glob Public Health *3*, e0001305. 10.1371/journal.pgph.0001305.
30. Van Boeckel, T.P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N.G., Gilbert, M., Bonhoeffer, S., and Laxminarayan, R. (2019). Global trends in antimicrobial resistance in animals in low- and middle-income countries. Science *365*. 10.1126/science.aaw1944.
31. Kasimanickam, V., Kasimanickam, M., and Kasimanickam, R. (2021). Antibiotics Use in Food Animal Production: Escalation of Antimicrobial Resistance: Where Are We Now in Combating AMR? Med Sci (Basel) *9*. 10.3390/medsci9010014.
32. Simjee, S., and Ippolito, G. (2022). European regulations on prevention use of antimicrobials from january 2022. Braz J Vet Med *44*, e000822. 10.29374/2527-2179.bjvm000822.
33. Ministry of Agriculture, Forestry and Fisheries, Japan (2025). 飼料及び飼料添加物の成分規格等に関する省令.
34. Ministry of Agriculture, Taiwan (2025). 動物用藥品使用準則.
35. Food and Agricultural Materials Inspection Center, Japan (2024). List of Feed Additives. http://www.famic.go.jp/ffis/feed/sub3_feedadditives_en.html.
36. The European Parliament and the Council of the European Union (2022). Regulation (EU) 2019/6 of the European Parliament and of the Council on veterinary medicinal products and repealing Directive 2001/82/EC.
37. Castanon, J.I. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. Poult Sci *86*, 2466-2471. 10.3382/ps.2007-00249.
38. The European Commission (2022). Commission Implementing Regulation (EU) 2022/1255 of 19 July 2022 designating antimicrobials or groups of antimicrobials reserved for treatment of certain infections in humans, in accordance with Regulation (EU) 2019/6 of the European Parliament and of the Council.
39. Ministry of Agriculture, Forestry and Fisheries, Japan (2014). 農林水産省令第 7 号.
40. Ministry of Agriculture, Forestry and Fisheries, Japan (2017). 農林水産省令第 71 号.
41. Ministry of Agriculture, Forestry and Fisheries, Japan (2018). 農林水産省令第 83 号.
42. Ministry of Agriculture, Forestry and Fisheries, Japan (2019). 農林水産省令第 38 号.
43. Ministry of Agriculture, Forestry and Fisheries, Japan (2019). 農林水産省令第 37 号.
44. Ministry of Agriculture, Taiwan (2014). 農防字第 1031472328 號.
45. Ministry of Agriculture, Taiwan (2015). 農防字第 1041473480 號.
46. Ministry of Agriculture, Taiwan (2016). 農防字第 1051472565 號.
47. Ministry of Agriculture, Taiwan (2022). 農防字第 1111470620 號.
48. Ministry of Agriculture, Taiwan (2023). 農防字第 1121470886 號.
49. Agency for Food, Environmental and Occupational Health & Safety, France (2025). Index des Médicaments vétérinaires autorisés en France. https://www.ircp.anmv.anses.fr.

doi:10.6342/NTU202503336

50. National Veterinary Assay Laboratory, Japan (2025). 動物用医薬品等データベース. https://www.vm.nval.go.jp.
51. Animal and Plant Health Inspection Agency, Taiwan (2025). 動物用藥品許可證查詢. https://amdrug2.aphia.gov.tw/license-query.
52. Global Antibiotic Research & Development Partnership (2025). AntibioticDB. https://antibioticdb.com.
53. Agency for Food, Environmental and Occupational Health & Safety, France (2023). Sales of Veterinary Medicinal Products Containing Antimicrobials in France in 2022.
54. Hosoi, Y., Matsuda, M., Kawanishi, M., Harada, S., Kumakawa, M., Sekiguchi, H., Asai, T., and Sekiya, T. (2024). Antimicrobial Use in the Animal Sector in Japan from 2011 to 2022. Antibiotics (Basel) *13*. 10.3390/antibiotics13121204.
55. Ministry of Agriculture and Food, France (2024). Écoantibio 3 : réduire les risques d'antibiorésistance et promouvoir le bon usage des antimicrobiens en médecine vétérinaire (plan national 2023-2028). https://agriculture.gouv.fr/le-plan-ecoantibio-3-2023-2028.
56. Zhao, C., Wang, Y., Mulchandani, R., and Van Boeckel, T.P. (2024). Global surveillance of antimicrobial resistance in food animals using priority drugs maps. Nat Commun *15*, 763. 10.1038/s41467-024-45111-7.
57. Emes, D., Naylor, N., Waage, J., and Knight, G. (2022). Quantifying the Relationship between Antibiotic Use in Food-Producing Animals and Antibiotic Resistance in Humans. Antibiotics (Basel) *11*. 10.3390/antibiotics11010066.
58. Durazzi, F., Pezzani, M.D., Arieti, F., Simonetti, O., Canziani, L.M., Carrara, E., Barbato, L., Onorati, F., Remondini, D., and Tacconelli, E. (2023). Modelling antimicrobial resistance transmission to guide personalized antimicrobial stewardship interventions and infection control policies in healthcare setting: a pilot study. Sci Rep *13*, 15803. 10.1038/s41598-023-42511-5.
59. Statistics and Forecasting Department of the Ministry of Agriculture and Food, France (2023). Statistical Book 2022.
60. Japan, P.S.o.O.S.o. (2024). 畜産統計調査.
61. Ministry of Agriculture, Taiwan. (2022). 畜牧類農情統計調查結果(含產值)（110年）.
62. Central Intelligence Agency, U.S. (2025). The World Factbook. https://www.cia.gov/the-world-factbook/.
63. Munk, P., Brinch, C., Møller, F.D., Petersen, T.N., Hendriksen, R.S., Seyfarth, A.M., Kjeldgaard, J.S., Svendsen, C.A., van Bunnik, B., Berglund, F., et al. (2022). Genomic analysis of sewage from 101 countries reveals global landscape of antimicrobial resistance. Nat Commun *13*, 7251. 10.1038/s41467-022-34312-7.
64. Martiny, H.M., Pyrounakis, N., Petersen, T.N., Lukjančenko, O., Aarestrup, F.M., Clausen, P., and Munk, P. (2024). ARGprofiler-a pipeline for large-scale analysis of antimicrobial resistance genes and their flanking regions in metagenomic datasets. Bioinformatics *40*. 10.1093/bioinformatics/btae086.
65. Martiny, H.-M., Pyrounakis, N., Lukjančenko, O., Petersen, T.N., Aarestrup, F.M., Clausen, P.T.L.C., and Munk, P. (2024). PanRes - Collection of antimicrobial resistance genes. 10.5281/zenodo.8055115.
66. Ruscheweyh, H.J., Milanese, A., Paoli, L., Karcher, N., Clayssen, Q., Keller, M.I., Wirbel, J., Bork, P., Mende, D.R., Zeller, G., and Sunagawa, S. (2022). Cultivation-independent genomes greatly expand taxonomic-profiling

doi:10.6342/NTU202503336

capabilities of mOTUs across various environments. Microbiome *10*, 212. 10.1186/s40168-022-01410-z.
67. Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139-140. 10.1093/bioinformatics/btp616.
68. van Lingen, H.J., Suarez-Diez, M., and Saccenti, E. (2024). Normalization of gene counts affects principal components-based exploratory analysis of RNA-sequencing data. Biochim Biophys Acta Gene Regul Mech *1867*, 195058. 10.1016/j.bbagrm.2024.195058.
69. Pérez-Cobas, A.E., Gomez-Valero, L., and Buchrieser, C. (2020). Metagenomic approaches in microbial ecology: an update on whole-genome and marker gene sequencing analyses. Microb Genom *6*. 10.1099/mgen.0.000409.
70. Wang, B., Sun, F., and Luan, Y. (2024). Comparison of the effectiveness of different normalization methods for metagenomic cross-study phenotype prediction under heterogeneity. Sci Rep *14*, 7024. 10.1038/s41598-024-57670-2.
71. Daruka, L., Czikkely, M.S., Szili, P., Farkas, Z., Balogh, D., Grézal, G., Maharramov, E., Vu, T.H., Sipos, L., Juhász, S., et al. (2025). ESKAPE pathogens rapidly develop resistance against antibiotics in development in vitro. Nat Microbiol *10*, 313-331. 10.1038/s41564-024-01891-8.
72. Maeda, T., and Furusawa, C. (2024). Laboratory Evolution of Antimicrobial Resistance in Bacteria to Develop Rational Treatment Strategies. Antibiotics (Basel) *13*. 10.3390/antibiotics13010094.
73. De Oliveira, D.M.P., Forde, B.M., Kidd, T.J., Harris, P.N.A., Schembri, M.A., Beatson, S.A., Paterson, D.L., and Walker, M.J. (2020). Antimicrobial Resistance in ESKAPE Pathogens. Clin Microbiol Rev *33*. 10.1128/cmr.00181-19.
74. Kritsotakis, E.I., Lagoutari, D., Michailellis, E., Georgakakis, I., and Gikas, A. (2022). Burden of multidrug and extensively drug-resistant ESKAPEE pathogens in a secondary hospital care setting in Greece. Epidemiol Infect *150*, e170. 10.1017/s0950268822001492.
75. Breijyeh, Z., Jubeh, B., and Karaman, R. (2020). Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. Molecules *25*. 10.3390/molecules25061340.
76. Elshobary, M.E., Badawy, N.K., Ashraf, Y., Zatioun, A.A., Masriya, H.H., Ammar, M.M., Mohamed, N.A., Mourad, S., and Assy, A.M. (2025). Combating Antibiotic Resistance: Mechanisms, Multidrug-Resistant Pathogens, and Novel Therapeutic Approaches: An Updated Review. Pharmaceuticals (Basel) *18*. 10.3390/ph18030402.
77. Elshamy, A.A., Aboshanab, K.M., Yassien, M.A., and Hassouna, N.A. (2020). Prevalence of plasmid-mediated resistance genes among multidrug-resistant uropathogens in Egypt. Afr Health Sci *20*, 190-198. 10.4314/ahs.v20i1.24.
78. Bennett, P.M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol *153 Suppl 1*, S347-357. 10.1038/sj.bjp.0707607.
79. Molano, L.G., Hirsch, P., Hannig, M., Müller, R., and Keller, A. (2025). The PLSDB 2025 update: enhanced annotations and improved functionality for comprehensive plasmid research. Nucleic Acids Res *53*, D189-d196. 10.1093/nar/gkae1095.
80. Alcock, B.P., Huynh, W., Chalil, R., Smith, K.W., Raphenya, A.R., Wlodarski, M.A., Edalatmand, A., Petkau, A., Syed, S.A., Tsang, K.K., et al. (2023). CARD 2023: expanded curation, support for machine learning, and resistome prediction

doi:10.6342/NTU202503336

at the Comprehensive Antibiotic Resistance Database. Nucleic Acids Res *51*, D690-d699. 10.1093/nar/gkac920.
81. Yusuf, I., Skiebe, E., and Wilharm, G. (2023). Evaluation of CHROMagar Acinetobacter and MacConkey media for the recovery of Acinetobacter baumannii from soil samples. Lett Appl Microbiol *76*. 10.1093/lambio/ovac051.
82. Sigma-Aldrich (2018). 50875 GSP Agar (Pseudomonas Aeromonas Selective Agar acc. to Kielwein, Glutamate Starch Phenol Red Agar).
83. Prasad, M., Shetty, S.K., Nair, B.G., Pal, S., and Madhavan, A. (2022). A novel and improved selective media for the isolation and enumeration of Klebsiella species. Appl Microbiol Biotechnol *106*, 8273-8284. 10.1007/s00253-022-12270-w.
84. Jung, B., and Hoilat, G.J. (2025). MacConkey Medium. In StatPearls, (StatPearls Publishing Copyright © 2025, StatPearls Publishing LLC.).
85. Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., and Hordijk, J. (2018). Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J Antimicrob Chemother *73*, 1121-1137. 10.1093/jac/dkx488.
86. Wyres, K.L., and Holt, K.E. (2018). Klebsiella pneumoniae as a key trafficker of drug resistance genes from environmental to clinically important bacteria. Curr Opin Microbiol *45*, 131-139. 10.1016/j.mib.2018.04.004.
87. Lam, M.M.C., and Hamidian, M. (2024). Examining the role of Acinetobacter baumannii plasmid types in disseminating antimicrobial resistance. NPJ Antimicrob Resist *2*, 1. 10.1038/s44259-023-00019-y.
88. Cazares, A., Moore, M.P., Hall, J.P.J., Wright, L.L., Grimes, M., Emond-Rhéault, J.G., Pongchaikul, P., Santanirand, P., Levesque, R.C., Fothergill, J.L., and Winstanley, C. (2020). A megaplasmid family driving dissemination of multidrug resistance in Pseudomonas. Nat Commun *11*, 1370. 10.1038/s41467-020-15081-7.
89. Letunic, I., and Bork, P. (2024). Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. Nucleic Acids Res *52*, W78-w82. 10.1093/nar/gkae268.
90. European Committee on Antimicrobial Susceptibility Testing. (2025). Data from the EUCAST MIC distribution website. https://www.eucast.org.
91. Zalewska, M., Błażejewska, A., Czapko, A., and Popowska, M. (2021). Antibiotics and Antibiotic Resistance Genes in Animal Manure - Consequences of Its Application in Agriculture. Front Microbiol *12*, 610656. 10.3389/fmicb.2021.610656.
92. Wang, F.H., Qiao, M., Chen, Z., Su, J.Q., and Zhu, Y.G. (2015). Antibiotic resistance genes in manure-amended soil and vegetables at harvest. J Hazard Mater *299*, 215-221. 10.1016/j.jhazmat.2015.05.028.
93. Șchiopu, P., Toc, D.A., Colosi, I.A., Costache, C., Ruospo, G., Berar, G., Gălbău Ș, G., Ghilea, A.C., Botan, A., Pană, A.G., et al. (2023). An Overview of the Factors Involved in Biofilm Production by the Enterococcus Genus. Int J Mol Sci *24*. 10.3390/ijms241411577.
94. Hunashal, Y., Kumar, G.S., Choy, M.S., D'Andréa É, D., Da Silva Santiago, A., Schoenle, M.V., Desbonnet, C., Arthur, M., Rice, L.B., Page, R., and Peti, W. (2023). Molecular basis of β-lactam antibiotic resistance of ESKAPE bacterium E. faecium Penicillin Binding Protein PBP5. Nat Commun *14*, 4268. 10.1038/s41467-023-39966-5.

doi:10.6342/NTU202503336

95. Alexander, J.A.N., Worrall, L.J., Hu, J., Vuckovic, M., Satishkumar, N., Poon, R., Sobhanifar, S., Rosell, F.I., Jenkins, J., Chiang, D., et al. (2023). Structural basis of broad-spectrum β-lactam resistance in Staphylococcus aureus. Nature *613*, 375-382. 10.1038/s41586-022-05583-3.
96. Peng, Q., Tang, X., Dong, W., Sun, N., and Yuan, W. (2022). A Review of Biofilm Formation of Staphylococcus aureus and Its Regulation Mechanism. Antibiotics (Basel) *12*. 10.3390/antibiotics12010012.
97. Sharp, S.E., and Searcy, C. (2006). Comparison of mannitol salt agar and blood agar plates for identification and susceptibility testing of Staphylococcus aureus in specimens from cystic fibrosis patients. J Clin Microbiol *44*, 4545-4546. 10.1128/jcm.01129-06.
98. Russo, A., Tarantino, U., d'Ettorre, G., Della Rocca, C., Ceccarelli, G., Gasbarra, E., Venditti, M., and Iundusi, R. (2021). First report of spondylodiscitis caused by Bacillus circulans in an immunocompetent patient: Clinical case and review of the literature. IDCases *23*, e01058. 10.1016/j.idcr.2021.e01058.
99. Ministry of Health and Welfare, Taiwan (2024). 國家級防疫一體抗生素抗藥性管理行動計畫.
100. Li, L.G., Yin, X., and Zhang, T. (2018). Tracking antibiotic resistance gene pollution from different sources using machine-learning classification. Microbiome *6*, 93. 10.1186/s40168-018-0480-x.
101. Partridge, S.R., Kwong, S.M., Firth, N., and Jensen, S.O. (2018). Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev *31*. 10.1128/cmr.00088-17.
102. Loos, D., Filho, A., Dutilh, B.E., Barber, A.E., and Panagiotou, G. (2024). A global survey of host, aquatic, and soil microbiomes reveals shared abundance and genomic features between bacterial and fungal generalists. Cell Rep *43*, 114046. 10.1016/j.celrep.2024.114046.
103. Food Safety Commission, Japan (2011). 動物用医薬品・飼料添加物評価書: アビラマイシン.
104. Dos Santos, D.F., Istvan, P., Quirino, B.F., and Kruger, R.H. (2017). Functional Metagenomics as a Tool for Identification of New Antibiotic Resistance Genes from Natural Environments. Microb Ecol *73*, 479-491. 10.1007/s00248-016-0866-x.
105. MacFadden, D.R., McGough, S.F., Fisman, D., Santillana, M., and Brownstein, J.S. (2018). Antibiotic Resistance Increases with Local Temperature. Nat Clim Chang *8*, 510-514. 10.1038/s41558-018-0161-6.
106. Rodrigues da Costa, M., and Diana, A. (2022). A Systematic Review on the Link between Animal Welfare and Antimicrobial Use in Captive Animals. Animals (Basel) *12*. 10.3390/ani12081025.

doi:10.6342/NTU202503336

Appendix

1. Inclusion of Outlier Samples in Metagenomic Analysis

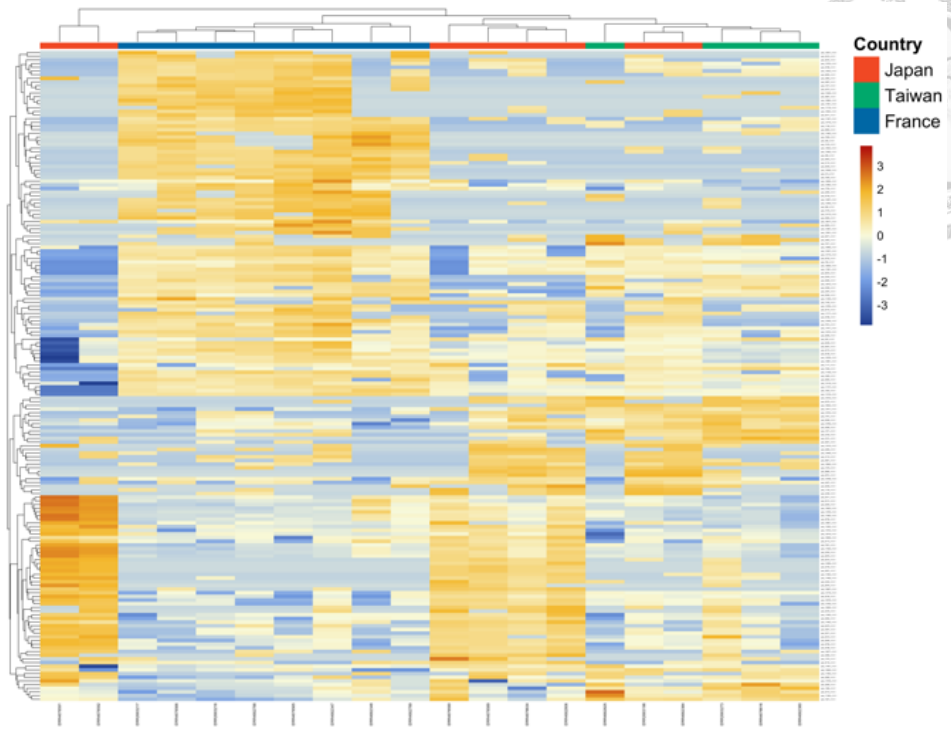
The two Japanese wastewater samples (ERR4678591 and ERR4678592) included in this appendix were part of the original dataset utilized in the resistome study. However, they were later excluded from the final analysis due to their evident outlier behavior, as indicated by the results shown in the heatmap and PCA figures presented below.

As shown in the hierarchical clustering heatmap and PCA plot that was generated using the same methods described in the main analysis (Figures 5 and 6), the two outlier samples from Japan exhibit clear separation from the rest of the Japan cohort. The heatmap shows that the two samples diverge furthest from the others. This is possibly due to an overexpression of several ARGs relative to the rest of the dataset (Figure i). The PCA plot further supports this trend, as the two samples are located away from the major Japan cluster, indicating distinct ARG profiles (Figure ii).

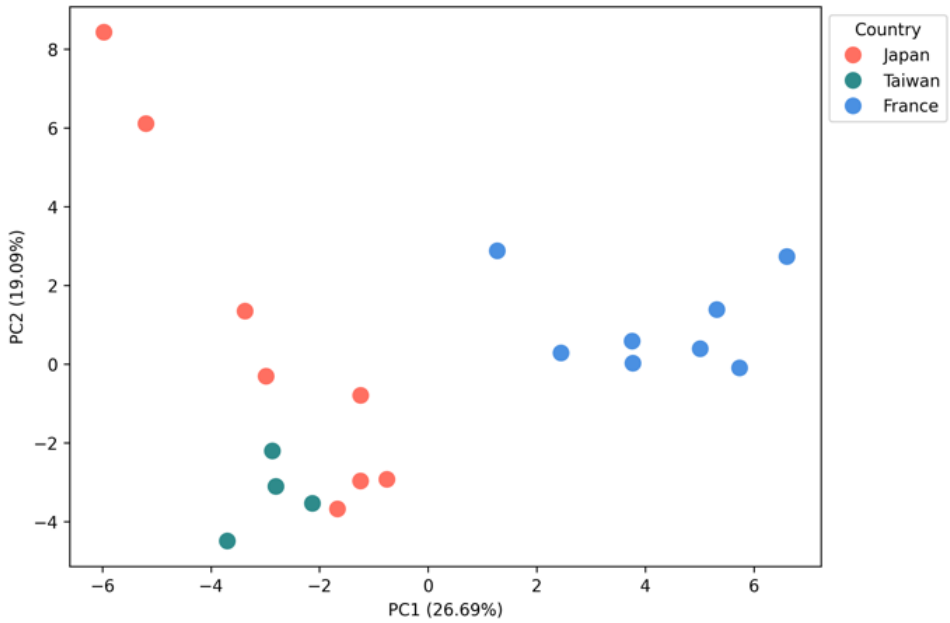
The observed patterns may indicate site-specific or environmental factors that influenced the composition of the resistome in these samples. The ENA metadata offers limited background information, such as wastewater type and local environmental conditions, complicating the identification of the precise factors influencing these deviations.

This extended visualization acts as a comparative reference to compare and justify the exclusion of these samples from the main analysis. It also serves as an indicative example to demonstrate the complexity of factors, where the presence and absence of outlier samples considerably shift the clustering compositions, and the importance of providing detailed metadata to analyze the potential factors contributing to the outlier results.

doi:10.6342/NTU202503336



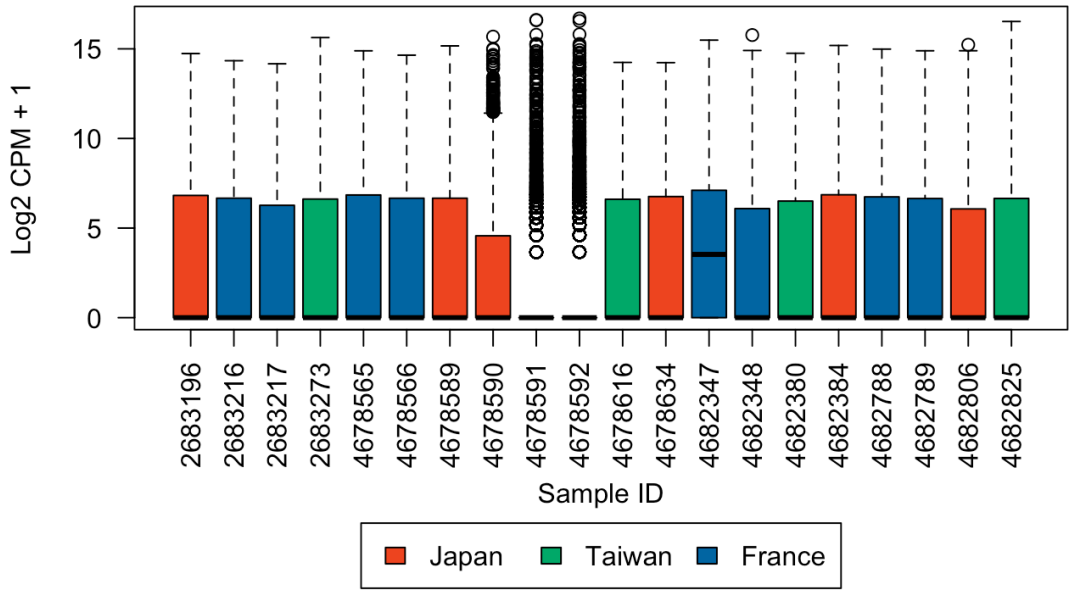
Appendix Figure i. Heatmap showing the ARGs (rows) and samples (columns) grouped by countries distinguished by color-coding, with the same methods applied in the main report (Figure 5). Two outlier samples from Japan are included at the leftmost of the two columns in this heatmap.



Appendix Figure ii. PCA plot based on ARG abundance, with the same methods applied in the main report (Figure 6). Two outlier samples from Japan are included in this plot, displayed above the value 4 in PC2.

doi:10.6342/NTU202503336

2. TMM Normalization Result for ARG Fragment Counts



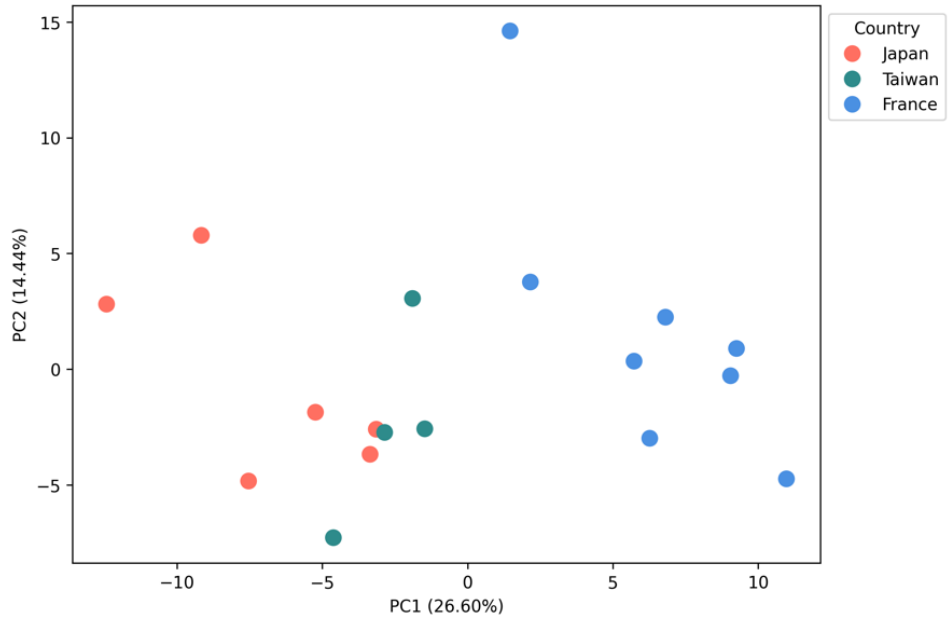
Appendix Figure iii. Box plot of TMM normalization result for the 20 wastewater samples studied in the main report. The "ERR" prefix from the original accession numbers is omitted from the sample IDs displayed in this figure. Sample IDs "4678591" and "4678592" are samples from Japan that displayed consistent outlier behaviors mentioned in Appendix 1 and were excluded from the analysis in the main report.

3. PCA Analysis with Original Multi-Drug Resistance Grouping

In the main report, multi-drug resistance annotations were grouped into a single category to reduce feature complexity. This appendix presents an alternative PCA plot of resistome composition based on drug class-level ARG abundance. Unlike the PCA shown in the main report, where all ARGs conferring resistance to multiple drug classes were grouped into a single "multi-drug" category, the analysis here retains the original annotation of each multi-drug resistance group, such as "beta-lactam/polymyxin."

doi:10.6342/NTU202503336

The analytical workflow described in the main report was employed. Two Japanese samples (ERR4678591 and ERR4678592), formerly classified as outliers, were excluded from this analysis.



Appendix Figure iv. PCA plot based on ARG abundance, with the same methods applied in the main report (Figure 6). Genes that confer more than one resistance class are grouped individually with their original drug class combinations in this plot, instead of categorizing all the classes that confer more than one resistance class into a single "multi-drug" group.

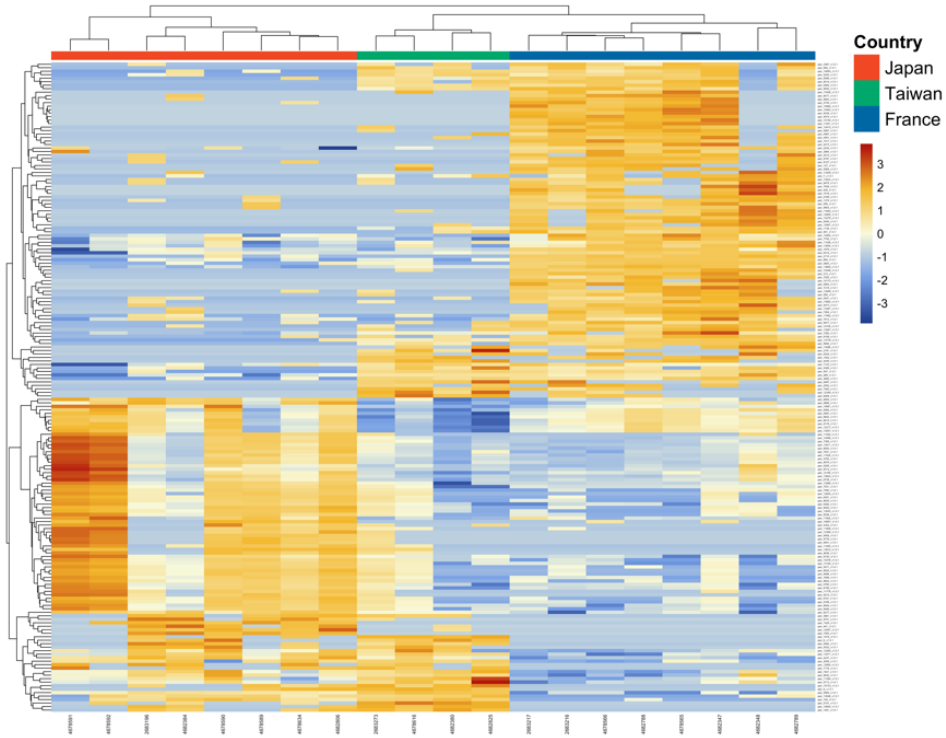
The result of the PCA plot reveals less distinct clustering compared to the main analysis (Figure 6). Nevertheless, separation by continental origin remains visible along the PC1, with the same result of France grouping separately from Japan and Taiwan (Figure iv). This observation still supports the continental variation in resistome profiles, even though it is with reduced resolution.

These findings reinforce the influence of ARG classification granularity on clustering patterns and highlight the importance of analytical consistency in comparative resistome studies.

doi:10.6342/NTU202503336

4. Impact of Non-Antibiotic Resistance Genes on Clustering Dynamics

To discover more about the broader co-selection dynamics that shape national resistome profiles, an additional hierarchical clustering analysis was performed by incorporating genes not only resistant to antibiotics but also to non-antibiotics such as metals and biocides. This analysis followed the same methodology used in the main report.



Appendix Figure v. Heatmap showing the ARGs (rows) and samples (columns) grouped by countries distinguished by color-coding, with the same methods applied in the main report (Figure 5). Two outlier samples from Japan are included in the two leftmost columns in this heatmap. Non-antibiotic resistance genes, such as metal and biocide resistance genes, are also included.

The result displayed that the inclusion of non-antibiotic resistance genes altered the clustering pattern observed in the main report. When these genes were considered, the cluster of samples from Taiwan shifted closer to those from France (Figure v), whereas in the antibiotic-only analysis, Taiwan had clustered more closely with Japan (Figure 5).

doi:10.6342/NTU202503336

This shift suggests that in certain situations, environmental factors such as metal contamination or other chemical stressors may have a greater impact on the composition of resistance genes than antibiotics alone.

The result demonstrates the importance of expanding resistome surveillance from antibiotic to non-antibiotic ARGs. Integrating non-antibiotic resistance elements into AMR profiling could improve our understanding of the ecological and anthropogenic pressures driving resistance patterns and highlight the multifactorial nature of AMR dissemination across countries.

5. Colistin Combination Products in the Human Sector

To assess the relevance of beta-lactam and polymyxin combination ARGs identified in non-clinical metagenomic samples, it is important to first evaluate whether such drug combinations are commonly used in human medicine. To this end, national drug registration databases from Taiwan, France, and Japan were consulted to identify authorized colistin-containing products and their corresponding formulations[1-3]. All data were retrieved on March 17th, 2025.

In Taiwan, the official Western medicine database currently lists 14 approved colistin-containing antibiotic products. Among these, a total of 12 products contain only colistin as the active ingredient. The rest of the 2 products include one with the combination of colistin and tetracycline, and another with colistin and erythromycin. Both combinations of products are used for ophthalmic infections. Additionally, 3 of the single-active-ingredient colistin products and both combination formulations are currently not in production.

doi:10.6342/NTU202503336

In France, drug authorization data were retrieved from the national drug database. As of the current search, 17 approved human-use antibiotic products contain colistin, and all of these products list colistin as the only active substance. No authorized human drugs in France combine colistin with beta-lactam antibiotics.

In Japan, the Pharmaceuticals and Medical Devices Agency database lists 6 approved antibiotic products containing colistin for human use. Among these, 1 product contains a combination of colistin and erythromycin, while 2 include colistin and chloramphenicol. All 3 combination products are used for ophthalmic infections. The remaining 3 products contain only colistin as the active ingredient. Additionally, 2 of the single-active-ingredient colistin products and 1 combination formulation are currently not in production.

Overall, the results show that colistin is most often used by itself in clinical settings, with only a few non-beta-lactam combinations observed. This supports the idea that beta-lactam/polymyxin combination resistance could be a potential indicator of veterinary or environmental origin.

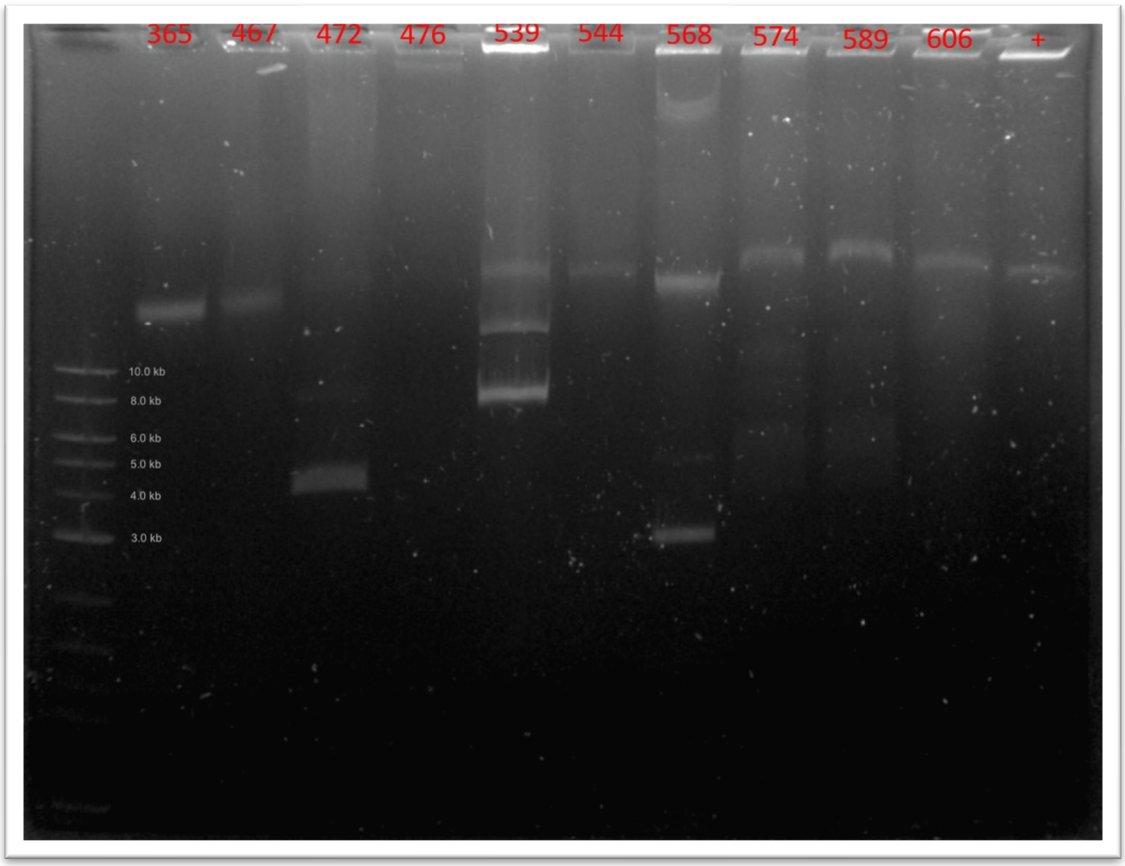
1. Taiwan Food and Drug Administration. 西藥許可證查詢. https://lmspiq.fda.gov.tw/web/DRPIQ/DRPIQLicSearch
2. France National Agency for the Safety of Medicines and Health Products. https://agence-prd.ansm.sante.fr/php/ecodex/index.php
3. Japan RAD-AR Council. 病院・クリニックで処方される医療用医薬品を検索できます. https://www.rad-ar.or.jp/siori/

doi:10.6342/NTU202503336

6. Gel Electrophoresis Image of Plasmid Extraction Result

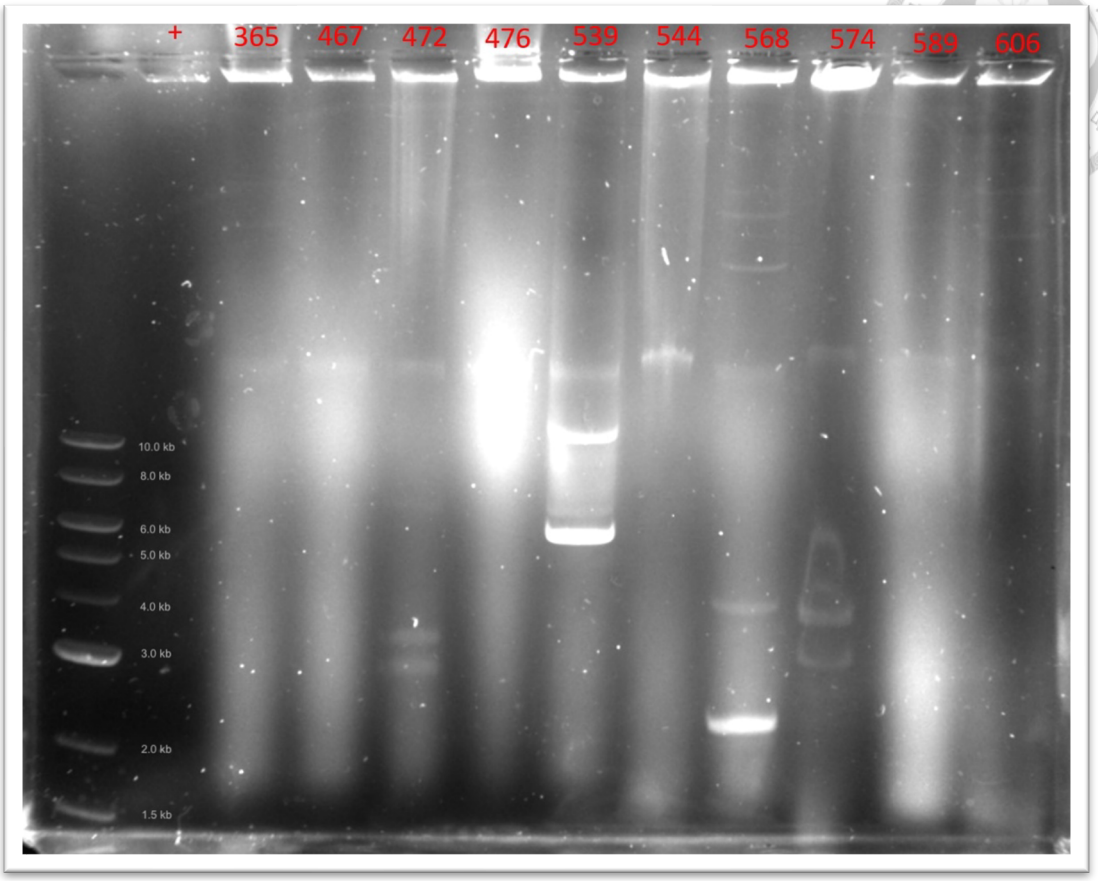
Based on the following gel images, 21 sample collections with ID 472, 475, 476, 539, 540, 541, 568, 570, 571, 572, 574, 589, 606, 648, 649, 650, 651, 688, 755, 770, and 777 were categorized as collections with visible plasmid bands via visual inspection.

a.

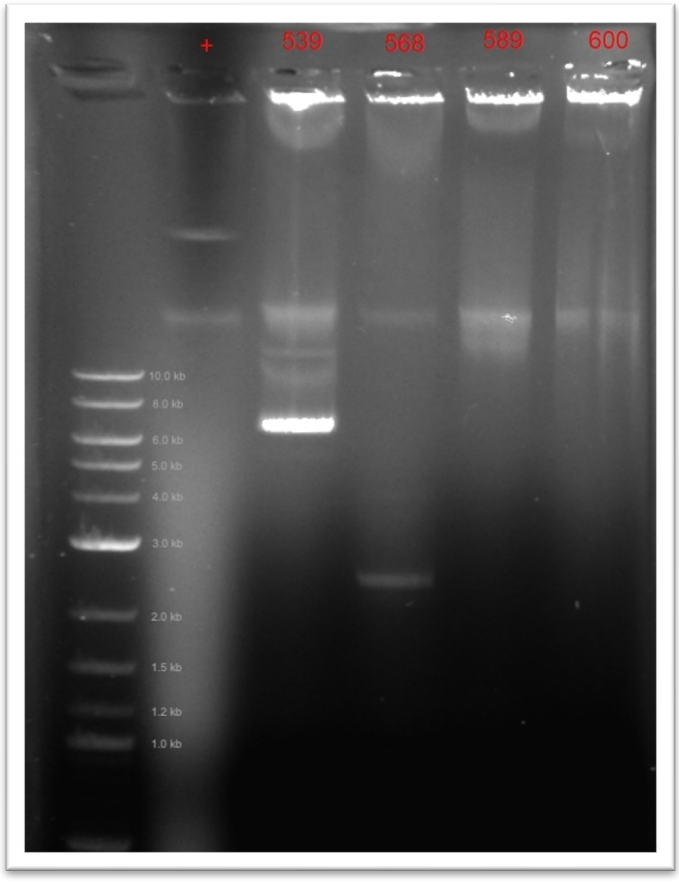


doi:10.6342/NTU202503336

b.

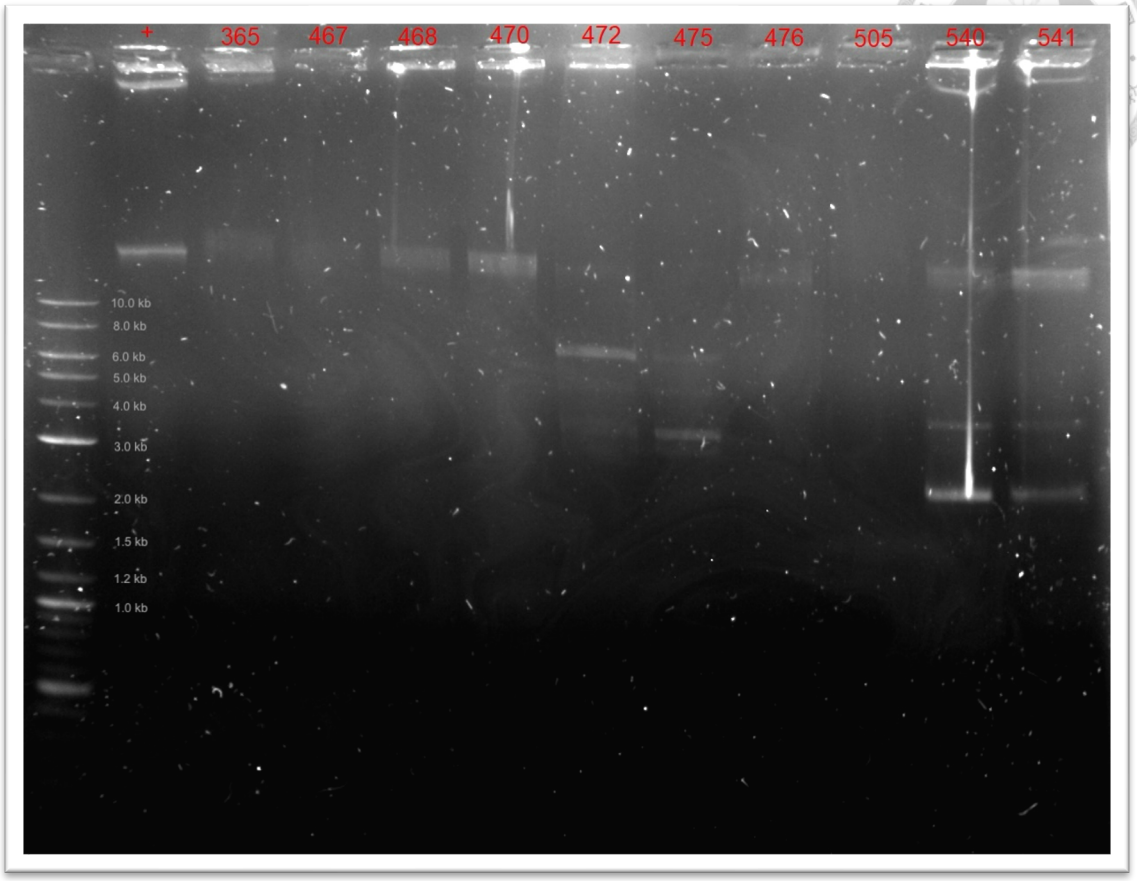


c.

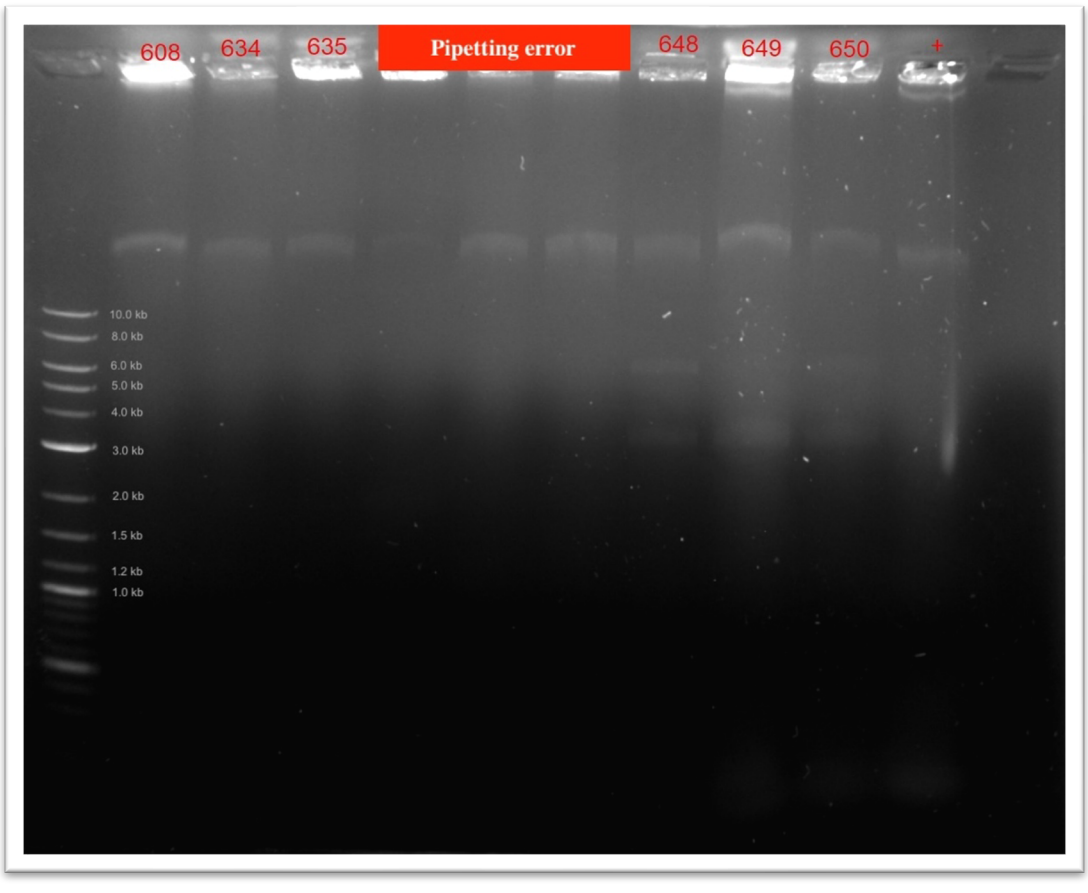


doi:10.6342/NTU202503336

d.

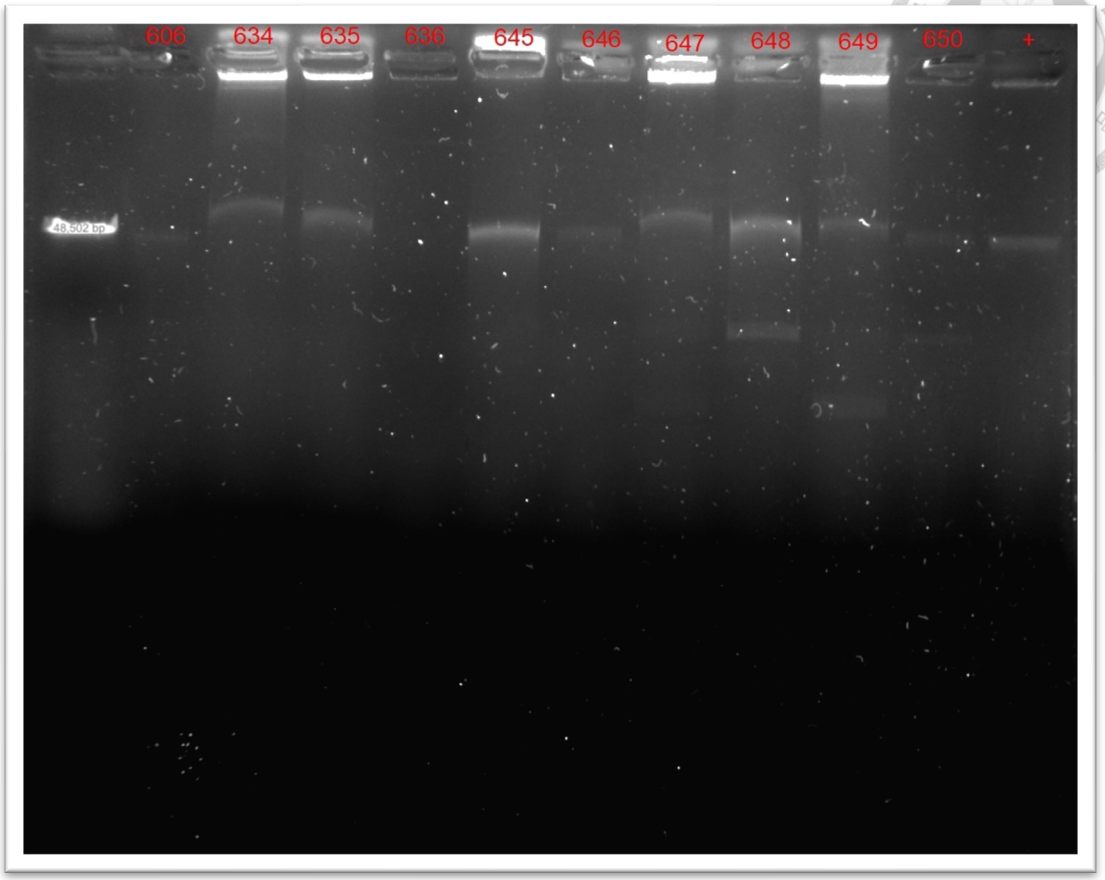


e.

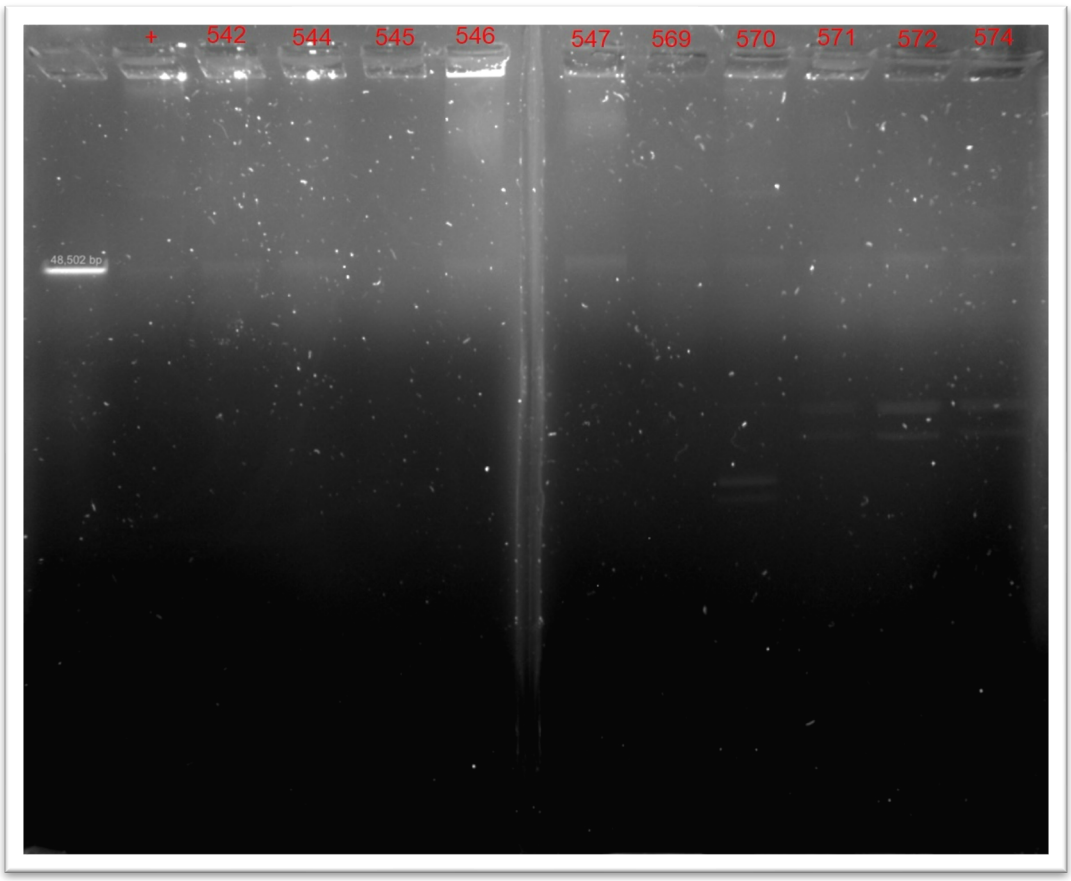


doi:10.6342/NTU202503336

f.

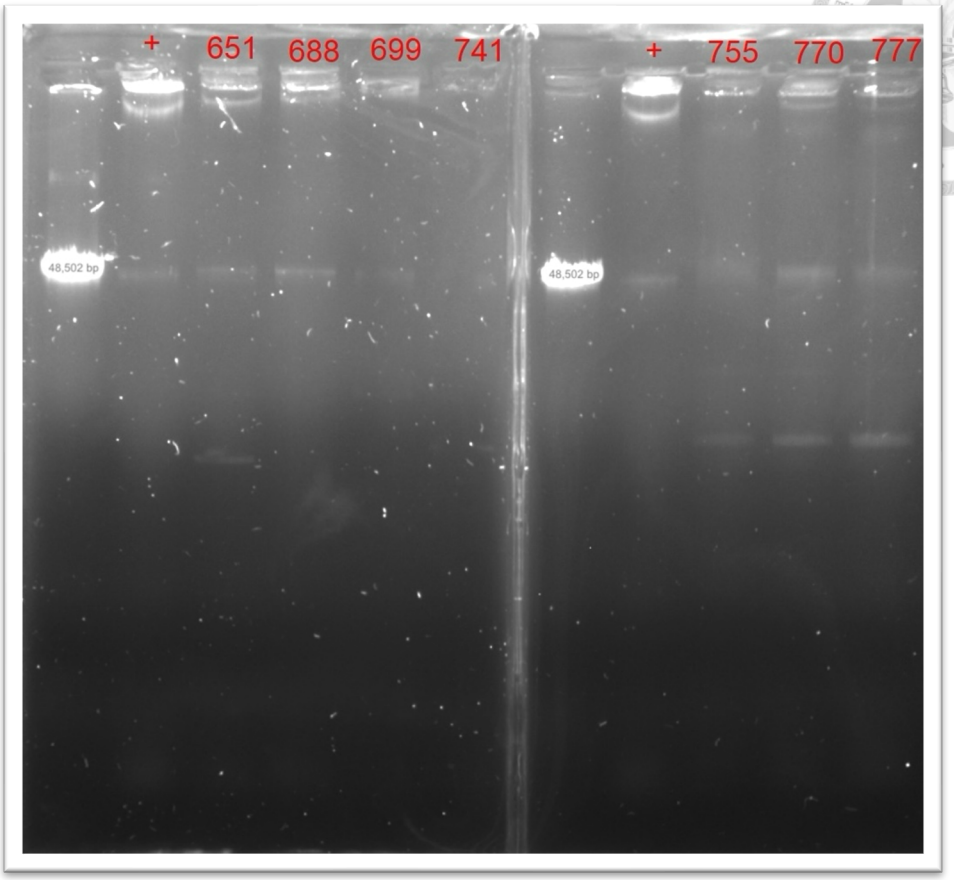


g.



doi:10.6342/NTU202503336

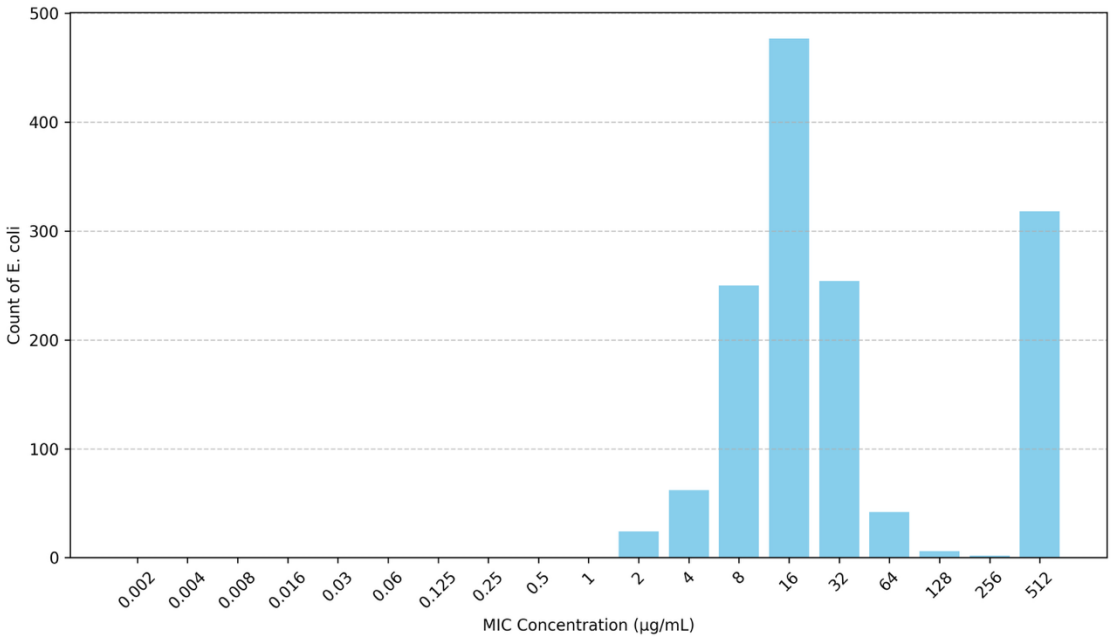
h.



Appendix Figure vi. Gel electrophoresis images of plasmid extraction results. For every image, the leftmost lane of the gel is the DNA marker as a control. For the rest of the lanes in the images, each lane is marked with its sample ID on top of the loading well. The lanes marked with "+" represent lanes for positive control, which is an *Escherichia coli* strain with a 65 kb length of plasmid. For subfigures a. to e., the marker lanes were loaded with 1 kb Plus DNA Ladder from New England Biolabs. The ladder was served as a control purpose only, it cannot be served as an accurate size reference for plasmids, since the 1 kb Plus DNA ladder contains linear DNA, while plasmids are circular and migrate differently based on their shape and conformation during gel electrophoresis For subfigures f. to h., the marker lanes were loaded with Lambda DNA from New England Biolabs. The position of the chromosomal DNA debris on gels usually migrates to the same position as Lambda DNA, which is 48,502 base pairs in length. Sample ID 505 in subfigure d. doesn't belong to the 40 *Escherichia coli* collections tested in the study. Subfigure e. includes three lanes that cannot be used as references due to pipetting errors. Gel images were produced by Bio-Rad Gel Doc™ EZ Imager.

doi:10.6342/NTU202503336

7. *Escherichia coli* MIC Distribution for Sulfamethoxazole



Appendix Figure vii. Number of *Escherichia coli* distribution according to their sulfamethoxazole MIC concentration. The distribution data was retrieved from the EUCAST Antimicrobial Wild Type Distributions of Microorganisms Database on July 22nd, 2025[1]. According to the database, the sulfamethoxazole MIC concentration for *Escherichia coli does* not yet have a referable ECOFF value due to insufficient data. In this report, we set an unofficial, estimated value of 32 µg/mL for *Escherichia coli* to distinguish if the strain is wild or non-wild type for sulfamethoxazole, to ensure the antibiotic has a comparable result to the other antibiotics. However, the result for sulfamethoxazole has to be treated with extra caution since the reference concentration is a pure estimation.

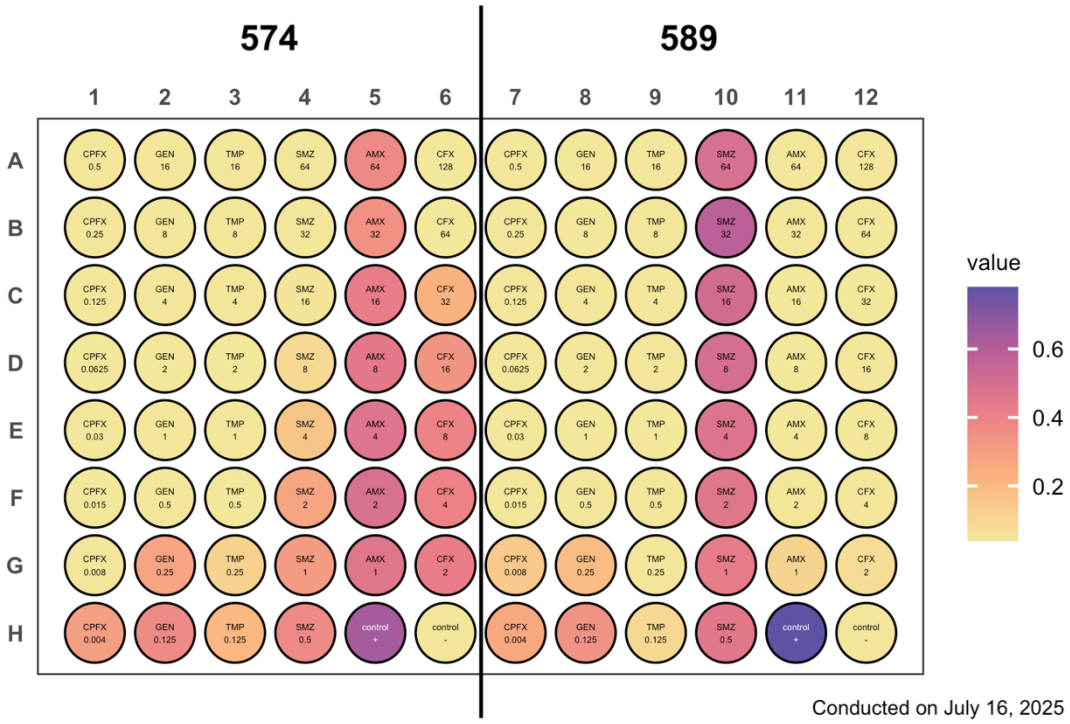
1. European Committee on Antimicrobial Susceptibility Testing. Data from the EUCAST MIC distribution website. https://www.eucast.org.

doi:10.6342/NTU202503336

8. MIC Results for *Escherichia coli* Collections

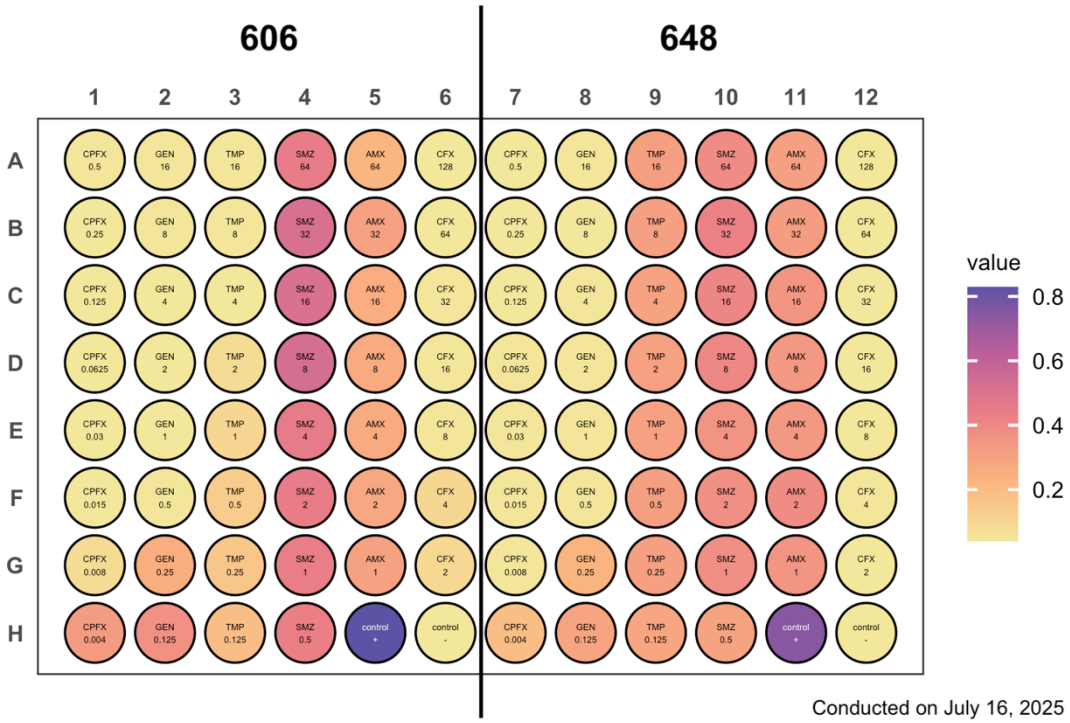
The MIC test for this report was conducted by the broth microdilution method with 96-well plates. Ciprofloxacin, gentamicin, trimethoprim, sulfamethoxazole, amoxicillin, and cefoxitin were tested in the following results.

a.

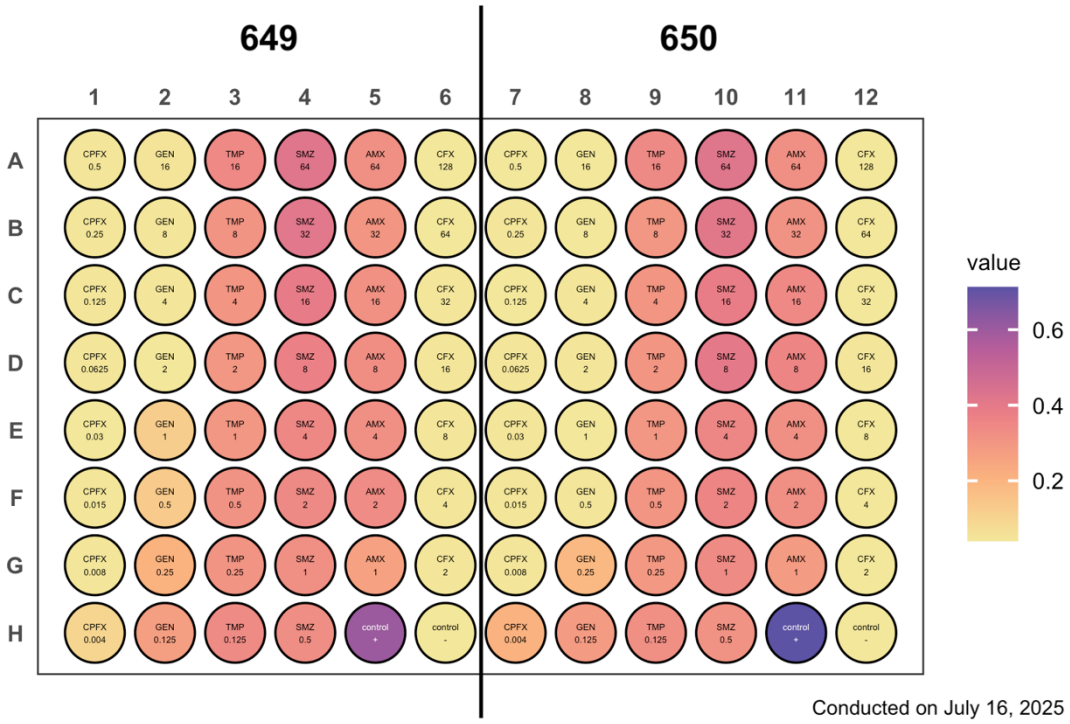


doi:10.6342/NTU202503336

b.

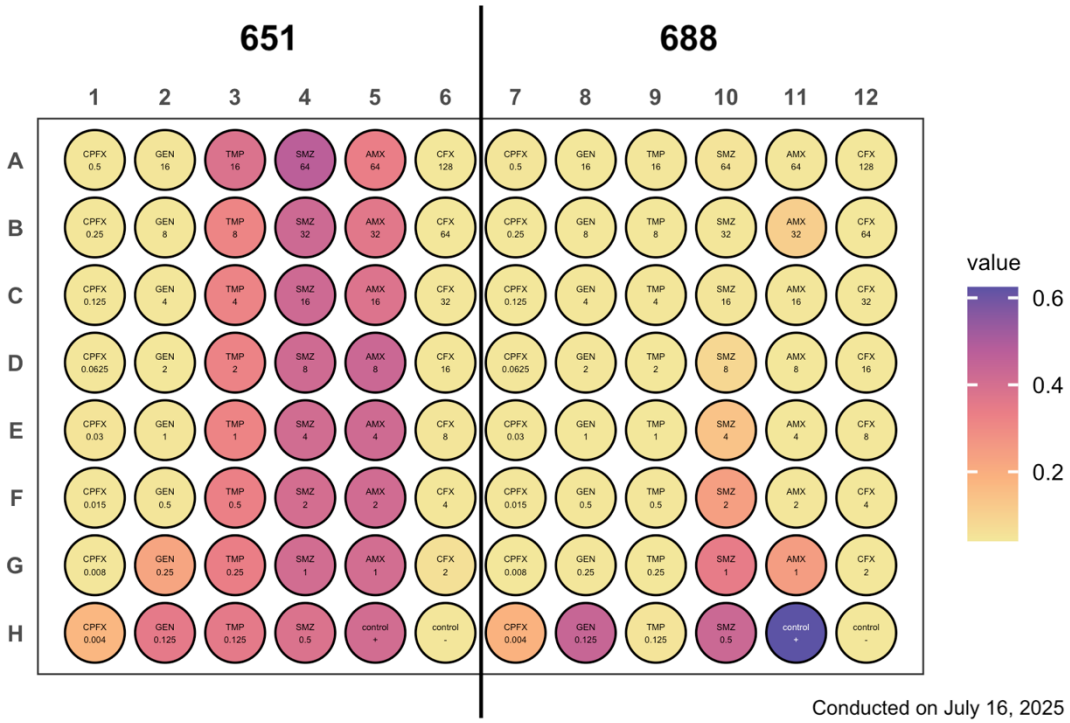


c.

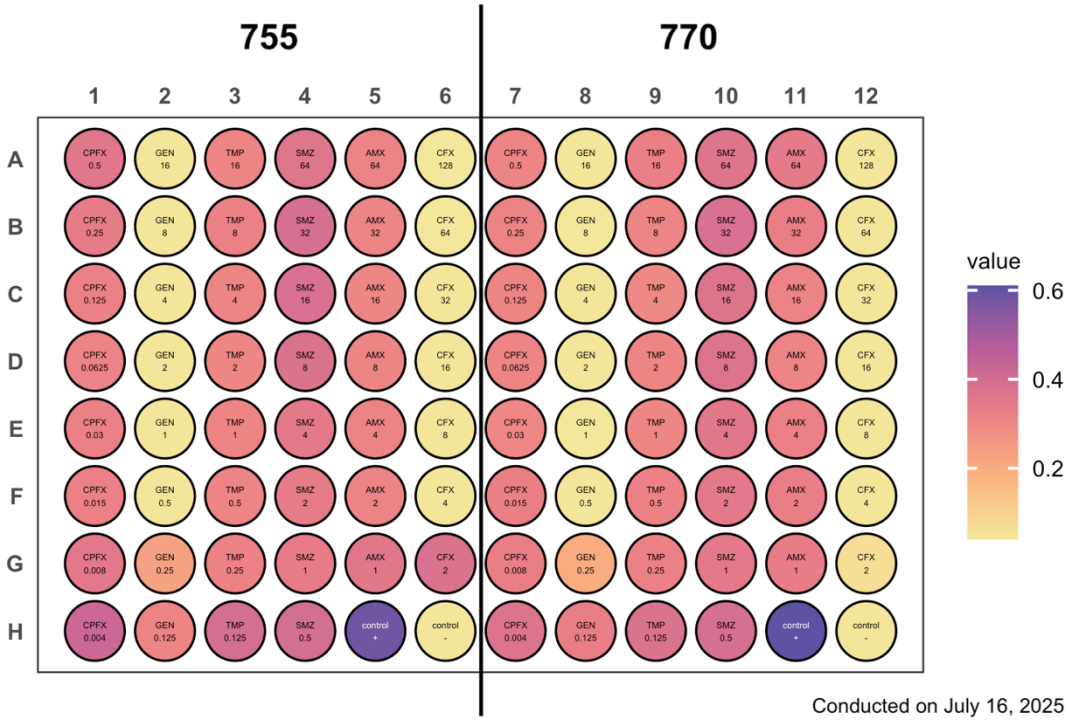


doi:10.6342/NTU202503336

d.

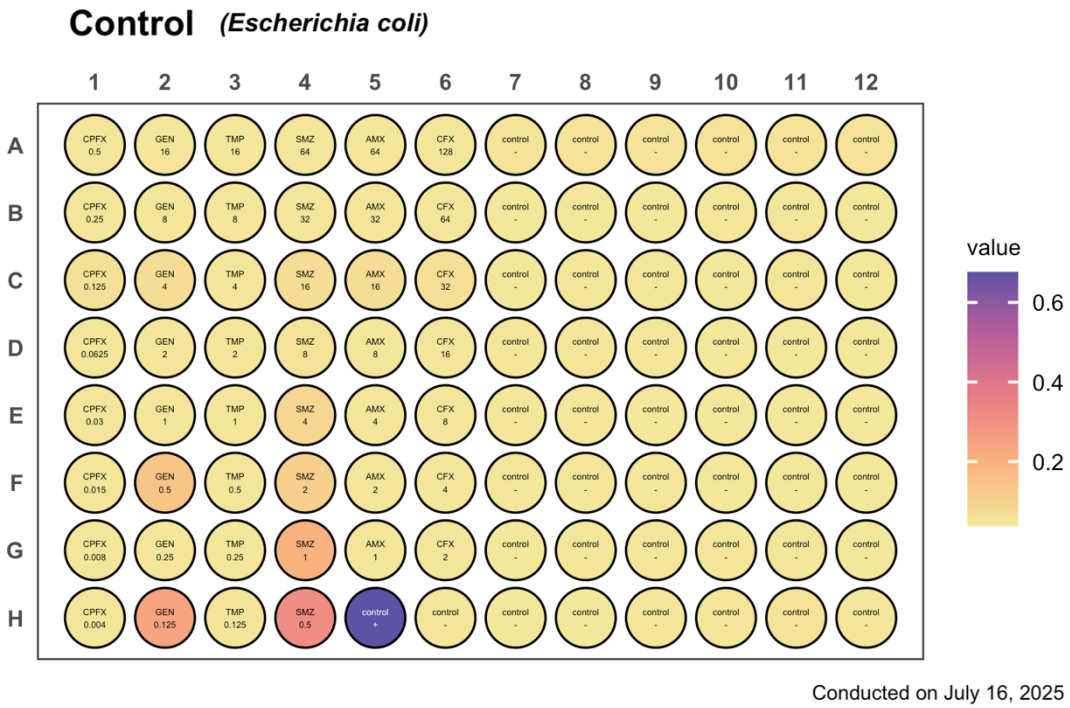


e.



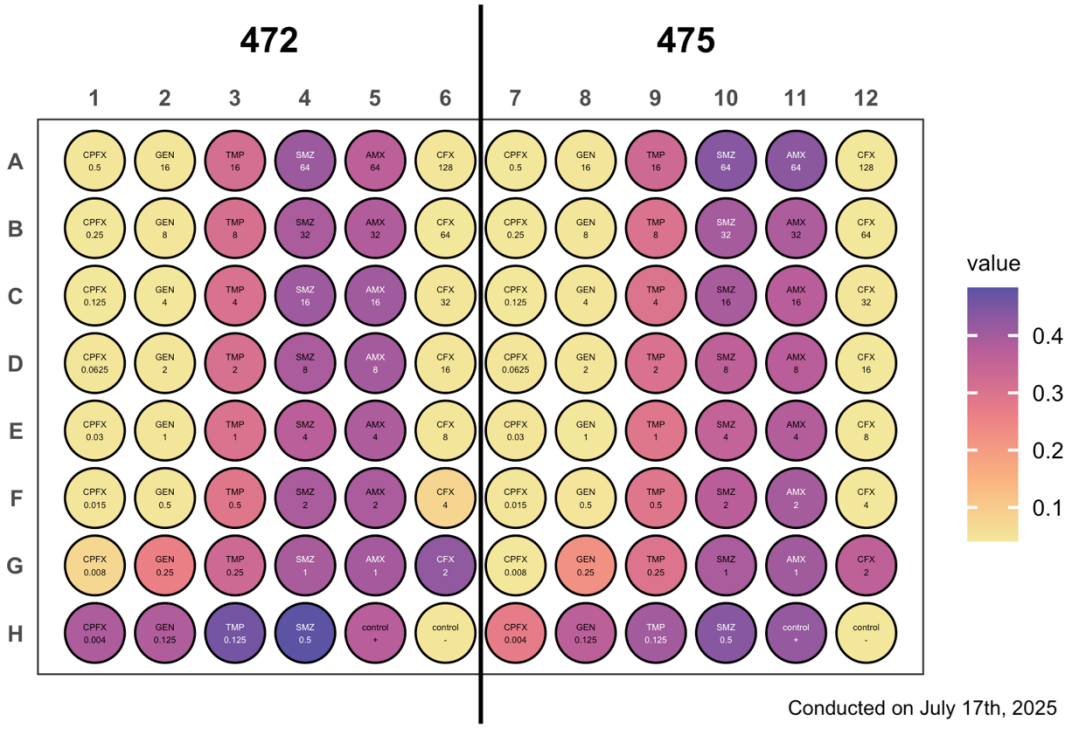
doi:10.6342/NTU202503336

f.



Conducted on July 16, 2025

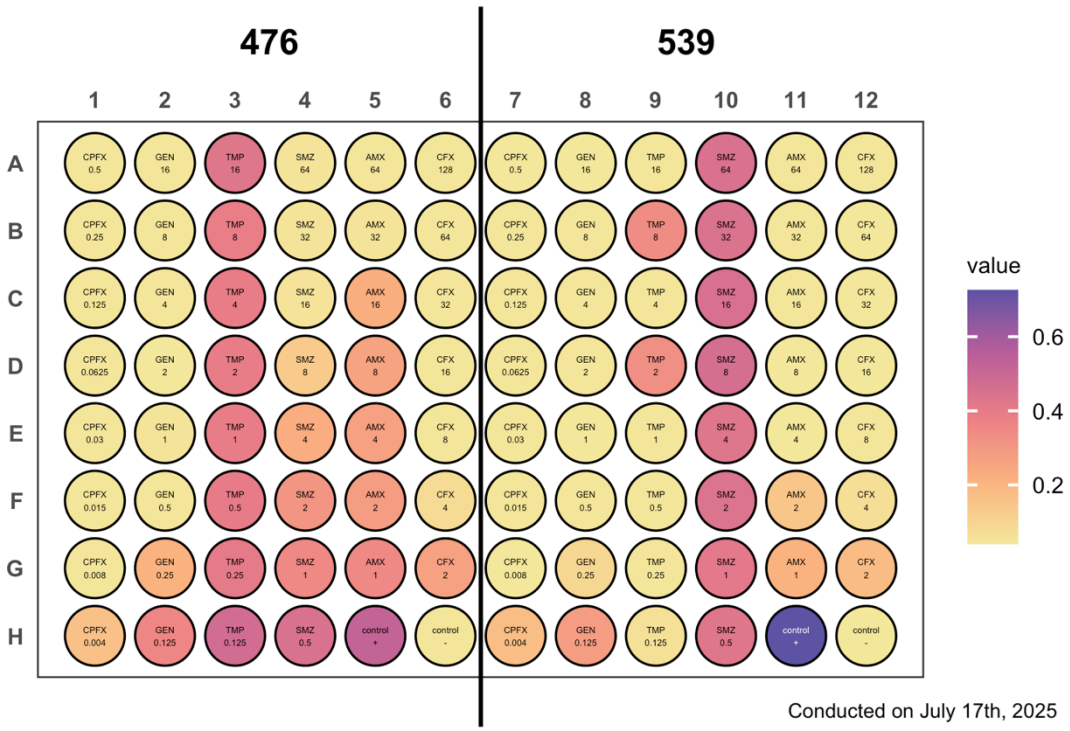
g.



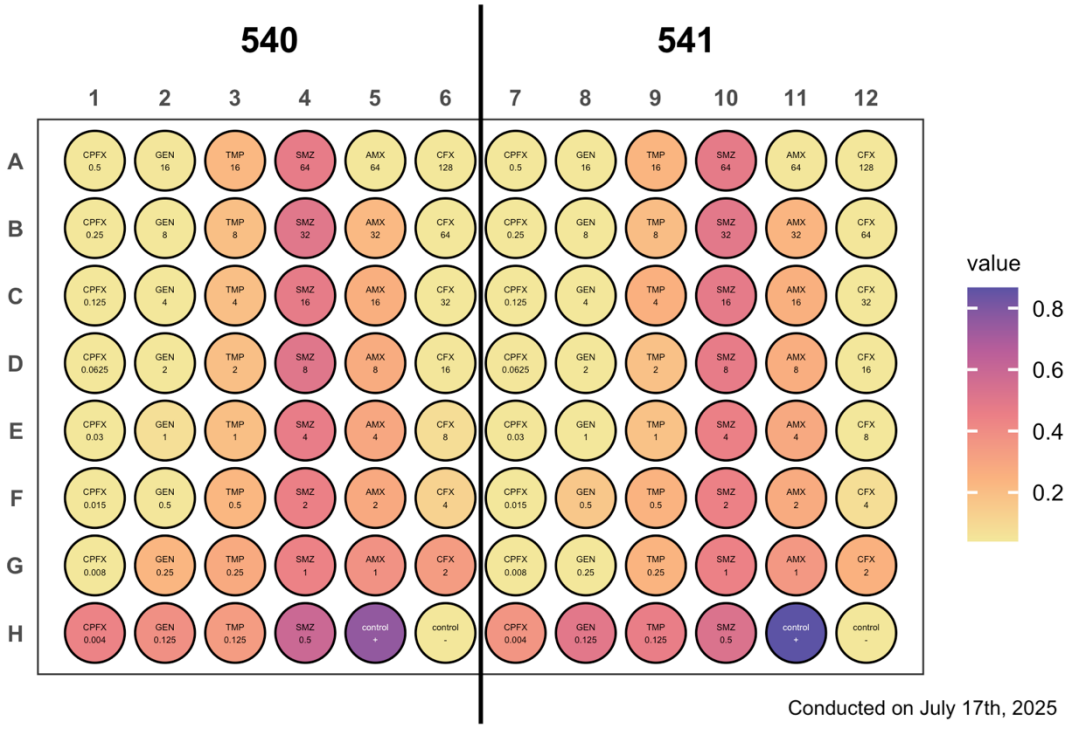
Conducted on July 17th, 2025

doi:10.6342/NTU202503336

h.

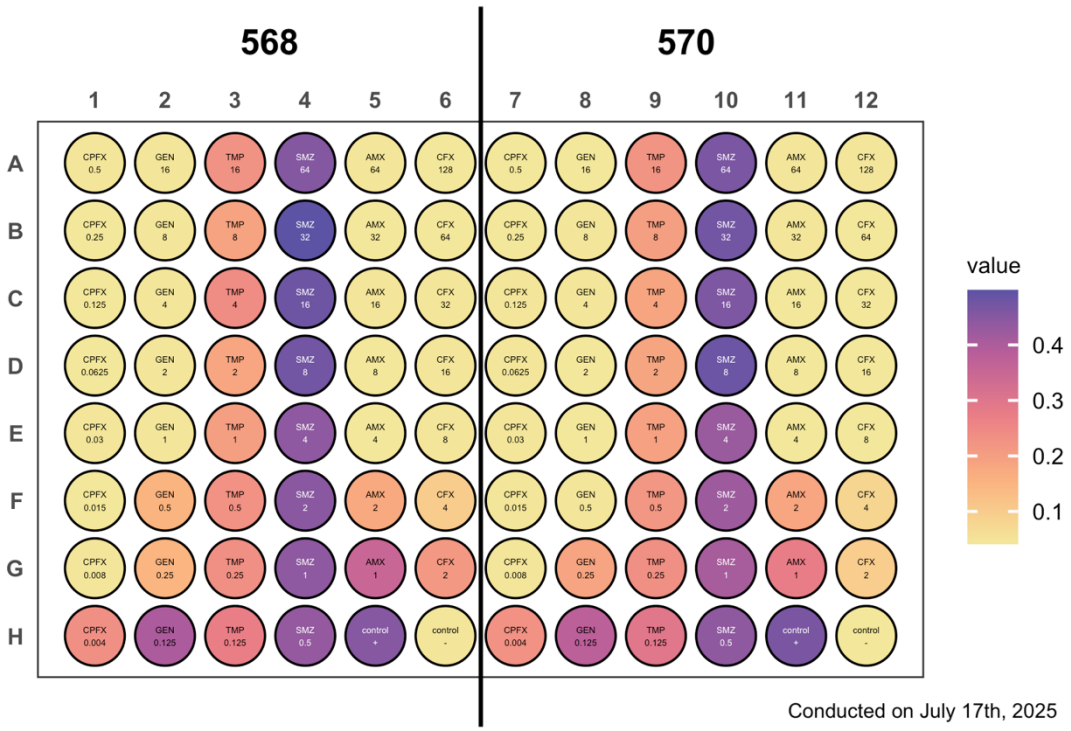


i.

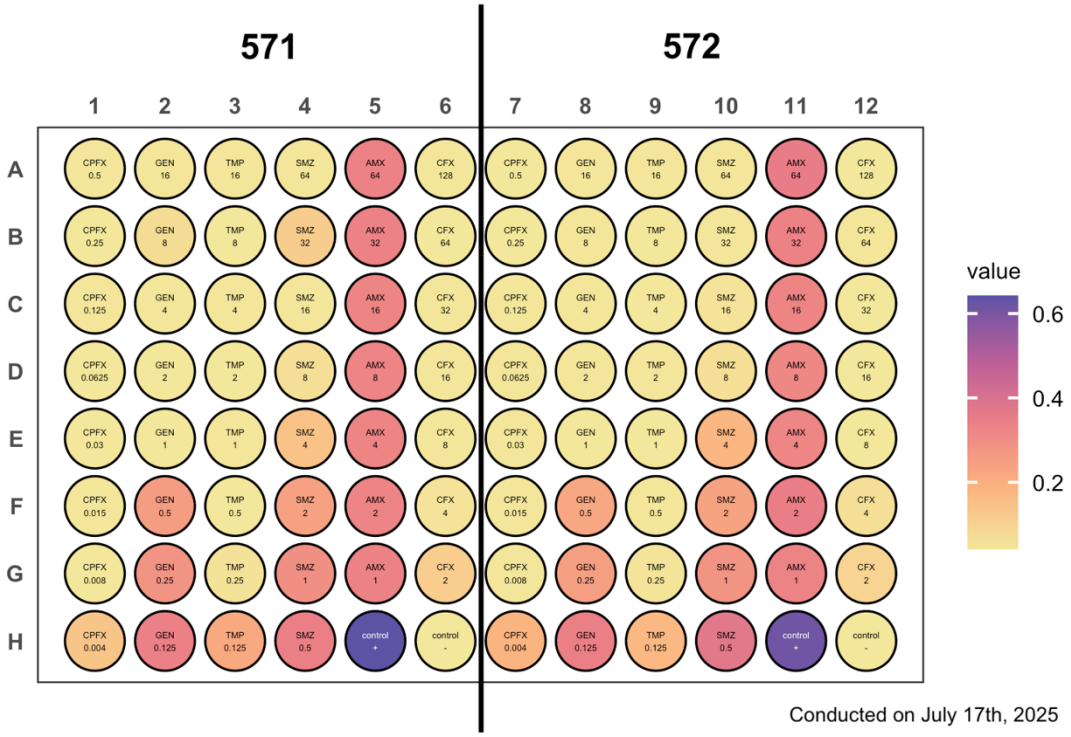


doi:10.6342/NTU202503336

j.

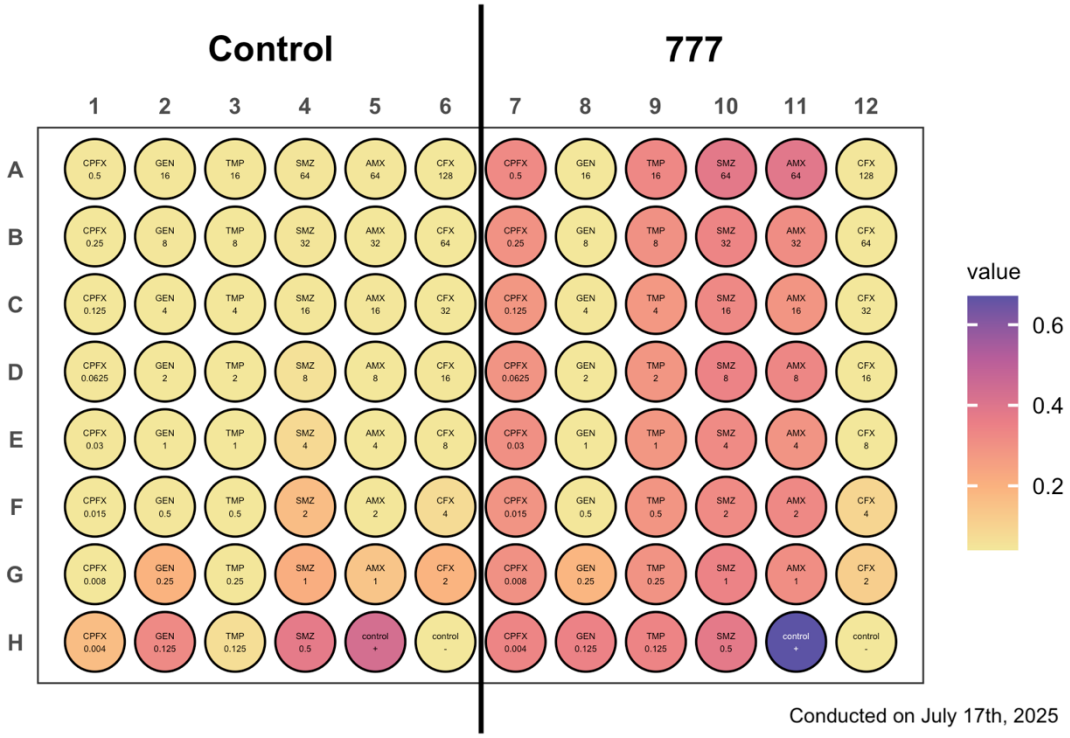


k.



doi:10.6342/NTU202503336

l.



Appendix Figure viii. MIC result of *Escherichia coli* Collections with 6 antibiotics tested. 2 collections were tested in each 96-well plate by separating the plate vertically in the middle. Ciprofloxacin, gentamicin, trimethoprim, sulfamethoxazole, amoxicillin, and cefoxitin were placed in each column in order, followed by 2-fold dilutions from the top to bottom row. The last row for amoxicillin was set for positive control, while the last row for cefoxitin was set for negative control. The ECOFF concentrations are in row D for all antibiotics except sulfamethoxazole. Subfigures a. to e. were conducted in the same batch of experiment with subfigure f. as their corresponding control, while subfigures g. to k. were another batch of experiment with subfigure l. as their corresponding control. The *Escherichia coli* control in subfigures f. and l. is a strain known to be susceptible to ciprofloxacin (≤ 0.06 μg/mL), gentamicin (≤ 1 μg/mL), sulfamethoxazole (≤ 32 μg/mL), amoxicillin (≤ 4 μg/mL), and cefoxitin (≤ 4 μg/mL). The absorbance of the wells was measured at the wavelength of 600nm with the precision mode. The result values in each plate were visualized through the R package ggplate.

doi:10.6342/NTU202503336